THE EFFECT OF HEAT ON THE STRUCTURE AND AGGREGATION BEHAVIOUR OF BOVINE β-LACTOGLOBULINS A, B AND C.

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Massey University.

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Errata.

1) The citation Elofsson *et al.* (1996b) should read Elofsson (1996b).

2) The errors shown for the ΔG_{app} values in Chapters 5 and 7 were calculated incorrectly and are smaller than the true errors.

Abstract.

The bovine milk protein β-lactoglobulin (BLG) possesses a thiol group which becomes solvent exposed at elevated temperatures, leading to the formation of disulphide-linked milk protein aggregates. This phenomenon is of interest to the dairy industry because milk is heat-treated in many modern processes. This study is concerned with how the structure of BLG is altered during and as a consequence of heat treatment and how aggregates are formed. Bovine BLG exhibits genetic polymorphism and the A, B and C variants, present in New Zealand milks, differ in their susceptibilities to heat-induced structural change and aggregate formation, and their response to heat treatment is examined in the present study.

This study used the following techniques: near and far UV CD, intrinsic protein fluorescence, hydrophobic probe fluorescence, thiol group solvent-exposure and both native-PAGE and SDS-PAGE.

Spectroscopic and thiol exposure results suggest that the tertiary structure of BLG is altered during and as a consequence of heat treatment and that the amount of β-sheet in this protein does not alter appreciably as a consequence of heat treatment. PAGE results indicate that BLG forms a mixture of non-covalently-linked and disulphide-linked aggregates during heating, and that disulphide-linked dimers in particular are associated into larger non-covalently-linked aggregates. These non-covalently-linked aggregates are intermediates and large disulphide-linked aggregates are the end product of the BLG aggregation pathway. β-Lactoglobulin A forms aggregates, particularly large disulphide-linked aggregates, more slowly than BLGs B and C. Spectroscopic and thiol availability results suggest that the "intrinsic thermostability" of BLG C is appreciably greater than that of BLG B, which is slightly greater than that of BLG A. Furthermore, the extent of "irreversible structural change" in molecules of BLG C which occurs as a consequence of heat treatment is less than that in molecules of BLG A, which is less than that in molecules of BLG B. In the case of BLGs A and B this reflects the slower rate at which aggregates of BLG A form compared to that of BLG B. The present study has advanced the understanding of the BLG aggregation mechanism and how the A, B and C variants differ in their response to heat treatment.

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LIST OF ABBREVIATIONS.

ANS - 1-anilinonaphthalene-8-sulphonate
BLG - β-lactoglobulin
BLG A/B - a 1:1 mixture of BLGs A and B
BSA - bovine serum albumin
CSA - (+)-10-camphorsulphonic acid
DEAE - diethylaminoethyl cellulose
DLS - dynamic light scattering
DSC - differential scanning calorimetry
DTNB - 5,5'-dithio-bis(2-nitrobenzoic acid)
ΔC_p - change in heat capacity
$\Delta H°$ - change in enthalpy
far UV CD - far ultra-violet circular dichroism
FPLC - fast protein liquid chromatography
Gdn-HCl - guanidine hydrochloride
HPLC - high performance liquid chromatography
I_{ANS} - ANS fluorescence emission intensity
I_{Trp} - tryptophan fluorescence emission intensity
K - equilibrium constant
NATA - N-acetyltryptophanamide
near UV CD - near ultra-violet circular dichroism
NEM - N-ethylmaleimide
NMR - nuclear magnetic resonance
ODNB - 5-(octyldithio)-2-nitrobenzoic acid
PAGE - polyacrylamide gel electrophoresis
PCS - photon correlation spectroscopy
PMT - photomultiplier tube
Q - fluorescence quantum yield
RET - radiationless (or resonance) energy transfer
S - Svedberg unit (measure of sedimentation velocity)
SDS - sodium dodecyl sulphate
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMUF - simulated milk ultrafiltrate
TCA - trichloroacetic acid
TEMED - N,N,N',N'-tetramethylethylenediamine
T_m - midpoint temperature
T_{max} - denaturation temperature (DSC)
TNB - thionitrobenzoate
3D - three dimensional

Chapter 1.

INTRODUCTION.

Non-human milk has been an important part of the human diet for over 4000 years. However, during the last 100 years or so, the range of food products which contain dairy ingredients has diversified rapidly. Today, dairy based food products are prepared using sophisticated processing techniques, many of which involve heating milk. This can have a detrimental effect on the structures and reactivities of the proteins in milk. Therefore, to further improve modern dairy products, a detailed understanding of how milk proteins are affected by heat treatment is required. The study presented and discussed in this thesis was initiated to gain a greater understanding of how the structure of bovine β-lactoglobulin (BLG), one of the whey proteins present in bovine milk, is affected by heat and how aggregates of this protein form during heat treatment.

The effect of heat on the structure of BLG is of interest to the dairy industry because this protein possesses a free thiol group which becomes solvent-exposed at elevated temperatures. This solvent-exposed thiol group can then participate in thiol-disulphide interchange reactions with the disulphide bonds of other BLG molecules and also other milk proteins such as α-lactalbumin, bovine serum albumin, the immunoglobulins and κ-casein. The end products of these reactions are high molecular weight polymers of BLG and milk protein heteropolymers.

The formation of milk protein polymers during heat treatment is both a liability and an asset to dairy product manufacture. For example, in the preparation of ultra high temperature (UHT) milk and milk powder, this phenomenon is a liability. Amorphous protein polymers are produced during heat treatment and form a deposit on the metal heat-exchanger plates used to heat milk. This phenomenon, known as fouling, reduces the efficiency of heat transfer. Periodically, the foulant must be removed from the heat-exchanger plates and this interrupts production. In contrast, the formation of whey protein gels can be exploited in food formulation. Whey protein gels are a particular class of milk protein polymer which can be described as a three dimensional matrix of cross-linked linear polymers of protein molecules in which water is trapped. Convenience foods such as processed meats and quiches rely on the gelling properties of whey proteins.

Bovine BLG exhibits genetic polymorphism and the study in this thesis was made using the A, B and C variants, all of which have been identified in the milk of New Zealand's dairy herds. The research concerns how the thermostabilities and aggregation behaviour of these variants differ from each other. The relationship between

heat-induced structural change and aggregation was also investigated. β-Lactoglobulins A and B, which are found in most New Zealand bulk milk in a 1:1 ratio, are known to differ in a number of properties. For example, when milk containing mainly BLG A is heat-treated, fouling occurs more rapidly than when milk containing mainly BLG B is heat-treated and the results in this thesis may provide an explanation for why this is so.

The BLG C gene occurs at a frequency of 0.06 in the New Zealand Jersey cow population. In geographical regions where the population density of Jersey cows is high (e.g. Taranaki), the C variant may therefore have an appreciable effect on the preparation and properties of dairy products. For this reason, the research described here also includes a study of the thermostability and aggregation behaviour of BLG C.

In 1993, when this study was commenced, a number of sporadic reports pertaining to the effect of heat on the structure and aggregation of BLGs A and B had already been published. Although many of these studies are significant in their own right, they do not offer an overall understanding of how the structures of these variants are affected by heat. Furthermore, from the results obtained in these early studies, it is difficult to identify the relationship between heat-induced structural change and aggregate formation. Studies published prior to the end of 1993 (and also those of McSwiney *et al.*, 1994a, b; Havea *et al.*, 1998) are considered in the Review of Literature (Chapter 2). Since 1993, several studies in which the effect of heat on the structure and aggregation behaviour of BLG are examined have appeared. Because these reports were published after the study in this thesis had been planned, they are discussed in the appropriate Results and Discussion sections of Chapters 4, 5 and 6 and not in the Review of Literature.

Chapter 2.

REVIEW OF LITERATURE.

2.1. β-LACTOGLOBULIN.

β-Lactoglobulin is present in the milk of most mammals, except for that of rodents and humans, and is usually the most abundant whey protein (Hambling *et al.*, 1992). For example, the total whey protein concentration of bovine milk normally ranges from 5 - 7 mg/mL, of which, between 2 and 4 mg/mL is BLG (Swaisgood, 1982). The combined concentration of casein and whey protein species in bovine milk usually ranges from 30 mg/mL to 35 mg/mL (Swaisgood, 1982). Thus, between 7% and 11% of the protein in bovine milk is BLG.

In the milk of monogastric species, for example dolphins, horses and pigs, BLG usually exists in a monomeric form of approximate M_r 18 000 (Hambling *et al.*, 1992). However, in the milk of ruminants, for example cows, sheep and reindeer, two monomers usually associate to form dimers (Hambling *et al.*, 1992).

β-Lactoglobulin binds certain hydrophobic molecules with high affinity (Hambling *et al.*, 1992), for example retinol ($K_a = 5 \times 10^7\ M^{-1}$), retinoic acid ($K_a = 3.9 \times 10^8\ M^{-1}$), alkanes ($K_a = 10^3\ M^{-1}$) and triglycerides ($K_a$ unknown). In addition, free fatty acids and sodium dodecyl sulphate (SDS) may bind to bovine BLG non-specifically (Hambling *et al.*, 1992). Hydrophobic fluorescent probes, for example 1-anilinonaphthalene-8-sulphonate (ANS) and 6-propionyl-2-dimethylaminonaphthalene (PRODAN), also bind to BLG (Creamer, unpublished results). Furthermore, because the fluorescence emission characteristics of such probes are dependent on environment, they can be used to examine structure and also structural change.

Bovine BLG is remarkably resistant to denaturation at low pH (Ananthanarayanan *et al.*, 1977; Kella and Kinsella, 1988a). Conversely, the protein is readily denatured at pH values above 8 (Groves *et al.*, 1951; Casal *et al.*, 1988). Bovine BLG also exhibits considerable resistance to digestion by gastric proteases (Miranda and Pelissier, 1983), and is more resistant to tryptic digestion when fatty acids are bound to it (Calvo *et al.*, 1993).

Although BLG has been studied since 1934, its biological function is still not known. However, because BLG binds lipids, is acid-stable and is resistant to gastric digestion, its primary role may be to facilitate the transport of retinol or fatty acids to the intestinal tract of the neonate. From preliminary results, Papiz *et al.* (1986) reported the presence of receptors for the BLG-retinol complex in the intestinal mucosa of calves, which is consistent with this postulated role. β-Lactoglobulin, which stimulates the activity of pre-gastric lipase by binding the liberated fatty acids that would otherwise lead to inhibition (Perez *et al.*, 1992), may also aid in the metabolism of milk lipids (Calvo *et al.*, 1993). In addition, Flower (1994) has suggested that BLG may help regulate intracellular retinoid concentrations because retinoic acid, for example, stimulates gene expression (Blomhoff *et al.*, 1990) and its concentration is carefully regulated *in vivo* (Flower, 1994).

β-Lactoglobulin also inhibits the activities of phosphoprotein phosphatases which in turn regulate the concentration of inorganic phosphate in milk by dephosphorylating casein and other organic compounds which contain phosphate groups (Farrell and Thompson, 1990).

2.2. THE PRIMARY STRUCTURE OF BOVINE β-LACTOGLOBULIN.

There are 162 amino acid residues in the mature polypeptide chain of bovine BLG. The amino acid sequences of BLGs A, B and C (Eigel *et al.*, 1984) are shown in Fig. 2.2.1.

The high resolution crystal structure of BLG A/B of Brownlow *et al.* (1997) and those of BLGs A, B and C of Bewley *et al.* (1998) indicate that these variants contain two disulphide bonds (Cys^{66}-Cys^{160}) and (Cys^{106}-Cys^{119}), and a thiol group, that of Cys^{121}. Using radioactive labelling techniques, Brittan (1997) has also shown that the thiol group of BLGs A, B and C is that of Cys^{121}. Exposure of the thiol of Cys^{121} to solvent occurs as a consequence of structural change in BLG molecules (Larsen and Jenness, 1952; Gough and Jenness, 1962; McSwiney *et al.*, 1994a, b).

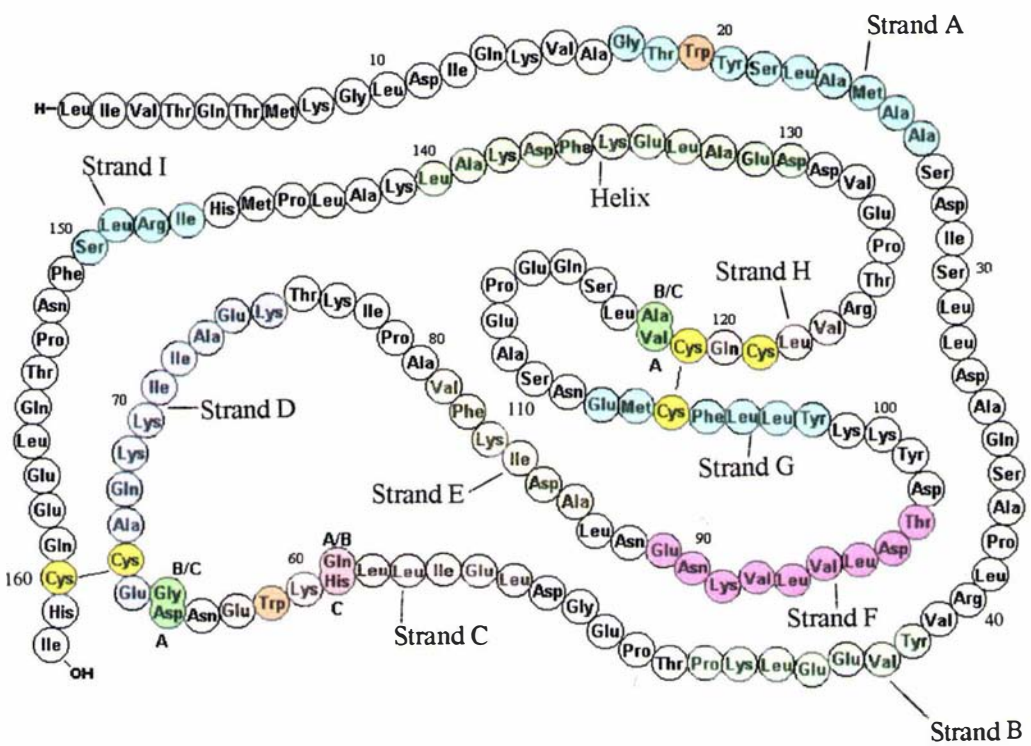


Fig. 2.2.1. The primary structure of bovine BLGs A, B and C (Eigel *et al.*, 1984). The five cysteine and two tryptophan residues are coloured yellow and tan respectively. Positions at which the amino acid substitutions in BLGs A and B occur are coloured green, while that in BLGs B and C is coloured pink. The residues which form β-strands A, B, C, D, E, F, G, H, the α-helix, and β-strand I are indicated (Section 2.4, Brownlow *et al.*, 1997).

2.3. GENETIC VARIANTS OF BOVINE β-LACTOGLOBULIN.

Bovine BLG exhibits genetic polymorphism and to date twelve genetic variants of this protein have been identified: BLGs A, B, C, D, E, F, G (Eigel *et al.*, 1984), H (Lodes, 1995) I, J (Godovac-Zimmermann *et al.*, 1996), W (Lodes, 1995) and Dr, which is N-glycosylated (Bell *et al.*, 1970). The sequence differences of these variants are shown in Table 2.3.1.

Table 2.3.1. Sequence Differences of the Twelve Known Genetic Variants of Bovine β-Lactoglobulin.

Variant	Variable Amino Acid Position												
	28	45	50	56	59	64	78	108	118	126	129	130	158
A	D	E	P	I	Q	D	I	E	V	P	D	D	E
B	D	E	P	I	Q	G	I	E	A	P	D	D	E
C	D	E	P	I	H	G	I	E	A	P	D	D	E
D	D	Q	P	I	Q	G	I	E	A	P	D	D	E
E	D	E	P	I	Q	G	I	E	A	P	D	D	G
F	D	E	S	I	Q	G	I	E	A	P	D/Y*	D/Y*	G
G	D	E	P	I	Q	G	M	E	A	P	D	D	G
H	‡												
I	D	E	P	I	Q	G	I	G	S	P	D	D	E
J	D	E	P	I	Q	G	I	E	A	L	D	D	E
W	D	E	P	L	Q	G	I	E	A	P	D	D	E
Dr	N#	E	P	I	Q	D	I	E	V	P	D	D	E

Data for variants A-G and Dr from Hambling *et al.* (1992)

Data for variants I and J from Godovac-Zimmermann *et al.* (1996)

Data for variants H and W from Lodes (1995).

* Not clear which residue is at which position since the changes in E, F and G reported by Bell *et al.* (1981) are inferred (Hambling *et al.*, 1992).

‡ The amino acid substitution(s) of this variant are not known (Lodes, 1995).

This Asn is glycosylated (Hambling *et al.*, 1992).

In New Zealand dairy herds, three of these genetic variants have been identified: BLGs A and B, which are found in the milk of both Friesian and Jersey cows, and BLG C, which is only found in the milk of Jersey cows (Paterson *et al.*, 1995a). Amongst New Zealand Friesian cows, the genes for BLGs A and B are found in 45% and 55% of the population respectively. The genes for BLGs A, B and C are found in 41 %, 53 % and 6 % respectively of the New Zealand Jersey cow population (Paterson *et al.*, 1995b). The concentration of BLG in bovine milk is variant-dependent (Paterson *et al.*, 1995b, Table 2.3.2).

Table 2.3.2. Dependence of the Relative Concentration of β-Lactoglobulin in Milk on β-Lactoglobulin Phenotype*.

Phenotype	Relative BLG Concentration
AA	100
AB	95
AC	80
BB	71
BC	64

*Data from Paterson *et al.* (1995b).

Because the population of cows with the BLG CC phenotype amongst New Zealand herds is small, statistically significant data could not be collected from milk containing only BLG C.

Although many of the properties of BLG are common to all genetic variants, there are several important differences in their chemical and physical properties. For example, the electrophoretic mobilities of BLGs A, B and C, differ. In a native agar gel system at pH 8.6, BLGs A, B and C migrate the greatest, intermediate and shortest distances respectively (Aschaffenburg, 1965). A difference in charge between the A and B variants at pH 8.6 is the most likely explanation for the difference in the electrophoretic mobility of these variants (Aschaffenburg, 1965).

The solubilities of the A, B and C variants differ; at pH 5.2, BLG A is the least soluble, while BLG C is the most soluble (Bell and McKenzie, 1967). Differences have also been identified in the optical rotation properties of these three variants (Bell and McKenzie, 1967). In addition, the isoelectric points of BLGs A, B and C differ (5.1 for BLG A, 5.2 for BLG B and 5.3 for BLG C, Tanford *et al.*, 1959; Basch and Timasheff, 1967; Ghose *et al.*, 1968).

β-Lactoglobulins A and B also exhibit different susceptibilities to heat-induced structural change. These differences will be discussed in detail in Section 2.8.

2.4. THE THREE-DIMENSIONAL STRUCTURE OF BOVINE β-LACTOGLOBULIN.

The crystal structure of bovine BLG was determined at medium resolution by Papiz *et al.* (1986) and Monaco *et al.* (1987), and at high resolution by Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998). In the study of Papiz *et al.* (1986), crystallographic data were acquired from lattice Y crystals (orthorhombic unit cell, space group $B22_12$, with cell dimensions approximately a = 55.4 Å, b = 67.2 Å, c = 82.0 Å) grown at pH 7.6 and the structure was determined at a resolution of 2.8 Å. Monaco *et al.* (1987), using lattice Z crystals (trigonal unit cell, space group $P3_221$, with cell dimensions approximately a = b = 54.0 Å, c = 112.7 Å) grown at pH 7.8, determined the structure of BLG at a resolution of 2.5 Å. In both of these models, several amino acid residues are located in unusual environments. For example, two prolines, normally found in loops and β-turns, are located in β-sheets. In addition, several hydrophobic residues, normally found in β-sheets, appear in β-turns and loops. Furthermore, Monaco *et al.* (1987) reported that the retinol binding site of BLG was on the outside of the calyx, a mode of binding not observed in other lipocalins.

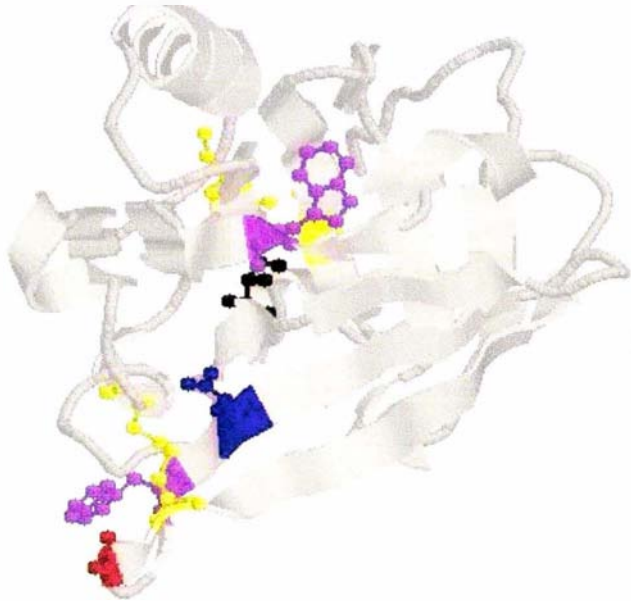
A revised structure of BLG A/B has recently been presented (Brownlow *et al.*, 1997), at a resolution of 1.8 Å based on data collected from lattice X crystals (triclinic unit cell, space group P1, with cell dimensions approximately a = 37.8 Å, b = 49.6 Å, c = 56.6 Å) grown at pH 6.5. This structure corrected the anomalies that had been apparent in those of Papiz *et al.* (1986) and Monaco *et al.* (1987). Crystal structures for BLGs A, B and C have also been determined (Bewley *et al.*, 1998), at 1.80 Å, 1.95 Å and 1.80 Å resolution respectively using data acquired from lattice Y crystals grown at pH values between 7.3 and 7.6. Qin *et al.* (1998) have determined the structure of BLG A at 2.56 Å, 2.21 Å and 2.49 Å using data acquired from lattice Z crystals grown at pH 6.2, pH 7.1 and pH 8.2 respectively. The high resolution BLG crystal structures of Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998) indicate that in the medium resolution BLG crystal structures (Papiz *et al.*, 1986; Monaco *et al.*, 1987), several β-strands were misaligned with the sequence.

To date, the high resolution structure of monomeric BLG at pH 2.2 has been partially solved by nuclear magnetic resonance (NMR) spectroscopy (Uhrínová *et al.*, 1998). They have found that the secondary structure elements in the NMR structure of an expression artefact of BLG, where residue -3 is Glu, -2 is Ala, -1 is Glu, 1 is Ala, and 2 is Tyr, are very similar to those in the high resolution crystal structures. Uhrínová *et al.* (1998) have also found that the two disulphide bonds of BLG form between Cys^{66} and Cys^{160} and between Cys^{106} and Cys^{119} and that the thiol group is that of Cys^{121}, in agreement with the high resolution crystal structures.

The native conformation of bovine BLG contains approximately 50 % β-sheet and approximately 10 % α-helix and is based on an 8-stranded antiparallel β-barrel. However, this barrel is somewhat distorted, and the 3D structure of BLG is better described as a flattened cone or calyx, the interior of which is hydrophobic (Brownlow *et al.*, 1997). The 3D structure of BLG A/B of Brownlow *et al.* (1997), depicted in various ways, is shown in Figs 2.4.1 - 2.4.4.

The eight β-strands which make up the β-barrel of BLG are designated A-H (Brownlow *et al.*, 1997). β-Strands A-D form one side of the calyx, while β-strands E-H and also part of β-strand A form the opposite side. β-Strands B-H are connected through short loops at the narrow end (i.e. the bottom) of the calyx and larger loops at the other end. However, β-strand A is connected to β-strand B via a long flexible loop (loop AB) which contains an α-helical turn. Loop AB forms part of the dimer interface and several of its residues form hydrogen bonds with residues in loop AB of the corresponding monomer. The eleven residues between the N-terminus and the N-terminal end of β-strand A form a loop which contains a 3_{10}-helical turn. This loop closes over the bottom of the calyx. At the C-terminal end of β-strand H, the polypeptide chain doubles back along the outside of the calyx and then forms a 3-turn α-helix. This helix is amphipathic, and packs against β-strands E-H on the exterior of the calyx. After a short loop region on the C-terminal side of the α-helix, the polypeptide chain forms another β-strand, labelled I. Between the C-terminal end of β-strand I and the C-terminus, the polypeptide chain forms an extremely flexible loop which contains an α-helical turn. This loop is anchored to the exterior of the calyx via the disulphide bond Cys^{66}-Cys^{160}. In the primary structure of BLG (Fig. 2.2.1), the amino acids involved in the various elements of bovine BLG secondary structure are indicated. The pattern of hydrogen bonds in a monomer of BLG A at pH 7.1 is shown in Fig. 2.4.5.

Fig. 2.4.1. Cartoon representation of the structure of BLG A/B of Brownlow et al. (1997). The structure in panel b is rotated approximately 80 degrees and 120 degrees in the y and z axes respectively relative to that in panel a.



The structure in panel c is rotated approximately 80 degrees, -20 degrees and 180 degrees in the x, y and z axes respectively relative to that in panel a. All of the figures in Section 2.4 can be viewed in 3D.

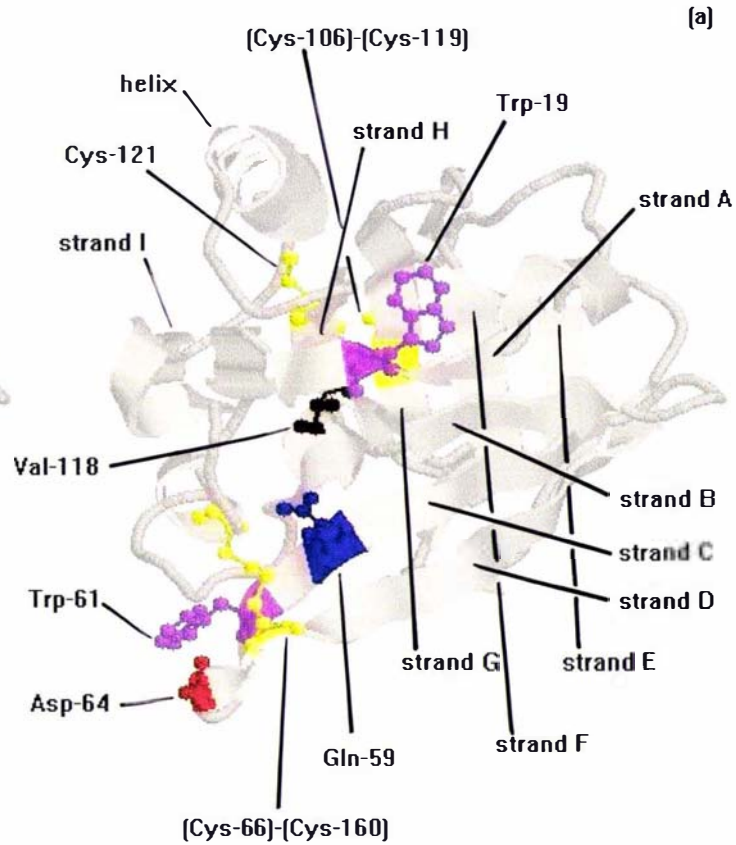


Fig. 2.4.1b. Caption as per Fig. 2.4.1.a.

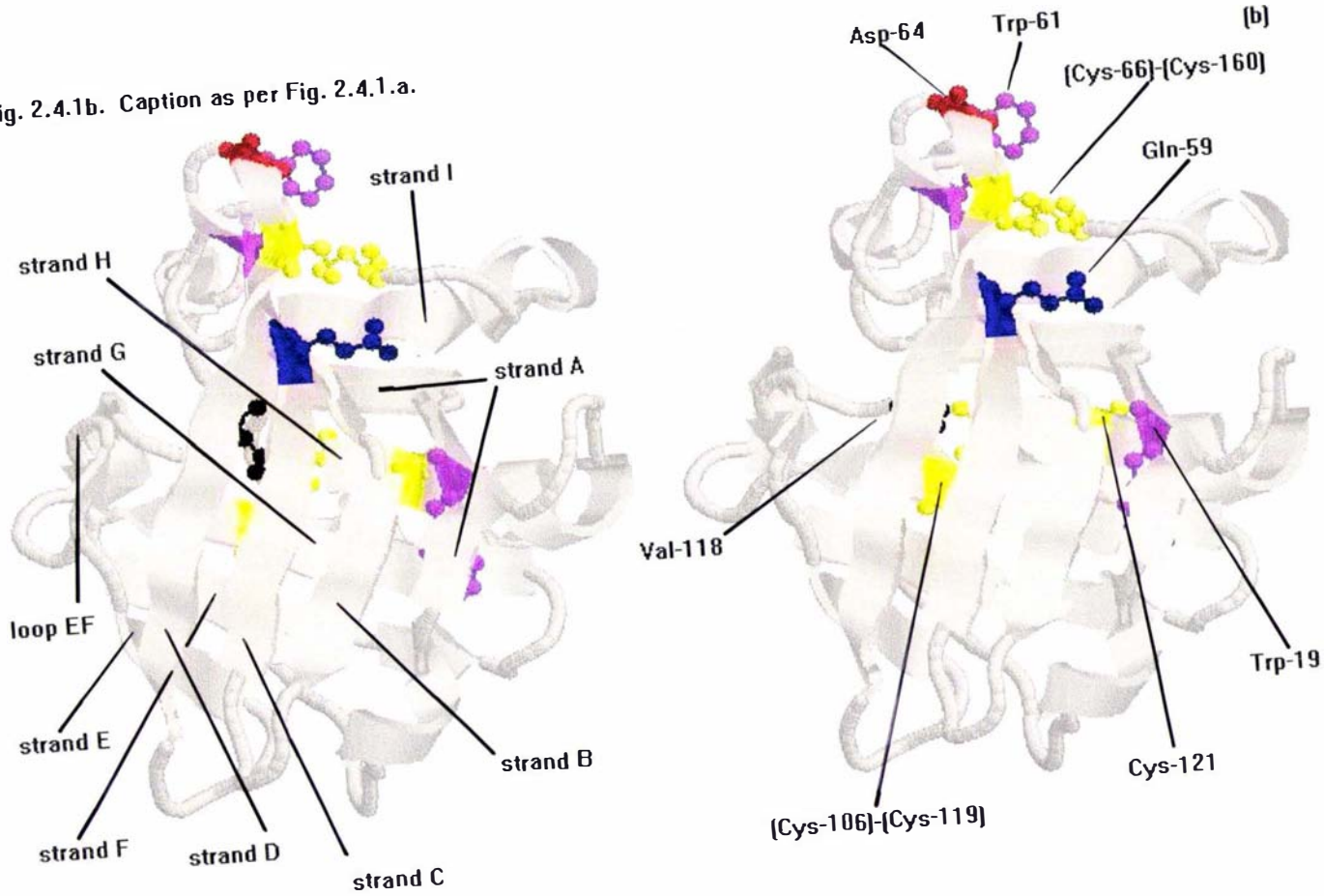
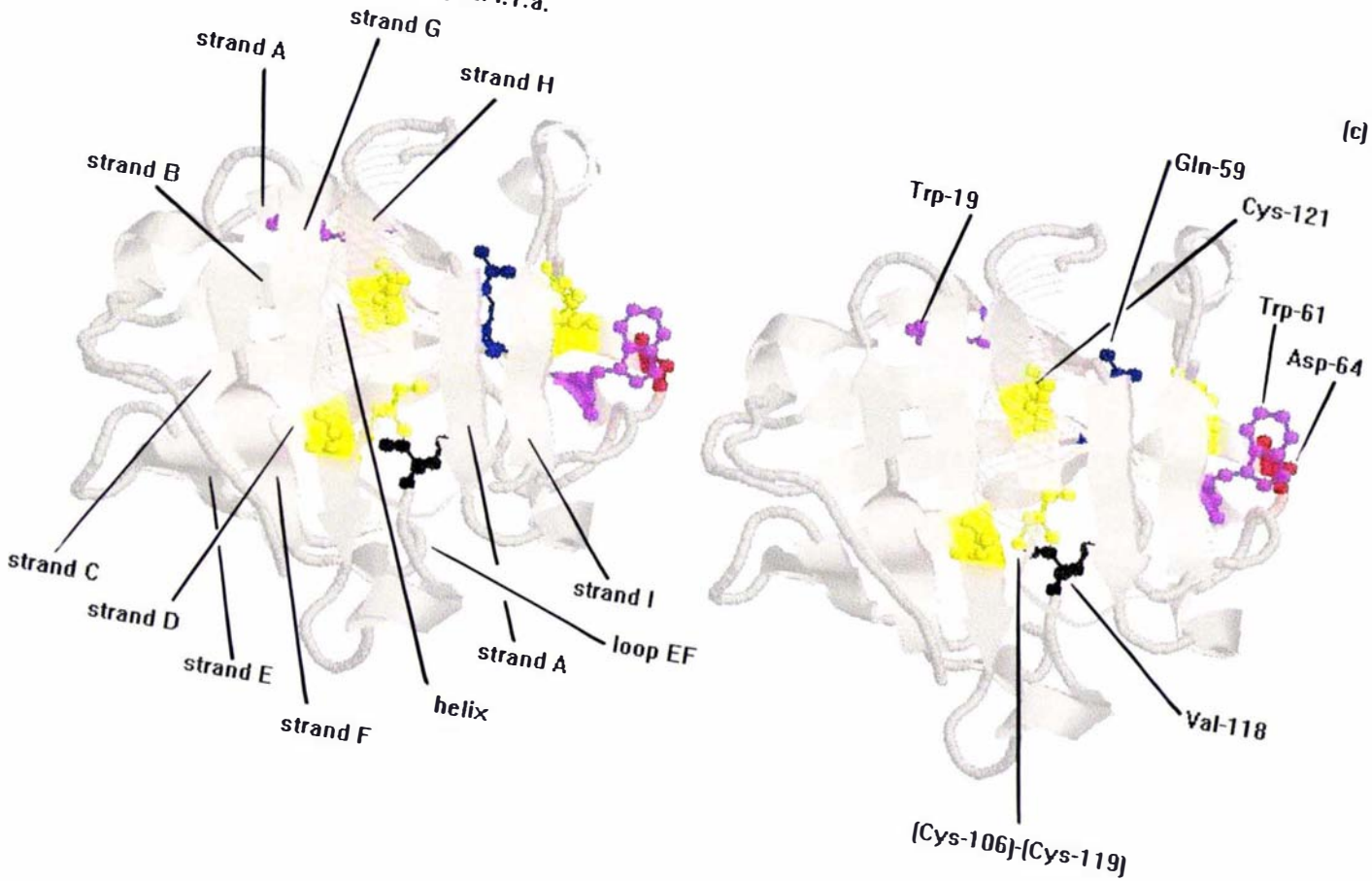


Fig. 2.4.1c. Caption as per Fig. 2.4.1.a.



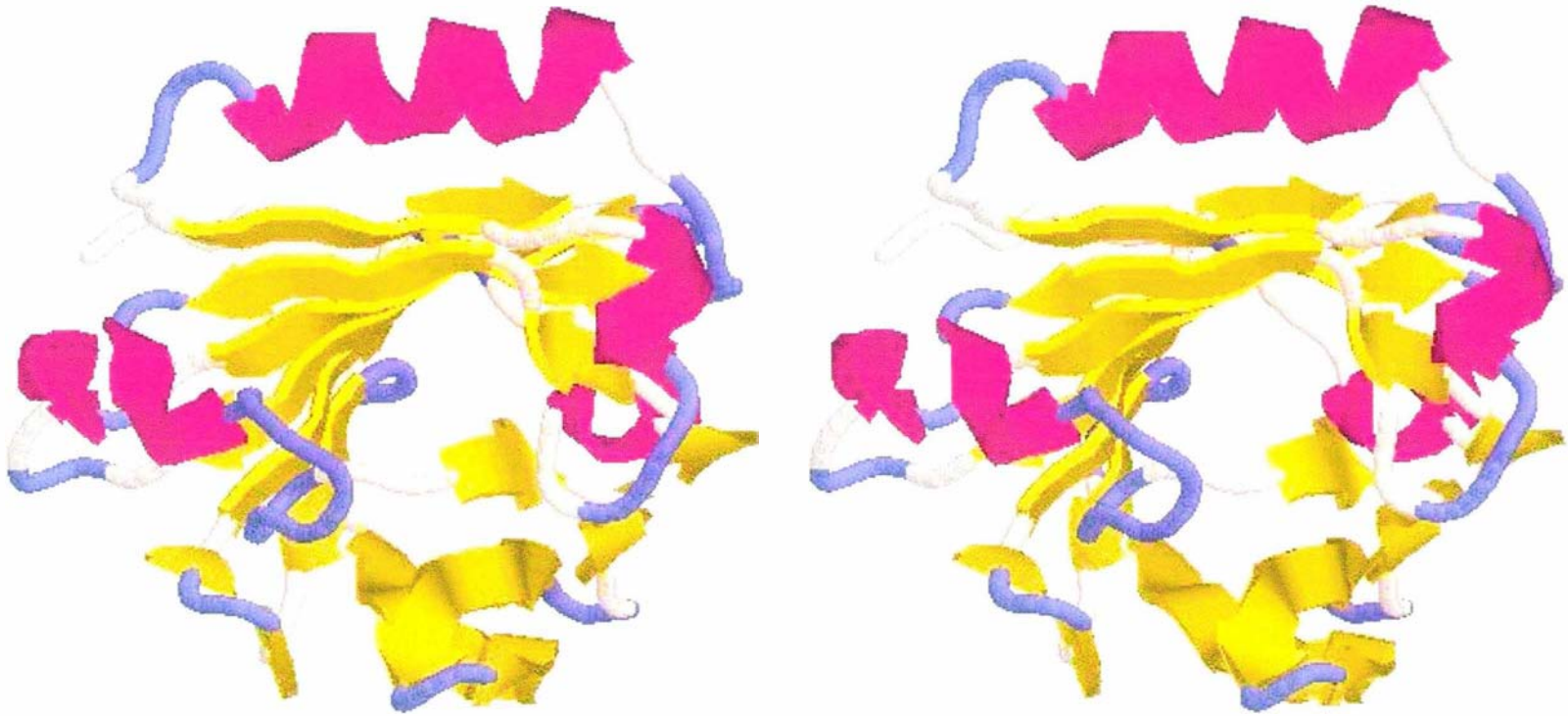


Fig. 2.4.2. Cartoon representation of the structure of BLG A/B of Brownlow et al. (1997). Stretches of amino acid sequence involved in different structural elements are indicated as follows: beta-strands (yellow), the alpha-helix and alpha-helical turns (magenta), beta-turns (blue) and irregular regions (white).

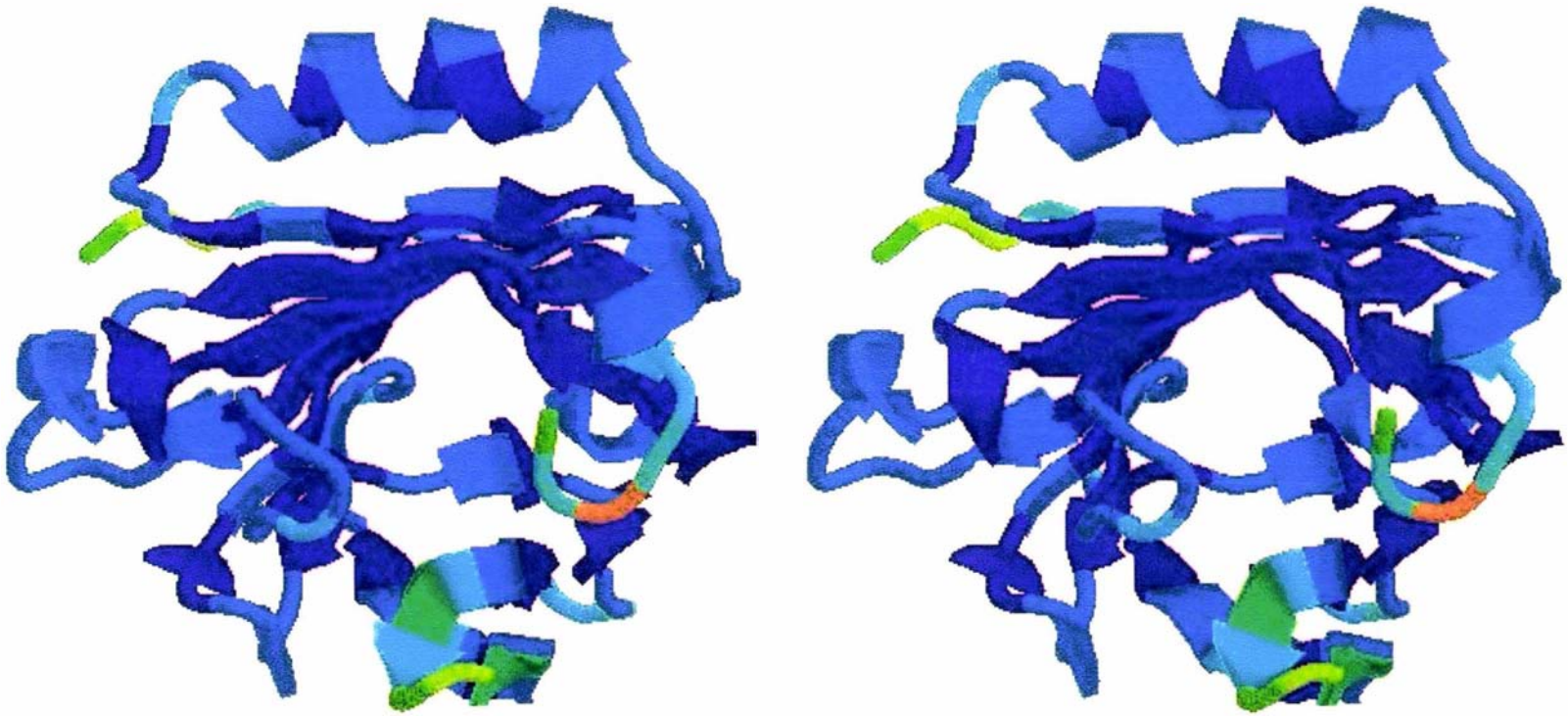


Fig. 2.4.3. Cartoon representation of the structure of BLG A/B of Brownlow et al. (1997). The relative flexibilities of different regions of this structure are colour-coded according to the anisotropic temperature (beta) value stored in the protein data bank file, giving a measure of the mobility/flexibility of a given atom's position (Rasmol user manual, Sayle, 1993). The colour coding is as follows: dark blue, mid blue, pale blue, pale green, green, yellow, orange; less flexible - more flexible.

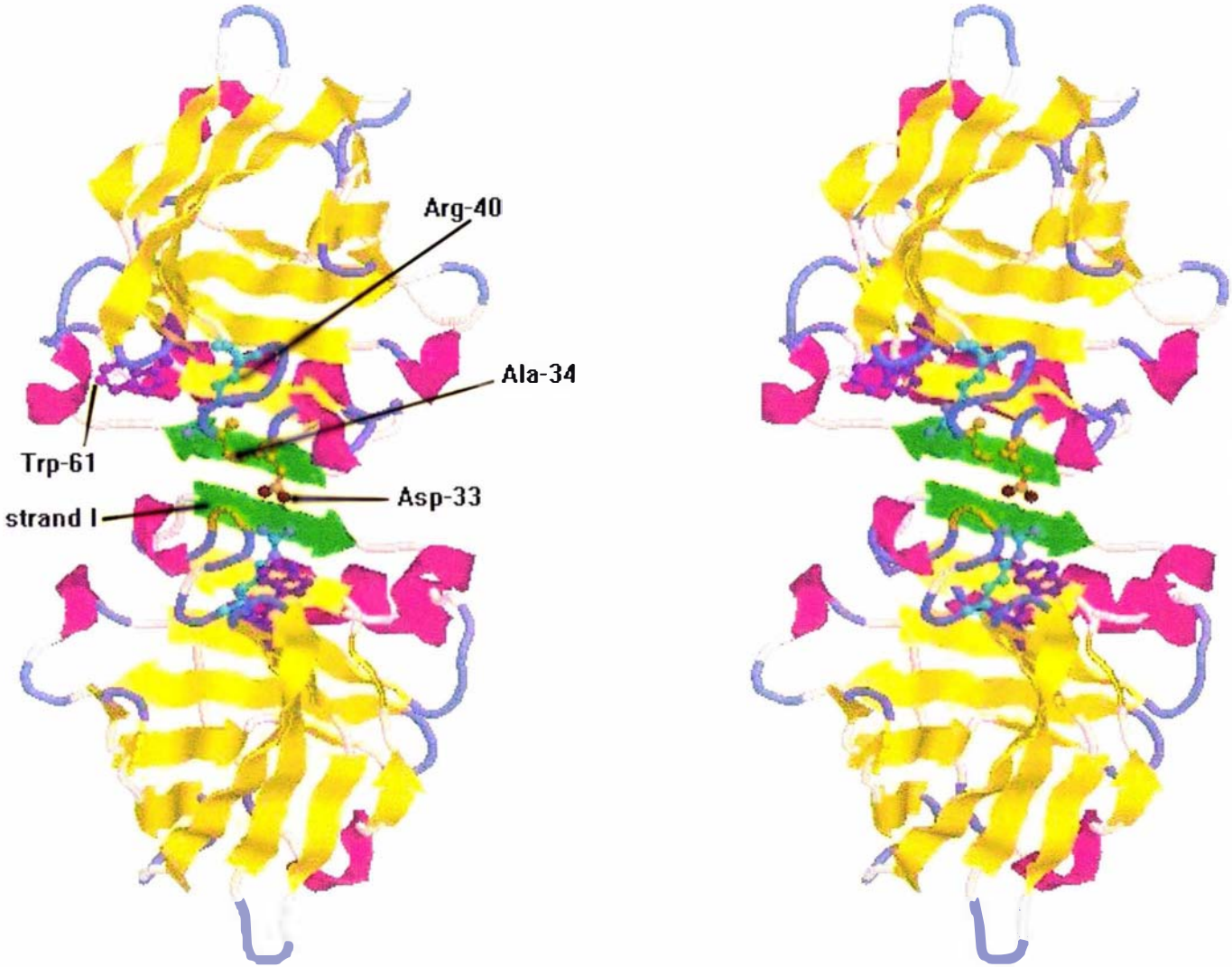


Fig. 2.4.4. Cartoon representation of the structure of a dimer of BLG A/B (Brownlow et al., 1997). Except for strand I, stretches of amino acid sequence involved in different structural elements are coloured as described in Fig. 2.4.2.

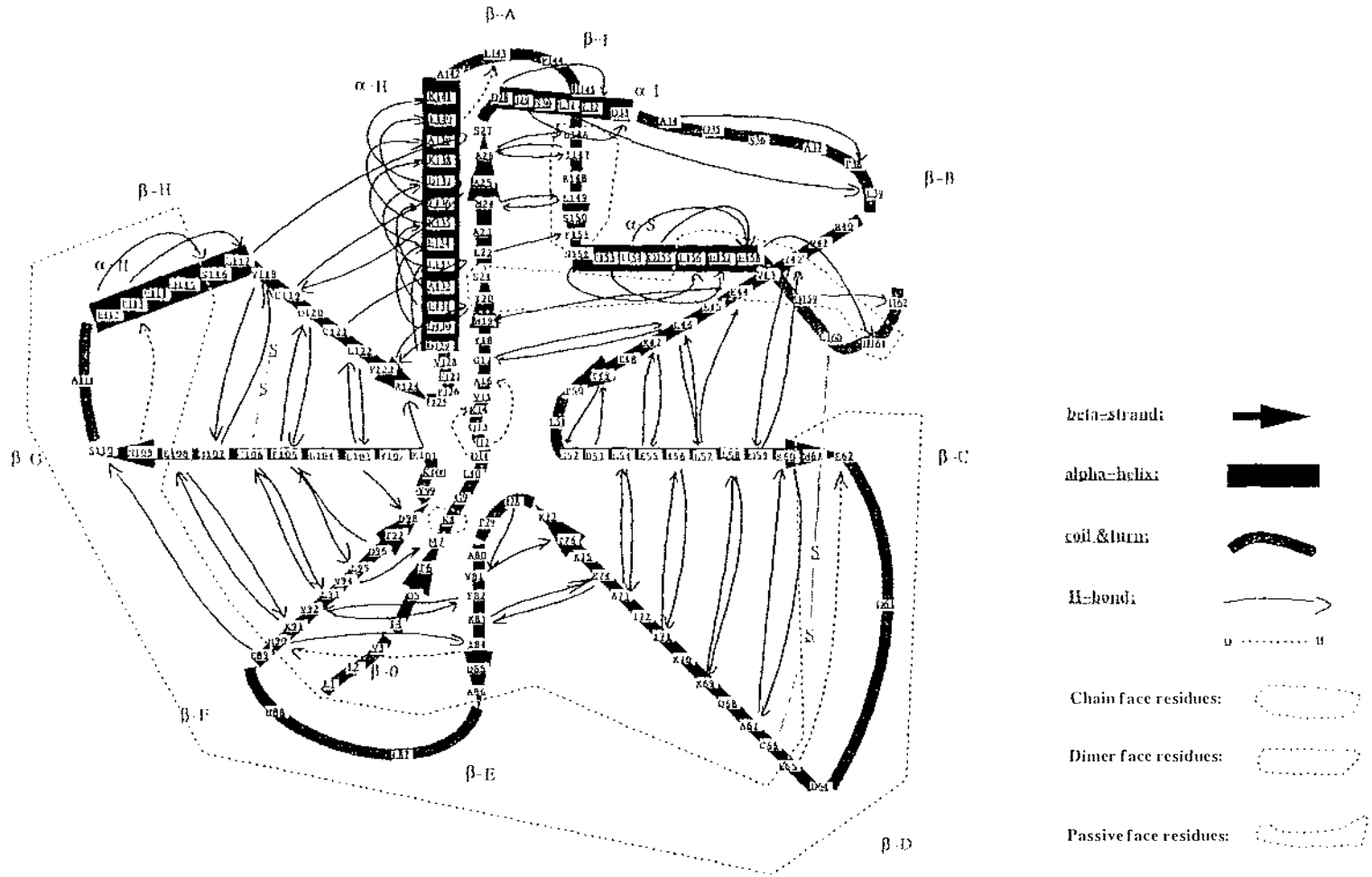


Fig. 2.4.5. Topology expression of the hydrogen bonds in BLG A at pH 7.1, redrawn from Qin *et al.* (1998).

2.4.1. ENVIRONMENTS OF AROMATIC SIDE CHAINS.

Bovine BLG possesses a number of aromatic residues: two tryptophans, four tyrosines and four phenylalanines; it therefore produces both near UV CD and fluorescence emission spectra. Structural change in molecules of BLG can therefore be examined by following changes in these spectra. The aromatic residues of BLG include: Tyr^{20}, Tyr^{42}, Tyr^{99}, Tyr^{102}, Phe^{82}, Phe^{105}, Phe^{136}, Phe^{151}, Trp^{19}, Trp^{61}, and also His^{146} and His^{161} (Fig. 2.2.1). In addition to these residues, Gln^{59} is replaced by His^{59} in bovine BLG C. As will be discussed in later sections of this thesis, the side chains of Trp^{19} and Trp^{61} are particularly important chromophores and fluorophores.

The crystal structure of BLG A/B (Brownlow *et al.*, 1997) shows that the solvent-accessibilities of the aromatic side chains of BLG differ. With respect to tyrosine side chains, that of Tyr^{42} is completely buried, while that of Tyr^{102} is only partially buried. These residues are located at the ends of β-strands B and G respectively. The side chains of Tyr^{20} and Tyr^{99} are solvent-accessible and are located close to the bend of β-strand A and in the FG loop respectively.

The four phenylalanine side chains of BLG are also located in different environments (Brownlow *et al.*, 1997). The residues Phe^{82} and Phe^{105} are located on β-strands E and G respectively, and their side chains point into the hydrophobic interior of the calyx. The side chain of Phe^{136} is sandwiched between the calyx exterior and the α-helix, and is thus also located in a hydrophobic environment. In contrast, Phe^{151} is in the flexible C-terminal loop, and its side chain is solvent-exposed and approximately 8 Å from the dimer interface.

The residue His^{146} forms part of the dimer interface, while His^{161} is completely solvent-exposed. Residue 59 is located on β-strand C and the side chain of His^{59} of BLG C forms a salt bridge with Glu^{44}, and is approximately 5 Å from the disulphide bond Cys^{66}-Cys^{160} (Bewley *et al.*, 1998, Section 2.4.4).

In the crystal structure of BLG A/B (Brownlow *et al.*, 1997) the side chain of Trp^{19} is partially solvent-inaccessible, while that of Trp^{61} is solvent-exposed. The side chain of Trp^{19}, located at the bend in β-strand A, faces into the interior of the calyx (Fig. 2.4.6), while that of Trp^{61} is at the end of β-strand C and points out into the solvent (Fig. 2.4.7). Mills (1976) suggested that in native BLG B, Trp^{61} fluorescence is quenched by a disulphide bond. This suggestion is consistent with the crystal

structure of Brownlow *et al.* (1997), in which the side chain of Trp^{61} is approximately 6 Å from the disulphide bond Cys^{66}-Cys^{160}. The suggestion of Mills (1976) is also consistent with the results of Cho *et al.* (1994), which indicate that the replacement of Trp^{19} with Ala^{19}, using site-directed mutagenesis, decreases tryptophan fluorescence intensity by approximately 80 %.

Brownlow *et al.* (1997) have suggested that in wild-type BLG, the fluorescence from Trp^{19} may be quenched by Arg^{124} because the guanido group of this side chain is only 3 - 4 Å from the face of the Trp^{19} indole ring. This suggestion requires further investigation because results obtained in protein unfolding studies, for example Brems *et al.* (1985), indicate that quenching of protein tryptophan fluorescence does not occur in the presence of concentrated guanidine hydrochloride (Gdn-HCl).

2.4.2. CYSTEINE RESIDUES.

The residues Cys^{106} and Cys^{119} are located in β-strands G and H respectively and form a disulphide bridge. Providing the α-helix remains packed against the exterior of the calyx, this disulphide bridge is not solvent-accessible, and is shielded from the thiol of Cys^{121} by the side chains of Phe^{136}, Ala^{139} and Leu^{140}, as indicated in Fig. 2.4.8. As mentioned above, the second disulphide bridge of BLG, Cys^{66}-Cys^{160}, fastens the C-terminal loop to the exterior of the calyx (Brownlow *et al.*, 1997, Fig. 2.4.9). In the crystal structure of Brownlow *et al.* (1997), these two disulphide bridges are quite well defined. The side chain of Cys^{121}, with its free thiol group, is sandwiched between the exterior of the calyx and the α-helix (Fig. 2.4.8), and is therefore completely buried in the structure of native BLG (Brownlow *et al.*, 1997).

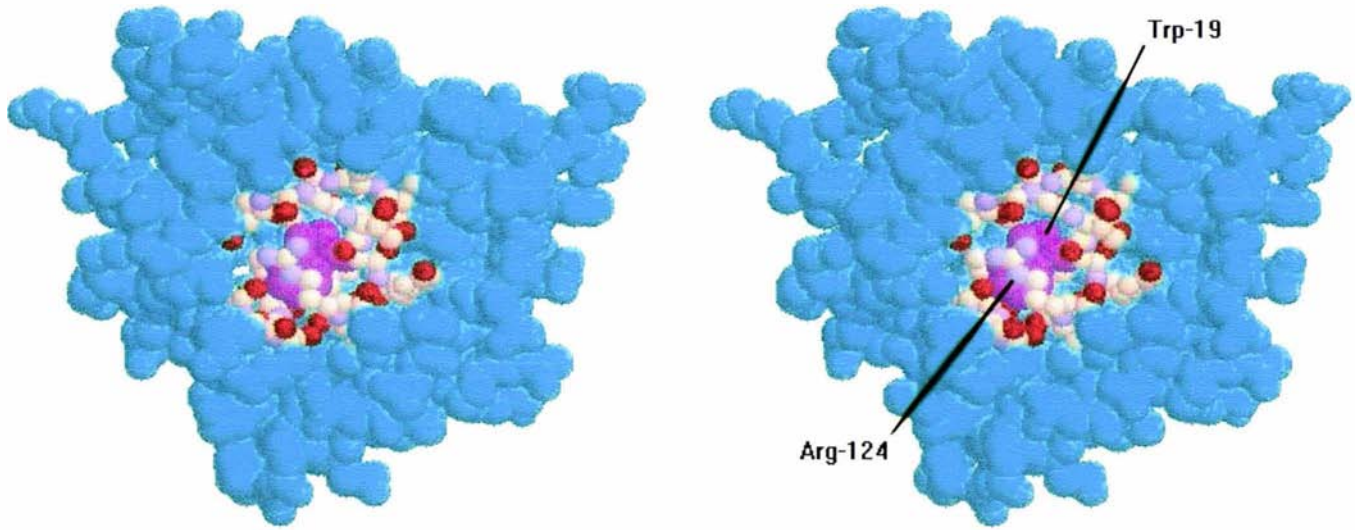


Fig. 2.4.6. The environment of Trp-19 in the structure of BLG A/B of Brownlow et al. (1997). The carbon, oxygen and nitrogen atoms within 6.0 angstroms of the side chain of Trp-19 are coloured grey, red and blue respectively and their sizes have been reduced to avoid obscuring the view of this side chain.

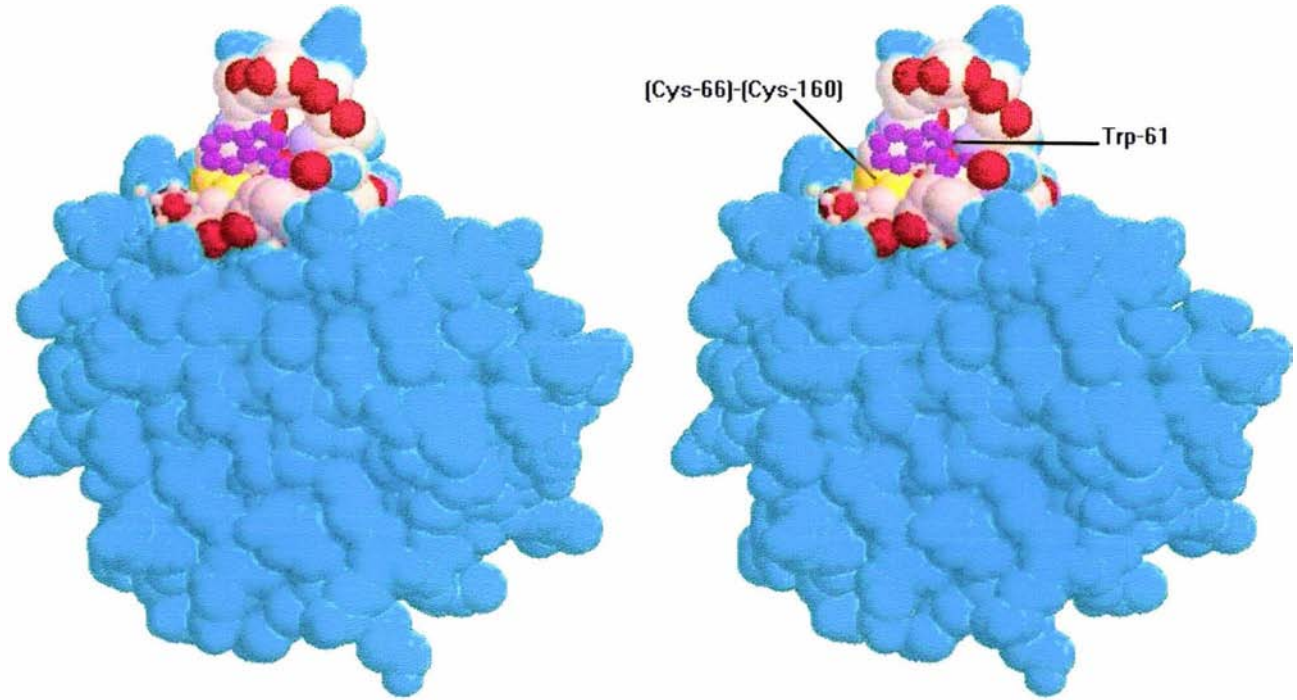
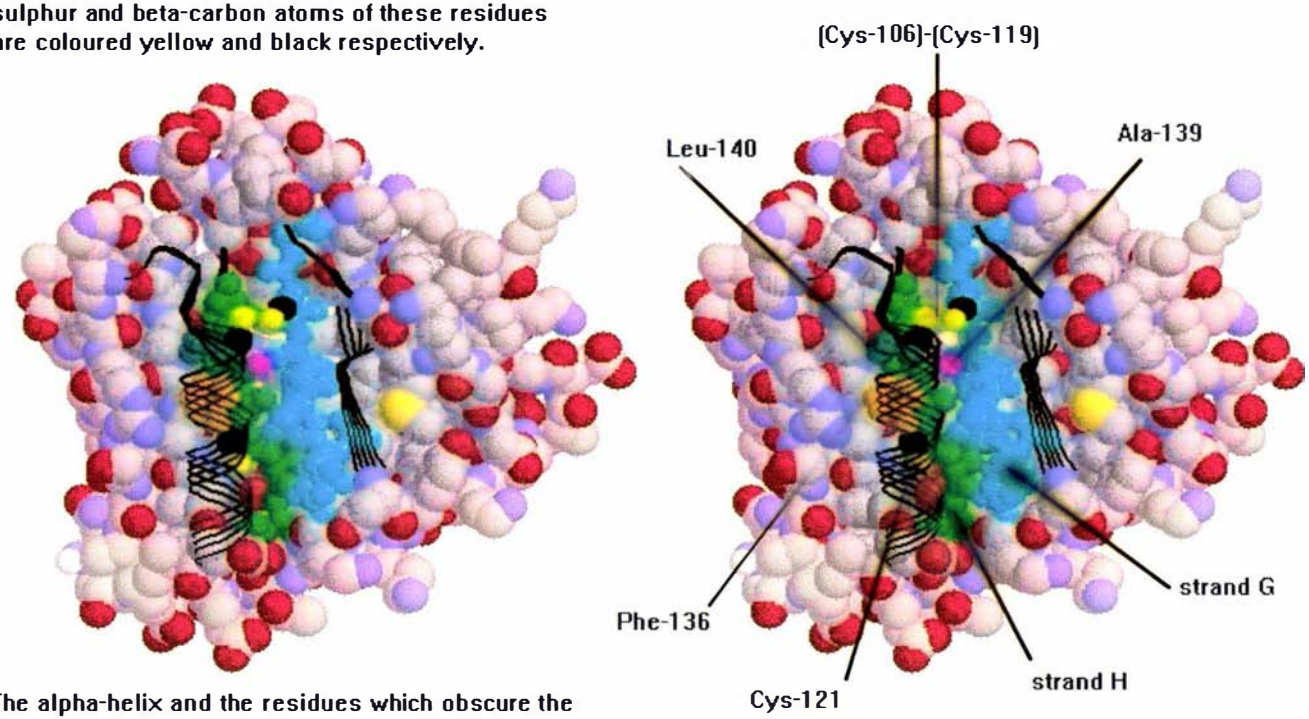


Fig. 2.4.7. The environment of Trp-61 in the structure of BLG A at pH 6.2 of Qin et al. (1998). The carbon, oxygen, nitrogen and sulphur atoms within 6.0 angstroms of the side chain of this residue are coloured grey, red, blue and yellow respectively.

Fig. 2.4.8. The environment of Cys-121 and the disulphide bond (Cys-106)-(Cys-119) in the structure of BLG A/B (Brownlow et al., 1997). The sulphur and beta-carbon atoms of these residues are coloured yellow and black respectively.



The alpha-helix and the residues which obscure the view of the labelled side chains and strands are depicted as ribbons.

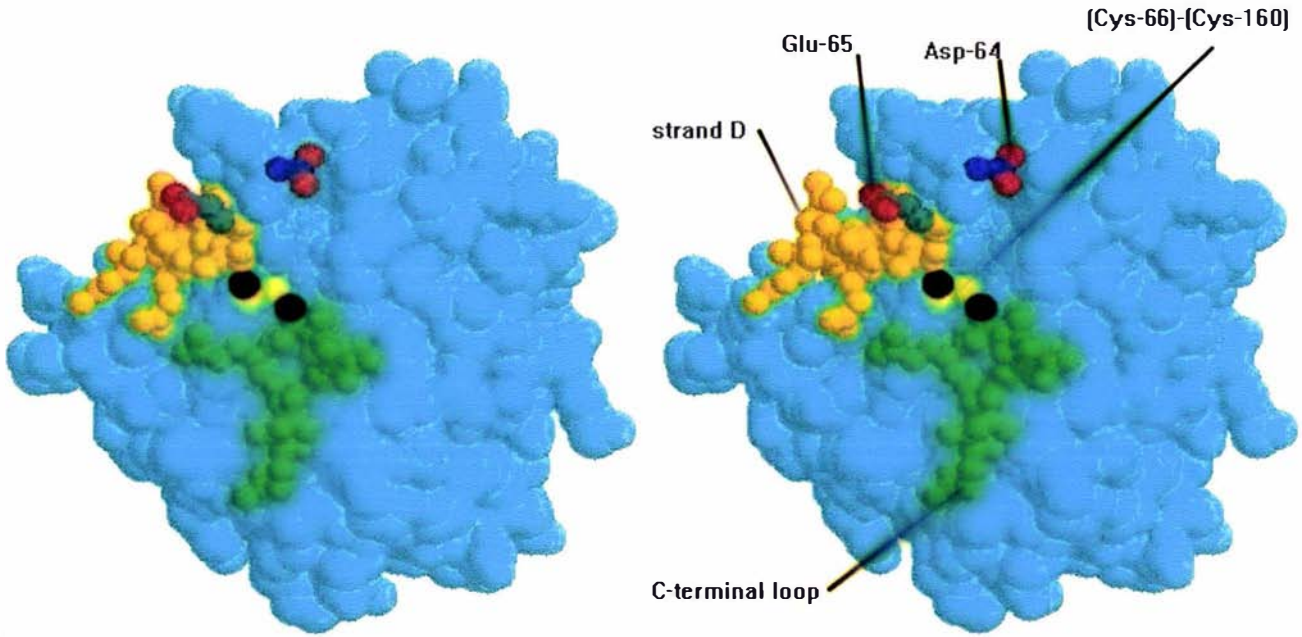


Fig. 2.4.9. The environment of the disulphide bond (Cys-66)-(Cys-160) in the structure of BLG A at pH 6.2 of Qin et al. (1998). The sulphur and beta-carbon atoms of these residues are coloured yellow and black respectively. The oxygen atoms of the side chains of Asp-64 and Glu-65 are coloured red. The C-terminus is located at the right-hand end of the arch of the C-terminal loop. The side chain of Trp-61, which obscures the view of the disulphide bond (Cys-66)-(Cys-160), is depicted in ball and stick format.

2.4.3. DIMER INTERFACE.

In the crystal structure of BLG A/B (Brownlow *et al.*, 1997), there are twelve hydrogen bonds at the BLG dimer interface, four of which are formed between main chain atoms of the two β-I strands of the dimer. The remaining eight are formed between atoms in the side chains of residues in the two AB loops. Those regions where the intermolecular hydrogen bonds form in dimeric BLG are shown in Fig. 2.4.4. The conformations of both polypeptide chains in a dimer pair are equivalent within the limits of the resolution of the structure (Brownlow *et al.*, 1997).

Lontie and Preaux (1966) have suggested that in solution the thiol group of BLG is located close to the dimer interface, and that processes which cause BLG dimers to dissociate also lead to thiol exposure. They found that when native BLG was treated with N-ethylmaleimide (NEM), monomers were unable to re-associate. However, in lattice X crystals, the two Cys^{121} side chains of BLG dimers are 25.6 Å apart (Brownlow *et al.*, 1997), showing that they are not located close to the dimer interface.

2.4.4. GENETIC VARIANTS.

Residue 64 of BLG, which is an aspartate in BLG A and a glycine in BLGs B and C, is located in loop CD (Bewley *et al.*, 1998, Fig. 2.4.10), and is completely solvent-exposed. Although loop CD is poorly defined in the crystal structure (Bewley *et al.*, 1998), it is likely that the side chain of Asp^{64} in BLG A points away from the other side chains in this loop. In BLG A, the carboxyl of Asp^{64} is approximately 11.5 Å from the disulphide bond Cys^{66}-Cys^{160} (Bewley *et al.*, 1998).

Residue 118, which is valine in BLG A and alanine in BLGs B and C, is located at the end of β-strand H, on the opposite side of this β-strand to the disulphide bond Cys^{119}-Cys^{106} and approximately 7.1 Å from it (Brownlow *et al.*, 1997, Fig. 2.4.11). The side chain of Val^{118} is located in a hydrophobic environment. Bewley *et al.* (1998) have reported that when Val^{118} is replaced with Ala^{118} several hydrophobic side chains shift slightly, reducing the size of the space previously occupied by the former.

Residue 59, the position of the BLG B/BLG C substitution, is located in the centre of β-strand C, which is poorly defined in the structures of Bewley *et al.* (1998). This suggests that the structure of BLG in the vicinity of $residue^{59}$ is quite flexible. Therefore, although the side chain of residue 59 appears to be only partially solvent-accessible in the crystal structures of Bewley *et al.* (1998), it may be considerably more solvent-exposed in solution. In BLG B, Gln^{59} forms a hydrogen bond with Glu^{44}, while in BLG C a salt bridge forms between His^{59} and Glu^{44} (Fig. 2.4.12).

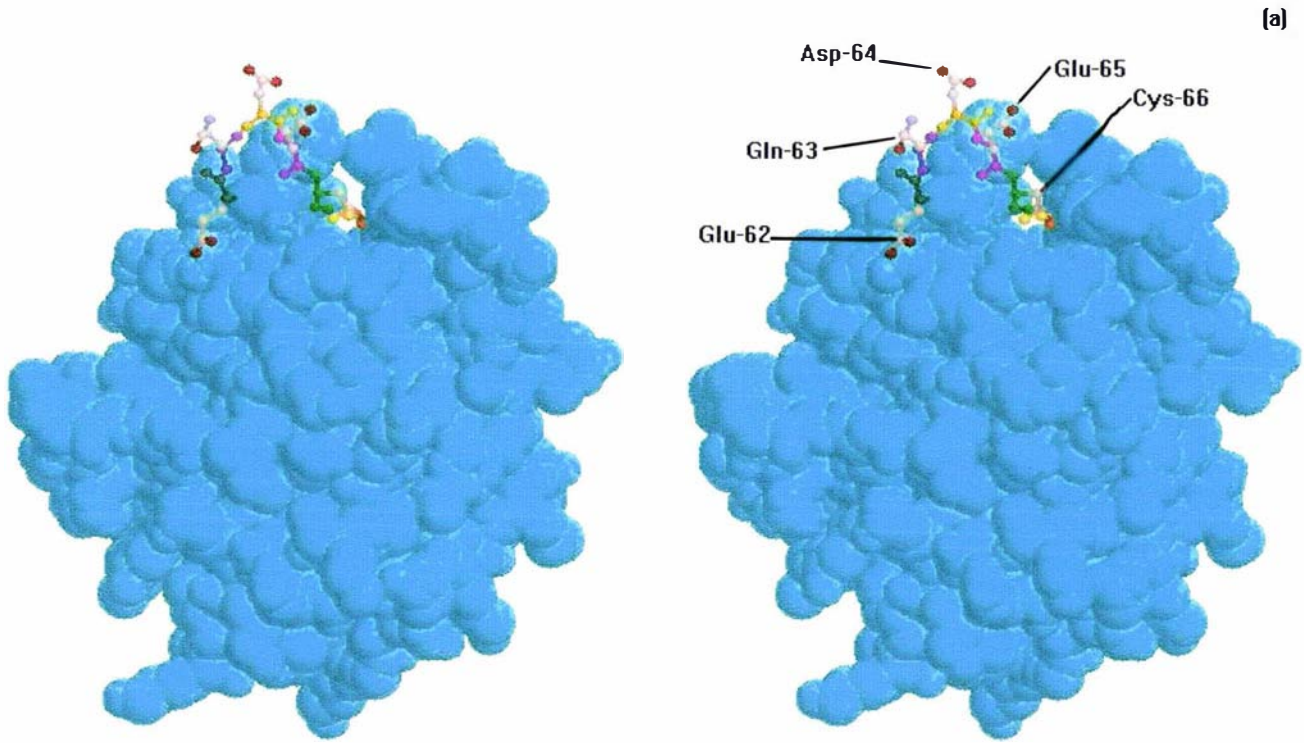
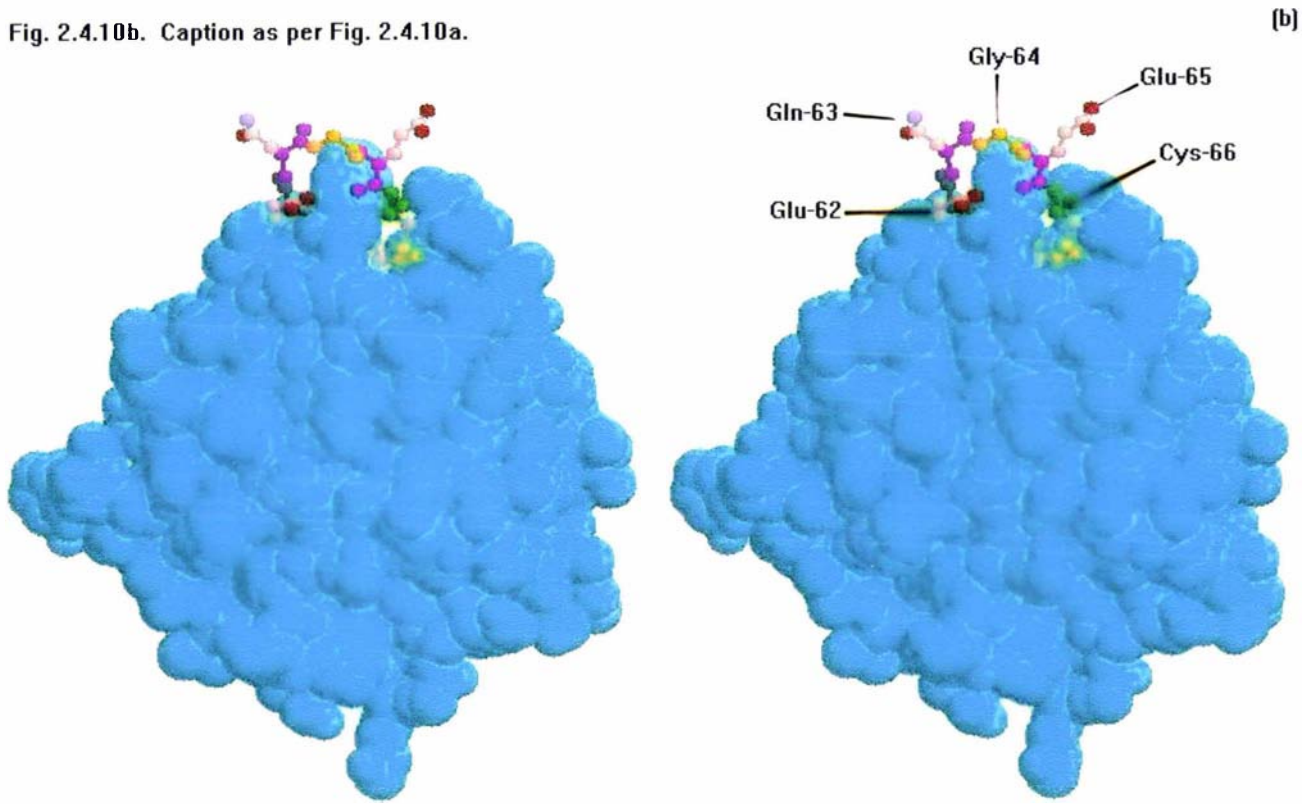


Fig. 2.4.10. The environment of residue-64 in the structures of BLG of Bewley et al. (1998). BLGs A and B are shown in panels a and b respectively. The carbon, oxygen, nitrogen and sulphur atoms of the labelled side chains are coloured grey, red, blue and yellow respectively.

Fig. 2.4.10b. Caption as per Fig. 2.4.10a.



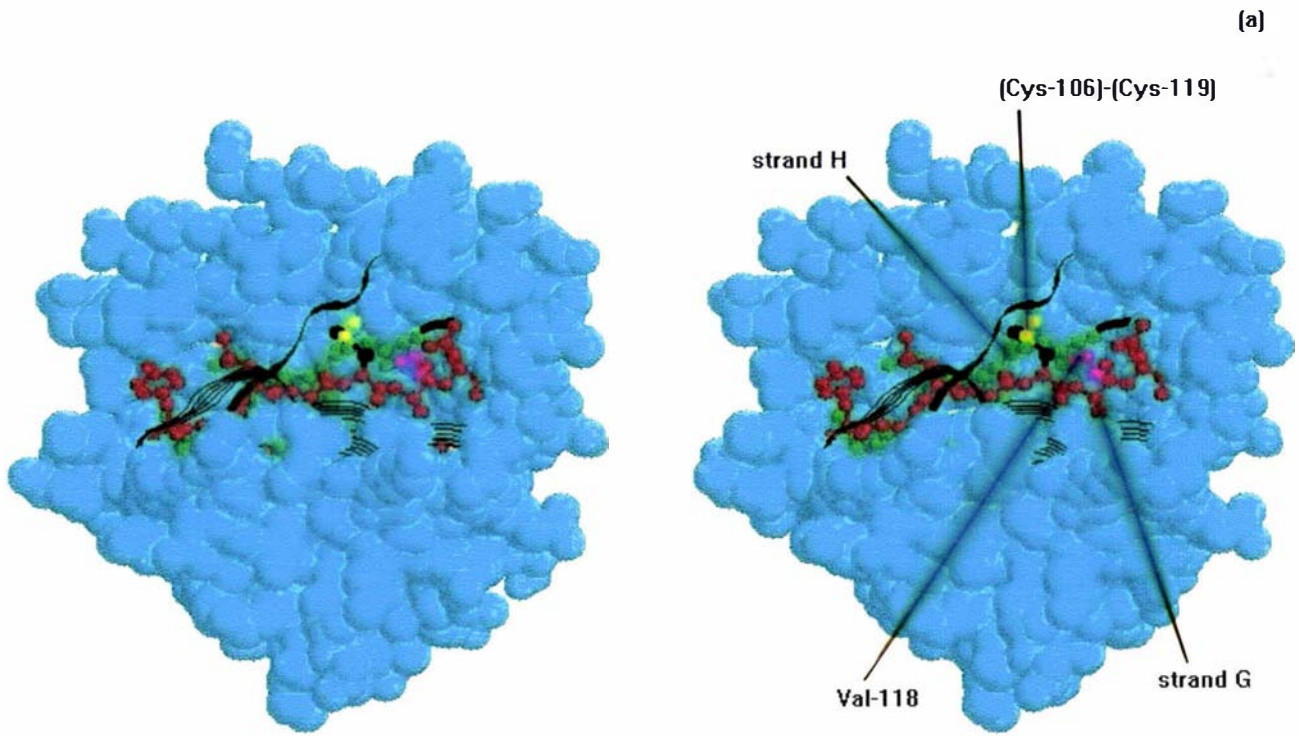
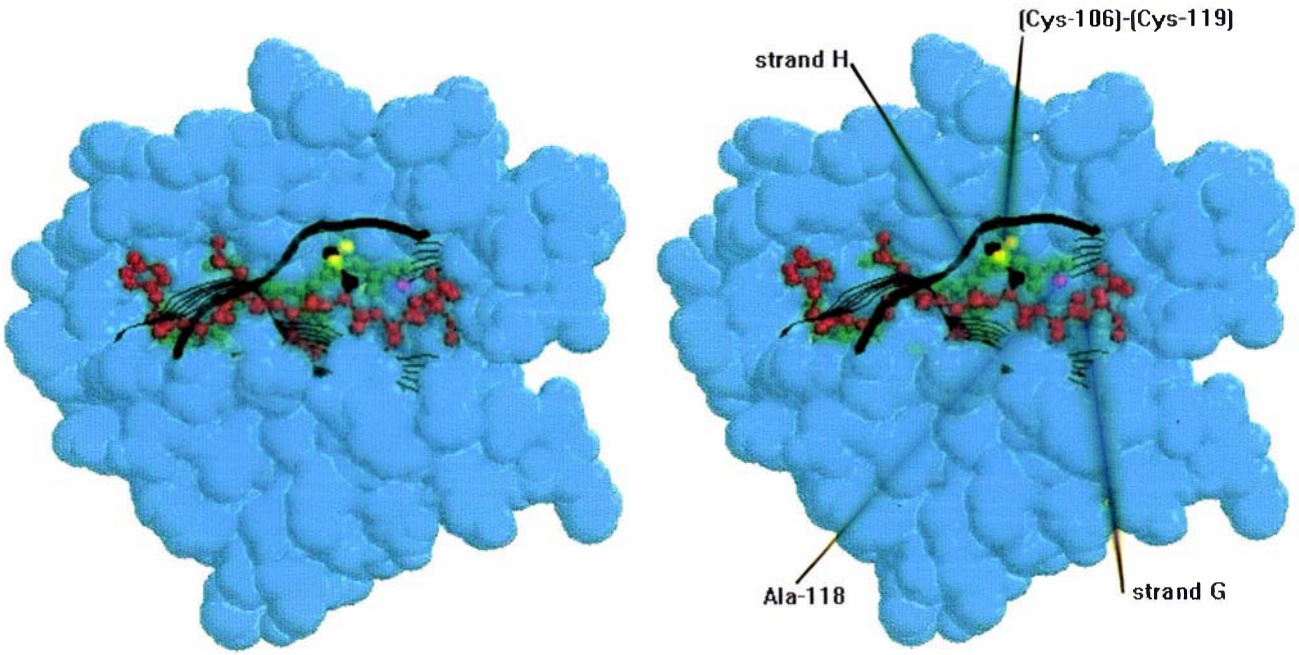


Fig. 2.4.11. The environment of residue-118 in the structures of BLG of Bewley et al. (1998). BLGs A and B are shown in panels a and b respectively. Residues which obscure the view of the labelled side chains and strands are depicted as ribbons.

Fig. 2.4.11b. Caption as per Fig. 2.4.11a.

(b)



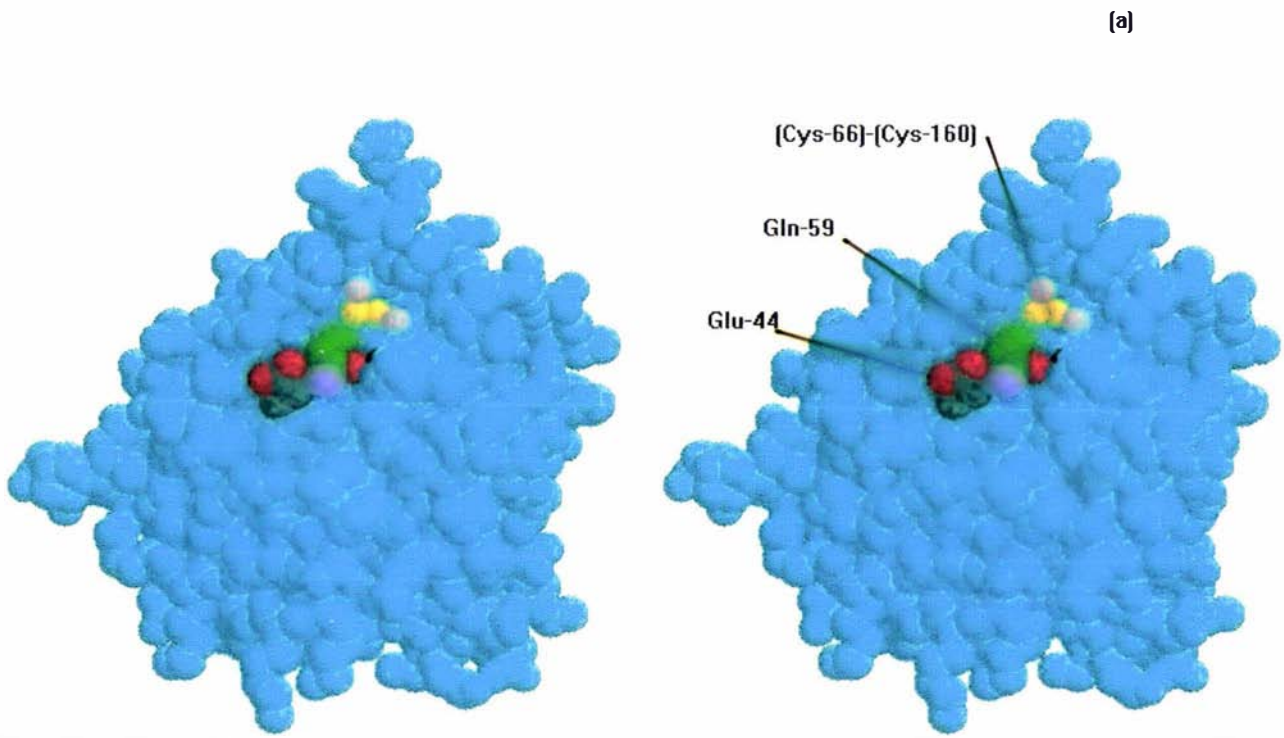
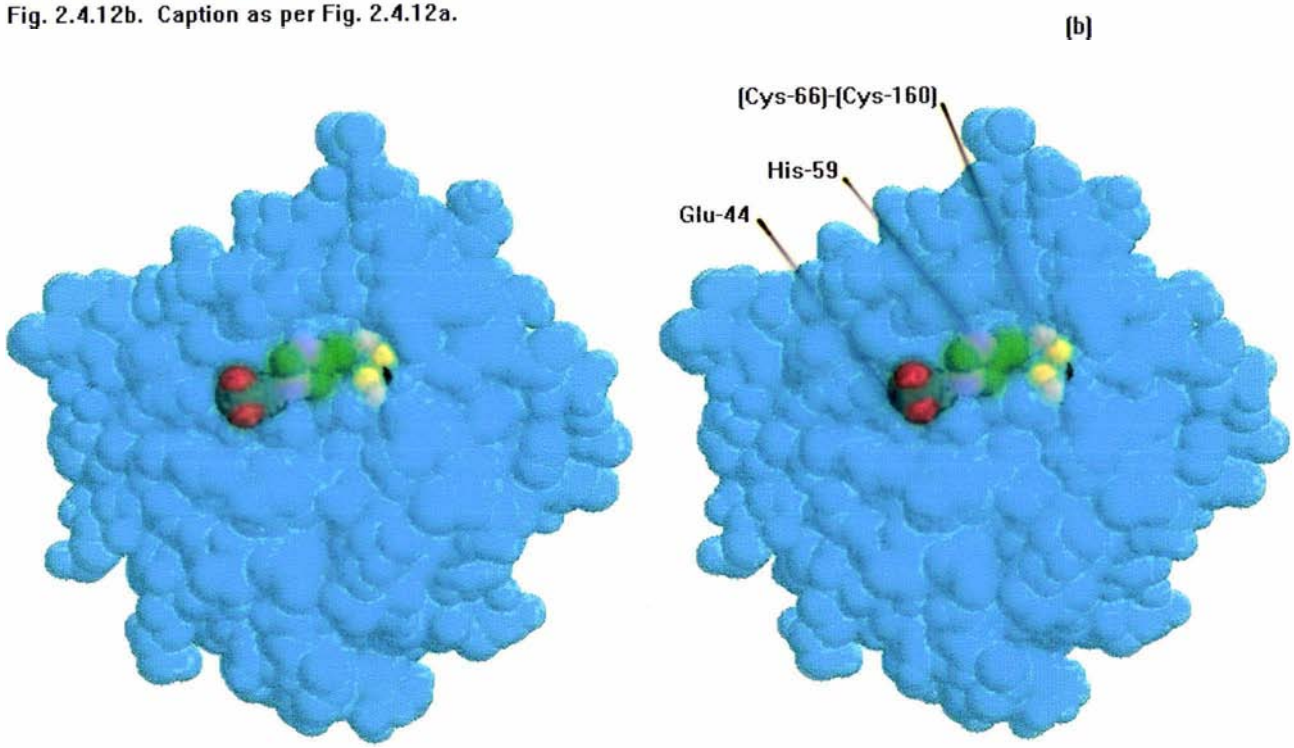


Fig. 2.4.12. The environment of residue-59 in the structures of BLG of Bewley et al. (1998). The side chain of Glu-44 forms a hydrogen bond and a salt bridge with residue-59 in BLGs B (panel a) and C (panel c) respectively. The carbon, oxygen, nitrogen and sulphur atoms of the labelled side chains are coloured grey, red, blue and yellow respectively.

Fig. 2.4.12b. Caption as per Fig. 2.4.12a.



2.4.5. LIGAND BINDING SITE OF β-LACTOGLOBULIN.

As mentioned in Section 2.1, BLG binds retinol and a number of other hydrophobic ligands with high affinity. However, despite several attempts to crystallise BLG with a ligand bound (Sawyer, 1995, Baker, 1996 and Jameson, 1997, personal communications), none of the crystallographic data published to date indicate the exact location of the high affinity ligand binding site. Although ligands may be bound within the calyx, the standard mode of ligand binding amongst the lipocalins (Flower, 1996), neither Papiz *et al.* (1986) nor Monaco *et al.* (1987) observed electron density within the calyx interior. Additionally, the crystal structures of Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998) are those of delipidated BLG and therefore do not indicate how ligands bind to this protein. Nevertheless, although aspects of the structure of Monaco *et al.* (1987) are incorrect, their suggestion that retinol binds between the α-helix and the exterior of the calyx is worthy of consideration. In contrast to this suggestion, the results from the modelling study of Papiz *et al.* (1986) indicate that retinol can be accommodated within the calyx of BLG. When the β-ionone ring of this ligand is inserted into the calyx first, they found that only the tip of the hydroxyl group protrudes into the solvent. This is consistent with the structures of Qin *et al.* (1998).

2.4.6. LIPOCALIN FAMILY AND CALYCIN SUPERFAMILY.

The 3D structure of bovine BLG (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.* 1998) is similar to those of a number of other small proteins which bind low M_r hydrophobic molecules, for example human retinol binding protein (hRBP), which transports retinol (Monaco *et al.*, 1987), murine urinary protein, which is thought to bind pheromones (North, 1989) and odorant binding protein (Monaco *et al.*, 1996). All of these proteins are members of the lipocalin family (Flower *et al.*, 1996). Their 3D structures are generally highly conserved, with an 8-stranded antiparallel β-barrel as the basic fold (Flower *et al.*, 1996). All but one of the β-strands are connected through short loops, while the remaining β-strand is connected to the others via a large loop, which in some lipocalins closes over the mouth of the calyx. The structures of BLG and hRBP are particularly similar (Flower *et al.*, 1996). In contrast to 3D structure, sequence identity amongst the lipocalins is quite low, generally less than 20 % (Cho *et al.*, 1994; Flower *et al.*, 1996).

A structural homology between the lipocalins and two other families of lipid binding proteins, the fatty acid binding proteins (FABPs) and the avidins, has been reported (Flower, 1996). These three protein families, make up the "lipocalycin" superfamily (Flower, 1996). The FABPs fold to form a 10-stranded antiparallel β-clam, a very flattened cone, and contain two α-helices (Flower, 1996). These proteins are usually involved in the intracellular trafficking of low M_r hydrophobic molecules, for example, retinol (Flower *et al.*, 1993). Members of the FABP family include cellular retinol binding protein and P2-myelin protein (Cowan *et al.*, 1993).

The avidins fold to form an 8-stranded antiparallel β-barrel (Salemme *et al.*, 1989; Livnah *et al.*, 1993). However, avidins do not possess an α-helix and the AB loop is much shorter than it is in lipocalins (Flower 1996). Furthermore, the β-barrel of the avidins is more circular in cross-section than those of the lipocalins (Flower, 1996). Members of the avidin family include avidin and streptavidin. These proteins, which are found in the white of bird eggs and the bacterium *Streptomyces avidinii* respectively, bind biotin with extremely high affinity ($K_a = 10^{15}$ M, Salemme *et al.*, 1989; Livnah *et al.*, 1993).

2.5. THE DISSOCIATION OF BOVINE β-LACTOGLOBULIN DIMERS AND CONFORMATIONAL TRANSITIONS.

2.5.1. DIMER DISSOCIATION.

As mentioned in Section 2.1, bovine BLG exists primarily as a dimer under physiological conditions. However, the equilibrium between dimer and monomer is influenced by a number of factors. Firstly, at room temperature dimers do not form below pH 3.5 (Townend and Timasheff, 1960) or above pH 8 (McKenzie and Sawyer, 1967). Secondly, the monomer-dimer equilibrium shifts in the direction of monomers as the temperature of BLG solutions at pH 7.0 is increased, and as the ionic strength of BLG solutions is decreased (Aymard *et al.*, 1996). Thirdly, the proportion of BLG present in solution as dimers increases with increasing protein concentration (Timasheff and Townend, 1962). Fourthly, dimers of BLG A associate more weakly than those of BLG B (Timasheff and Townend, 1961), which in turn associate more weakly than those of BLG C (McKenzie and Sawyer, 1967). Finally, it is known that BLG A, in particular, reversibly octamerises close to 0 °C in the pH range 3.7 to 6.5 (Townend and Timasheff, 1960; Timasheff and Townend, 1961).

Outside the pH range at which dimers form at room temperature, the native structure of BLG is unstable. Mills and Creamer (1975) have suggested that a time-dependent change in the intensity of intrinsic protein fluorescence below pH 3.5 is indicative of conformational change. Groves *et al.* (1951) and Casal *et al.* (1988) found that at pH values 8 and greater BLG denatures at room temperature.

2.5.2. CONFORMATIONAL TRANSITIONS.

During pH increase from 4.0 to 7.8, bovine BLG undergoes two reversible conformational transitions. The first of these occurs between pH 4.0 and pH 6.0 and during pH increase in this range, BLG molecules are converted from the Q to the N conformation (Timasheff *et al.*, 1966). Between pH 6.5 and pH 7.8, the second conformational transition, the "Tanford transition", is observed (Tanford *et al.*, 1959). As pH values are increased from 6.5 to 7.8, BLG molecules are converted from the N to the R form (Tanford and Taggart, 1961).

2.5.2.1. The pH 4 to pH 6 Transition.

The first conformational transition is thought to arise as a consequence of the deprotonation of aqueous carboxyl groups (Timasheff *et al.*, 1966). This transition was identified from an unusually large change in the values for the ORD Moffit-Yang a_o parameter and sedimentation coefficient during pH change in the range 4.0 to 6.0. However, when compared to the Tanford transition, the extent of structural change which occurs during pH change in the range 4.0 to 6.0 is small (Timasheff *et al.*, 1966).

During pH increase from 4.0 to 6.0, BLGs A, B and C titrate differently (Timasheff *et al.*, 1966). The BLG B and BLG C transitions may involve the loss of one proton per monomer, while that for BLG A involves the loss of two protons per monomer. This suggests that Asp^{64} of the A variant participates in the pH 4.0 to pH 6.0 transition (Timasheff *et al.*, 1966). Furthermore, the titration curves for BLG C on the low and high pH sides of this transition resemble those for proteins containing 22 and 20 cationic groups per monomer respectively (McKenzie, 1971). From their results, Timasheff *et al.* (1966) suggested that in BLG C His^{59} may be involved in the pH 4.0 to pH 6.0 transition. This is consistent with the discussion presented by McKenzie (1971). The mechanism for the pH 4.0 to pH 6.0 transition does not, however, appear to have been as thoroughly studied as that of the Tanford transition.

2.5.2.2. The Tanford Transition.

The Tanford transition is characterised by a change in optical rotation ($[\alpha]_D$), from -25° to -48° (Tanford *et al.*, 1959), a change in sedimentation coefficient (expressed as Svedberg units, S), from 3.15×10^{13} S to 2.75×10^{13} S (Tanford *et al.*, 1959) and an increase in thiol group reactivity during pH increase from 6.5 to approximately 7.8. In the case of thiol group reactivity, Dunnill and Green (1965) found that the rate of reaction of the BLG thiol group with *p*-chloromercuribenzoic acid (PCMB) increases with increasing pH in a pH range similar to that in which the Tanford transition is observed. Dunnill and Green (1965) also suggested that the midpoint of the change in thiol group reactivity is at pH 7.4. Additionally, because the pK_a of the thiol group of native BLG is probably greater than 8.5, they concluded that thiol group ionisation is unlikely to be responsible for the magnitude of the observed increase in thiol reactivity. Furthermore, the rate of reaction at pH 4.6 of the thiol of BLG with PCMB determined by Boyer (1954) is slow compared with that of simple thiols with this reagent (Dunnill and Green, 1965).

Additional evidence in support of a conformational transition between pH 6.5 and pH 7.8 comes from titration curves. On the low and high pH sides of the Tanford transition, the pH titration curve for BLG follows that expected for 50.5 and 52.5 carboxyl groups per dimer respectively (Tanford *et al.*, 1959). This indicates that two carboxyl groups in each BLG dimer with unusually high pK_a values (i.e. $pK_a \approx 7.3$) become exposed and are deprotonated in the pH range of the Tanford transition (Tanford *et al.*, 1959). The $\Delta H°$ for carboxyl group exposure is positive, suggesting that on the low pH side of the Tanford transition the two buried carboxyl groups are hydrogen bonded to other groups (Tanford and Taggart, 1961).

In the crystal structures of BLG A of Qin *et al.* (1998), the number of hydrogen bonds formed by Glu^{89}, the conformation of loop EF and the length of β-strand F are all affected by the Tanford transition. They report that at pH 6.2 the carboxyl group of Glu^{89} is protonated and forms a hydrogen bond with the peptide oxygen atom of Ser^{116}. Furthermore, loop EF packs across the wide opening of the calyx at pH 6.2. The hydrogen bond between the carboxyl of Glu^{89} and Ser^{116} is, however, not present at pH 8.2 (Qin *et al.*, 1998). This lengthens β-strand F by one hydrogen bond and shifts loop EF away from the wide opening of the calyx.

The increase in the reactivity of the thiol of BLG which occurs as a consequence of pH increase in the range of the Tanford transition cannot be explained in terms of conformational change, because Qin *et al.* (1998) have found that the environments of the thiol group of Cys^{121} are similar in the crystals formed at pH 6.2 and pH 8.2.

Georges *et al.* (1962), who used light scattering to study dimer dissociation of BLG, have suggested that a Tanford-like thermal transition occurs after the heat-induced dissociation of BLG dimers. They reported that van't Hoff plots for BLG dimer dissociation (i.e. *ln*K for dimer dissociation versus 1/T) exhibited an upwards curvature which remained after the electrostatic contribution to the free energy of dimer

dissociation had been subtracted. Because the slope of a van't Hoff plot is equal to $-\Delta H/R$ for the process in question, the plots for BLG (Georges *et al.*, 1962) suggest that the reaction enthalpy for dimer dissociation shows an unusually strong dependence on temperature. Georges *et al.* (1962) suggested that this curvature reflects a change in the contribution of a conformational change to the overall enthalpy of BLG dimer dissociation. Dupont (1965) has also suggested that a conformational transition is linked to the temperature-dependent dissociation of BLG dimers and that eventually, this conformational change is irreversible.

2.6. STRUCTURAL CHANGE IN MOLECULES OF BOVINE β-LACTOGLOBULIN.

The structure of bovine BLG is non-native at high temperatures (Larsen and Jenness, 1952; Gough and Jenness, 1962; Sawyer *et al.*, 1971; Mills, 1976; Ananthanarayanan *et al.*, 1977; de Wit and Klarenbeek, 1981; Park and Lund, 1984; Kella and Kinsella, 1988a; Griffin *et al.*, 1993), at high pH (Groves *et al.*, 1951; Timasheff *et al.*, 1966; Purcell and Susi, 1984; Casal *et al.*, 1988), in the presence of urea (Pace and Tanford, 1968) and guanidine hydrochloride (Gdn-HCl, Ananthanarayanan *et al.*, 1977; Griffin *et al.*, 1989), in organic solvents (Tanford and De, 1961; Dufour and Haertlè, 1990), and at high pressure (Dufour *et al.*, 1994; Iametti *et al.*, 1997; Futenberger *et al.* 1997). As mentioned in Section 2.2, a thiol group, that of Cys^{121}, becomes solvent-exposed when BLG molecules undergo structural change (Larson and Jenness, 1952; Gough and Jenness, 1962; Watanabe and Klostermeyer, 1976) and exposure of this thiol group has a profound effect on the nature of these structural changes. If structural change in BLG molecules occurs under conditions which allow the thiol group to participate in intramolecular and intermolecular thiol-disulphide interchange reactions, then these structural changes can be irreversible (Larson and Jenness, 1952; Gough and Jenness, 1962; McKenzie and Ralston, 1973). For example, McKenzie and Ralston (1973) reported that thiol-disulphide interchange reactions occurred when BLG was unfolded in 7 M urea at pH 3.5 and pH 5.2. However, if structural change in BLG molecules occurs below pH 3.5, protonated thiol groups are less likely to participate in thiol-disulphide interchange reactions, and structural change is usually reversible. If thiol-disulphide interchange reactions do not occur, then the equilibrium between native and non-native BLG can be established. For example, Ananthanarayanan *et al.* (1977) reported that the extent of thiol-disulphide interchange was minimal at pH values below 2. Similarly, Pace and Tanford (1968) reported that between pH 2.5 and pH 3.5, the unfolding of BLG in urea was reversible. In this section, a review of both reversible and irreversible structural change in BLG molecules induced by urea, Gdn-HCl and heat is presented.

2.6.1. THE REVERSIBLE UNFOLDING OF β-LACTOGLOBULIN.

The urea-induced and heat-induced unfolding of BLG A has been examined in the pH range 2.5 to 3.5 by Pace and Tanford (1968), where this process is reversible (see introduction to BLG structural change, above). Furthermore, below pH 3.5, most BLG molecules will be monomers (Section 2.5.1) in the Q form (Section 2.5.2.1). Thus, by examining structural change at pH values below 3.5, thermodynamic parameters for the unfolding process can be determined with the greatest accuracy. Pace and Tanford (1968) found, from results obtained in a preliminary sedimentation study, that at room temperature at a BLG concentration of 5 mg/mL, dimers could not form in urea solutions at concentrations 3 M and higher. Therefore, because Pace and Tanford (1968) found that the unfolding transition occurs at urea concentrations between 4 M and 5 M, they concluded that the majority of BLG A molecules unfolded from the monomeric form.

Some of the properties of the unfolded state were also characterised by Pace and Tanford (1968). In 8 M urea, the optical rotation of BLG A solutions decreased with increasing temperature in a near-linear manner, which suggests that BLG molecules had assumed a random coil conformation. They justified their interpretation of these results on the basis of those of Kauzmann and Eyring (1941), who reported that for random coil proteins optical rotation decreases linearly with increasing temperature as the freedom of rotation about single bonds becomes greater.

To determine whether stable intermediates were formed along the BLG unfolding pathway, time-dependent changes in optical rotation at room temperature at several different urea concentrations were followed. Pace and Tanford (1968) reported that in all instances, first order kinetic plots with linear slopes were obtained, consistent with an unfolding pathway on which intermediates were not formed. They interpreted this as indicating that the unfolding of BLG is a 2-state process. Upon removal of urea, optical rotation values for BLG samples were restored to those for samples that had not been previously exposed to this denaturant, leading Pace and Tanford (1968) to conclude that unfolding was reversible under their experimental conditions.

The results from a study of the heat-induced unfolding of BLG A in the pH range 2.5 to 3.5 by Pace and Tanford (1968) indicated that there is a large difference between the heat capacities of the folded and the unfolded protein (ΔC_p = 8.79 $kJdeg^{-1}mol^{-1}$). The ΔC_p value for the unfolding of a protein is related to the number of hydrophobic contacts in its interior (Privalov and Gill, 1988), and is not dependent on conditions such as those used to induce unfolding (Pace and Tanford, 1968). The ΔC_p value for the unfolding of BLG A is similar to those for a number of other small single domain

proteins which unfold via a 2-state process; for example, ribonuclease (10.88 kJdeg^{1}mol^{-1}, Brandts, 1965), myoglobin (5.86 kJdeg^{-1}mol^{-1}, Hermans and Acampora, 1967) and lysosyme (9.46 kJdeg^{-1}mol^{-1}, Hawkes *et al.*, 1984).

In a study similar to that of Pace and Tanford (1968), Ananthanarayanan *et al.* (1977) used CD, ORD, viscosity change and optical rotation to follow the Gdn-HCl-induced and heat-induced unfolding of BLG A at pH 2. Unfolding profiles, prepared from data acquired using these techniques, were all sigmoidal in shape and all midpoint Gdn-HCl concentrations coincided (Ananthanarayanan *et al.*, 1977). From these results, Ananthanarayanan *et al.* (1977) suggested that the Gdn-HCl-induced unfolding of BLG A is a 2-state process, in agreement with the results obtained in the urea-induced unfolding study of Pace and Tanford (1968). Similar conclusions were drawn by Griffin *et al.* (1989), from their study of the Gdn-HCl-induced unfolding of BLG A. The optical rotation results of Ananthanarayanan *et al.* (1977) suggested that the midpoint Gdn-HCl concentration for the unfolding of BLG A at pH 2 was 3.4 M. Similarly, Griffin *et al.* (1989) reported that at pH 2.52, the midpoint Gdn-HCl concentration for BLG A is 3.11 M. Ananthanarayanan *et al.* (1977) also studied the thermal unfolding of BLG A and reported that midpoint temperatures determined from CD, ORD, viscosity change and optical rotation data were all approximately 78 °C.

Ananthanarayanan *et al.* (1977) also found that between 15 % and 20 % of the native structure was still intact in molecules of thermally denatured BLG A. Furthermore, Ananthanarayanan *et al.* (1977) observed a second unfolding transition when thermally denatured BLG A was treated with Gdn-HCl, indicating that the residual structure in thermally denatured molecules was lost after addition of Gdn-HCl.

To determine whether or not thermal unfolding was reversible, Ananthanarayanan *et al.* (1977) measured the CD, ORD, optical rotation and viscosity of BLG A solutions prior to temperature increase, and then again upon return to room temperature. Although heating temperatures and times were not specified, they reported that data obtained before and after heat treatment were equivalent, provided that solutions were not held at elevated temperatures for too long. This suggests that high temperatures, *per se*, did not lead to irreversible structural change in BLG molecules.

2.6.2. IRREVERSIBLE STRUCTURAL CHANGE IN MOLECULES OF β-LACTOGLOBULIN.

The effect of heat treatment on the structure of BLG at neutral pH has been studied extensively using several spectroscopic techniques (Sawyer *et al.*, 1971; Mills, 1976; Kella and Kinsella, 1988a; Griffin *et al.*, 1993). In most of these studies, data were acquired under conditions where heat-induced structural change is irreversible, thus preventing the determination of thermodynamic parameters for unfolding. Nevertheless, from the results obtained in these studies, the structural changes in molecules of BLG which occur during or as a consequence of heat treatment at pH values similar to those in milk (i.e. neutral pH) can be described in appreciable detail. Unless stated otherwise, all of the studies discussed below were made using BLG solutions at concentrations 10.00 mg/mL and less.

2.6.2.1. Structural Change in the Vicinity of β-Lactoglobulin Tryptophan Side Chains.

Heat-induced structural change in the vicinity of tryptophan side chains has been extensively studied using the techniques of near UV CD spectroscopy, intrinsic protein fluorescence and UV difference spectroscopy. In the crystal structure of Brownlow *et al.* (1997), Trp^{19} is surrounded by a greater number of side chains than Trp^{61} (Figs 2.4.6 and 2.4.7). When packed against other side chains, aromatic side chains usually produce strong CD bands because they are located in chiral environments (Strickland, 1974). Therefore, it is likely that the contribution from Trp^{19} to the near UV CD spectrum of BLG is greater than that from Trp^{61}. Furthermore, because Trp^{61} is close to the disulphide bond Cys^{66}-Cys^{160}, the extent of quenching of fluorescence from this tryptophan side chain is probably greater than that from Trp^{19} (Section 2.4.1).

2.6.2.1.1. Near UV CD Spectroscopy:

The near UV CD spectra of unheated BLG A at pH 7.0 (Griffin *et al.*, 1993) and BLG A/B at pH 6.7 (Arakawa, 1989) are dominated by two troughs, at 293 nm and 287 nm. This is consistent with the above suggestion that the side chain of Trp^{19} is located in a chiral environment.

From measurements made at elevated temperatures, Arakawa (1989) and Griffin *et al.* (1993) found that the intensities of spectral bands, particularly those at 287 nm and 293 nm, decreased with increasing temperature. In both of these studies, it was suggested that a decrease in the intensities of these spectral bands is indicative of a change in tertiary structure, particularly in the vicinity of tryptophan side chains.

Furthermore, Griffin *et al.* (1993) reported that structural change is only observed at temperatures approximately 50 °C and greater, while Arakawa (1989) only observed structural change at temperatures greater than 59 °C. From their near UV CD results, Griffin *et al.* (1993) concluded that the heat-induced unfolding of BLG is a 2-state process.

In the study of Griffin *et al.* (1993), measurements were made on solutions of BLG A at temperatures up to 90 °C. Because the extent of spectral change which occurred as a consequence of temperature increase from 85 °C to 90 °C was small, they concluded that no further change in tertiary structure occurs when the temperatures of BLG A solutions are raised above 85 °C. Nevertheless, even at temperatures as high as 90 °C, BLGs A (Griffin *et al.*, 1993) and A/B (Arakawa, 1989) produce weak CD bands. This suggests that at least some of the aromatic side chains of BLG are located in semi-chiral environments at approximately neutral pH at temperatures of up to 90 °C.

2.6.2.1.2. Tryptophan Fluorescence:

The fluorescence emission spectrum of BLG B also exhibits a dependence on temperature (Mills, 1976). As the temperatures of BLG B solutions at pH 6.5 were gradually increased from 20 °C to 90 °C, the λ_{max} of the tryptophan emission peak was red shifted from 328 nm to 338 nm. Furthermore, Mills (1976) reported that the width of the emission peak at half height ($W_{1/2}$) increased from 50 nm to 61 nm as the temperatures of BLG solutions were raised from 20 °C to 90 °C.

Mills (1976) also determined the heating temperature above which these spectral changes became irreversible. He found that when BLG B solutions previously heated to temperatures less than 70 °C were cooled to room temperature, values for emission λ_{max} and $W_{1/2}$ were restored to their initial values of 328 nm and 50 nm respectively. In contrast, when BLG B solutions previously heated to temperatures greater than 70 °C were cooled to room temperature, values for emission λ_{max} and $W_{1/2}$ decreased to 333 nm and 54 nm respectively (Mills, 1976). Furthermore, the intensities of tryptophan emissions from samples of BLG B previously heat-treated at temperatures 70 °C and higher were greater than those from unheated BLG B samples.

The results of Mills (1976) can be interpreted in the following manner. The $W_{1/2}$ results suggest that in molecules of unheated BLG B, either both tryptophan side chains are located in similar environments or the emission intensity from one tryptophan side chain is negligible compared to that of the other (Mills, 1976). The high resolution crystal structures (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998) indicate that the latter must be the correct interpretation because the side chain of Trp^{61} is approximately 6 Å from the disulphide bond Cys^{66}-Cys^{160} (Fig. 2.4.9).

The low tryptophan emission λ_{max} value for native BLG B, which indicates that one tryptophan side chain of BLG is located in a moderately hydrophobic environment (Mills, 1976), is also consistent with the crystal structures, which indicate that Trp^{19} is in a more hydrophobic environment than Trp^{61}. Mills (1976) suggested that the heat-induced increase in tryptophan emission λ_{max} is consistent with an increase in the solvent-accessibility of one or both tryptophan side chains. In contrast to some fluorophores, tryptophan emission intensity does not vary appreciably with solvent polarity (Lakowicz, 1983). Therefore, it is unlikely that the increase in tryptophan emission intensity observed by Mills (1976) reflected a change in the solvent-accessibility of Trp^{19}. Increases in tryptophan emission intensity may, however, occur as a consequence of an increase in the distance between the side chain of Trp^{61} and the disulphide bond Cys^{66}-Cys^{160}.

Mills (1976) also reported that the above spectral changes became irreversible at heating temperatures greater than 70 °C and from these results concluded that heat-induced structural change is irreversible above this temperature. Furthermore, he reported that heat treated samples contained a component which migrated more slowly than native BLG B on polyacrylamide gels, and suggested that during heat-treatment at temperatures 80 °C and higher, BLG B becomes irreversibly polymerised.

Results from intrinsic protein fluorescence measurements made by Mills (1976) in the presence of the quencher BrO_3^- offer additional insight into the effects of temperature increase on the environments of BLG B tryptophan side chains. Quenching of tryptophan fluorescence was observed at temperatures 50 °C and greater in the presence of BrO_3^-. Mills (1976) concluded from these results that at least one BLG B tryptophan side chain is solvent-exposed at temperatures 50 °C and greater and this is consistent with the high resolution crystal structures which indicate that the side chain of Trp^{61} is solvent-exposed. The temperature 50 °C is similar to that at which Griffin *et al.* (1993) first observed changes in the near UV CD spectrum of BLG (Section 2.6.2.1.1). Mills (1976) also found that at room temperature tryptophan fluorescence from BLG B solutions that had been previously heat treated at 90 °C for 2 hr could be quenched using BrO_3^-. This suggests that at least one of the tryptophan side chains of BLG B becomes irreversibly solvent-exposed as a consequence of heat treatment under these conditions (Mills, 1976).

In the presence of BrO_3^-, the λ_{max} of tryptophan emissions from BLG B increased from 328 nm at 60 °C to 335 nm at 90 °C (Mills, 1976). However, when emission spectra recorded in the presence of BrO_3^- were subtracted from those recorded in the absence of BrO_3^-, the λ_{max} in the resulting difference spectra were 335 nm at 60 °C and 340 nm at 90 °C. This indicates that although one tryptophan side chain is solvent-exposed at temperatures 50 °C and higher, the other tryptophan side chain does not become solvent-exposed until a temperature higher than approximately 50 °C is attained.

The tryptophan fluorescence results of Mills (1976) may reflect the oxidation of tryptophan side chains by BrO_3^- rather than fluorescence quenching (Creamer, personal communication). However, because neither of these processes can occur until BLG tryptophan side chains have become solvent-exposed, the effect of the oxidation of tryptophan side chains on the interpretation of the results of Mills (1976) should be minimal.

Mills (1976) reported that tryptophan emission intensity per mole of BLG B increases by approximately 30 % with decreasing protein concentration between 0.72 and 0.02 mg/mL, even after correction for the absorption of incident light by BLG (i.e. for an inner filter effect). Therefore, the quantum yield of tryptophan in monomeric BLG B appears to be greater than that in the corresponding dimeric species. This in turn suggests that the extent of tryptophan fluorescence quenching in dimeric BLG B is greater than that in monomeric BLG B and that the environment of at least one tryptophan side chain is altered as a consequence of dimer dissociation (Mills, 1976). Using the K_d for BLG dimer dissociation at pH 6.5 determined by Georges *et al.* (1962), Mills (1976) estimated that the quantum yield of monomeric BLG was approximately twice that of the dimer. In the high resolution crystal structure of BLG A/B (Brownlow *et al.*, 1997), Trp^{61} is close to the dimer interface (i.e. within approximately 18 Å, Fig.2.4.4) and a decrease in the extent of the quenching of the fluorescence from this side chain could therefore occur as a consequence of dimer dissociation.

2.6.2.1.3. UV Difference Spectroscopy:

In the UV difference spectroscopic study of Kella and Kinsella (1988a), it was found that the A_{293} of BLG A/B decreased with increasing temperature. Gueguen *et al.* (1985) found that the values for ΔA in the wavelength ranges 278 nm - 280 nm, 287 nm - 288 nm and 292 nm - 293 nm for pea legumin increased with decreasing pH and increasing ionic strength and interpreted these results in terms of an increase in the polarities of the environments of tryptophan and tyrosine side chains. Thus, the results of Kella and Kinsella (1988a) indicate that the polarities of the environments of Trp^{19} and Trp^{61} of BLG increase with increasing temperature. At pH 7.0, Kella and Kinsella (1988a) found that the decrease in A_{293} occurred between 45 °C and 75 °C, a temperature range similar to that in which changes in the near UV CD spectrum of BLG A at pH 7.0 are observed (Griffin *et al.*, 1993). Kella and Kinsella (1988a) concluded that the heat-induced unfolding of BLG was a 2-state process. The near UV CD results of Griffin *et al.* (1993) support this conclusion (Section 2.6.2.1.1). The similarity in the conclusions of Kella and Kinsella (1988a) and Griffin *et al.* (1993) probably reflects the experimental techniques used by these two groups. Because the side chain of Trp^{61} is solvent-accessible while that of Trp^{19} partially solvent-inaccessible in the structure of native BLG, both near UV CD and UV difference spectroscopic results probably reflect a structural change in the vicinity of Trp^{19}.

2.6.2.2. Secondary Structure Change.

Changes in BLG secondary structure which occur during or as a consequence of heat-treatment have been examined using far UV CD spectroscopy (Sawyer *et al.*, 1971; Lapanje and Poklar, 1989; Griffin *et al.*, 1993). In the spectra of unheated BLG A/B (Lapanje and Poklar, 1989) and BLG A (Griffin *et al.*, 1993), a peak at 194 nm and a trough at 216 nm, each of moderate and similar intensity were observed, in agreement with the results of Townend *et al.* (1967). Sawyer *et al.* (1971) made measurements down to only 200 nm and were therefore unable to observe the peak at 194 nm. Townend *et al.* (1967) estimated from spectral data similar to those obtained in the above three studies that native BLG contains approximately 40 % β-sheet and approximately 10 % α-helix. This estimate is reasonably consistent with the crystal structures of Papiz *et al.* (1986), Monaco *et al.* (1987), Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998), which indicate that BLG contains approximately 50 % β-sheet and approximately 10 % α-helix.

Although the far UV CD spectra of unheated BLGs obtained by Sawyer *et al.* (1971), Lapanje and Poklar (1989) and Griffin *et al.* (1993) are similar, the results obtained at elevated temperatures or at room temperature after heat treatment by these three groups differ.

Sawyer *et al.* (1971) made measurements at room temperature using samples of BLG A/B that had been previously heat-treated at temperatures between 60 °C and 80 °C at pH 6.8. They found that the intensity of ellipticity at 216 nm in the spectra of the heat-treated samples was approximately 1.8 × greater than that in the spectra of the unheated samples. Sawyer *et al.* (1971) concluded that, in dilute solutions, the amount of β-sheet structure in molecules of BLG A/B increases markedly as a consequence of heat treatment.

Lapanje and Poklar (1989) made far UV CD measurements at elevated temperatures. They reported that as the temperatures of solutions of BLG A/B at pH 5.5 and at pH 2.0 were raised from 28 °C to 81 °C, and from 26 °C to 73 °C respectively, the intensity of ellipticity at 216 nm decreased. Their results therefore suggest that the proportion of β-sheet in molecules of BLG A/B decreases with increasing temperature. Lapanje and Poklar (1989) also reported that when solutions of BLG A/B at elevated temperatures at pH 2.0 were cooled to room temperature, values for ellipticity at 216 nm were restored to their initial values, and suggested that at pH 2.0, heat-induced structural change in molecules of BLG A/B is reversible.

Griffin *et al.* (1993) also made far UV CD measurements at elevated temperatures. They reported that as the temperature of solutions of BLG A at pH 7.0 was increased, ellipticity at 207 nm intensified, while that at 216 nm remained approximately constant. This suggests that the amount of β-sheet structure in molecules of BLG A does not change appreciably during temperature increase (Griffin *et al.*, 1993).

2.6.2.3. Thiol Exposure.

Irreversible structural change in molecules of BLG which occurs as a consequence of heat treatment has also been studied by measuring extents of irreversible thiol exposure (Larson and Jenness, 1952; Gough and Jenness, 1962; Watanabe and Klostermeyer, 1976). Larson and Jenness (1952) heated solutions of BLG A/B at pH values between 6.6 and 6.9 at different temperatures for different times in the presence and absence of Gdn-HCl and determined concentrations of solvent-exposed thiol groups. Their results indicate that a maximum of one thiol group per BLG monomer becomes solvent-exposed as a consequence of heat treatment, and that upon exposure, thiol groups are oxidised. Gough and Jenness (1962) found that when solutions of BLG A/B are heat-treated at 73 °C at pH 6.7, the concentration of irreversibly solvent-exposed BLG thiol groups available for quantification decreased with increasing heating time. Their results therefore suggest that the extent of thiol oxidation increases with increasing heating time.

In a study similar to those of Larson and Jenness (1952) and Gough and Jenness (1962), Watanabe and Klostermeyer (1976) also reported that for BLG A over a range of pH values between 3.0 and 9.8, a maximum of one thiol group per monomer became exposed as a consequence of heat treatment. They also found that the concentration of irreversibly solvent-exposed thiol groups decreased with both increasing pH and increasing heat treatment temperature. This suggests that the extent of thiol oxidation increases with both increasing heat treatment time and increasing pH (Watanabe and Klostermeyer, 1976).

The extent of heat-induced disulphide bonding in molecules of BLG appears to increase with increasing heat treatment temperature (Watanabe and Klostermeyer, 1976). However, the magnitude of the decrease in the concentration of solvent-exposed thiols is not proportional to the magnitude of the increase in the extent of disulphide bonding, suggesting that some solvent-exposed BLG thiol groups are oxidised to sulphenic and sulphinic groups during heat treatment (Watanabe and Klostermeyer, 1976). Oxidation of exposed BLG thiols can, however, be minimised if protein samples are heated in an anaerobic environment (Watanabe and Klostermeyer, 1976).

2.6.3. STRUCTURAL CHANGE IN MOLECULES OF β-LACTOGLOBULIN AT HIGH pH.

Structural change in molecules of BLG at high pH has been examined by Groves *et al.* (1951), Townend *et al.* (1967) and Casal *et al.* (1988). Groves *et al.* (1951) reported that the rate of denaturation of BLG A/B at 25 °C increased with increasing pH above 8. These findings are consistent with the results obtained in the far UV CD study of Townend *et al.* (1967), who found that as the pH values of BLG solutions were raised from 5 to 13, the intensity of ellipticity at approximately 201 nm increased much more markedly than at 216 nm. Townend *et al.* (1967) concluded that the amount of random structure in molecules of BLG is greater at high pH than at approximately neutral pH, suggesting that at high pH, the structure of BLG is partially unfolded. They also concluded that the amount of β-structure in molecules of BLG at high pH is similar to that at approximately neutral pH. Casal *et al.* (1988) reported, from infrared spectroscopic results, that the alkaline denaturation of BLG occurs in two stages. During pH increase from 8 to 11, the α-helical content of BLG decreases dramatically and some β-sheets unfold (Casal *et al.*, 1988). As pH values are increased from 11 to 13, the remaining β-sheet unfolds, although not completely. The results of Casal *et al.* (1988) therefore appear inconsistent with those of Townend *et al.* (1967), but this inconsistency may reflect the different experimental techniques used by these two groups. Far UV CD spectroscopy is more suited to following changes in the amount of β-sheet in proteins than infrared spectroscopy.

2.6.4. EFFECT OF pH AND β-LACTOGLOBULIN CONCENTRATION ON THERMOSTABILITY.

The susceptibility of BLG to heat-induced structural change increases with increasing pH between 1.5 and 9.0 (Mills, 1976; de Wit and Klarenbeek, 1981; Park and Lund, 1984; Kella and Kinsella, 1988a; Lapanje and Poklar, 1989; Griffin *et al.*, 1993). Mills (1976) examined the effect of pH on the thermostability of BLG B by following changes in tryptophan fluorescence emission intensity during temperature increase from 20 °C to 90 °C. In thermal unfolding profiles prepared using emission intensity data acquired at pH 5.6, pH 6.4 and pH 7.2, a point of inflexion was observed, the temperature of which decreased with increasing pH (Mills, 1976). From these results, Mills (1976) concluded that the environments of tryptophan side chains become more susceptible to heat-induced structural change as pH values are increased from 5.6 to 7.2.

Kella and Kinsella (1988a) used plots of ΔA_{293} versus temperature, thermal unfolding profiles, to compare the thermostabilities of BLG at different pH values. Values for ΔA_{293} were determined from UV difference spectra, recorded over a range of elevated temperatures using unbuffered 1.0 mg/mL solutions of BLG A/B at pH values between 1.0 and 3.0 and between 6.5 and 7.5. Kella and Kinsella (1988a) did not make measurements between pH 3.0 and pH 6.5, because in this pH range close to 0 °C, BLG can octamerise (Section 2.5.1). They reported that all thermal unfolding profiles were sigmoidal in shape and their midpoint temperatures (T_m) increased with decreasing pH between 7.5 and 1.5. However, the T_m at pH 1.0 was lower than that at pH 1.5. From these results, Kella and Kinsella (1988a) concluded that the thermostability of BLG A/B increases with decreasing pH in the range 7.5 to 1.5. The results of Kella and Kinsella (1988a) are therefore consistent with those of Mills (1976). In a study similar to those of Mills (1976) and Kella and Kinsella (1988a), Griffin *et al.* (1993) found, using near UV CD spectroscopy, that the thermostability of BLG A increases with decreasing pH between 7.5 and 6.5.

The effect of pH on the thermostability of BLG has also been examined using differential scanning calorimetry (DSC). de Wit and Klarenbeek (1981) reported that the denaturation temperature (T_d or T_{max}, the temperature of maximum heat transfer or enthalpy change after extrapolation to zero protein concentration) of BLG A/B increased with decreasing pH between 8.0 and 6.0. Furthermore, the denaturation temperature at pH 3.5 was greater than that at pH 6.0. de Wit and Klarenbeek (1981) found that DSC curves obtained at pH values between 3.5 and 6.0 were less reproducible (and therefore were not presented) than those obtained outside this pH range. The DSC results of de Wit and Klarenbeek (1981) therefore appear consistent with those obtained in the

thermal unfolding studies of Mills (1976), Kella and Kinsella (1988a) and Griffin *et al.* (1993). Park and Lund (1984), also using DSC, reported that the thermostability of BLG A/B between pH 5.0 and 8.0 was greater than that at either pH 4.0 or pH 9.0.

Amongst other DSC studies, Lapanje and Poklar (1989) reported that the denaturation temperature of BLG A/B at pH 2.0 was higher than that at pH 5.5. They concluded that the observed differences in denaturation temperatures at pH 2.0 and pH 5.5 may indicate that BLG is more stable at the lower pH value, in agreement with the results of both de Wit and Klarenbeek (1981) and Kella and Kinsella (1988a).

The variation in the net charge on BLG at different pH values above the isoelectric point (5.1 for BLG A, 5.2 for BLG B and 5.3 for BLG C, Tanford *et al.*, 1959; Basch and Timasheff, 1967; Ghose *et al.*, 1968) may influence thermostability. As pH values are increased from 6.5 to 7.5, the net charge on BLG molecules increases (McKenzie, 1971). This in turn may cause the strength of electrostatic repulsions between the solvent-exposed charged side chains of BLG to increase, leading to a decrease in thermostability.

2.7. THE AGGREGATION AND GELATION OF BOVINE β-LACTOGLOBULIN.

Bovine BLG forms aggregates and gels when heated. Gels may be categorised as a sub-class of aggregates which will form only under specific solution conditions. For example, McSwiney *et al.* (1994b) reported that at 80 °C at pH 7.0, both BLGs A and B form gels at protein concentrations of 50 mg/mL and greater. At lower protein concentrations BLG still forms aggregates but gelation does not occur.

2.7.1. MODELS FOR THE HEAT-INDUCED AGGREGATION OF β-LACTOGLOBULIN.

2.7.1.1. McKenzie (1971).

Amongst the earlier models for the heat-induced aggregation of BLG, that of McKenzie (1971) is the most detailed (Fig. 2.7.1).

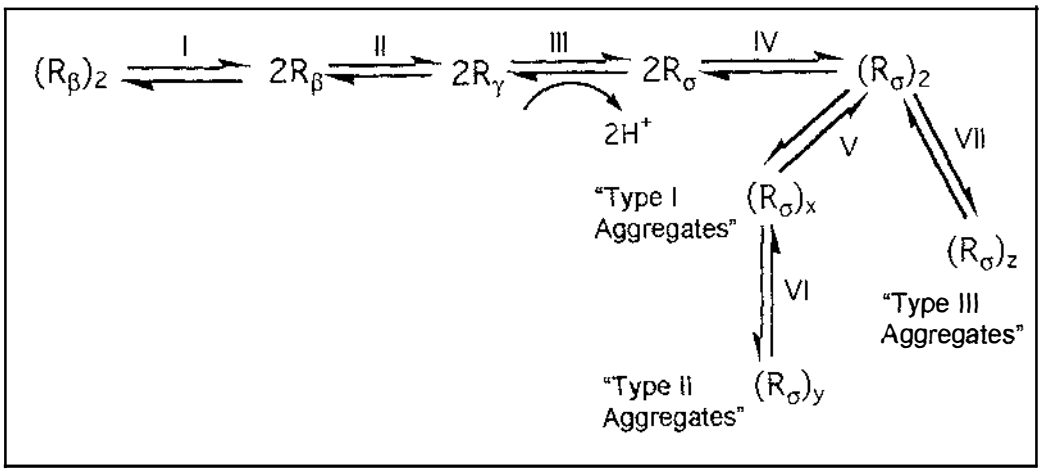


Fig. 2.7.1. The model for the heat-induced aggregation of bovine BLG of McKenzie (1971).

In this model, reaction I depicts dimer dissociation. From results obtained in a light scattering study, Stauff and Ühlein (1955) proposed that dimer dissociation precedes aggregate formation. McKenzie (1971) suggested that after dimer dissociation, BLG monomers are converted, via reaction II, to an ionisable form (R_γ, Fig. 2.7.1) and then ionised via reaction III. The ionised species (R_σ) may then form aggregates (reactions IV-VII). Reactions II and III represent an interpretation of the results of Dupont (1965) and Georges *et al.* (1962), who reported that a conformational change in BLG molecules occurs as a consequence of heat-induced dimer dissociation (Section 2.5.2.2).

Reactions V, VI and VII in this aggregation model depict the formation of different types of BLG aggregate species, as reported by Briggs and Hull (1945) and Sawyer (1968). In these studies (Briggs and Hull, 1945; Sawyer, 1968), low M_r aggregates ("Type I" aggregates formed via reaction V in the model of McKenzie (1971) shown in Fig. 2.7.1) were prepared by heat-treating solutions of BLG A/B at neutral pH at temperatures 97.5 °C and higher. Sawyer (1968) reported that the low M_r aggregates (a sedimentation coefficient of 3.7 S) are disulphide-linked. Briggs and Hull (1945) and Sawyer (1968) prepared high M_r aggregates by heat-treating the BLG solutions described above at temperatures 80 °C and lower. These high M_r aggregates (approximately 29 S) do not appear to be stabilised by intermolecular disulphide links (Sawyer, 1968). In the aggregation model of McKenzie, "Type II" aggregates are formed from "Type I". The formation of low and high M_r aggregates of BLG is discussed in greater detail in Section 2.7.4.

Sawyer (1968) also found that aggregates were formed when solutions of BLG A/B at pH 7.0 were heat-treated at 75 °C in the presence of either NEM or 2-ME. This is depicted as the formation of "Type III" aggregates via reaction VII in the model of McKenzie (1971). One may therefore deduce that "Type III" aggregates are stabilised by non-covalent forces.

The BLG aggregation model of Mulvihill and Donovan (1987) is very similar to that of McKenzie (1971). However, Mulvihill and Donovan (1987) do not include a step in which the ionised BLG species forms a dimer before forming either Type I or Type III aggregates.

2.7.1.2. Griffin *et al.* (1993).

The model of Griffin *et al.* (1993) is shown in Fig. 2.7.2. In agreement with the model of McKenzie (1971), Griffin *et al.* (1993) suggested that during heat treatment BLG dimers dissociate to monomers (reaction I), and are then converted to species 2A', which has a tendency to aggregate (reaction II).

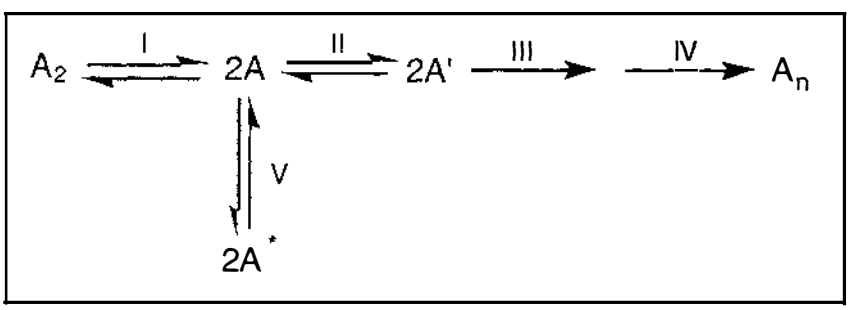


Fig. 2.7.2. The model for the heat-induced aggregation of bovine BLG A of Griffin *et al.* (1993).

Species 2A' in the model of Griffin *et al.* (1993) is therefore analogous to the species $(R\sigma)_2$ from which "Type I and III" aggregates are formed in the BLG aggregation model of McKenzie (1971). However, in contrast to McKenzie (1971), Griffin *et al.* (1993) suggested that only one reaction must occur before BLG monomers are converted to the form which has a tendency to aggregate.

Griffin *et al.* (1993) used near and far UV CD to study the structure of BLG A at elevated temperatures, and found that, although the tertiary structure of BLG was affected by heating, most of the secondary structure was not (Section 2.6.2.2). The far UV CD results therefore suggest that molecules of BLG A do not unfold when heated (Griffin *et al.*, 1993). However, near UV CD results suggest that heating leads to a conformational change which makes molecules of BLG A more susceptible to aggregate formation (Griffin *et al.*, 1993).

Reactions III and IV in the model shown in Fig. 2.7.2 depict the formation of aggregates of BLG A. However, in contrast to the model of McKenzie (1971), Griffin *et al.* (1993) suggest that under particular heat treatment conditions, low M_r polymers formed via reaction V (i.e. those analogous to the "Type I" aggregates described by McKenzie, 1971) do not aggregate further. Reaction V in the model of Griffin *et al.* (1993) will be discussed in greater detail in Section 2.7.4.

Griffin *et al.* (1993) suggested, from photon correlation spectroscopy (PCS) results, that during the initial stages of aggregation BLG molecules associate in a manner which leads to the formation of rod-like particles. They also suggested that as aggregation progresses, these rod-like particles associate to produce aggregate networks and that aggregates of BLG A may contain intermolecular β-sheet.

2.7.2. THE β-LACTOGLOBULIN AGGREGATION MODEL OF McSWINEY *et al.* (1994b) AND THE FORMATION OF NON-COVALENTLY-LINKED AGGREGATES DURING HEAT TREATMENT.

The results from the alkaline native[1] and non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (alkaline native-PAGE and SDS-PAGE) studies of McSwiney *et al.* (1994a) clearly demonstrate that BLG forms both covalently-linked and non-covalently-linked aggregates during heat treatment. When samples of BLG that had been heat-treated for different times at pH 7.0 were run on alkaline native and non-reducing SDS gels, the concentration of monomeric BLG on alkaline native gels was always less than that on non-reducing SDS gels. This suggests that BLG is present on alkaline native gels in a class of aggregates which run as monomers on non-reducing SDS gels (McSwiney *et al.*, 1994a). They concluded that covalently-linked aggregates appear on non-reducing SDS gels and that both covalently-linked and non-covalently-linked aggregates appear on alkaline native gels (Fig. 2.7.3).

McSwiney *et al.* (1994a) also reported that the decrease in the concentration of monomeric BLG with increasing heat treatment temperature was more marked on alkaline native gels than on non-reducing SDS gels (Fig. 2.7.3). From this result, they suggested that the non-covalently-linked aggregates are reaction intermediates, which would eventually be stabilised by covalent cross-links after longer heating times or after heat treatment at higher temperatures.

[1] The pH of these gels is 8.8. Native BLG dimers are therefore not observed because the majority of BLG molecules will be monomeric at pH 8.8.

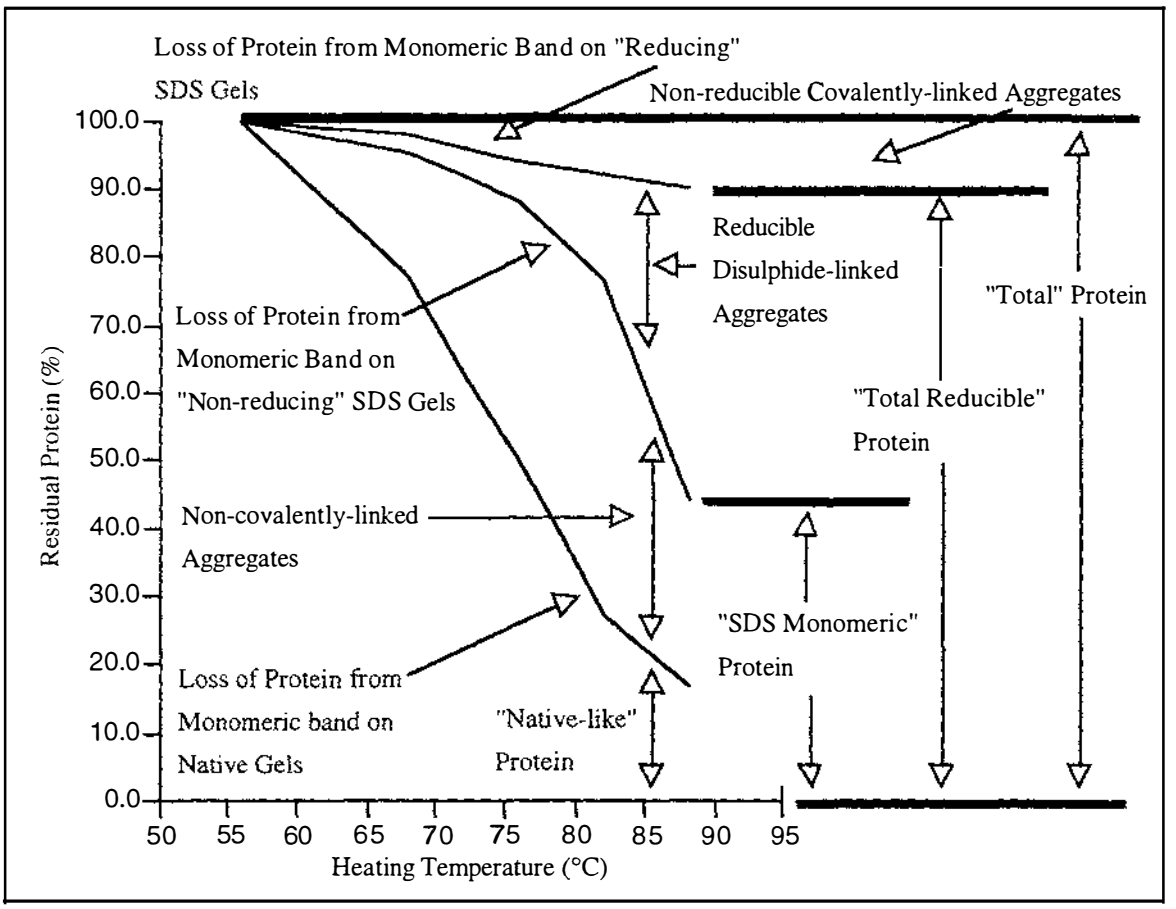


Fig. 2.7.3. The use of SDS-reduced-PAGE, SDS-non-reduced-PAGE and alkaline native-PAGE to follow temperature-dependent changes in the concentration of monomeric BLG and different classes of BLG aggregate species. The lines without arrows depict the temperature-dependent decrease in the percentage of BLG present in the monomer band of an SDS-reduced, SDS-non-reduced or alkaline native gel. The vertical distances between these lines represent the temperature-dependent increase in the concentration of non-covalently-linked aggregates, disulphide-linked aggregates and aggregates stabilised by non-disulphide covalent links. This figure is a modified version of Fig. 1 of Havea *et al.* (1998).

Sawyer (1968) concluded that covalently-linked aggregates of BLG, formed as a consequence of heat treatment at temperatures less than 100 °C, are stabilised almost exclusively by intermolecular disulphide bonds because he found that only non-covalently-linked aggregates were formed during heat treatment in the presence of NEM. Similarly, McSwiney *et al.* (1994a, b) found that when samples of heat-treated BLG known to contain aggregates were run on SDS gels in the presence of 2-ME the only band observed was that of the monomeric species. From these results, they drew a similar conclusion to that of Sawyer (1968).

The results of McSwiney *et al.* (1994b) demonstrate that some experimental techniques may not allow differentiation of aggregates of similar M_r stabilised in different ways. For example, dimers held together by disulphide links, non-covalent links, or by the hydrogen bonding network formed between native monomers may be difficult to separate using gel permeation chromatography at approximately neutral pH. The results obtained by McSwiney *et al.* (1994b) may therefore indicate that the aggregation of BLG is best studied using several techniques and under several sets of conditions, each of which cause a different class of aggregates to dissociate.

Aggregation Model.

From the results obtained in their PAGE studies, McSwiney *et al.* (1994b) have suggested that BLG undergoes different reactions (Fig. 2.7.4) from those in the aggregation models of McKenzie (1971) and Griffin *et al.* (1993), which are discussed in Sections 2.7.1.1 and 2.7.1.2.

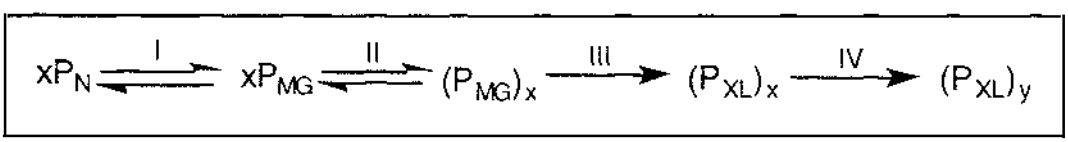


Fig. 2.7.4. The model for the heat-induced aggregation of bovine BLG of McSwiney *et al.* (1994b).

In their model, McSwiney *et al.* (1994b) suggest that native BLG (xP_N) is converted to a molten globule-like species (xP_{MG}) via reaction I, which then associates to form non-covalently-linked aggregates ($(P_{MG})_x$) via reaction II. These aggregates are then stabilised (reaction III) by disulphide links ($(P_{XL})_x$). McSwiney *et al.* (1994b) also reported, from results obtained in a series of rheological measurements, that BLG gel strength continued to increase after all of the monomeric protein had been converted to covalently-linked aggregates. This increase may reflect molecular rearrangements within the BLG gels (McSwiney *et al.* 1994b), which are depicted as reaction IV in Fig. 2.7.4.

2.7.3. THE RELATIONSHIP BETWEEN HEAT-INDUCED STRUCTURAL CHANGE IN β-LACTOGLOBULIN MOLECULES AND THE COMMENCEMENT OF AGGREGATION.

Conditions which lead to structural change in BLG molecules (e.g. elevated temperatures or concentrated urea solutions) usually lead also to aggregate formation. Although there is little experimental evidence which proves that aggregate formation is dependent on structural change, Griffin *et al.* (1993) have tentatively identified a linkage between these two events. They reported that in BLG A samples previously heat-treated at temperatures approximately 70 °C and higher, extents of both structural change in BLG A molecules (far UV CD results) and aggregate formation (PCS results) were more marked than in those previously heat-treated at temperatures lower than approximately 70 °C. From this comparison, they suggested that aggregation is dependent on structural change in BLG molecules.

2.7.4. COMPLEXITY OF THE β-LACTOGLOBULIN AGGREGATION MECHANISM.

The size of BLG aggregates shows some dependence on heat treatment temperature. Briggs and Hull (1945) and Sawyer (1968) reported that low M_r aggregates are formed as a consequence of heat treatment at temperatures 97.5 °C and higher, while high M_r aggregates are formed as a consequence of heat treatment at temperatures 80 °C and lower (Section 2.7.1.1). More recently, evidence for a dependence of aggregate size on heat treatment temperature was found by Griffin *et al.* (1993). At pH 7.0, the size of BLG A aggregates increased with increasing heat treatment temperature between 73 °C and 86 °C, but at temperatures above 86 °C, the opposite was true. A similar trend was observed at pH 7.8, although at this pH value particle size was greatest in samples that had been heat treated at approximately 80 °C. Griffin *et al.* (1993) believe that the observed maximum in the plots of particle size versus heat treatment temperature indicates that at least two chemical processes were operating under the conditions used in their study, one of these was dominant at low temperatures, while the other was dominant at high temperatures. They also suggested that the aggregates formed at low and high temperatures may be analogous to the high and low M_r aggregates identified by Briggs and Hull (1945) and Sawyer (1968).

2.8. COMPARISON OF THE SUSCEPTIBILITIES OF BOVINE β-LACTOGLOBULINS A, B AND C TO HEAT-INDUCED STRUCTURAL CHANGE AND AGGREGATION.

As well as exhibiting differences in electrophoretic mobility and solubility (Section 2.3), BLGs A and B vary slightly in thermostability (Gough and Jenness, 1962; Sawyer *et al.*, 1971; Hillier and Lyster, 1979; Imafidon *et al.*, 1991; Parris *et al.*, 1993; McSwiney *et al.*, 1994b). Furthermore, the results of Sawyer (1968) suggest that the thermostability of BLG C is different to those of BLGs A and B. In the majority of studies published prior to 1993, differences in the thermostabilities of these variants were examined by comparing rates and extents of loss of native BLG and formation of BLG aggregates which occurred during, or as a consequence of, heat treatment at different temperatures. At the commencement of the study discussed in this thesis, a detailed comparison of the thermostabilities of BLGs A, B and C had not been made.

2.8.1. RATES AND EXTENTS OF HEAT-INDUCED STRUCTURAL CHANGE IN MOLECULES OF β-LACTOGLOBULINS A AND B.

Gough and Jenness (1962), Sawyer (1968) and Sawyer *et al.* (1971) all suggested that at concentrations 10 mg/mL and lower, BLG A is more thermostable than BLG B. A similar conclusion was drawn by Hillier and Lyster (1979) for the heat treatment of skim milk and cheese whey (BLG concentrations of approximately 2-3 mg/mL) at temperatures less than 95 °C, although they also reported that BLG A is less thermostable than BLG B at temperatures greater than 95 °C. Gough and Jenness (1962) followed the time-dependent loss of native BLGs A and B during heating at 73 °C by measuring, at room temperature, the concentration of protein soluble at pH 5.0, the thiol availability and the optical rotation of samples previously heat treated for different times. They reported that these time-dependent changes were less marked for BLG A than for BLG B. Hillier and Lyster (1979) followed the time-dependent decrease in the concentration of native BLGs A and B during heat treatment by measuring, at room temperature, the concentration of BLG soluble at pH 4.6 in samples that had been previously heat treated for different times. They reported that this time-dependent decrease was less marked for BLG A than for BLG B. Sawyer *et al.* (1971) used ultracentrifugation to measure extents of the heat-induced increase in the

sedimentation coefficient of BLG species in solutions that had been heat-treated for different temperatures. They found that the extent of the increase in sedimentation coefficient with increasing heat treatment temperature was less marked for BLG A than for BLG B. Sawyer (1968) reported that because solutions of BLG A are less turbid than those of BLG B after heat treatment at 97.5 °C for 10 min, the thermostability of the A variant is greater than that of the B variant.

Imafidon *et al.* (1991) and McSwiney *et al.* (1994b) on the other hand have suggested that at higher concentrations (100 mg/mL) BLG A is less thermostable than BLG B. From PAGE results, McSwiney *et al.* (1994b) found that during heat treatment the rate of decrease in the concentration of monomeric BLG B was slower than that for monomeric BLG A. The DSC results of Imafidon *et al.* (1991) suggest that the denaturation temperature for BLG B is approximately 1 °C higher than that for BLG A. The heat treatment temperatures, pH, buffer and salt concentrations used in the studies discussed in this section are summarised in Table 2.8.1.

2.8.2. COMPARISON OF THE NATURE OF AGGREGATES FORMED BY β-LACTOGLOBULINS A AND B.

The physicochemical properties of aggregates of BLGs A and B differ (Parris *et al.*, 1993; McSwiney *et al.*, 1994b). For example, after heat treatment of sweet whey at temperatures between 65 °C and 85 °C in the presence of SDS, aggregates of BLG B are both smaller and more soluble than those of BLG A (Parris *et al.*, 1993).

The mechanical properties of gels formed from solutions of BLGs A and B also differ. Gels of BLG A are more rigid and viscoelastic than those of BLG B (McSwiney *et al.*, 1994b). Furthermore, McSwiney *et al.* (1994b) found that the stiffness of BLG A gels increases appreciably with increasing protein concentration between 50 mg/mL and 200 mg/mL, whereas that of BLG B gels does not. McSwiney *et al.* (1994b) also reported that the stiffness of BLG B gels is affected appreciably by pH, but that of BLG A is not. Gel stiffness is dependent on the extent of intermolecular disulphide bonding (Mulvihill and Kinsella, 1987).

Table 2.8.1. Summary of Experimental Conditions used in Studies in which the Thermostabilities of β-Lactoglobulins A, B and C were Compared.

Study	[BLG]	Heat Treatment Temperature	pH	[Buffer]	[Salt]
Gough and Jenness (1962)	10.0 mg/mL	73 °C	6.7	phosphate buffer (I = 0.1)	
Sawyer (1968)	4.8 mg/mL	75 °C	7.0	phosphate buffer (I = 0.1)	
Sawyer *et al.* (1971)	5 mg/mL	60 °C - 95 °C	6.6	60 mM cacodylate (I = 0.1)	50 mM NaCl (I = 0.1)
Hillier and Lyster (1979)	skim milk and cheese whey (i.e. ~2-3 mg/mL)	70 °C - 150 °C	~ 6.7	SMUF*	
Imafidon *et al.* (1991)	100 mg/mL	DSC	6.5 and 6.8	water, 20 mM PIPES, phosphate and SMUF*	
Parris *et al.* (1993)	sweet whey (i.e. ~ 2-3 mg/mL)	35 °C, 65 °C, 75 °C and 85 °C	~ 6.7	SMUF*	
McSwiney *et al.* (1994b)	100 mg/mL	70 °C, 75 °C, 80 °C and 85 °C	7.0	20 mM imidazole	100 mM NaCl

*SMUF (Simulated Milk Ultra Filtrate) contains: 146 mM lactose, 11.6 mM phosphate, 9.6 mM citrate, 1.0 mM sulphate, 2.2 mM carbonate, 32.4 mM Cl^-, 3.2 mM Mg^{2+}, 9.0 mM Ca^{2+}, 39.4 mM K^+, 18.3 mM Na^+.

2.9. Research Objectives.

2.9.1. Summary of the Review of Literature.

The structure of BLG in the vicinities of Trp^{19} and Trp^{61} is altered during and as a consequence of heat treatment (Mills, 1976; Kella and Kinsella, 1988a; Griffin *et al.*, 1993). Furthermore, reactions which involve the thiol group of BLG occur at elevated temperatures (Larson and Jenness, 1952; Gough and Jenness, 1962; Watanabe and Klostermeyer, 1976). Because the thiol group of Cys^{121} is solvent-inaccessible in native BLG, these results suggest that structural change in the vicinity of this side chain, which is a considerable distance from both Trp^{19} and Trp^{61} (Fig. 2.4.1 a - c), occurs during heat treatment.

The far UV CD results of Sawyer *et al.* (1971), Lapanje and Poklar (1989) and Griffin *et al.* (1993), describe how the β-sheet structure of BLG is affected by heat treatment. Sawyer *et al.* (1971) reported that the amount of β-sheet increased appreciably as a consequence of heat treatment, whereas Griffin *et al.* (1993) reported that the amount of β-sheet in this protein does not change appreciably during heat treatment. The results of Lapanje and Poklar (1989) suggest that BLG possesses less β-sheet at elevated temperatures than at room temperature, in contrast to the conclusions of both Sawyer *et al.* (1971) and Griffin *et al.* (1993).

McSwiney *et al.* (1994a, b) have shown that both non-covalently-linked and disulphide-linked aggregates of BLG are formed during heat treatment. McSwiney *et al.* (1994b) suggested that the non-covalently-linked aggregates are intermediates on the BLG aggregation pathway because only large disulphide-linked aggregates were left after extensive heating.

The results of Mills (1976), de Wit and Klarenbeek (1981), Park and Lund (1984), Kella and Kinsella (1988a) and Griffin *et al.* (1993) demonstrate that the thermostability of BLG decreases with increasing pH above approximately 6.0.

The results of Gough and Jenness (1962), Sawyer *et al.* (1971), those obtained by Hillier and Lyster (1979) at temperatures less than approximately 95 °C and possibly those of Sawyer (1968) all suggest that BLG A is less sensitive to heat treatment than BLG B. However, the results of McSwiney *et al.* (1994b) and possibly those of Imafidon *et al.* (1991) suggest that the opposite is true. Compared to BLGs A and B, the effect of heat treatment on the structure and aggregation behaviour of BLG C has not been studied as extensively.

The above summary demonstrated that:

1) The structural change in BLG molecules which occurs during and as a consequence of heat treatment required further characterisation.

2) The relationship between non-covalently-linked and disulphide-linked aggregates of BLG required further elucidation to advance understanding of the mechanism by which BLG forms aggregates during heat treatment.

3) A detailed comparison of the thermostabilities and aggregation behaviour of different BLG variants at different pH values had not been made.

4) In each of the studies published prior to 1994 only 1 or 2 techniques were used to measure extents of aggregation and structural change.

2.9.2. Objectives of Research.

1) To determine the events which occur during and as a consequence of heat treatment of BLG.

2) To investigate dissimilarities in how these events occur during and as a consequence of heat treatment of different variants and at different pH values.

2.9.3. Research Plan.

The following plan was adopted to meet the above objectives.

1) To obtain sufficient pure native BLGs A, B and C.

2) To characterise the aggregate species present in previously heat-treated solutions of BLGs A, B and C.

3) To characterise the structural changes in molecules of BLGs A, B and C which occur during heat treatment using a range of spectroscopic techniques.

4) To formulate a mechanism(s) for how aggregates of BLGs A, B and C are formed from the native species, and to integrate this mechanism with existing mechanisms in the literature.

2.9.4. Selection of Experimental Techniques.

Several spectroscopic techniques were used to determine how the entire structure of the BLG molecule is altered during and as a consequence of heat treatment. These were:

1) Far UV CD: provides information on the amounts of secondary structure (e.g. α-helix and β-sheet) and also "random" structure in proteins. "Random" structure is defined as that which is not α-helix, β-sheet or β-turn (Moore and Fasman, 1993).

2) Near UV CD: provides information on the environments of aromatic side chains and disulphide bonds in proteins.

3) Tryptophan Fluorescence: emission λ_{max} data provide information on the solvent-accessibilities of tryptophan side chains in proteins. Emission intensity data provide information on the proximity of tryptophan side chains to quenching groups, both intrinsic and extrinsic.

4) ANS Fluorescence: ANS is a hydrophobic probe which binds to proteins and ANS fluorescence data provide information on the structures of the solvent-accessible hydrophobic regions of a protein.

5) Thiol Availability: as determined by measuring the extent of reaction of BLG thiol groups with the thiol reagent DTNB.

In addition to the above, PAGE techniques similar to those described by McSwiney *et al.* (1994a, b), (Section 2.7.2) as well as 2D- (native- then non-reducing SDS-) PAGE were used to determine the number and type of aggregate species formed as a consequence of heat treatment.

The results obtained using the above techniques should give a detailed picture of the events which occur when BLGs A, B and C are heat-treated. These results should clarify how aggregates of BLGs A, B and C are formed from the corresponding native species.

Chapter 3.

MATERIALS AND GENERAL METHODS.

3.1. MATERIALS AND EQUIPMENT.

3.1.1. MATERIALS.

Glycine, glycerol, tris(hydroxymethyl)aminomethane (Tris), NaCl, K_2HPO_4 (anhydrous), $CaCl_2.2H_2O$, $NaH_2PO_4.H_2O$, $Na_2B_4O_7.10H_2O$, ethylenediaminetetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS), 2-mercaptoethanol (2-ME), ethanol (95 % (w/v)), NaOH and HCl, all analytical reagent grade, were from BDH Chemicals Ltd (Palmerston North, New Zealand). Trichloroacetic acid (TCA), analytical grade, was purchased from May and Baker Ltd (Dagenham, UK). Glacial acetic acid, industrial grade, was obtained from BP Chemicals. Isopropanol, industrial grade, was supplied by Shell Chemicals. Dry nitrogen, which was oxygen-free, was from BOC Gases (NZ) Ltd. (Palmerston North, New Zealand). Acrylamide, N,N'-methylene-bis-acrylamide, bromophenol blue, Coomassie Brilliant Blue R, ammonium persulphate, N,N,N',N'-tetramethylethylenediamine (TEMED), all of "Electrophoresis purity", were obtained from Bio-Rad Laboratories (Hercules, CA 94547, USA). Bovine BLG (3× crystallised, L 0130, lot 51H7210), reduced glutathione (G 6013, lot 35H00105), piperazine, dithioerythritol, N-acetyltryptophanamide (NATA), (+)-10-camphorsulphonic acid (CSA) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO 63178, USA). 5-(octyldithio)-2-nitrobenzoic acid (ODNB), (ammonium salt), was obtained from Fluka (Fluka Chemie AG, CH-9471 Buchs, Switzerland). 1-Anilinonaphthalene-8-sulphonate (ANS, "High purity"), was purchased from Molecular Probes Inc. (Eugene, OR 97402, USA). Artesian bore water was purified by reverse osmosis followed by carbon treatment and deionisation using Milli-Q apparatus (Millipore Corp., Bedford, MA 01730, USA). All buffers and solutions were prepared using this water.

3.1.2. EQUIPMENT.

3.1.2.1. Waterbath.

A Neslab model RTE-100 thermostatted waterbath (Neslab Instruments Inc., Newington, NH 03801, USA) was used to maintain the temperature of cell contents while spectroscopic measurements were made. The bath had a capacity of 5 L, and pumped water through approximately 1 m long inlet and outlet tubes at a flow rate of approximately 10 L/min. Inlet and outlet tubes were encased in 1 cm thick sponge insulation. These features helped to minimise the amount of heat dissipated as water flowed from the bath to cell holders. The bath also possessed a refrigeration unit, thus allowing precise temperature control below ambient temperature, as well as above. In addition, all samples of heat-treated BLG were prepared using this waterbath.

3.1.2.2. FPLC.

Crude preparations of BLGs A, B and C were purified further by size-exclusion chromatography using a Pharmacia FPLC system which consisted of the following components: two model P-500 pumps, a model P-1 pump, a model LCC-500 Plus controller, a model VWM 2141 detector, a model REC 102 multi-channel chart recorder, a model Frac-200 fraction collector and a column of Superdex-75 (50 × 600 mm, 1178 mL volume, 13 μm average bead size, preparation grade). This column was water-jacketed and separations were made at 4 °C. The FPLC system, controlled from the LCC-500 Plus controller, was completely automated.

The absorbance of the eluate was monitored at 280 nm and recorded using the chart recorder for the duration of each run. On another channel of the chart recorder the times where the fraction collector changed tubes were recorded. Thus, the fractions from a particular run which contained BLG could be identified from chart recordings.

3.1.2.3. HPLC.

The purity of BLG in some preparations were determined using analytical ion-exchange chromatography. Proteins were separated on a 1 mL Resource Q ion-exchange column (Mono Q medium, 6.4 × 30 mm, 15 μm average bead size, Pharmacia LKB Biotechnology, S-751 82, Uppsala, Sweden) using a Waters HPLC system consisting of the following components: two model 6000A pumps, a WISP 7108 automatic injector, and a model 490 absorbance detector. The apparatus, controlled from an IBM-compatible 486 personal computer via the Millennium 2010 data acquisition and manipulation system, was completely automated. Proteins were eluted from the ion-exchange column using a salt gradient, created using two pumps, A and B,

which controlled the flow of salt-free buffer and salt-containing buffer respectively. Before these solutions reached the column, they were mixed thoroughly in a mixing chamber. Chromatographic data, initially displayed on a CRT monitor as plots of A_{205} versus time, were stored as computer files.

3.1.2.4. Spectroscopy Cells.

a) Near UV CD: A stoppered 10 mm path length cylindrical quartz cell (Starna, Hainault, Essex, UK) was used for all near UV CD measurements at room temperature.
A stoppered water-jacketed 10 mm path length cylindrical quartz cell (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan) was used for all CD measurements at elevated temperatures.
b) Far UV CD: Either a stoppered 1 mm or a stoppered 0.5 mm path length cylindrical quartz cell (Starna), neither of which were water-jacketed, was used for all far UV CD measurements.
c) Fluorimetry: All fluorimetric measurements were made using stoppered square 4 mL quartz cells (W × D × H = 10 mm × 10 mm × 40 mm, Starna) which had three polished sides.
d) UV/Visible Spectroscopy: All spectrophotometric measurements were made using unstoppered square 4 mL quartz cells (W × D × H = 10 mm × 10 mm × 40 mm, Starna) which had two polished sides .

3.1.2.5. Spectrophotometer.

All spectrophotometric measurements were made using a Shimadzu model UV-260 double-beam spectrophotometer (Shimadzu, 1-Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604, Japan). The spectrophotometer was computer controlled and all measured absorbance values and spectra were displayed on a CRT monitor. Instrumental parameters and absorbance results were recorded on a plotter.

The spectrophotometer was housed in a room maintained at 20 °C and cell contents were left to equilibrate to this temperature by placing cells on a bench in this room for 15 min.

β-Lactoglobulin thiol availability at elevated temperatures was also studied using the Shimadzu spectrophotometer. However, in these studies cell contents were heated using a temperature controlled cell holder which was connected to the Neslab RTE-100 waterbath. The cell holder had 4 sample cell slots, but only the one closest to where water entered the cell holder was used.

3.1.2.6. Spectropolarimeter.

CD measurements were made using a Jasco model J-720 spectropolarimeter. This instrument was also housed in the room maintained at 20 °C, and was controlled from an IBM-compatible 486 personal computer via software purchased from Jasco. Spectra were initially displayed on a CRT monitor and then stored as computer files. Most spectropolarimetric measurements were also made at 20 °C and cell contents were equilibrated to this temperature as described in Section 3.1.2.5.

A very intense light source (450 W xenon lamp), capable of emitting radiation from 800 nm to 170 nm was used for the acquisition of CD data. However, in the presence of strong UV light, oxygen is converted to ozone. The formation of ozone was a concern because many of the mirrors in the spectropolarimeter were made of polished aluminium, which is particularly susceptible to oxidation by ozone. Therefore, prior to lamp ignition, the interior of the spectropolarimeter was flushed for 6 min using a 9 L/min flow of dry oxygen-free nitrogen. Vents directed the flow of gas onto the aluminium mirrors. Once the lamp had been started, the flow rate of dry oxygen-free nitrogen was decreased to 3 L/min. This flow rate was maintained until after the instrument had been switched off. In addition, the lamp was cooled by a 2 L/min flow of cold tap water during operation. The interior of the spectropolarimeter was also flushed for 6 min using a 9 L/min flow of dry oxygen-free nitrogen prior to making each far UV CD measurement. Flushing was necessary because far UV measurements were made below 240 nm, the wavelength at which oxygen starts to absorb UV light. The spectrum of each sample was scanned 5 times and these were incorporated into a single scan file. Thus, the final spectrum was the average of several sequential scans.

In addition to controlling data acquisition, the J-270 software was used for data manipulation and correction. For example, "baseline" buffer spectra were routinely subtracted from protein spectra "point-by-point" using the J-720 software. The software was also used to convert units of optical rotation from millidegrees to $\Delta\varepsilon$ or $[\theta]$. Once again, these corrections were made on a complete spectrum "point-by-point". Values for CD or absorbance at a particular wavelength could also be determined using the J-720 software. A vertical line was positioned on the wavelength of interest (x axis) and the CD or absorbance (y axis) was recorded.

Up to eight spectra could be electronically overlaid using the J-720 software. Furthermore, by multiplying selected spectra in overlays by constants, it was possible to examine extents of superposition. These features of the J-720 software were used when comparing the spectra of BLG samples which had been prepared using different purification protocols. CD spectra could be electronically smoothed using the J-720 software. Finally, the J-720 spectropolarimeter could also be used to monitor continuously the CD at a particular wavelength.

The "basic functions" mode of the J-720 software was used for all of the data acquisition and manipulation described above. However, additional modes were available, one of which permitted the automated collection of CD spectra at a series of elevated temperatures. For these measurements, BLG solutions were heated in the water-jacketed cell (Section 3.1.2.4) which was connected to the Neslab waterbath. This waterbath was controlled using the J-720 software via a Neslab M-RS-232 bath/computer interface. However, individual spectra in a particular file could not be corrected separately. Therefore, it was not possible to subtract the phosphate buffer "baseline" spectrum from each protein spectrum in a particular series.

3.1.2.7. Fluorimeter.

Fluorimetric measurements were made using a Perkin-Elmer MPF-2A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) and all fluorimetric data were stored as chart recordings.

The temperature of cell contents was carefully maintained at 20 °C during all fluorimetric measurements. Precise temperature control was needed primarily because the quantum yield of fluorophores is strongly dependent on temperature. Fluorimeter cell contents were maintained at 20 °C by a flow of water at the same temperature through the cell holder of the fluorimeter. Water was both pumped and maintained at 20 °C using the Neslab waterbath. During fluorescence data collection, this waterbath was used to both heat and cool water. Ambient temperatures were usually less than 20 °C at the beginning of data collection periods, but always climbed above this value during the course of measurements. The Neslab waterbath was also used to heat cell contents during elevated temperature fluorescence measurements.

The temperature-controlled cell holder of the fluorimeter could accommodate up to 4 cells, each of which was shifted into the light beam by rotating the cell holder 90°, 180° or 270°. Different cell holder positions were used in different fluorescence data collection protocols. The cell block positions used for each fluorescence study are discussed in the appropriate section.

3.1.2.8. Laser Densitometer.

Polyacrylamide gels were scanned using a Molecular Dynamics Personal Densitometer (Molecular Dynamics, Sunnyvale, CA 94086, USA). The instrument was controlled from an IBM-compatible 386 personal computer via ImageQuant data acquisition and manipulation software. Absorbance from protein bands was measured at 633 nm using a He-Ne laser with a beam diameter of 50 μm. The resolution of the scanned image was 100 μm. The gel mounting tray of the densitometer was retractable, and could therefore be thoroughly cleaned before gels were scanned.

Scanned images were initially displayed on the CRT monitor and then stored as computer files. The concentrations of species in samples were calculated from gel band intensities, which were determined using ImageQuant.

3.1.2.9. Miscellaneous Equipment.

An Amicon stirred cell ultrafiltration system was used to concentrate solutions of BLG. The system consisted of the following components: a YM-10 membrane (10 000 M_r cut-off) and a 400 mL stirred cell (Amicon Inc., Beverly, MA 01915, USA). All protein solutions and buffers were filtered through 0.2 μm pore size Sartorius Minisart Plus syringe filters (Sartorius AG, P O Box 32 43, W-3400 Goettingen, Germany).

Temperatures were measured using a Kane-May model 455-XP chromel/alumel wire thermocouple (Comark, Welwyn, Garden City, Herts, UK). All pH measurements were made using a using a Radiometer model PHM 64 Research pH meter (Radiometer Copenhagen A/S, DK-2400 Copenhagen NV, Denmark) equipped with a Schott-Geräte model N 61 probe (Schott-Geräte GmbH, Postfach 1130, D-6238 Hofheim a.Ts., Germany).

A milk centrifuge (Elecrem Buttermaschinen, HÄKA GmbH, Stutensee, Germany) was used to remove fat from whole milk. A Sorval Model RC2-B refrigerated centrifuge (Dupont, Newtown, CT 16470, USA) was used in all BLG preparations.

All electrophoretic runs were made using the Bio-Rad Mini Protein II system in conjunction with a Bio-Rad model 1000/500 power supply unit (Bio-Rad Laboratories).

3.2. GENERAL METHODS.

3.2.1. ALKALINE NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS.

Alkaline native polyacrylamide gel electrophoresis (alkaline native-PAGE), utilising a discontinuous buffer system, was used for several purposes. Firstly, BLG purities at various stages of preparation were assessed using this technique. Secondly, size-exclusion chromatography fractions were run on native gels to determine which ones contained pure BLG. Thirdly, alkaline native-PAGE was used to study aggregation in samples of heat-treated BLG.

3.2.1.1. Preparation of Polyacrylamide Gels.

The resolving gel solution (15 % (w/v) total acrylamide (T), which contained 97.4 % (w/w) acrylamide and 2.6 % (w/w) of the cross-linking agent N,N'-methylene-bis-acrylamide, i.e. acrylamide: cross-linker ratio 37.5:1) was prepared by mixing 7.5 mL H_2O, 2.5 mL stock alkaline native resolving gel buffer (3.0 M Tris, pH 8.8) and 10 mL stock solution of acrylamide (30 % (w/v) T, acrylamide:cross-linker ratio 37.5:1), all previously warmed to 20 °C, in a Büchner flask. The gel solution was then degassed for 15 min at 20 °C using a water pump. During degassing, the solution was stirred rapidly using a magnetic stirrer. After degassing, 100 µL freshly prepared ammonium persulphate solution (440 mM) and 10 µL TEMED were added and quickly mixed into the gel solution. An aliquot (3.3 mL) was then poured between two glass plates and overlaid with water. Once the gel had set, the water was poured off. Water remaining near the surface of the gel was then blotted by inserting a wedge of filter paper between the two glass plates.

The stacking gel solution was prepared by mixing 6.25 mL H_2O, 2.5 mL stock alkaline native stacking gel buffer (500 mM Tris, pH 6.8) and 1.25 mL stock acrylamide solution (30 % (w/v) T, acrylamide:cross-linker ratio 37.5:1), in a Büchner flask. The gel solution was degassed for 15 min as described above, after which 50 µL freshly prepared ammonium persulphate solution and 10 µL TEMED were added and quickly mixed in. Stacking gel solution was then poured on top of the resolving gel, until it had reached the top of the glass plates. Sample loading wells were created by inserting either a 15-prong or a 10-prong teflon comb into the top portion of the stacking gel immediately after the solution had been poured into the casting apparatus. After the stacking gel solution had been poured, gels were left to set at room temperature for 1 hr and were then transferred to a cold room (4 °C). Gels were cast at least 16 hr before use.

3.2.1.2. Sample Preparation and Electrophoresis Run.

Protein samples were diluted, as required, with native gel electrophoresis sample buffer. This buffer was prepared by mixing 300 mL water, 100 mL stock stacking gel buffer, 40 mL glycerol and 10 mL 0.1 % (w/v) bromophenol blue (1.6 g bromophenol blue dissolved in approximately 7 mL 100 mM NaOH, then diluted to 1.6 L with water), and then adjusting the pH to 6.8.

In preparation for electrophoresis, a pair of gels was fitted to the Mini Protean II assembly. Alkaline native-PAGE electrode buffer (24 mM Tris, 192 mM glycine, pH 8.3) was then used to fill the upper and lower buffer chambers, after which aliquots (10 μL) of the diluted protein solutions were loaded into the sample wells of the gel through this electrode buffer.

Gels were run with the voltage, current and power set at upper limits of 210 V, 70 mA and 3.25 W per gel respectively to minimise the generation of heat during electrophoresis. Electrophoresis runs were stopped approximately 5 min after the bromophenol blue tracking dye had moved out of the bottom of the gel. Gels were then removed from between the glass plates and transferred to clean plastic containers (L × W × D = 14.0 × 9.5 × 5.0 cm).

3.2.1.3. Staining and Destaining.

Each gel was stained with 50 mL Coomassie Brilliant Blue R stain solution (1.0 g Coomassie Brilliant Blue R dissolved in a mixture of 1300 mL water, 500 mL isopropanol and 200 mL glacial acetic acid). Gel containers were rocked to ensure uniform staining. After 1 hr, all but approximately 2.5 mL of the stain was removed and replaced with 100 mL of destaining solution (a mixture of 80 mL water, 10 mL isopropanol and 10 mL glacial acetic acid). The destaining solution was replaced after 1 hr with fresh destaining solution and left to rock for a further 19 hr.

3.2.1.4. Photography.

After destaining, all gels were photographed using a purpose-designed photographic station. This station was housed in a darkened room and consisted of a light box and a Pentax K100 camera. The camera, equipped with green and orange filters and 100 ASA T-Max black and white film, was mounted on a clamp above the upper surface of the light box.

The light box (L × W × H = 270 mm × 220 mm × 105 mm) contained 4 fluorescent tubes and its interior was parabolic in shape and painted white. This helped to reflect light towards the upper surface of the box. The vertical distance between the fluorescent tubes and the upper surface of the box was less than the horizontal distance between tubes. In addition, a slab of semi-transparent white glass was mounted

immediately below the upper surface. Both of these features helped to minimise variation in light intensity at the upper surface, on which gels were placed.

The 100 ASA T-Max film was selected for photographing protein bands on gels stained with Coomassie Brilliant Blue R because it is very sensitive to light in the wavelength range in which this stain absorbs (595 nm, Darbre, 1986). However, this film is also very sensitive to light at other wavelengths. Green and orange filters were therefore used to reduce the intensity of light outside the wavelength range of the Coomassie Brilliant Blue R absorption peak. Gels were photographed at f 8 using a 1/15 shutter speed, which ensured that photographs were not taken in between light pulses from the fluorescent tubes.

3.2.1.5. Laser Densitometry.

Gels were scanned using the Molecular Dynamics Personal Densitometer. Prior to scanning, the upper and lower surfaces of the retractable glass gel mounting tray were thoroughly cleaned using a damp tissue. Gels were then placed on this tray. After removal of air bubbles, a clean glass plate was carefully placed on top of each gel. Once again, care was taken to prevent the entrapment of air bubbles. The tray was then returned to the interior of the densitometer, after which gels were scanned. Band intensities were determined from stored images using the software package ImageQuant as described in Section 3.1.2.8.

3.2.2. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS.

Aggregation in samples of heat-treated BLG was studied using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples, prepared for electrophoresis in the presence (i.e. 2-ME-treated) or the absence (i.e. 2-ME-untreated) of 2-mercaptoethanol (2-ME), were run on 2-ME-free SDS gels using a discontinuous buffer system.

3.2.2.1. Preparation of SDS-polyacrylamide Gels.

The resolving gel solution (15 % (w/v) T, acrylamide:cross-linker ratio 37.5:1) was prepared by first mixing and then degassing 2.02 mL H_2O, 2.5 mL stock SDS resolving gel buffer (1.5 M Tris, pH 8.8), and 5.3 mL stock acrylamide solution (30 % (w/v) T, acrylamide:cross-linker ratio 37.5:1, see Section 3.2.1.1). After degassing, 100 µL stock SDS solution (350 mM), 50 µL freshly prepared ammonium persulphate solution (440 mM) and 5 µL TEMED were quickly mixed into the gel solution. The gel was then poured according to the procedure described in Section 3.2.1.1.

The stacking gel was prepared by mixing and then degassing 6.1 mL H_2O, 2.5 mL stock SDS stacking gel buffer (500 mM Tris, pH 6.8) and 1.3 mL stock acrylamide solution (30 % (w/v) T, acrylamide:cross-linker ratio 37.5:1, see Section 3.2.1.1). To the degassed gel solution, 100 μL stock SDS, 50 μL freshly prepared ammonium persulphate solution and 10 μL TEMED were quickly mixed in. SDS gel preparation was then completed, using degassed SDS stacking gel solution, according to the procedure described in Section 3.2.1.1. The storage protocol described in Section 3.2.1.1 was also used for SDS gels.

3.2.2.2. Sample Preparation.

Samples not requiring disulphide bond reduction (i.e. 2-ME-untreated) were prepared for SDS-PAGE by diluting protein solutions with SDS sample buffer to concentrations equivalent to those used for native-PAGE (Section 3.2.1.2). These SDS-PAGE samples were not heated prior to electrophoresis. Sample buffer for SDS-PAGE was prepared by mixing 400 mL water, 160 mL stock SDS solution (Section 3.2.2.1), 100 mL stock SDS stacking gel buffer, 80 mL glycerol and 20 mL 0.1 % (w/v) bromophenol blue solution (Section 3.2.1.2).

Samples requiring disulphide bond reduction (i.e. 2-ME-treated) were diluted to protein concentrations equivalent to those used for alkaline native-PAGE (Section 3.2.1.2) with SDS sample buffer, treated with 2-ME (20 μL per 1 mL sample), and then heated at 100 °C for 4 min in tightly capped vials. After heat treatment, samples were cooled to room temperature.

3.2.2.3. Electrophoresis Run.

Gels were prepared for electrophoresis using a procedure similar to that described in Section 3.2.1.2. However, SDS-PAGE electrode buffer (24 mM Tris, 192 mM glycine, 4 mM SDS, pH 8.3) was used to fill the upper and lower buffer chambers. SDS gels were then loaded and run in a manner similar to that described in Section 3.2.1.2, except that SDS gel electrophoresis runs were stopped as soon as the tracking dye band had moved out of the bottom of the gel. The staining and destaining procedure used for SDS-PAGE was the same as that used for alkaline native-PAGE (Section 3.2.1.3).

3.2.3. BUFFER SOLUTIONS.

3.2.3.1. Phosphate Buffer (26.1 mM, pH 6.7, pH 7.0 or pH 7.4, with 68.4 mM NaCl).

Sodium dihydrogen phosphate monohydrate (3.6 g) and sodium chloride (4.0 g) were dissolved in approximately 950 mL water. The pH of the solution was then adjusted to 6.7, 7.0 or 7.4, as required, with 2 M NaOH. After pH adjustment, buffers were diluted to 1.0 L with water. For dialysis, 5.0 L volumes were prepared. For thiol availability studies, pH 7.0 buffer was degassed daily, in a Büchner flask, using a water pump.

3.2.3.2. Concentrated Phosphate/Borate Buffer (58.5 mM phosphate, 58.5 mM borate, pH 8.1, with 308 mM NaCl).

Sodium dihydrogen phosphate monohydrate (1.61 g), sodium borate decahydrate (4.46 g) and sodium chloride (3.60 g) were dissolved in approximately 75 mL water. The pH was then adjusted to 8.1 with 2 M NaOH, after which the buffer was diluted to 200 mL with water.

3.2.3.3. Dilute Phosphate/Borate Buffer (13.1 mM phosphate, 13.1 mM borate, pH 8.1, with 68.4 mM NaCl).

Sodium dihydrogen phosphate monohydrate (1.81 g) sodium borate decahydrate (5.0 g) and sodium chloride (4.0 g) were dissolved in approximately 950 mL water. The pH was then adjusted to 8.1, after which the buffer was diluted to 1.0 L with water.

3.2.3.4. Phosphate Buffer (20 mM, pH 6.0, with 30 mM NaCl).

Sodium dihydrogen phosphate monohydrate (13.8 g) and sodium chloride (8.7 g) were dissolved in approximately 4.8 L water. The pH was then adjusted to 6.0 with 2.0 M NaOH, after which the buffer was diluted to 5.0 L with water.

3.2.4. CIRCULAR DICHROISM SPECTROSCOPY.

3.2.4.1. Instrument Calibration Checks.

The wavelength and also CD intensity calibration of the spectropolarimeter were checked periodically. The wavelength calibration of the spectropolarimeter was checked by comparing experimentally determined and standard λ_{max} values of peaks in the absorbance spectra of neodymium glass (Jasco) and benzene vapour. Absorbance spectra, as plots of photomultiplier tube (PMT) voltage versus wavelength, were obtained using the J-720 software. A cell containing benzene vapour was prepared by placing a drop of benzene in the bottom of a 10 mm path length cell, which was then sealed with a teflon bung.

The spectrum of neodymium glass was recorded at a scan speed of 10 nm/min, using a band width of 0.2 nm, a time constant of 0.25 s and a step resolution of 0.05 nm. Measurements were made from 550 nm to 600 nm. The exact position of the absorption peak close to the standard value of 585.9 nm (Jasco J-720 manual) was then determined. The spectrum of benzene vapour was recorded using similar instrument settings. However, measurements were made from 230 nm to 270 nm. The exact position of the absorption peak close to the standard value of 266.7 nm (Creamer, personal communication) was then determined.

The calibration of intensity of $\Delta\varepsilon$ was checked by comparing the experimentally determined value for the ratio $\Delta\varepsilon_{192}$:$\Delta\varepsilon_{290}$ in the spectrum of a freshly prepared solution of (+)-10-camphorsulphonic acid (CSA, approximately 1.0 mg/mL) to that given by Johnson (1990). Exact concentrations (between 1.01 mg/mL and 1.03 mg/mL) were determined specrophotometrically using the standard absorbance value, A_{285} (1.0 mg/mL CSA, 50 mm path) = 0.743 (Johnson, 1990). Measurements were made using a similar procedure to that described in Section 3.2.4.2 below, except that a 10 mm path length cell was used and spectra were recorded from 190 nm to 350 nm.

3.2.4.2. Data Acquisition Protocol: Near UV CD Spectroscopy.

Near UV CD spectra of BLG were recorded at 20 °C using 1.00 mg/mL solutions. Protein samples were diluted to this concentration with the buffer against which the protein had originally been dialysed. Both the protein and the buffer were filtered prior to sample dilution.

All near UV CD measurements were made using the 10 mm path length cell described in Section 3.1.2.4. Prior to filling, the cell was rinsed with 8 volumes of the appropriate filtered buffer, delivered and withdrawn using two clean glass Pasteur pipettes. After the fourth volume had been withdrawn, the first pipette was discarded and rinsing was continued using the second pipette. The cell was then rinsed with approximately 200 µL of the BLG solution of interest, emptied, and then filled to

capacity. The stopper of the cell was also rinsed regularly. After filling, the cell was stoppered and placed in the cell holder of the spectropolarimeter. The spectrum was recorded at a scan speed of 50 nm/min using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. Measurements were made from 250 nm to 350 nm. Each spectrum recorded represented the average of five scans.

Treatment of Data.

The spectrum of each protein sample was manipulated using a two-step procedure with the J-720 software. In step one, the "baseline" spectrum of the appropriate buffer (the average of 5 scans) was subtracted from each protein spectrum. In step two, the units of optical rotation were converted from millidegrees to molar circular dichroism ($\Delta\varepsilon$) using equation 3.2.4.1, where d is the rotation in degrees, MW is the molecular weight of protein and c is the protein concentration in mg/mL.

$$\Delta\varepsilon = 0.0304 \times d \times MW \times c^{-1} \qquad (3.2.4.1)$$ Creamer *et al.* (1981)

The values for $\Delta\varepsilon$ at 293 nm and 270 nm ($\Delta\varepsilon_{293}$ and $\Delta\varepsilon_{270}$ respectively) in manipulated spectra were then determined.

3.2.4.3. Data Acquisition Protocol: Far UV CD Spectroscopy.

Far UV CD measurements were made using either 1.0 mm or 0.5 mm path length cells. Prior to filling, the appropriate cell was rinsed with filtered water, using the procedure described in Section 3.2.4.2. Liquids were removed from these narrow path length cells using a flicking motion. However, the last 10 % of the volume was very difficult to remove. Thus, thorough rinsing was needed before cells were filled with each new protein solution. Cell stoppers were also rinsed regularly.

The BLG samples prepared in Section 3.2.4.2 were used for far UV CD spectroscopy also. However, prior to making far UV CD measurements, these solutions were diluted either 10-fold or 6-fold with filtered water and then placed in 1 mm and 0.5 mm path length cells respectively. After filling, cells were stoppered, placed in the cell holder of the spectropolarimeter, and the sample compartment of the instrument was then flushed with dry oxygen-free nitrogen as described in Section 3.1.2.6. Measurements were then made using instrument settings similar to those described in Section 3.2.4.2, but at a scan speed of 20 nm/min from 185 nm to 250 nm. Each protein spectrum recorded represented the average of 5 scans. For far UV CD, the "baseline" spectrum recorded was that of water, and represented the average of 15 scans.

Treatment of Data.

The spectrum of each protein sample was manipulated with the J-720 software using a three-step procedure similar to that described in Section 3.2.4.2 . In step one, the "baseline" spectrum of water was subtracted from each protein spectrum. Secondly, the units for optical rotation were converted from millidegrees to molar circular dichroism ($\Delta\varepsilon$) using equation 3.2.4.1, and then to molar ellipticity [θ] by multiplying by either 33 000 (data collected using the 1.0 mm path length cell) or 66 000 (data collected using the 0.5 mm path length cell), (Strickland, 1974). Finally, a low pass filter, which removed high frequency spectral components, was used to decrease the amount of noise in each spectrum (Jasco J-720 manual). Values for [θ] at 216 nm and 205 nm ($[\theta]_{216}$ and $[\theta]_{205}$ respectively) in manipulated spectra were then determined.

3.2.5. FLUORIMETRY.

3.2.5.1. Instrument Calibration Checks.

The excitation and emission wavelength calibration of the Perkin-Elmer model MPF-2A fluorescence spectrophotometer was initially verified by comparing experimentally determined and standard excitation and emission λ_{max} values of six commercially prepared fluorescent samples (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, England). The six standards, each embedded in a matrix of polymethylmethacrylate, were: naphthalene (6 μM), anthracene (1 μM), ovalene (0.1 μM), *p*-terphenyl (0.3 μM), tetraphenylbutadiene (0.1 μM) and rhodamine B (3 μM). Excitation and emission spectra were recorded, using the instrument settings shown in columns 1 and 2 of Table 3.2.5.1, at a scan speed of 25 nm/min in a wavelength range which encompassed the λ_{max} value of interest. Experimentally determined excitation and emission λ_{max} values were then compared with those shown in columns 3 and 4 of Table 3.2.5.1.

Table 3.2.5.1. Excitation and Emission Wavelength Calibration of the MPF-2A Fluorimeter using Six Commercially Prepared Standards.

Compound*	Spectral Band Widths (nm)		λ_{max} values (nm)	
	Excitation	Emission	Emission λ_{max} for Recording Excitation Spectra	Excitation λ_{max} for Recording Emission Spectra
Naphthalene	5	5	330	290
Anthracene	5	5	402	360
Ovalene	3	5	482	342
p-terphenyl	3	5	338	295
Tetraphenylbutadiene	3	5	422	348
Rhodamine B	3	5	573	562

*Provided by Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, England.

3.2.5.2. Data Acquisition Protocol.

Fluorimetric measurements were made using the cells described in Section 3.1.2.4. Prior to filling, these cells were rinsed with 8 volumes of H_2O, then two volumes of 95 % (w/v) ethanol, another 8 volumes of H_2O, and finally with approximately 200 μL of BLG solution. Teflon stoppers were rinsed in a similar manner. After the final rinse, residual solution was removed from the interior of the cell using a glass Pasteur pipette. Cells were filled, stoppered, placed in the cell holder of the fluorimeter and left to equilibrate to 20 °C for 5 min. Unless stated otherwise, all data were acquired from cells placed in the same position (i.e. position 4) of the fluorimeter cell holder to eliminate systematic errors resulting from differences in temperature and cell alignment between the four positions. Specific data acquisition procedures will be described in later sections.

3.2.5.3. Calibration of Sensitivity Ranges.

Emission intensity data acquired using the MPF-2A fluorescence spectrophotometer could be recorded at six different sensitivities. However, before data sets were recorded using a combination of these emission intensity sensitivity settings, precise conversion factors for interchange between settings were determined. The fluorescent material used for this calibration was a 0.2 μM solution of N-acetyltryptophanamide (NATA). Emission intensities at 345 nm were measured using a 3 mL aliquot of diluted NATA held at 20 °C and excited at 287 nm.

Initially, the emission slit width was set at a value which gave an emission intensity at the highest sensitivity (setting 6) of 90 % of full scale. The sensitivity was then switched to setting 5 and the ratio of emission intensities (setting 6: setting 5) was calculated. The emission band width was then increased until the emission intensity at setting 5 had been increased to 90 % of full scale. The sensitivity was then switched to setting 4 and the new ratio of emission intensities (setting 5: setting 4) was calculated. Conversion factors between remaining sensitivity settings were determined in a similar manner.

Chapter 4.

PREPARATION OF β-LACTOGLOBULIN.

4.1 INTRODUCTION.

Because the studies presented and discussed in later chapters of this thesis are concerned with the characterisation of the structural changes in BLG molecules which occur during and as a consequence of heat treatment, it was very important that the starting material used in these studies was both pure and native. Furthermore, because these studies were made over a period of several years, it was important that the preparative protocol used to obtain pure native BLG was repeatable. However, the conditions used in some published preparative protocols may lead to irreversible structural change in BLG molecules. For example, Sigma BLG, which is prepared using the method of Aschaffenburg and Drewry (1957) described below, contains disulphide-linked dimers (Iametti *et al.*, 1996). Furthermore, Elofsson *et al.* (1996a) and Hoffmann *et al.* (1996) have found that during heat treatment, rates of particle size increase in solutions of Sigma BLG are faster than those in solutions of freshly prepared BLG. Griffin *et al.* (1993) have suggested that aggregation may also occur during the preparation of Sigma BLG. In all instances, this is a strong indication that irreversible structural change occurs at some stage during the preparation of Sigma BLG. In the preparative protocol of Aschaffenburg and Drewry (1957), the whey proteins are fractionated at pH 2.0 and this may lead to irreversible structural change in BLG molecules. Furthermore, irreversible structural change in BLG molecules in Sigma preparations may occur during lyophilisation or during subsequent storage periods. In the preparative protocol of Fox *et al.* (1967), TCA, a protein denaturant (White *et al.*, 1959), is used to separate BLG from the other whey proteins. Therefore BLG samples purified using this procedure may also contain appreciable quantities of non-native material. In the discussion presented below, a number of BLG preparative procedures are reviewed; the aim being to select one suitable for preparing gram quantities of pure native protein for use in the studies in later chapters of this thesis. Furthermore, because bound retinol and palmitic acid affect the thermostability of BLG (Puyol *et al.*, 1994), it was important that protein prepared using the selected preparative protocol was lipid-free.

The procedure developed by Palmer (1934) is not commonly used today. Nonetheless, in the procedure used to prepare BLG for use in structural change and aggregation studies which is presented in this thesis, whey proteins were concentrated in a manner similar to that described by Palmer (1934). In the procedure of

Palmer (1934), caseins are precipitated at pH 4.6, filtered and discarded, and the resulting whey frozen. Once frozen, lumps of whey are spread evenly on a piece of muslin stretched tightly over the mouth of a large jar, and then left to thaw at 4 °C. The first quarter of the total whey volume to thaw is retained and the remaining solid, mostly water, is then discarded.

The oldest method for preparing BLG that is still commonly used today is that of Aschaffenburg and Drewry (1957). For example, the BLG used in the studies of Griffin *et al.* (1993) and Brownlow *et al.* (1997) was prepared using this method. Fat and caseins are precipitated simultaneously from whole milk using Na_2SO_4. After filtration, the pH of the resulting whey is adjusted to approximately 2 to precipitate proteins other than BLG. The pH of the resulting filtrate is then adjusted to approximately 6 and BLG is precipitated by adding more Na_2SO_4. After filtration, the retained BLG precipitate is re-dissolved in water and then dialysed against water.

Protocols for preparing BLG were reviewed and modified by Armstrong *et al.* in 1967. The major drawback of the method of Aschaffenburg and Drewry (1957) is the use of Na_2SO_4 as a protein precipitant (Armstrong *et al.*, 1967). Before the required masses of this salt will dissolve, solutions must be warmed to approximately 40 °C. Furthermore, Na_2SO_4 precipitates if solutions are not maintained at this temperature. In the study of Armstrong *et al.* (1967), $(NH_4)_2SO_4$ was used in place of Na_2SO_4. Fractionation procedures where the pH of preparations was only lowered to 3.5 were also examined by Armstrong *et al.* (1967).

Two sets of methodologies were published by Armstrong *et al.* (1967). In the first, $(NH_4)_2SO_4$ is used to precipitate the caseins from either skim or whole milk, after which the pH of the resulting whey is lowered, causing proteins other than BLG to precipitate. After filtration, BLG is precipitated with additional $(NH_4)_2SO_4$. For each protocol in this set, only one factor was changed.

In the second set of methodologies published by Armstrong *et al.* (1967), all of the proteins in whey that has been prepared as described above are precipitated with $(NH_4)_2SO_4$. β-Lactoglobulin is then selectively re-dissolved by suspending the precipitate in water. After lowering the pH of the suspension, whey protein which failed to re-dissolve is removed by centrifugation. The pH of the resulting supernatant is then adjusted to 6.0 and BLG is precipitated by adding extra $(NH_4)_2SO_4$. For each of the protocols in this set of methodologies, only one factor was changed.

Armstrong *et al.* (1967) did not state if the purities of BLG samples prepared using their methods were any greater than those of samples prepared using the method of Aschaffenburg and Drewry (1957). However, they stressed that the conditions used in some of their preparative protocols were less severe than those used in the method of Aschaffenburg and Drewry (1957).

Mailliart and Ribadeau-Dumas (1988) developed a salt fractionation procedure based on that of Aschaffenburg and Drewry (1957), which can be scaled up for industrial application. In this protocol, caseins are precipitated at pH 4.6. After the precipitate has been removed, whey is concentrated between 10- and 15-fold by ultrafiltration. The pH of the concentrated whey is then adjusted to 2.0, after which all proteins except BLG are precipitated using NaCl. Precipitated protein is then removed by centrifugation and BLG is precipitated by increasing the NaCl concentration in the retained supernatant. After centrifugation, the BLG pellet is re-dissolved in water, and then dialysed against pH 6.0 phosphate buffer.

Mailliart and Ribadeau-Dumas (1988) were able to show that the protocol which they endorse gave optimal separation of BLG from the other milk proteins. For example, they found that when whey protein concentrations were increased between 10- and 15-fold, an improved separation of BLG from the other whey proteins was achieved. Furthermore, they reported that the extent of α-lactalbumin contamination in the BLG fraction was less than 1% after fractionation at pH 2.0 using 70 g/L NaCl.

In the method of Fox *et al.* (1967), TCA is used to separate BLG from the other proteins in whey previously adjusted to pH 4.6. After TCA treatment, traces of non-BLG whey protein are removed from the resulting supernatant using dilute $(NH_4)_2SO_4$. β-Lactoglobulin is then precipitated from the resulting supernatant by increasing the $(NH_4)_2SO_4$ concentration to saturation point. The precipitate is then re-dissolved and dialysed against water.

Because TCA is commonly used as a protein denaturant (White *et al.*, 1959), Fox *et al.* (1967) made a series of intrinsic viscosity, electrophoretic and specific rotation measurements to determine whether irreversible structural change occurred during preparation. They reported that the physicochemical properties of BLG prepared using their protocol were indistinguishable from those of BLG prepared using other techniques. A 3° decrease in specific rotation was, however, observed when BLG prepared using TCA was treated with this precipitant for a second time (Fox *et al.*, 1967). Unfortunately, Fox *et al.* (1967) did not make similar measurements on BLG samples that had been prepared by other means. Therefore, it is impossible to tell whether measurable changes in the physicochemical properties of BLG are brought about by the use of TCA.

β-Lactoglobulin has also been prepared using protocols based on column chromatography. For example, in the procedure of Monaco *et al.* (1987), fat is removed from whole milk by centrifugation, after which the pH of the resulting skim milk is adjusted to 6.6 with HCl. Caseins are then precipitated using $CaCl_2$ solution.

After centrifugation and dialysis, whey proteins are separated by ion-exchange chromatography, using a column of DEAE-cellulose. β-Lactoglobulin fractions obtained from the ion-exchange column are then purified further by size-exclusion chromatography, using a column of Sephadex G-100.

Affinity chromatography, a technique where the elution of selected proteins is retarded by ligands immobilised on a solid support, has also been used to prepare pure BLG. Jang and Swaisgood (1990) developed a protocol whereby BLG was purified from whey using a column of immobilised retinal. In 0.1 M phosphate buffer, only the elution of BLG was retarded by the bound retinal. Jang and Swaisgood (1990) were also able to separate native and denatured BLG using their retinal column.

In the method of Chiancone and Gattoni (1991), BLG was purified from whey using a column of immobilised monomeric BLG. Whey was percolated through the column in "associating buffer" (0.1 M acetate, pH 4.65), and the bound BLG was then eluted in "dissociating buffer" (0.1 M NaCl/HCl, pH 2.0).

The salt fractionation method of Mailliart and Ribadeau-Dumas (1988) used in conjunction with size-exclusion chromatography (Method I in Table 4.1.1) appeared suitable for preparing gram quantities of pure BLG. Furthermore, because the whey proteins are fractionated at pH 2.0 in this protocol, the purified BLG should be lipid-free (Diaz de Villegas *et al.*, 1987). However, pH adjustment to 2.0 may lead to irreversible structural change in BLG molecules. To determine whether or not this occurs, the results obtained from a series of near and far UV CD and fluorescence measurements made on samples of BLG prepared using Method I were compared to those obtained from BLG samples prepared using other methods.

The method of Fox *et al.* (1967) followed by size-exclusion chromatography looked equally suitable for preparing gram quantities of pure BLG (Method III in Table 4.1.1). However, because the use of TCA in this method may lead to irreversible structural change in BLG molecules, a spectroscopic comparison of BLG prepared using Method III with BLG prepared using other methods was made. The method of Monaco *et al.* (1987) appeared suitable for preparing milligram quantities of pure native BLG (Method II in Table 4.1.1) and the spectroscopic results obtained from BLG samples prepared using this procedure were compared with those obtained using Methods I and III. To determine how structural change in BLG molecules affects spectroscopic results, a series of spectroscopic measurements were also made on solutions of previously heat-treated BLG.

The objective of the study described below was to determine which of the three methods summarised in Table 4.1.1 would give pure native BLG suitable for use in the studies presented and discussed in later chapters of this thesis.

Table 4.1.1. The Protocols used to Prepare β-Lactoglobulin.

Method Number	Preparative Techniques		
Method I	Concentrated Acid Whey (section 4.2.1.2)	"NaCl Fractionation" (Mailliart and Ribadeau-Dumas, 1988) (section 4.2.1.3)	Size-Exclusion Chromatography (section 4.2.1.5)
Method II	"$CaCl_2$ Fractionation" (Monaco *et al.*, 1987) (section 4.2.1.6)	Size-Exclusion Chromatography (section 4.2.1.5.)	
Method III	Concentrated Acid Whey (section 4.2.1.2)	"TCA" Fractionation (Fox *et al.*, 1967) (section 4.2.1.4)	Size-Exclusion Chromatography (section 4.2.1.5)

4.2. METHODS.

4.2.1. PREPARATION OF β-LACTOGLOBULIN FROM WHOLE MILK.

4.2.1.1. Supply of Milk.

The raw material for all preparations was fresh unpasteurised whole milk. Phenotyping results, obtained as described by Anema and Creamer (1993), were used to select cows with the BLG phenotype AA or BB. Similarly, results from the phenotyping study of Paterson *et al.* (1995b) were used for identification of cows containing the BLG CC phenotype. Where possible, milk from the same group of cows was used for all preparations.

Selected cows were usually milked in the morning, and the preparation of BLG was initiated approximately 4 hr after collection of milk. Occasionally, cows were also milked in the evening prior to the commencement of BLG preparation. Milk collected in the evening was stored at 4 °C overnight. Usually, three 15 L - 20 L volumes of milk, containing one of the three genetic variants, were collected. Alkaline native-PAGE was used to confirm that the milk samples each contained the correct genetic variant of BLG.

4.2.1.2. Preparation of Concentrated Acid Whey.

The milk centrifuge described in Section 3.1.2.9 was used to remove fat from whole milk pre-warmed to 40 °C. After fat removal, the skim milk was re-warmed to 40 °C, and the pH was adjusted, with constant stirring, to 4.6 with 1 M HCl. Precipitated caseins were then separated from the whey by filtration through a pad of fabric with a loose weave (4 layers of terylene curtain material).

Whey proteins were then concentrated using a freeze-thaw technique similar to that used by Palmer (1934), (Section 4.1). Whey was transferred to 1.25 L transparent plastic soft drink bottles which had a height:width ratio of 11:4, frozen, and then thawed. During thawing, containers were kept upright and stationary, which permitted a concentration gradient to form. The lower 1/2 - 2/3 of the volume was then removed by siphoning. This fraction, and the upper fraction was retained, transferred to two separate sets of containers, and then frozen, thawed and siphoned for a second time. After the second siphoning, fractions were categorised according to their origins: concentrated (the lower portion of a volume derived from the original lower fraction), medium (the upper portion of a volume derived from the original lower fraction and the lower portion of a volume derived from the original upper fraction) and dilute (the upper portion of a volume derived from the original upper fraction). Dilute fractions were discarded. This categorisation of fractions is presented in Fig. 4.2.1. The freeze-thaw-siphon protocol was then repeated a further five times (i.e. 7 complete cycles), after which the total volume of whey was approximately 1/5 of the initial volume. For example, on one occasion, an initial whey volume of approximately 20 L was decreased to 4.3 L. Each freeze-thaw-siphon cycle took 1 day. Typically, whey was siphoned early in the morning, re-frozen during late morning/early afternoon and then set out to thaw in the late afternoon.

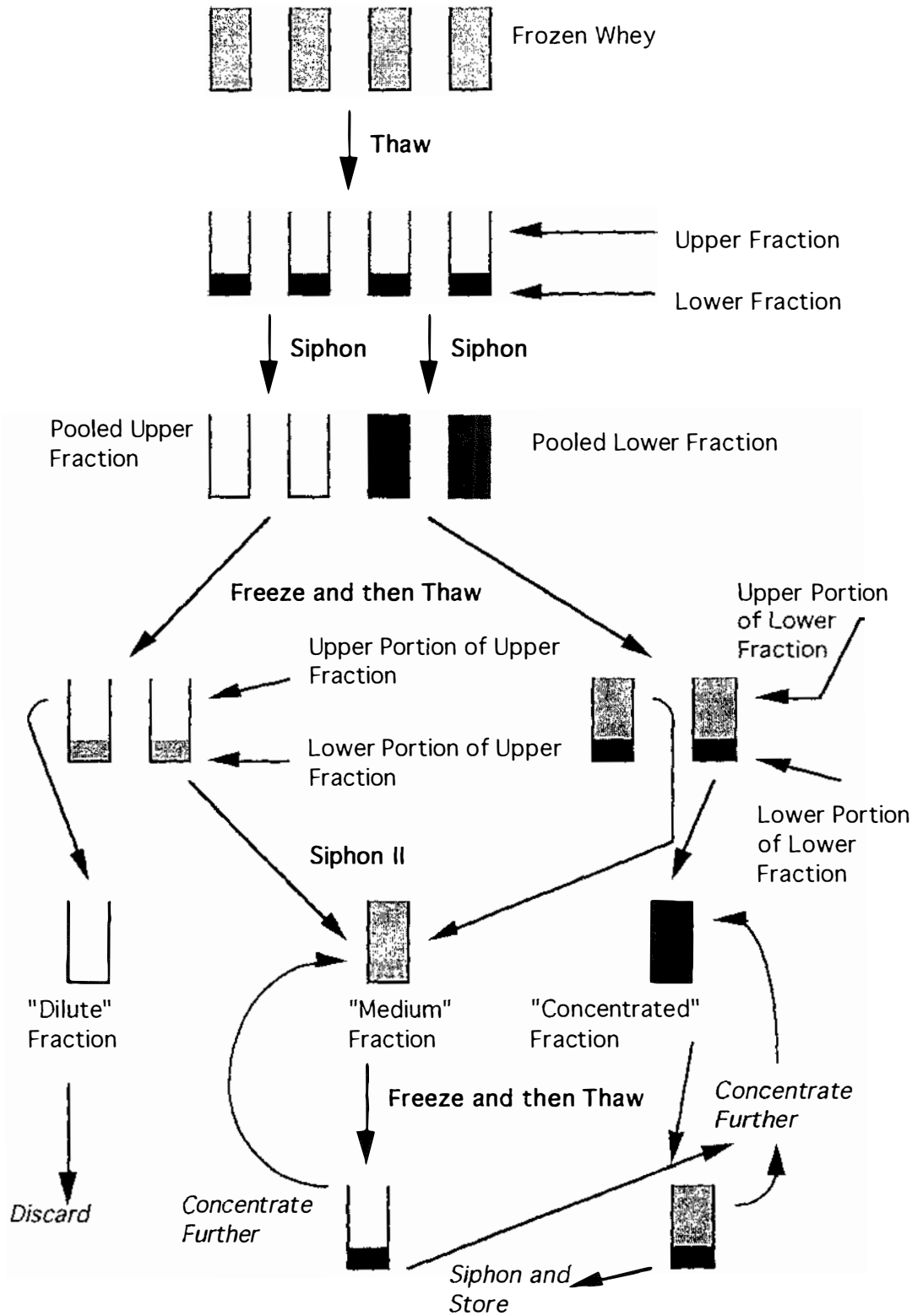


Fig. 4.2.1. Schematic representation of the freeze-thaw-siphon protocol used to concentrate whey proteins.

No specific procedure was used for the removal of crystalline lactose from the whey. However, significant quantities were removed passively during the freeze-thaw-siphon cycles. Lactose formed a dense pellet during thawing, and during whey siphoning only part of the total mass was transferred from one vessel to another. Thus, sedimented lactose remaining in containers could be re-suspended and then discarded.

4.2.1.3. Preparation of Crude β-Lactoglobulin from Concentrated Acid Whey Using NaCl.

β-Lactoglobulin was isolated from concentrated acid whey (section 4.2.1.2) using the procedure of Mailliart and Ribadeau-Dumas (1988). Concentrated whey was warmed to 40 °C, and the pH adjusted to 2.0 with 1 M HCl. The volume was then measured and, while maintaining constant slow stirring, NaCl (7 % (w/v)) was slowly added to the whey. After dissolution, the preparation was left to stir for 20 min. Precipitated proteins were then removed by centrifugation (10 000 × *g*, 20 min).

The supernatant was retained and the volume recorded. Additional NaCl (230 g per L supernatant, i.e. final NaCl concentration of 30 % (w/v)) was then slowly dissolved in the supernatant. The mixture was stirred slowly for a further 20 min. Precipitated proteins were then removed by centrifugation (10 000 × *g*, 20 min) and retained.

Immediately after centrifugation, the pellet was carefully re-dissolved in the minimum volume of water. Powdered anhydrous K_2HPO_4 was then slowly dissolved in the preparation to raise the pH to approximately 4.0. After this pH value had been attained, the preparation was dialysed against 5 changes of water. Alkaline native-PAGE was used to determine the purity of the BLG at various stages of the preparation. Protein solutions were stored frozen (-21 °C) in 25 mL containers for periods of up to 16 months. Solution volumes and NaCl masses required for a typical preparation are presented in Table 4.2.1.

Table 4.2.1. Solution volumes and NaCl masses required for a typical preparation of β-Lactoglobulin B.

Quantity	Volume or Mass
1) Acid Whey	20 L
2) Concentrated Whey	4.30 L
3) Concentrated Whey at pH 2.0	4.45 L
4) Mass NaCl for 7 % (w/v) solution	311 g
5) Supernatant from 7 % (w/v) fractionation	4.34 L
6) Mass NaCl for 30 % (w/v) precipitation	999 g
7) Solution volume of re-dissolved pellet	0.70 L

4.2.1.4. Preparation of Crude β-Lactoglobulin from Concentrated Acid Whey Using Trichloroacetic Acid.

β-Lactoglobulin B was purified from a 250 mL volume of concentrated acid whey (Section 4.2.1.2) using the procedure of Fox *et al.* (1967). Trichloroacetic acid (TCA, 8.55 g per 250 mL whey, dissolved in the minimum volume of water) was slowly added to the stirred volume of concentrated acid whey, which was held at 20 °C. The preparation, now containing 3.42 % (w/v) TCA, was then stirred for a further 30 min. After this time, precipitated protein was removed by centrifugation (10 000 × *g*, 20 min), and the supernatant (238 mL) was retained. A 1.0 mL volume of saturated ammonium sulphate solution was then mixed into the supernatant, giving a final ammonium sulphate concentration in the preparation of 0.021 M. Ammonium sulphate treatment ensured complete removal from solution of any non-BLG protein which did not precipitate in the presence of TCA alone. Although the turbidity of the solution did not visibly increase after ammonium sulphate addition, the preparation was centrifuged (10 000 × *g*, 20 min), and the supernatant (220 mL) was retained.

The concentration of ammonium sulphate in the preparation was then increased to 4.0 M (saturation) by the slow dissolution of crystalline ammonium sulphate (154.7 g) in the supernatant. The preparation was then stirred, at 20 °C, for 1 hr and then left to stand, at 20 °C, for a further 14 hr (i.e. overnight). Precipitated protein was then removed by centrifugation (10 000 × *g*, 20 min). The pellet was retained, washed in saturated ammonium sulphate solution, and then dissolved in the minimum volume of water (approximately 45 mL). Immediately after dissolution, the protein was dialysed against five changes of pH 6.0 phosphate buffer. The protein solution was then stored frozen (-21 °C) in 25 mL containers for seven months. Alkaline native-PAGE was used to determine the purity of the BLG at various stages of the preparation.

4.2.1.5. Preparative Size Exclusion Chromatography.

Size exclusion chromatography was used to further purify portions of the BLG A, B or C samples prepared as described in Sections 4.2.1.3 and 4.2.1.4. In the case of BLG prepared as described in Section 4.2.1.3, samples which had been stored frozen for more than 16 months were not used. Proteins were separated on the Superdex-75 column using the Pharmacia FPLC system described in Section 3.1.2.2. The column was equilibrated with pH 6.0 phosphate buffer and cooled to 4 °C prior to chromatography.

Typically, a 5 mL volume of a filtered protein solution was loaded onto the column for each run. Proteins were eluted at a flow rate of 2 mL/min and 4 mL fractions were collected. The A_{280} trace and tick marks from the chart recording of the first run of each day were used to identify the fractions which contained BLG. Fractions from both sides as well as the top of the BLG peak were then run on native gels to determine which ones contained pure BLG. Volumes (250 μL) of selected fractions were diluted 2-fold with native gel sample buffer and run on native gels using the procedure described in Section 3.2.1. Fractions were stored at 4 °C until alkaline native-PAGE results were available. After fractions had been pooled, purities were re-checked using alkaline native-PAGE. Usually twenty-four 5 mL volumes of a particular variant solution were chromatographed over a period of 1 week and approximately 3.0 g of pure BLG could be collected in this time. Protein solutions at pH 6.0 were stored frozen (-21 °C) in 25 mL containers.

4.2.1.6. Preparation of β-Lactoglobulin from Whole Milk using $CaCl_2$ and Size Exclusion Chromatography.

The procedure of Monaco *et al.* (1987) was used to purify BLGs A and B from 240 mL volumes of whole milk. Fat was removed from the whole milk by centrifugation ($2 \times 10\,000 \times g$, each for 20 min) at 4 °C. The skim milk (226 mL) was then warmed to 40 °C and, with constant stirring, adjusted to pH 6.6 with 1 M HCl. A 14.5 mL volume of aqueous 2.0 M $CaCl_2$ was then mixed into the skim milk, giving a final $CaCl_2$ concentration of 0.12 M, and the pH of the mixture was re-adjusted to 6.6 with 1 M NaOH. The preparation was then left to stand at 20 °C for 5 min. Precipitated protein was removed by centrifugation ($12\,000 \times g$, 30 min) and the supernatant (whey) was retained.

The pH of the whey was then adjusted to 5.9 and proteins were concentrated 4-fold (i.e. from 202 mL to 47 mL), at 4 °C, by stirred cell ultrafiltration. β-Lactoglobulin was then purified from the concentrated whey using the size exclusion chromatography procedure described in Section 4.2.1.5. Alkaline native-PAGE was used to determine the purity of the BLG at various stages of the preparation.

4.2.1.7. Ion-exchange Chromatographic Analysis of Purified β-Lactoglobulin.

In addition to alkaline native-PAGE, analytical ion-exchange chromatography, using the HPLC apparatus described in Section 3.1.2.3, was used to determine the purity of some freshly prepared BLG samples. Prior to chromatography, samples of BLGs A, B and C were concentrated to approximately 30 mg/mL by stirred cell ultrafiltration. The exact protein concentrations were then determined from measured A_{280} values using the standard absorbance value, A_{280} (10.0 mg/mL BLG) = 9.4 (Huang *et al.*, 1994a). Samples were diluted to 0.2 mg/mL with pH 5.5 piperazine buffer (20 mM, with 1 mM $CaCl_2$) and then centrifuged. Aliquots (100 μL) of 0.2 mg/mL BLG A, B or C solution were sequentially loaded onto the column and washed into the resin with 4 mL of pH 5.5 piperazine buffer (flow rate of 1.0 mL/min for 4 min). A linear gradient (0.0 M - 0.3 M NaCl, 0.3 M reached after 10 min) was used to elute proteins. In preparation for the next run, the salt concentration was increased over a period of 30 s to 1.0 M, maintained at this concentration for 30 s, and then decreased over a period of 30 s to 0.0 M. The column was then re-equilibrated with 4.5 mL salt-free pH 5.5 piperazine buffer. An overall flow rate of 1.0 mL/min was maintained at all stages of the chromatography run.

4.2.2. SPECTROSCOPIC ANALYSIS OF PURIFIED β-LACTOGLOBULIN SAMPLES.

Spectroscopic measurements were made using samples of BLGs A or B purified using the methods summarised in Table 4.1.1.

4.2.2.1. Near UV CD Spectroscopy.

β-Lactoglobulin solutions were filtered and then diluted to about 0.75 mg/mL with filtered pH 6.0 phosphate buffer in preparation for near UV CD spectroscopy. The spectrum of each protein solution was recorded and manipulated as described in Section 3.2.4.2. All of the spectra of unheated solutions of a particular variant were then standardised so that the intensities of $\Delta\varepsilon_{293}$ in each were equivalent. This was done by multiplying the spectra of BLG solutions prepared using Methods II and III by constants so that the intensities of $\Delta\varepsilon_{293}$ were equivalent to that in the spectrum of the solution prepared using Method I. These standardisations were made using the J-720 software, and assume that structural differences in BLG molecules are reflected in peak shapes as well as the intensity of $\Delta\varepsilon_{293}$. Normalised spectral pairs were then subtracted one from the other to show differences at other wavelengths.

4.2.2.2. Far UV CD Spectroscopy.

Prior to making far UV CD measurements, BLG solutions were diluted to about 0.75 mg/mL, as described in Section 4.2.2.1, then diluted to 0.075 mg/mL with water and spectra were recorded using the 1.0 mm path length cell as described in Section 3.2.4.3. The spectrum of each unheated BLG solution was then standardised using the two-step procedure described in Section 3.2.4.3. Large photomultiplier tube voltage values below 210 nm indicated low light intensity and hence diminished data quality (i.e. low signal:noise ratio) in this portion of far UV CD spectra. For this reason, spectra were adjusted, using a procedure similar to that described in Section 4.2.2.1, so that an overlay between 220 nm and 250 nm was achieved. Pairs of normalised spectra were then subtracted, one from the other, as described in Section 4.2.2.1.

4.2.2.3. Fluorimetry.

β-Lactoglobulin solutions were diluted to about 0.75 mg/mL with pH 6.0 phosphate buffer in preparation for fluorimetry. A filtered 0.2 μM solution of N-acetyltryptophanamide (NATA) was used for the control experiment.

Aliquots (3 mL) of either BLG or NATA solution, delivered into 3 mL cells, were then titrated with 1.41 mM 1-anilinonaphthalene-8-sulphonate (ANS) solution (4.2 mg ANS dissolved in 1 mL 95% ethanol, then diluted to 10 mL with H_2O) in 20 μL or 40 μL increments up to 140 μL. After each addition of ANS, the cell contents were mixed by inversion and returned to the fluorimeter cell holder. Solutions were then left to re-equilibrate to 20 °C for 5 min before measurements were made. Emission spectra were recorded between 310 nm and 510 nm using an excitation wavelength of 287 nm, excitation and emission slit widths of 8 nm, at a scan speed of 25 nm/min, and a chart speed of 1 cm/min. At the conclusion of each titration, the mole ratio ANS:monomeric BLG was approximately 1:1.

Additional Titrations.

The results obtained from a set of additional titrations were required for the correction of emission intensity data for the absorption of incident and emitted light by ANS molecules (inner filter effects). A 3.0 mL volume of water was titrated with ANS solution and the absorbance of the mixture at the fluorimetric λ_{ex} and emission λ_{max} values (287 nm and 480 nm respectively) was measured after each volume of ANS had been added. The Shimadzu spectrophotometer was used for these measurements.

Treatment of Data: ANS Emission Intensities.

The intensity of emissions from ANS was arbitrarily defined as the height (in cm) of the emission intensity peak which had a λ_{max} value near 477 nm. The zero emission intensity baseline, from which these measurements were made, was defined as the position of the pen on the chart paper when the emission shutter was closed.

Emission intensities were then corrected for inner filter effects using equation 4.2.2.1 (Lakowicz, 1983) and the results obtained in the additional titrations made as described above. In equation 4.2.2.1, F_{corr} is the corrected emission intensity, F_{obs} is the observed emission intensity, A_{ex} is the absorbance at the excitation wavelength and A_{em} is the absorbance at the emission λ_{max}.

$$F_{corr} \approx F_{obs} \times antilog \left(\frac{A_{ex} + A_{em}}{2} \right) \qquad (4.2.2.1)$$

Emission intensities were then corrected for variation in protein concentration between the samples. Corrections were made using equation 4.2.2.2. In this equation $F_{corr'}$ is the emission intensity corrected for variation in protein concentration among samples, F_{corr} is the emission intensity corrected using equation 4.2.2.1, $[BLG]_{high}$ is the BLG concentration of the most concentrated sample examined and $[BLG]_x$ is the BLG concentration of the sample of interest.

$$F_{corr'} = F_{corr} \times \frac{[BLG]_{high}}{[BLG]_x} \qquad (4.2.2.2)$$

This correction assumes that the magnitude of systematic error in all emission intensities measured from the more dilute sample of a pair is equivalent to the ratio of the two protein concentrations. Corrected ANS emission intensity profiles were then plotted against the mole ratio ANS:monomeric BLG.

4.2.2.4. Heat-treated β-Lactoglobulin.

Solutions of BLGs A and B prepared using Method II were heat-treated by placing stoppered glass test tubes, each containing a 5 mL aliquot of 0.75 mg/mL protein solution in a boiling water bath for 10 min. Solutions were then cooled to 20 °C, after which near UV CD and fluorimetric measurements were made, as described in Sections 4.2.2.1 and 4.2.2.3 respectively, without further dilution. Prior to far UV CD measurements, made as described in Section 4.2.2.2, solutions were diluted 10-fold with water.

4.3. RESULTS.

4.3.1. PREPARATION OF β-LACTOGLOBULIN.

4.3.1.1. Preparation of Concentrated Acid Whey (Methods I and II).

Qualitative alkaline native-PAGE results (Fig. 4.3.1.1) indicate that a precipitate of casein formed when the pH of skim milk was lowered to 4.6. On native gels, the caseins appear as broad "smears" in the lanes containing the skim milk samples while the whey proteins appear as narrow focused bands (Fig. 4.3.1.1).

Proteins remaining in the whey were then concentrated between 4- and 5-fold by the freeze-thaw-siphon protocol described in Section 4.2.1.2 in preparation for salt fractionation using the method of Mailliart and Ribadeau-Dumas (1988). Due to the opacity of the solutions, initial and final protein concentrations were difficult to determine.

4.3.1.2. Preparation of Crude β-Lactoglobulin from Concentrated Acid Whey Using the Salt Fractionation Procedure of Mailliart and Ribadeau-Dumas (1988), (Method I).

The alkaline native-PAGE results shown in Fig. 4.3.1.1 indicate that the precipitate (PI) formed as a consequence of treatment with 7 % (w/v) NaCl consisted of immunoglobulins, BSA, α-lactalbumin, and a significant quantity of BLG. However, only small quantities of immunoglobulins, BSA, and α-lactalbumin were retained in the corresponding supernatant fraction (SI, Fig. 4.3.1.1). Alkaline native-PAGE results (Fig. 4.3.1.1) also confirmed that the second precipitate (PII), which was formed when the NaCl concentration in SI was increased to 30% (w/v), was mostly BLG. However, some BLG was retained in the corresponding supernatant fraction (SII).

The final yields of BLG prepared as described in Sections 4.2.1.2 and 4.2.1.3 usually ranged from 50% to 65%. For example, in one preparation, 768 mL of 26 mg/mL BLG B solution (i.e. a total of approximately 20 g BLG) was prepared from 20 L whole milk (i.e. from an initial BLG mass of 40 g, assuming that the concentration of this protein in milk was 2 mg/mL).

To summarise, the results discussed above indicated that acceptable yields of moderately pure BLG could be obtained using the salt fractionation procedure of Mailliart and Ribadeau-Dumas (1988). However, because BLG free from other proteins was required for structural change and aggregation studies, the protein prepared using this procedure needed further purification.

Fig. 4.3.1.1. Alkaline native-PAGE electropherograms of the bovine milk protein fractions obtained when BLGs A, B and C were prepared using Method I. This method is summarised in Table 4.1.1. The fractions obtained from milk containing BLGs A, B and C are shown in gels 1, 2 and 3 respectively. From left to right gel lanes contain:

1) skim milk (1/40 dilution, BLGs B and C only)

2) whey (1/40 dilution)

3) concentrated whey (1/80 dilution)

4) protein soluble in 7 % (w/v) NaCl at pH 2.0 (fraction SI, 1/40 dilution)

5) protein soluble in 7 % (w/v) NaCl at pH 2.0 (fraction SI, 1/80 dilution)

6) protein precipitated in 7 % (w/v) NaCl at pH 2.0 (fraction PI, 1/40 dilution)

7) protein precipitated in 7 % (w/v) NaCl at pH 2.0 (fraction PI, 1/80 dilution)

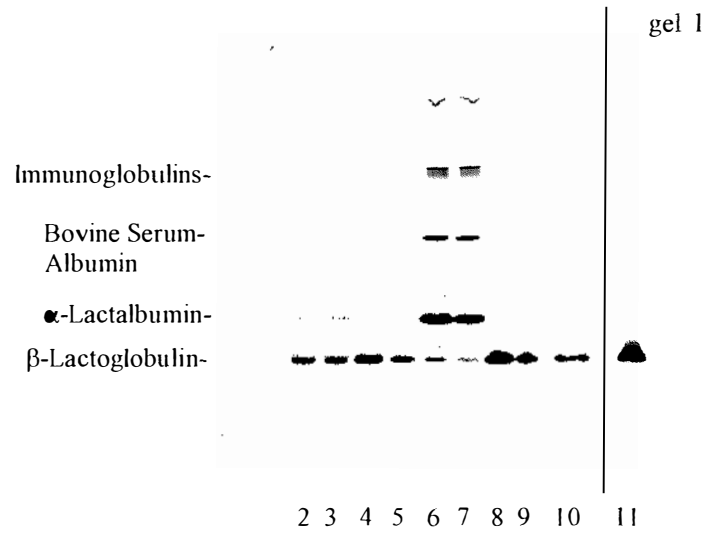
8) protein precipitated in 30 % (w/v) NaCl at pH 2.0 (fraction PII, 1/40 dilution)

9) protein precipitated in 30 % (w/v) NaCl at pH 2.0 (fraction PII, 1/80 dilution)

10) protein soluble in 30 % (w/v) NaCl at pH 2.0 (fraction SII, 1/2 dilution)

11) pooled BLG fractions obtained from size-exclusion chromatography (1/2 dilution).

All samples were diluted with alkaline native-PAGE sample buffer (Section 3.2.1.2), electrophoresed, stained/destained and photographed as described in Sections 3.2.1.2 - 3.2.1.4.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α-Lactalbumin-
β-Lactoglobulin-
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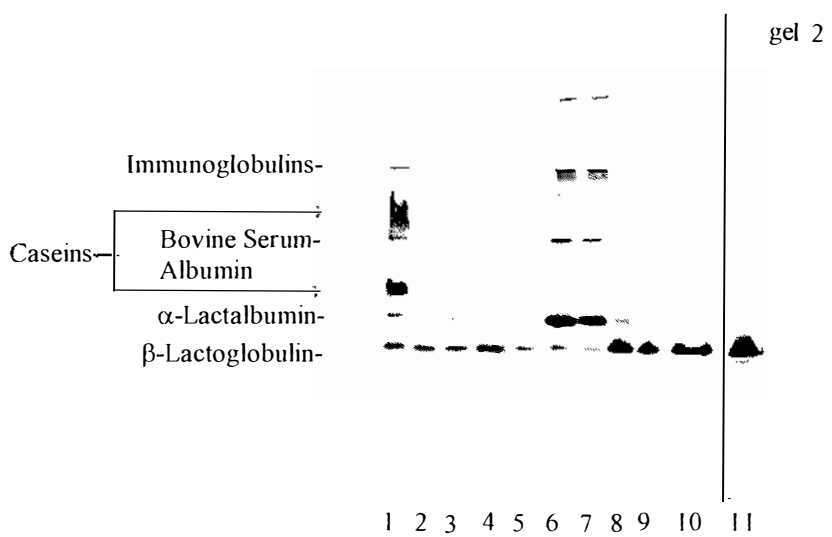
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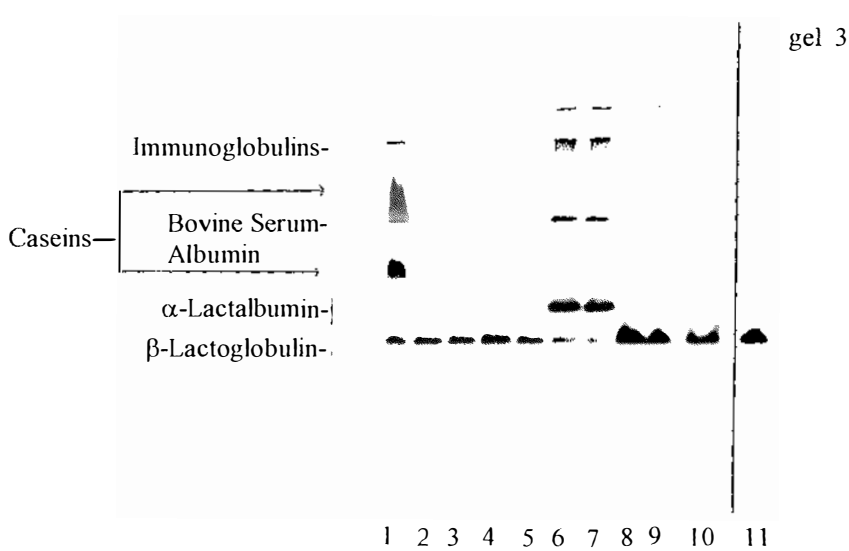
Immunoglobulins-
Caseins
Bovine Serum-
Albumin
α-Lactalbumin-
β-Lactoglobulin-
1 2 3 4 5 6 7 8 9 10 11


gel 3
Immunoglobulins-
Caseins
Bovine Serum-
Albumin
α-Lactalbumin-
β-Lactoglobulin-
1 2 3 4 5 6 7 8 9 10 11

4.3.1.3. Preparation of Crude β-Lactoglobulin B from Concentrated Acid Whey Using the TCA Fractionation Procedure of Fox *et al.* (1967) (Method III).

The precipitate which formed in 3.42 % (w/v) TCA (PI) consisted of immunoglobulins, BSA, α-lactalbumin and also a moderate quantity of BLG B (Fig. 4.3.1.2). β-Lactoglobulin was the only protein retained in the corresponding supernatant fraction (SI). When the 1 mL volume of saturated ammonium sulphate solution was mixed into fraction SI, no additional precipitation was observed.

A second precipitate (PII) was formed when the ammonium sulphate concentration of SI was increased to saturation point. Alkaline native-PAGE results (Fig. 4.3.1.2) confirmed that this precipitate contained only BLG B. Furthermore, the corresponding supernatant fraction SII was virtually devoid of protein.

The final yield of BLG B prepared from whole milk using the TCA fractionation procedure of Fox *et al.* (1967) was approximately 17%. From 250 mL concentrated acid whey containing approximately 11.5 mg/mL BLG (i.e. approximately 2.9 g BLG B), 85 mL of 5.80 mg/mL solution (i.e. approximately 493 mg BLG) was obtained. Thus, although pure BLG was obtained using the method of Fox *et al.* (1967), the yield was low compared to that obtained using the salt fractionation method of Mailliart and Ribadeau-Dumas (1988). To ensure the BLG prepared using the TCA fractionation procedure of Fox *et al.* (1967) was free from other proteins, it was chromatographed on a size-exclusion column before being used in spectroscopic studies.

4.3.1.4. Preparative Size-exclusion Chromatography (Methods I and III).

β-Lactoglobulin prepared using the methods of Mailliart and Ribadeau-Dumas (1988) and Fox *et al.* (1967), which are part of Methods I and III respectively (Table 4.1.1), was purified further by size-exclusion chromatography. Using chromatograms as well as alkaline native-PAGE results (Fig. 4.3.1.3), eluate fractions containing pure BLG were identified and pooled. In each run, there were usually five such fractions. Although many of the other fractions also contained BLG, they were contaminated with other proteins.

The alkaline native-PAGE results in Fig. 4.3.1.1 indicated that BLGs A and C could be purified to homogeneity using Method I. However, lanes containing samples of BLG B purified using Method I or III usually exhibited a band with a mobility similar to that of BLG A (Figs 4.3.1.1 and 4.3.1.2). This may indicate that preparations of BLG B were contaminated with BLG A. To determine whether or not this was true, the purities of samples of BLGs A, B and C were also determined by ion-exchange chromatographic analysis (Section 4.3.1.6).

Fig. 4.3.1.2. Alkaline native-PAGE electropherograms of the bovine milk protein fractions obtained when BLG B was prepared using Method III. This preparative protocol is summarised in Table 4.1.1. From left to right lanes in gel 1 contain:

1) concentrated whey (1/10 dilution)

2) concentrated whey (1/20 dilution)

3) protein soluble in 3.42 % (w/v) TCA (fraction SI, 1/10 dilution)

4) protein soluble in 3.42 % (w/v) TCA (fraction SI 1/20 dilution)

5) protein precipitated in 3.42 % (w/v) TCA (fraction PI, 1/10 dilution)

6) protein precipitated in 3.42 % (w/v) TCA (fraction PI 1/20 dilution)

7) protein precipitated in saturated ammonium sulphate (fraction PII, 1/10 dilution)

8) protein precipitated in saturated ammonium sulphate (fraction PII 1/20 dilution).

From left to right lanes in gel 2 contain:

1) protein soluble in saturated ammonium sulphate (fraction SII, 1/10 dilution)

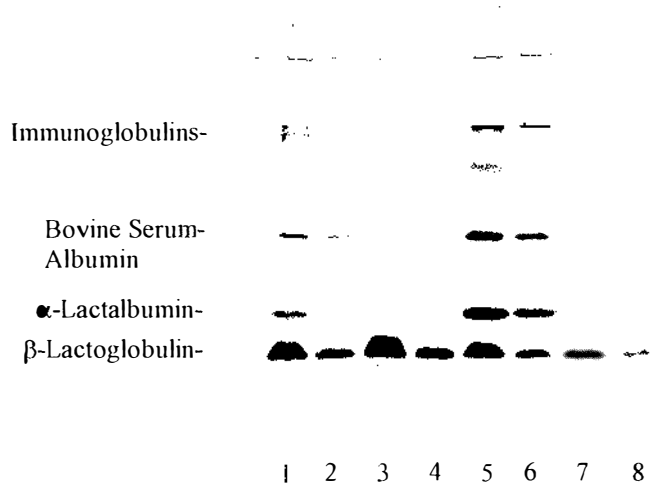
2) protein soluble in saturated ammonium sulphate (fraction SII 1/20 dilution)

3, 4) fraction PII washed in saturated ammonium sulphate solution (1/10 dilution)

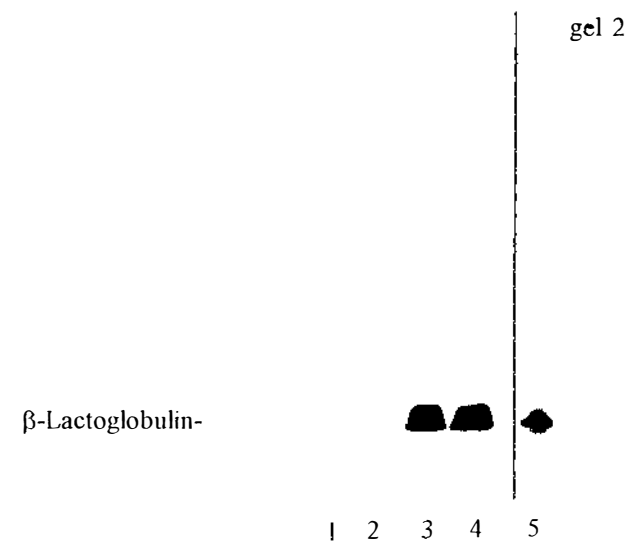
5) the crude BLG preparation purified further by size-exclusion chromatography (1/2 dilution).

All samples were diluted with alkaline native-PAGE sample buffer (Section 3.2.1.2), electrophoresed, stained/destained and photographed as described in Sections 3.2.1.2 - 3.2.1.4.

gel 1


Immunoglobulins-
Bovine Serum-
Albumin
α-Lactalbumin-
β-Lactoglobulin-
1 2 3 4 5 6 7 8

gel 2


β-Lactoglobulin-
1 2 3 4 5

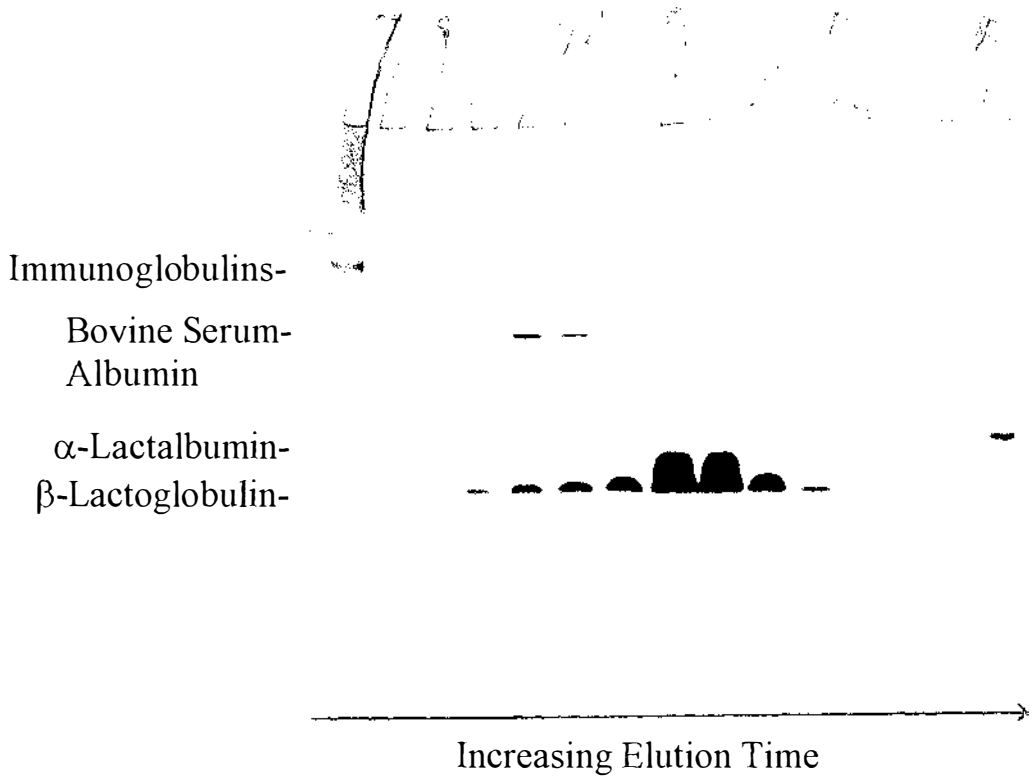


Fig. 4.3.1.3. Specimen alkaline native-PAGE electropherogram of eluate fractions obtained when crude preparations of BLG B were further purified by size-exclusion chromatography. Fractions, obtained as described in Section 3.2.1.5, are shown, from left to right, in order of increasing elution time. All samples were diluted 1/2 with alkaline native-PAGE sample buffer (Section 3.2.1.2), electrophoresed, stained/destained and photographed as described in Sections 3.2.1.2 - 3.2.1.4.

The yield of BLG obtained from each size-exclusion FPLC run was about 43%. For example, in one series of runs the approximate concentration of BLG in the crude preparation was 25 mg/mL (i.e. 125 mg BLG in the 5 mL volume loaded onto the column). After chromatography, the average BLG concentration in the five 4 mL fractions collected was 2.7 mg/mL (i.e. 54 mg BLG).

4.3.1.5. Preparation of β-Lactoglobulin from Whole Milk Using $CaCl_2$ and the Size-exclusion Chromatography Procedure of Monaco *et al.* (1987), (Method II).

When the $CaCl_2$ solution was mixed into the skim milk at pH 6.6, a white precipitate formed. For the reasons discussed in Section 4.3.1.1, alkaline native-PAGE results (Fig. 4.3.1.4) indicate that this precipitate consisted of casein.

After the caseins had been removed, the retained whey was concentrated by stirred cell ultrafiltration and then chromatographed by size-exclusion chromatography. The alkaline native-PAGE results obtained from these chromatographic runs were similar to those shown in Fig. 4.3.1.3. Furthermore, in agreement with the results shown in Figs 4.3.1.1 and 4.3.1.3, a band with a mobility similar to that of BLG A was observed in the lane containing the sample of BLG B purified using Method II (Fig. 4.3.1.4). This suggests that the BLG B sample prepared using Method II was also contaminated with BLG A.

The yields of BLG obtained from whey using size-exclusion chromatography were similar to those discussed in Section 4.3.1.4. For example, in the case of Method II, approximately 26 mg BLG was usually obtained from volumes of whey containing approximately 50 mg BLG. In the procedure of Monaco *et al.* (1987), on which Method II is based, whey proteins were separated using ion-exchange chromatography and then size-exclusion chromatography. The results shown in Fig. 4.3.1.4 suggest that the ion-exchange step is not necessary.

One of the disadvantages of Method II is that casein precipitates and fat are removed by centrifugation. Therefore, using six 250 mL GSA bottles only 1.5 L material can be processed in each 30 min spin, and a minimum time of approximately 7 hr would have been required to defat 20 L whole milk, the volume routinely used in Method I.

In summary, alkaline native-PAGE results (Fig. 4.3.1.4) indicate that pure BLG was obtained from whey in a single size-exclusion chromatography step. Furthermore, yields were equivalent to those obtained when crude preparations of BLG were chromatographed in the same manner. Overall though, Method II seems poorly suited as a protocol for preparing gram quantities of BLG from whole milk because several

Fig. 4.3.1.4. Alkaline native-PAGE electropherograms of the bovine milk protein fractions obtained when BLGs A and B were prepared using Method II. This procedure is summarised in Table 4.1.1. The fractions obtained from milk containing BLG A and milk containing BLG B are shown in gels 1 and 2 respectively. From left to right, gel lanes contain:

1) skim milk (1/20 dilution)

2) whey concentrated by stirred cell ultrafiltration (1/20 dilution)

3) whey concentrated by stirred cell ultrafiltration (1/10 dilution)

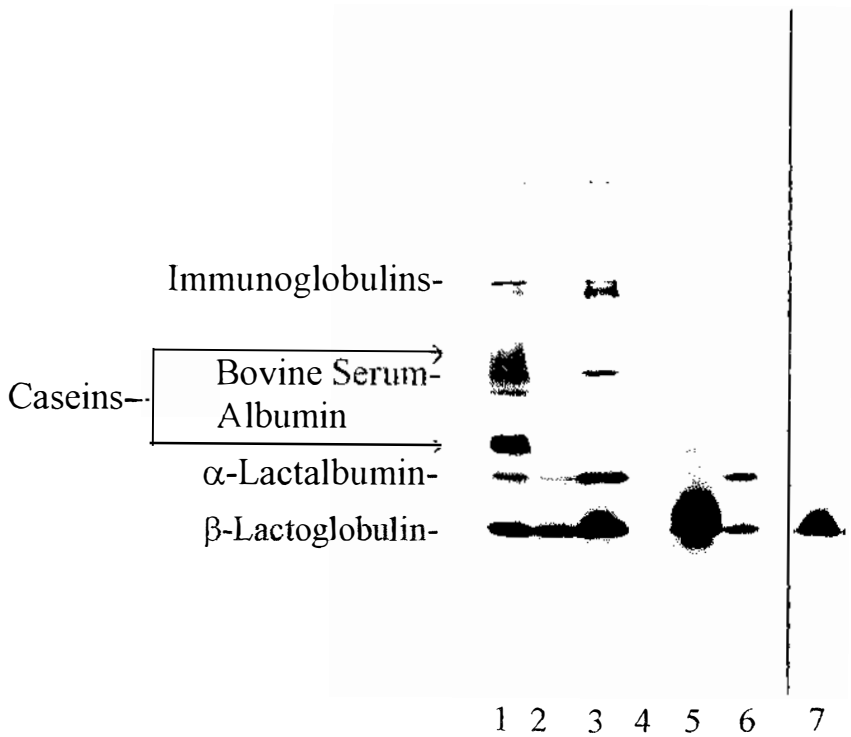
4) ultrafiltrate obtained from stirred cell ultrafiltration (1/2 dilution)

5) pooled BLG fractions obtained from size-exclusion chromatography (1/2 dilution)

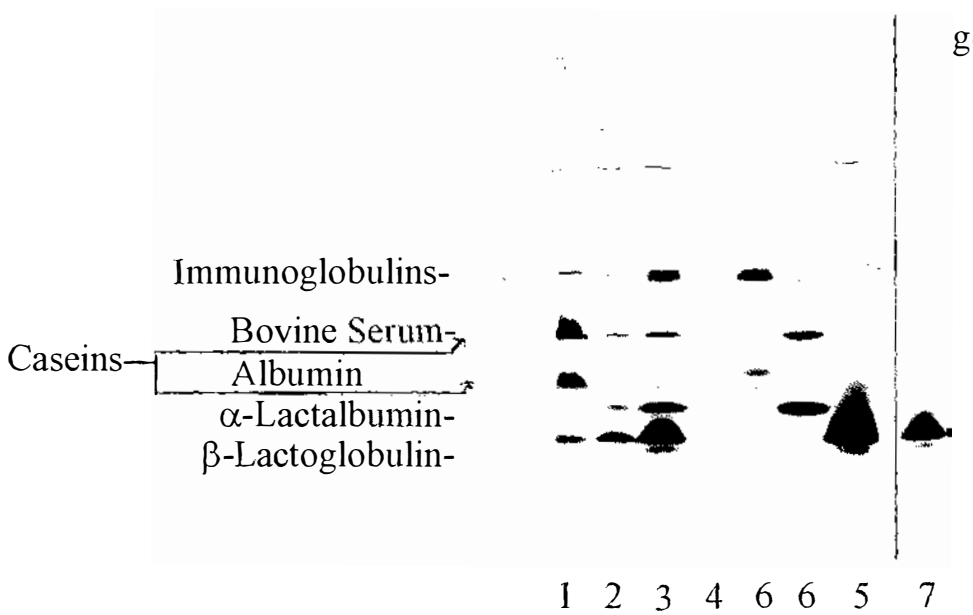
6) remaining fractions obtained from size-exclusion chromatography (1/2 dilution).

All samples were diluted with alkaline native-PAGE sample buffer (Section 3.2.1.2), electrophoresed, stained/destained and photographed as described in Sections 3.2.1.2 - 3.2.1.4.

gel 1


Immunoglobulins-
Caseins
Bovine Serum-
Albumin
α-Lactalbumin-
β-Lactoglobulin-
1 2 3 4 5 6 7

gel 2


Immunoglobulins-
Caseins
Bovine Serum-
Albumin
α-Lactalbumin-
β-Lactoglobulin-
1 2 3 4 6 6 5 7

centrifugation steps are required in the early stages of preparation when volumes of material are high. Furthermore, it is unlikely that the proteolytic enzymes in milk (e.g. the aspartate proteinase chymosin) become inactive when BLG is prepared using Method II because pH values are not lowered below 6.0. Therefore, if purified BLG fractions contain traces of these enzymes, then protein hydrolysis could occur during the subsequent long-term storage of frozen solutions of pure BLG.

4.3.1.6. Ion-exchange Chromatographic Analysis of β-Lactoglobulins A, B and C Prepared Using Method I.

The alkaline native-PAGE results shown in Figs 4.3.1.1, 4.3.1.2 and 4.3.1.4 suggested that samples of BLG B prepared using Methods I, II and III were contaminated with BLG A. To investigate this further, the purities of samples of BLGs A, B and C prepared using Method I were also determined by ion-exchange chromatographic analysis. The chromatograms shown in Fig. 4.3.1.5 indicate that BLGs A, B and C are eluted after 13.2 min, 12.0 min and 11.4 min respectively. Therefore, the absence of a peak or shoulder on the trailing side of the BLG B peak (Fig. 4.3.1.5) suggests that samples of BLG B prepared using Method I were not contaminated with BLG A.

In summary, the gels shown in Figs 4.3.1.1 and 4.3.1.2 and the chromatograms shown in Fig. 4.3.1.5 indicate that pure BLG can be obtained when material prepared using the methods of Mailliart and Ribadeau-Dumas (1988) and Fox *et al.* (1967), which are part of Methods I and III respectively (Table 4.1.1), is purified further using size-exclusion chromatography.

4.3.2. SPECTROSCOPY.

As discussed in Section 4.1, several spectroscopic techniques were used to determine whether the BLG molecules in samples prepared using Methods I, II and III were native. Near UV CD spectroscopy was used to identify structural change in the vicinities of aromatic side chains, far UV CD spectroscopy was used to identify changes in secondary structure and fluorescence from molecules of the probe ANS bound to BLG was used to show changes in the structures of the solvent-accessible hydrophobic surfaces of this protein. The aim of this spectroscopic study was to identify which BLG samples gave similar or different spectra, thus suggesting structural similarities or differences respectively. A more complete interpretation of spectroscopic results obtained from samples of BLG will be given in later chapters.

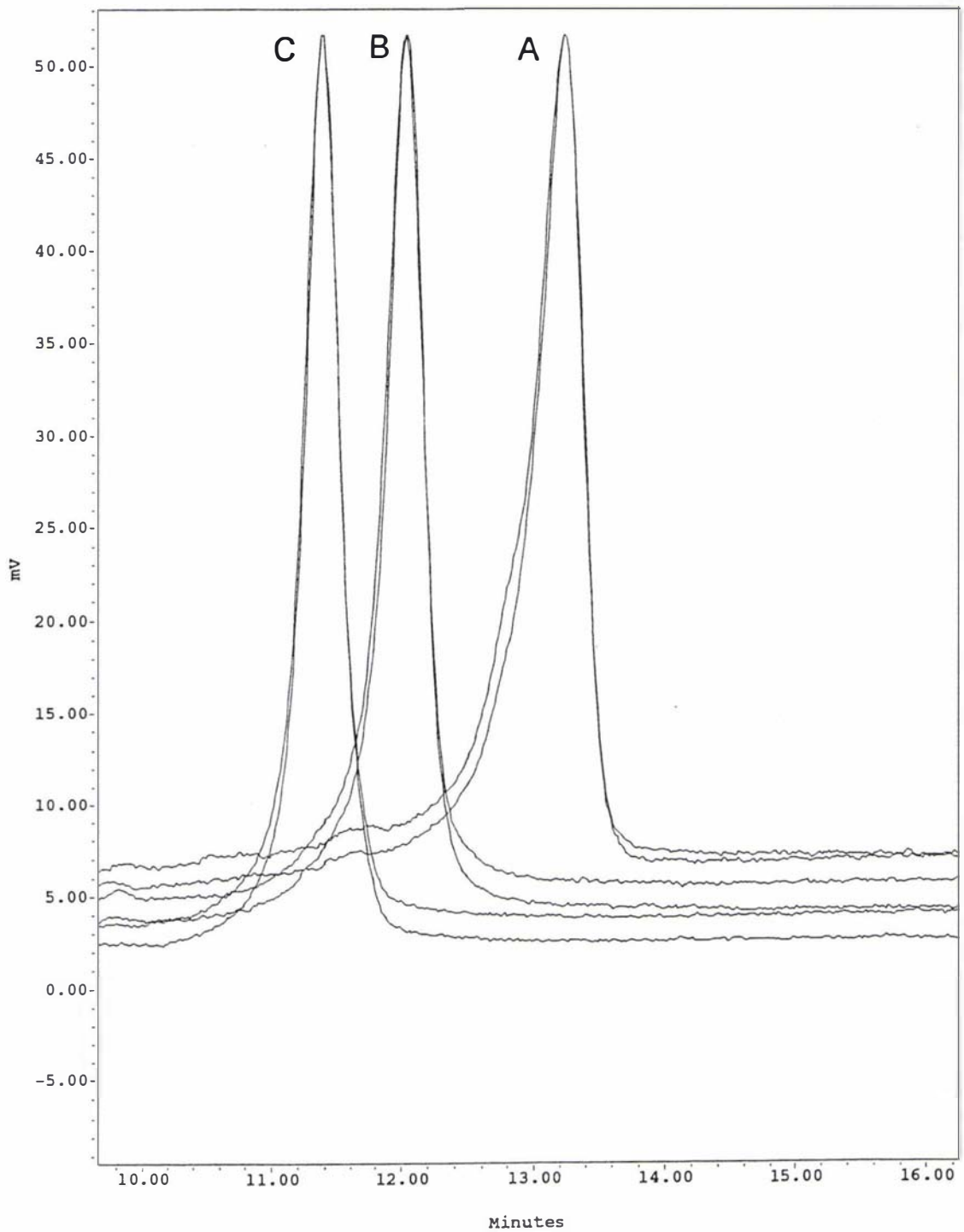


Fig. 4.3.1.5. Ion-exchange HPLC chromatograms of samples of BLGs A, B and C prepared using Method I. Duplicate traces for BLGs A, B and C are labelled A, B and C respectively. Proteins were separated on a 1 mL column of Resource-Q ion-exchange medium equilibrated with pH 6.0 piperazine buffer (20 mM piperazine, with 1 mM $CaCl_2$) using a linear gradient of NaCl (0.0 M-0.3 M NaCl, 0.3 M reached after 10 min, Section 4.2.1.7). The analyses were made using the HPLC system described in Section 3.1.2.3.

4.3.2.1. Near UV CD Spectroscopy.

The near UV CD spectra of BLGs A and B prepared using Methods I and II and BLG B prepared using Method III were standardised so that the intensities of $\Delta\varepsilon_{293}$ in all of the spectra of a particular variant were equivalent (Fig. 4.3.2.1a, b and c). Pairs of spectra were then overlaid and these overlays indicated that there was very little difference between the spectra of BLG samples prepared using Methods I and II (Fig. 4.3.2.1a and b for BLGs A and B respectively). For a particular variant, the standardised spectrum of the sample prepared using Method I was then subtracted from that of the sample prepared using Method II. In making this subtraction, it was assumed that non-zero values for CD in the resulting difference spectrum indicate that the structures of the BLG molecules in the two samples were different. Fig. 4.3.2.2 shows that positive and negative values for CD are distributed randomly about the line CD = 0 millidegrees. This suggests that for a particular variant, there is no significant difference between the spectra of samples prepared using Methods I and II. The significance of this will be discussed in Section 4.4.

There is also little difference between the standardised and overlaid spectra acquired from samples of BLG B prepared using Methods II and III (Fig. 4.3.2.1c). However, these two spectra do not superimpose particularly well in the vicinities of the bands at 267 nm and 277 nm. The significance of these spectral differences will be discussed in Section 4.4.

The near UV CD spectrum of BLG B prepared using Method II then heat-treated at 100 °C for 10 min (Fig. 4.3.2.1d) is different to those of the unheated samples. In the spectrum of the heat-treated sample, the intensities of the bands at 293 nm, 285 nm and 277 nm are less than those in the spectra of the unheated samples. Furthermore, in the spectrum of the heat-treated sample, the bands at 267 nm and 260 nm are not visible. These dissimilarities are emphasised in the difference spectrum (Fig. 4.3.2.2d) prepared by subtracting the spectrum of the heat-treated BLG B sample prepared using Method II from that of the unheated BLG B sample prepared using this method. In this difference spectrum, the values for CD are positive at all wavelengths. The spectrum of heat-treated BLG B shown in Fig. 4.3.2.1d therefore suggests that at least some of the aromatic side chains of this variant are shifted into less chiral environments as a consequence of heat treatment.

Fig. 4.3.2.1. Standardised near UV CD spectra of BLGs A and B. The spectra are as follows: BLG A prepared using Methods I (red) and II (green), panel a; BLG B prepared using Methods I (red) and II (green), panel b; BLG B prepared using Methods II (green) and III (red), panel c; BLG B prepared using Method II, both unheated (green) and heat-treated at 100 °C for 10 min (red), panel d. Spectra were acquired from approximately 0.75 mg/mL solutions at 20 °C in a 10 mm path length cell using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 50 nm/min, using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. Each final spectrum represents the average of five scans. The spectra were then manipulated as described in Section 3.2.4.2. Pairs of spectra of unheated samples were standardised as described in Section 4.2.2.1 using the J-720 software so that the intensities of CD at 293 nm in those of a particular variant were equivalent.

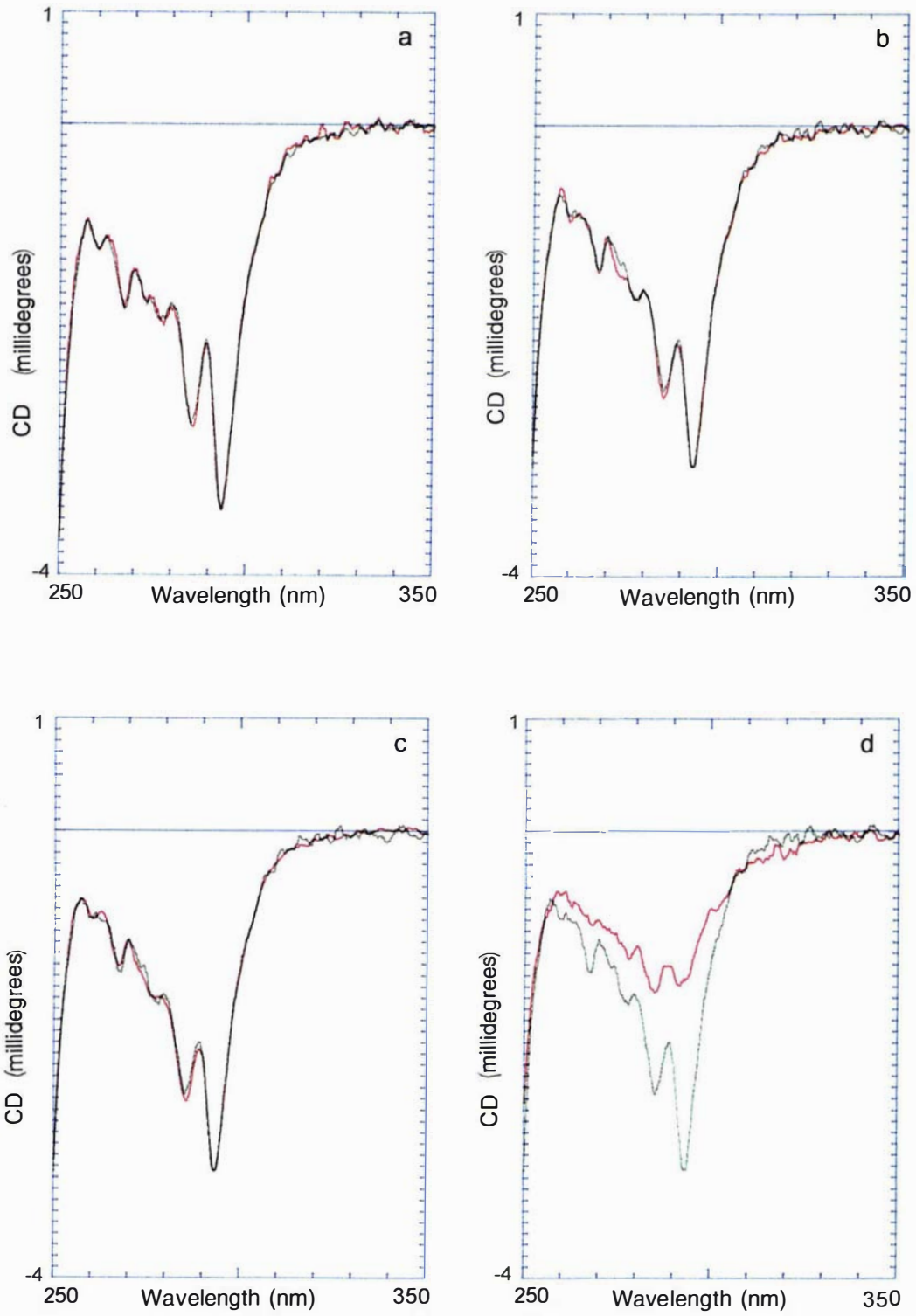
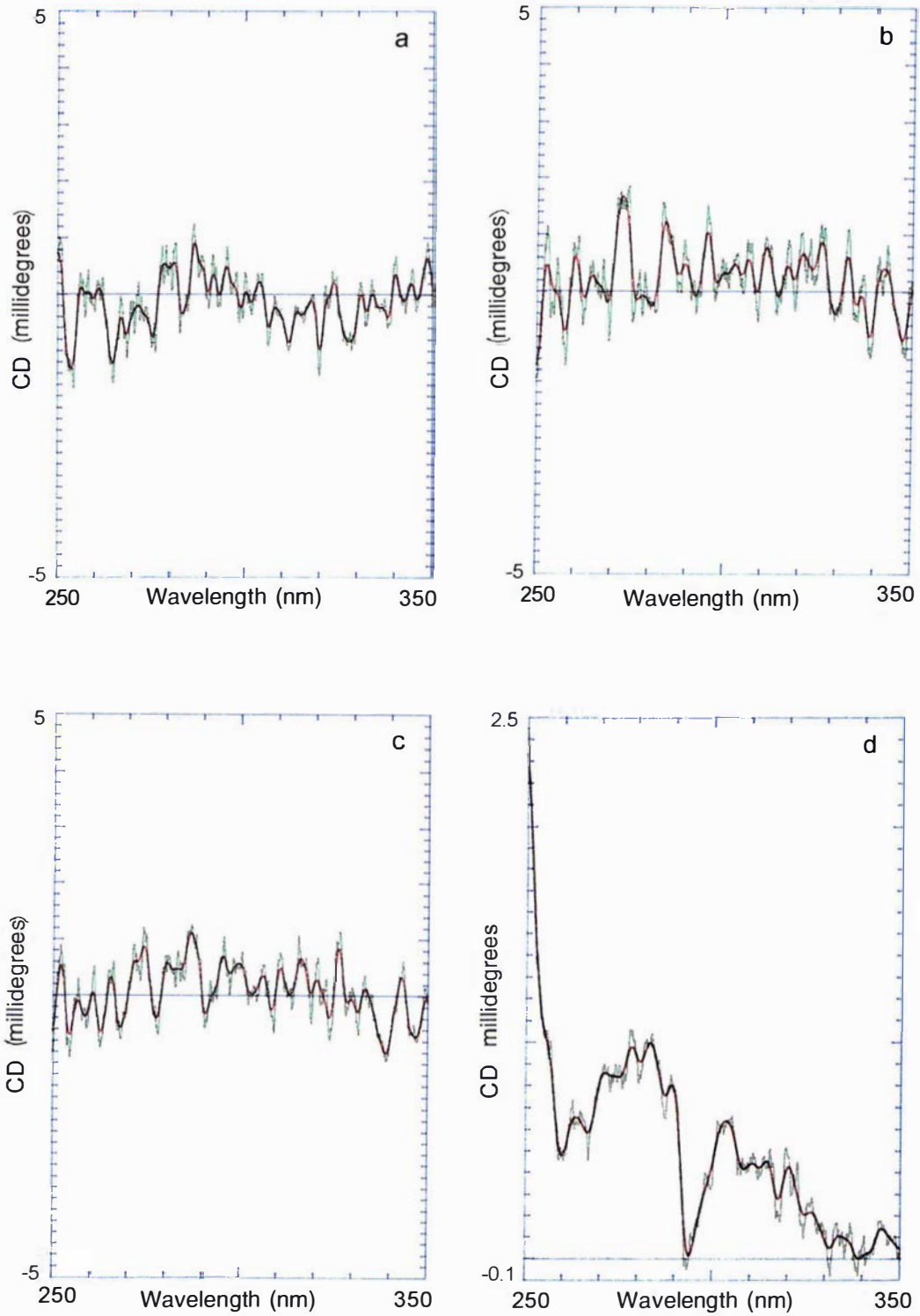

a
b
c
d
1
-4
CD (millidegrees)
250
Wavelength (nm)
350

Fig. 4.3.2.2. Difference near UV CD spectra for BLG A and for BLG B. The spectra are as follows: BLG A (Method II-Method I), panel a; BLG B (Method II-Method I), panel b; BLG B (Method II-Method III), panel c; BLG B (Method II, unheated-Method II, heat-treated), panel d. The green and red plots represent the raw and smoothed difference spectra respectively. The spectra of both unheated and heat-treated BLG samples were acquired, manipulated and standardised as described in Fig. 4.3.2.1 and then subtracted using the Jasco J-720 software (Section 4.2.2.1) and smoothed as described in Section 3.2.4.3.


a
5
-5
CD (millidegrees)
250
Wavelength (nm)
350
b
5
-5
CD (millidegrees)
250
Wavelength (nm)
350
c
5
-5
CD (millidegrees)
250
Wavelength (nm)
350
d
2.5
-0.1
CD millidegrees
250
Wavelength (nm)
350

4.3.2.2. Far UV CD Spectroscopy.

The far UV CD spectra of BLGs A and B prepared using Methods I and II and BLG B prepared using Method III were standardised so that the intensities of CD between 220 nm and 250 nm in all of the spectra of a particular variant were equivalent. These standardised spectra were then overlaid and overlays indicate that the spectra of samples prepared using Methods I and II (Fig. 4.3.2.3a and b for BLG s A and B respectively) are similar; only in the region from 196 nm to 185 nm do the spectra of a particular variant fail to superimpose. However, this may reflect the high absorbance of BLG solutions below approximately 200 nm. In the difference spectra for BLG A (Fig. 4.3.2.4a), values for CD between 250 nm and 196 nm are distributed randomly about the line CD = 0 millidegrees, while those in the wavelength range 196 nm-185 nm are all negative. The significance of these results will be discussed in Section 4.4.

The standardised far UV CD spectra of BLG B samples prepared using Methods II and III are quite different (Fig. 4.3.2.3c). The intensity of the positive band at approximately 194 nm in the spectrum of the sample prepared using Method III is less than that in the spectrum of the sample prepared using Method II. Furthermore, the λ_{min} of the negative band in the spectrum of the sample prepared using Method III may be at a wavelength lower than 218 nm, because between 215 nm and 205 nm, the spectrum of this BLG B sample is blue shifted by approximately 0.5 nm relative to those of the samples prepared using Methods I and II. The partial superposition of the spectra of BLG B samples prepared using Methods II and III is reflected in the corresponding difference spectrum (Fig. 4.3.2.4c), in which only positive values for CD are observed below 218 nm. The significance of this partial superposition will be discussed in Section 4.4.

In the far UV CD spectrum of the sample of BLG B prepared using Method II then heat-treated at 100 °C for 10 min, a new negative band with a λ_{min} at 205 nm is observed (Fig. 4.3.2.3d). Furthermore, the intensity of this new peak is greater than that of the one at 218 nm in the spectra of unheated BLG B samples prepared using Methods I, II and III. The intensity of the positive band at 194 nm in the spectrum of heat-treated BLG B is also less than that in the spectra of unheated samples. These two spectral differences cause the portion of the spectrum of the heat-treated sample between 215 nm and 185 nm to be blue shifted by 6 nm relative to that in the spectra of the unheated samples prepared using Methods I and II. In the difference spectrum (Fig. 4.3.2.4d), prepared by subtracting the spectrum of the heat-treated BLG B prepared using Method II from that of unheated BLG B prepared using Method II, a peak is observed at 200 nm. From these results and also those shown in Fig. 4.3.2.3d, it was concluded that the amount of random structure in this variant is increased irreversibly as a consequence of heat treatment.

Fig. 4.3.2.3. Standardised far UV CD spectra of BLGs A and B. The spectra are as follows: BLG A prepared using Methods I (red) and II (green), panel a; BLG B prepared using Methods I (red) and II (green), panel b; BLG B prepared using Methods II (green) and III (red), panel c; BLG B prepared using Method II, both unheated (green) and heat-treated at 100 °C for 10 min (red), panel d. Spectra were acquired from approximately 0.075 mg/mL solutions at 20 °C in a 1 mm path length cell using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 20 nm/min, using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. Each final spectrum represented the average of five scans. The spectra were then manipulated as described in Section 3.2.4.3. Pairs of spectra of unheated samples were standardised as described in Section 4.2.2.2 using the J-720 software so that the intensity of CD between 220 nm and 250 nm in those of a particular variant were equivalent.

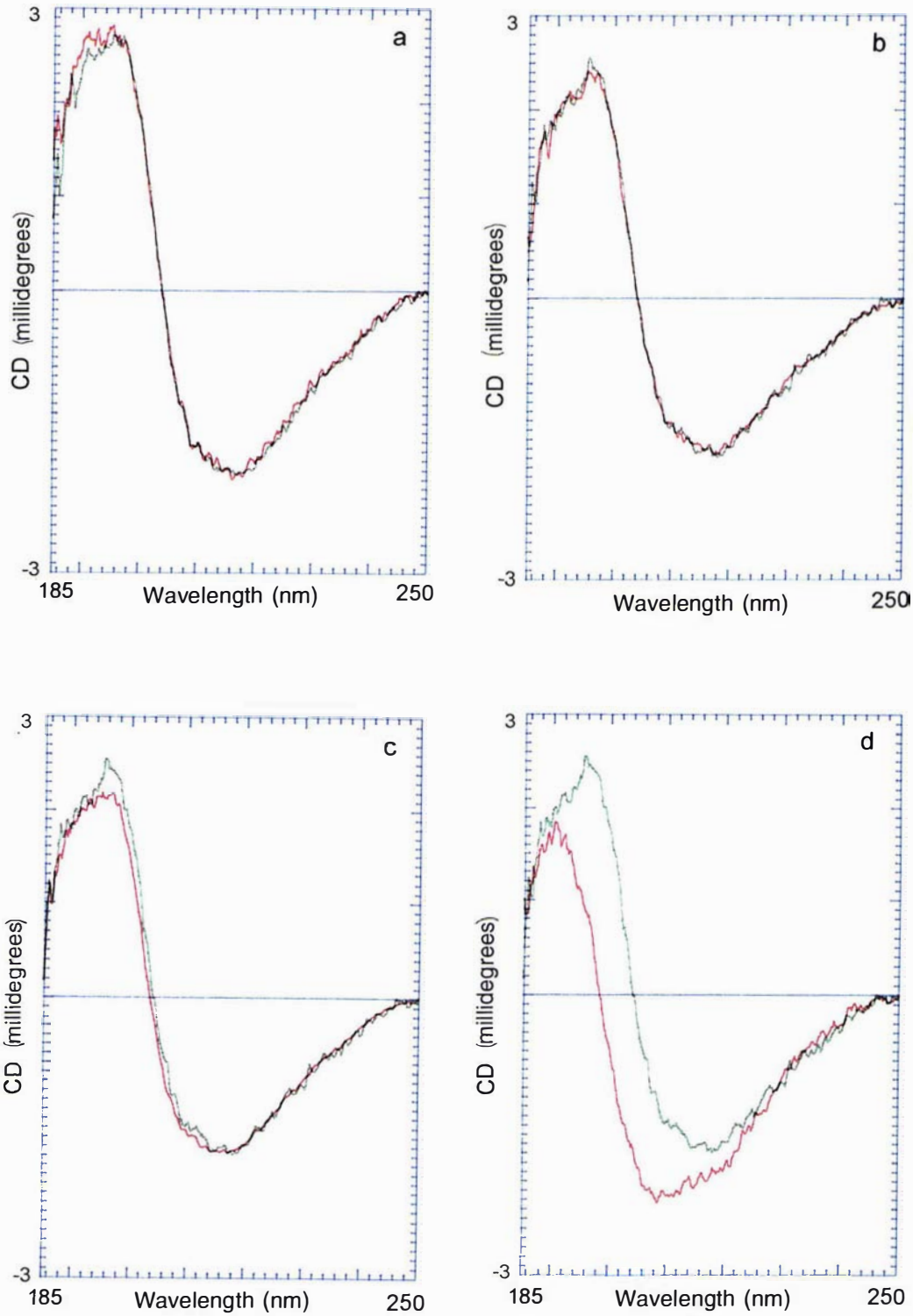
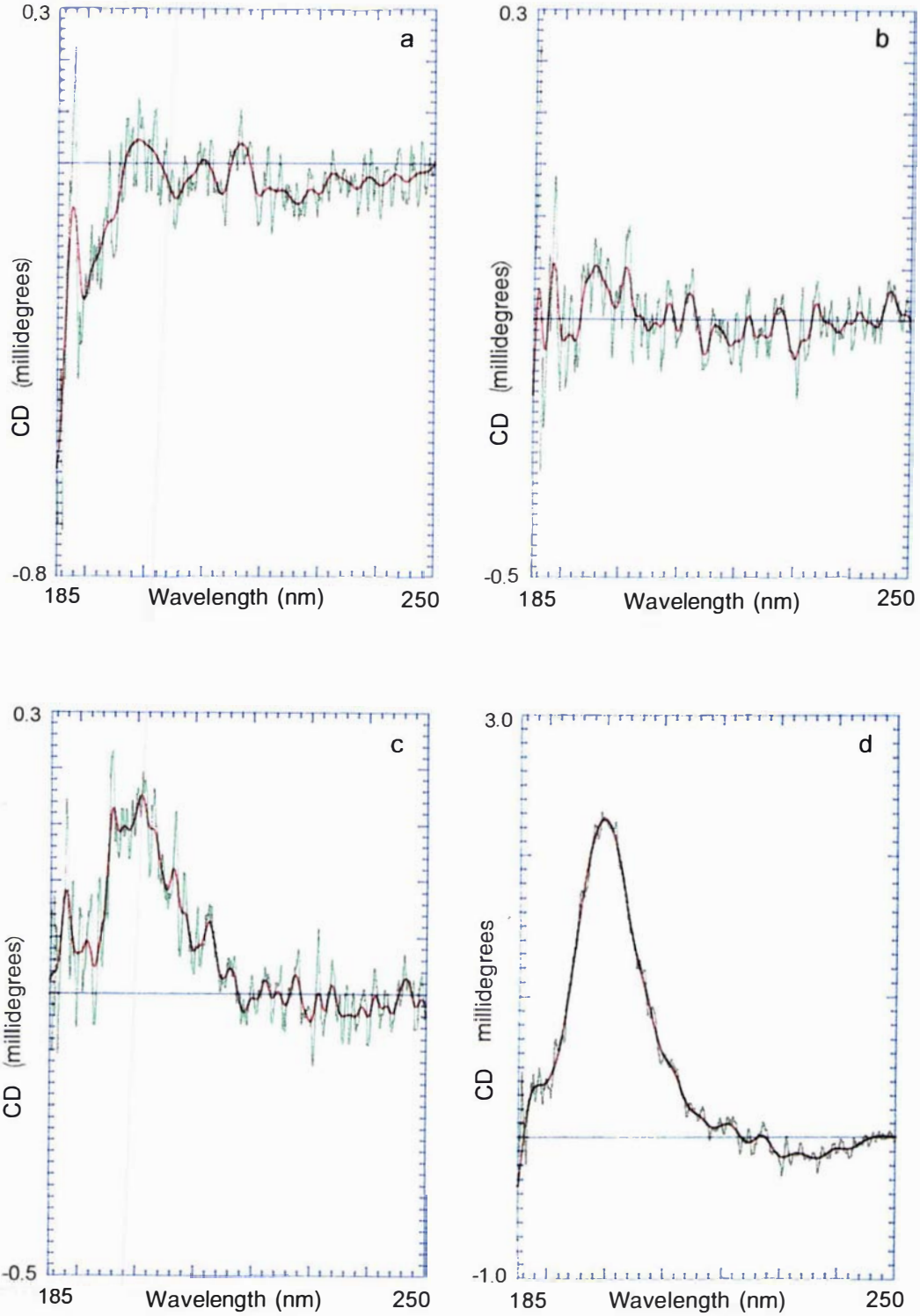

a
b
c
d
3
-3
CD (millidegrees)
185
Wavelength (nm)
250

Fig. 4.3.2.4. Difference far UV CD spectra for BLG A and for BLG B. The spectra are as follows: BLG A (Method II-Method I), panel a; BLG B (Method II-Method I), panel b; BLG B (Method II-Method III), panel c; BLG B (Method II, unheated-Method II, heat-treated), panel d. The green and red plots represent the raw and smoothed difference spectra respectively. The spectra of both unheated and heat-treated BLG samples were acquired, manipulated and standardised as described in Fig. 4.3.2.3 and then subtracted using the Jasco J-720 software (Section 4.2.2.2) and smoothed as described in Section 3.2.4.3.


a
0.3
-0.8
CD (millidegrees)
185
Wavelength (nm)
250
b
0.3
-0.5
CD (millidegrees)
185
Wavelength (nm)
250
c
0.3
-0.5
CD (millidegrees)
185
Wavelength (nm)
250
d
3.0
-1.0
CD millidegrees
185
Wavelength (nm)
250

4.3.2.3. ANS Fluorescence.

When mixtures of ANS and BLG were excited at 287 nm, an intense tryptophan emission peak (Lakowicz, 1983) was observed at approximately 330 nm (Fig. 4.3.2.5a, traces B and C). The other emission peak, which was centred at approximately 477 nm, was that of ANS (Slavìk, 1982).

During the titration of BLG with ANS, the intensity of the ANS emission peak increased as the mole ratio ANS:monomeric BLG increased (Fig. 4.3.2.5a, traces A, B and C). However, when a solution of aqueous tryptophan, as NATA, was titrated with ANS, no emission peak at approximately 477 nm was observed, indicating that the enhancement of ANS emission intensity was due to the presence of BLG. The fluorescence emission spectrum of NATA in the presence of 1.0 equivalent of ANS is shown in Fig. 4.3.2.5b.

When samples of BLGs A and B prepared using Methods I and II were titrated with ANS, emission intensity at approximately 477 nm increased in a near-linear manner with increasing mole ratio ANS:monomeric BLG (Fig. 4.3.2.6a and b). In addition, both the slopes and intercepts of the two plots are similar. Only the last point in the plot of data for the sample of BLG A prepared using Method II appears anomalous. The significance of these similarities will be discussed in Section 4.4.

Results from the titration of BLG B prepared using Methods II and III with ANS are displayed in Fig. 4.3.2.6c. In these plots also, ANS emission intensity increases in a near-linear manner with increasing mole ratio ANS:monomeric BLG. However, at a mole ratio ANS:monomeric BLG of 1:1, the emission intensity from ANS bound to BLG B prepared using Method III is approximately twice that of ANS bound to BLG B prepared using Method II. The significance of this will be discussed in Section 4.4.

ANS titration results obtained from samples of BLG B prepared using Method II, both unheated and heat-treated at 100 °C for 10 min, are presented in Fig. 4.3.2.6d. At a mole ratio ANS:monomeric BLG of 1:1, the intensity of emission from ANS bound to heat-treated BLG B was approximately 100 × greater than that from ANS bound to unheated BLG B prepared using Method II. This large difference suggests that heat treatment leads to irreversible structural change in molecules of BLG B.

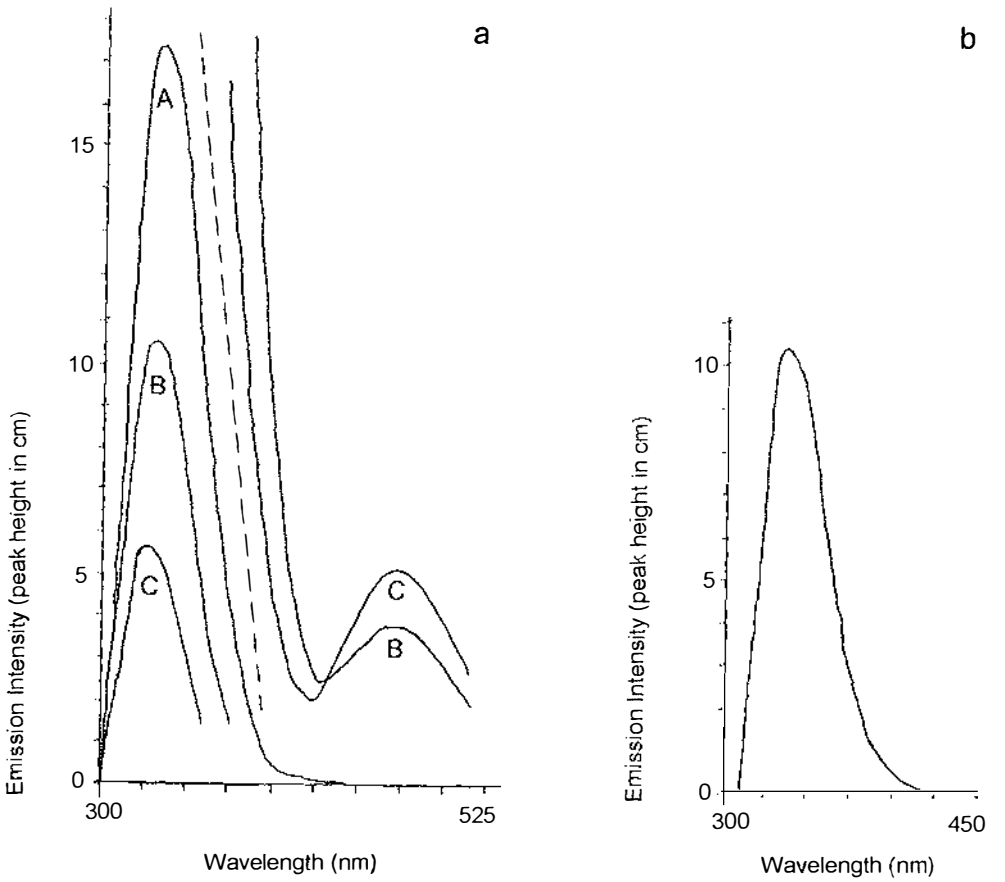


Fig. 4.3.2.5. Fluorescence emission spectra of BLG B in the absence and in the presence of 0.5 and 1.0 equivalents of ANS per BLG monomer, and that of N-acetyltryptophanamide (NATA) in the presence of 1.0 equivalent of ANS. The spectra of BLG B recorded in the absence, trace A; and in the presence of 0.5, trace B; and 1.0, trace C; equivalents of ANS are shown in panel a. The portions of spectra recorded at sensitivities 6 and 4 are shown on the left-hand and right-hand sides of the dotted line respectively. The spectrum of NATA in the presence of 1.0 equivalents of ANS is shown in panel b. Emission spectra were recorded between 310 nm and 510 nm using an excitation wavelength of 287 nm and excitation and emission band widths of 16 nm, at a scan speed of 25 nm/min and a chart speed of 1 cm/min.

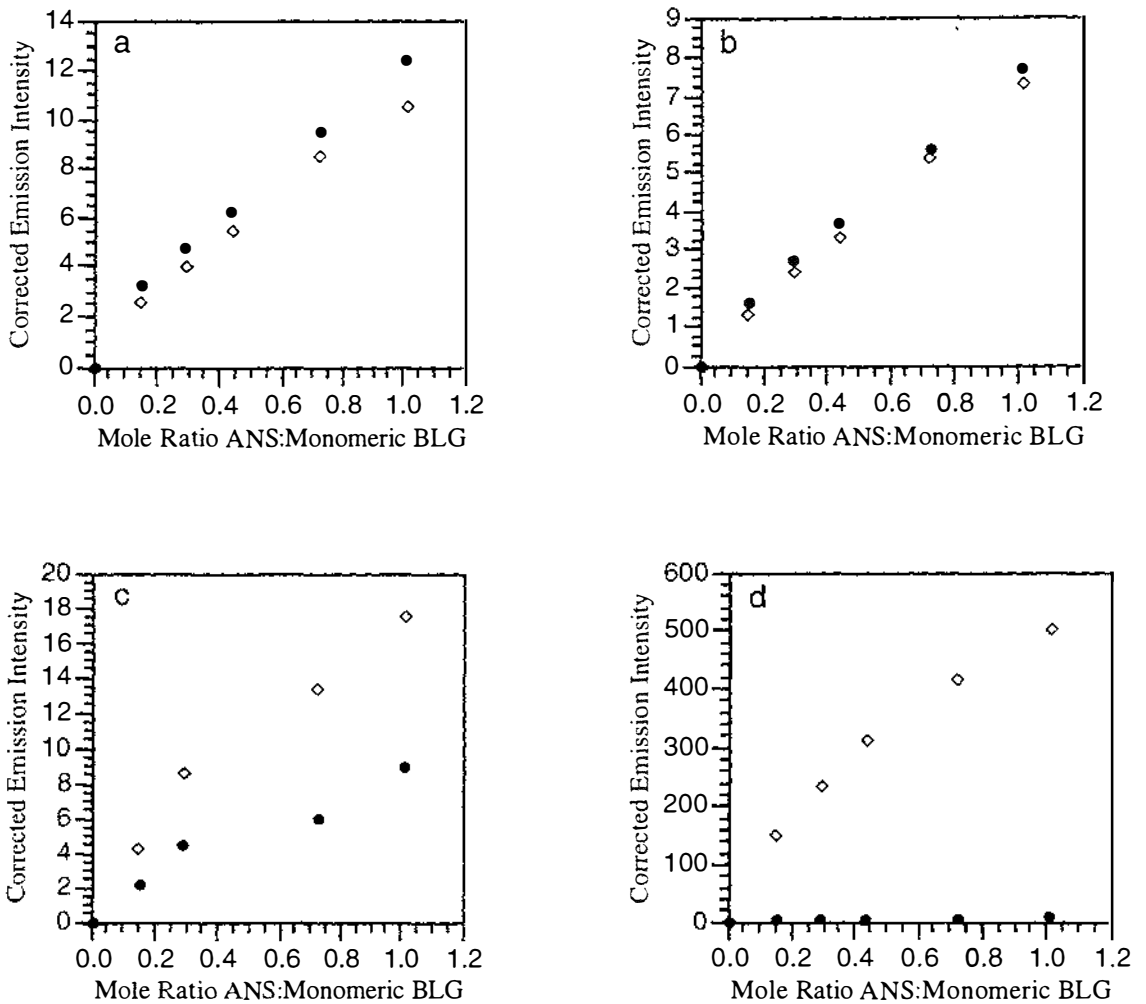


Fig. 4.3.2.6. Plots of ANS emission intensity at λ_{max} versus mole ratio ANS:monomeric BLG. The plots are as follows: BLG A prepared using Methods I (◇) and II (•), panel a; BLG B prepared using Methods I (◇) and II (•), panel b; BLG B prepared using Methods II (•) and III (◇), panel c; BLG B prepared using Method II, unheated (•) and heat-treated at 100 °C for 10 min (◇), panel d. ANS emission intensity values were determined from chart recordings (Section 4.2.2.3) and represent the height (in cm) of the emission peak at λ_{max} in spectra collected as described in Fig. 4.3.2.5. These emission intensity values were then corrected for the absorption of exciting and emitted radiation by ANS (inner filter effects) as described in Section 4.2.2.3 and variations in protein concentration amongst the samples.

4.4. DISCUSSION.

The standardised near and far UV CD spectra obtained from BLG samples prepared using Methods I and II are very similar (Figs 4.3.2.1a and b and Figs 4.3.2.3a and b). Furthermore, both the slopes and y intercepts of plots of ANS emission intensity versus mole ratio ANS:monomeric BLG for these samples are similar (Figs 4.3.2.6a and b). These results indicate that either the structures of the BLG molecules in these samples were equivalent, or the selected spectroscopic techniques were not sensitive enough to detect structural differences.

Significant differences were, however, identified in the far UV CD spectra of BLG B samples prepared using Methods II and III, λ_{min} being 218 nm and 215 nm respectively (Fig. 4.3.2.3c). Furthermore, at a mole ratio ANS:monomeric BLG of 1:1, the intensity of fluorescence from ANS bound to BLG prepared using Method III was approximately twice that from ANS bound to BLG prepared using Method II. Thus, a comparison of results obtained from BLG B samples prepared using Methods II and III suggests that the selected spectroscopic techniques were sensitive enough to detect structural differences in BLG molecules in samples prepared in different ways.

The differences between spectroscopic results obtained from unheated and heat-treated samples of BLG B prepared using Method II (Figs 4.3.2.1d, 4.3.2.2d, 4.3.2.3d, 4.3.2.4d and 4.3.2.6d) were similar to those between spectroscopic results obtained from samples of BLG B prepared using Methods II and III (Figs 4.3.2.1c, 4.3.2.2c, 4.3.2.3c, 4.3.2.4c and 4.3.2.6c), but the magnitude of differences in results were greater in the case of the former. Firstly, the bands between 277 nm and 267 nm in the near UV CD spectrum of unheated BLG B prepared using Method II were sharper than those in the spectrum of BLG B prepared using Method III, which were in turn sharper and more intense than those in the spectrum of BLG B prepared using Method II then heat-treated. Secondly, the standardised far UV CD spectrum of the heat-treated BLG B sample did not superimpose on that of BLG B prepared using Method II as well as that of BLG prepared using Method III. Thirdly, at the conclusion of ANS titrations, the intensity of emission from ANS bound to heat-treated BLG B and BLG B prepared using Method III was approximately 100 × and 2 × greater respectively than that from ANS bound to BLG B prepared using Method II. The similarities in the trends in these comparisons therefore suggest that minor irreversible structural change in BLG molecules occurs when this protein is prepared using the TCA fractionation method of Fox *et al.* (1967), which is part of Method III. In contrast, the spectroscopic results obtained from samples prepared using

Methods I and II are similar. Therefore, it is likely that the extent of irreversible structural change which occurs when BLG is prepared using the salt fractionation method of Mailliart and Ribadeau-Dumas 1988), which is part of Method I, is less than that which occurs when BLG is prepared using the method of Fox *et al.* (1967).

In conclusion, Method I, in which BLG is purified using the salt fractionation procedure of Mailliart and Ribadeau-Dumas (1988) and then purified further by size-exclusion chromatography, appears to be very appropriate for preparing gram quantities of pure native lipid-free BLG for use in the studies in later chapters of this thesis for the following reasons:

1) PAGE results (Fig. 4.3.1.1) indicate that BLG can be purified to homogeneity using this method.

2) Spectroscopic results (Figs 4.3.2.1 - 4.3.2.4 and 4.3.2.6) suggest that irreversible structural change does not occur when BLG is prepared using this method.

3) The adjustment of pH values to 2.0 during the salt fractionation portion of Method I ensures that the purified BLG is lipid-free (Section 4.1).

The spectroscopic results obtained for samples prepared using Methods I and II suggested that the structures of BLG molecules in these samples were similar. However, a number of time-consuming centrifugation steps are required in the initial stages of Method II, when volumes of material are still high. Furthermore, protein hydrolysis may occur during the long-term storage of frozen solutions of BLG prepared using Method II because the conditions used in this method do not lead to the inactivation of the proteolytic enzymes of milk (Section 4.3.1.5). For these reasons, Method II was considered inappropriate for preparing BLG for use in the studies in later chapters of this thesis. In the case of the measurements discussed in this chapter, protein hydrolysis was not considered a concern because spectroscopic data were acquired within 1 month of the conclusion of preparation.

Method III appears very appropriate for preparing gram quantities of pure lipid-free BLG. However, far UV CD results (Figs 4.3.2.3 and 4.3.2.4) suggest that some irreversible structural change occurs when BLG is prepared using this method. Thus, with respect to preparing BLG for use in the studies in later chapters of this thesis, there appeared to be no advantage in using Method III in preference to Method I, the selected method.

Chapter 5.

EXTENTS OF HEAT-INDUCED IRREVERSIBLE STRUCTURAL CHANGE IN β-LACTOGLOBULIN MOLECULES.

5.1. OVERVIEW OF DATA COLLECTION PROTOCOLS.

5.1.1. CD AND FLUORESCENCE MEASUREMENTS.

As part of the study presented and discussed in this chapter, CD and fluorimetry were used to study heat-induced irreversible structural change in molecules of BLGs A, B and C. Solutions at either pH 6.7 or pH 7.4 were heat-treated at different temperatures and then after cooling, spectra were recorded at 20 °C. All near and far UV CD, intrinsic protein and ANS fluorescence data for a particular variant at a particular pH value were acquired from the same set of heat-treated solutions over a period of one week. Typically, BLG solutions were heat-treated on Monday, near and far UV CD spectra were collected on Tuesday and Wednesday, and fluorimetric measurements were made on Thursday and Friday. Spectra were always recorded in order of increasing solution heat treatment temperature. A sufficient quantity of each heat-treated solution was prepared so that fluorimetric measurements, using 3.0 ml volumes of 1.00 mg/mL BLG solution, could be made in duplicate. For solutions heat-treated at a particular temperature, duplicate measurements were made on the same day. Because CD and fluorimetric measurements were made over two consecutive days, adjustment factors were used to standardise data collected on Day 2 relative to those collected on Day 1.

Extents of irreversible structural change in molecules of BLG A in solutions heat-treated at pH 8.1 were not measured using the protocol outlined above. At this pH value, BLG underwent slow time-dependent conformational changes at temperatures as low as 4 °C, and it was therefore important that the time between pH adjustment and data acquisition be kept to a minimum. This was achieved by raising the pH of solutions to 8.1, heat-treating, and recording spectra all on the same day. The complete set of data acquired from a particular series of heat-treated solutions was obtained over three consecutive days. Thus, for measurements made at pH 8.1, adjustment factors were used to standardise data collected on Days 2 and 3 relative to those collected on Day 1.

Data were collected from BLGs A, B and C over a period of 7 months, and in the following order: BLG A (pH 6.7, then pH 7.4 and lastly pH 8.1), then BLG B (pH 6.7, then pH 7.4), and lastly C (pH 6.7, then pH 7.4).

5.1.2. CONFIRMATION OF CD AND FLUORESCENCE RESULTS.

A number of additional CD and fluorimetric measurements were made to confirm that the results obtained as outlined in Section 5.1.1 were valid. For example, the observed differences in the thermostabilities of BLGs A, B and C may have been caused by variations in instrument performance over the 7 month data collection period, and not by differences in the structures of these three variants. Additionally, the need to standardise data collected on Day 2 relative to those collected on Day 1 may have affected the results obtained in the studies outlined in Section 5.1.1. To ensure that complete sets of data for all three variants at a particular pH value were collected in a single day, extents of irreversible structural change in BLG molecules in solutions of a particular variant previously heat-treated at only six different temperatures were measured. Solutions were heat-treated on Monday, CD spectra were recorded on Tuesday and fluorimetric measurements were made on Wednesday. Confirmatory measurements were made at various stages of the project using different batches of purified BLGs A, B and C.

5.1.3. THIOL AVAILABILITY.

The availability of the thiol group of BLG for reaction with DTNB was also used to measure extents of irreversible structural change in molecules of this protein in previously heat-treated solutions. All the data for a particular variant at a particular pH value were collected on the same day, and in duplicate. The six data sets were collected sequentially, over a one month period, in the following order: BLGs A then B then C at pH 6.7 and then BLGs A then B then C at pH 7.4.

5.1.4. CONFIRMATION OF THIOL AVAILABILITY RESULTS.

After the first set of thiol availability measurements had been made, additional confirmatory data were collected. These additional thiol availability measurements were made using solutions of each variant which had been heat-treated at only six different temperatures. However, in contrast to CD and fluorimetric confirmatory studies, thiol availability data for a particular variant at a particular pH value were collected on separate days, and in quadruplicate. The complete set of confirmatory measurements was made over a period of 1 week.

Both the measurements outlined in Section 5.1.3 and the confirmatory measurements discussed in this section were made towards the end of the project using the same batches of purified BLGs A, B and C. Confirmatory measurements were made in quadruplicate so that the magnitude of random error associated with thiol availability measurements could be determined.

The data collection protocols used in the confirmatory studies and the main study outlined in Section 5.1.3 were different. In the original protocol for determining extents of BLG thiol availability used in the main study (described in detail in Section 5.2.3), it was difficult to obtain consistent A_{412} values from solutions heat-treated at temperatures above approximately 80 °C. These inconsistencies were believed to be caused by evaporation of water from solutions during heat treatment. Thus, in the protocol used for confirmatory measurements, water lost through evaporation was replaced before A_{412} values were measured.

5.1.5. ALKALINE NATIVE AND SDS POLYACRYLAMIDE GEL ELECTROPHORESIS.

Extents of aggregation in BLG solutions heat-treated in a similar manner to those used in spectroscopic measurements were examined using alkaline native-PAGE and SDS-PAGE. Concentrations of covalently-linked aggregates were determined using disulphide-intact (i.e. non-reducing) SDS-PAGE, while concentrations of non-covalently-linked aggregates were determined by calculating differences between band intensities on alkaline native and disulphide-intact SDS gels. In addition, a 2D-PAGE technique (alkaline native-, then disulphide-intact SDS-PAGE) was used to resolve species with different M_r values from aggregates which contained both covalent and non-covalent links. Finally, disulphide-reduced (i.e. reducing) SDS-PAGE was used to confirm that all covalently-linked aggregates produced during heat treatment contained only disulphide bonds. Samples of BLGs A, B and C were heat-treated, and then electrophoresed, on both alkaline native and 2-ME-free SDS gels, within a 10 hr period. Measurements were made on pH 7.4 samples in a similar manner, but on a different day.

Alkaline native-PAGE and disulphide-intact SDS-PAGE were also used to study time-dependent changes in the concentrations of BLG monomer and aggregate species during a 2.5 hr heat treatment period. Measurements were made in a similar manner to that described above.

5.2. METHODS.

5.2.1. CD AND FLUORIMETRY.

5.2.1.1. Preparation of Protein for Heat Treatment.

a) pH 6.7 and pH 7.4:

Frozen solutions of BLG A, B or C were thawed at 4 °C, dialysed against 4 changes of either pH 6.7 or pH 7.4 phosphate buffer (Section 3.2.3.1) at 4 °C and then filtered. β-Lactoglobulin concentrations were then determined from measured A_{280} values as described in Section 4.2.1.7, and ranged from 2.90 mg/mL to 5.36 mg/mL.

b) pH 8.1.

Solutions of BLG A were dialysed against 4 changes of water and then filtered. At the beginning of days on which spectroscopic measurements were made, dialysed protein was then diluted 1.29-fold with concentrated pH 8.1 phosphate/borate buffer (Section 3.2.3.2). Thus, after dilution, the pH, the buffer and the salt concentrations of BLG A solutions had been raised to 8.1 ± 0.1 (preliminary results, data not shown), 26 mM and 68 mM respectively. β-Lactoglobulin concentrations were then determined from measured A_{280} values, and ranged from 2.78 mg/mL to 3.47 mg/mL.

5.2.1.2. Heat Treatment.

Between 21 and 26 aliquots (depending on the sample) of BLG solution were heat-treated at temperatures 2 °C apart in the range 40 °C - 94 °C. Each heat-treated solution was prepared by immersing a stoppered 5 mL glass test tube, containing between 1.50 mL and 2.75 mL BLG solution, in the Neslab waterbath pre-equilibrated to the required temperature. The temperature of the BLG solution in each tube was monitored continuously during heat treatment, using the Kane-May thermocouple. Prior to the immersion of each tube in the waterbath, the position of the tip of the probe was adjusted to ensure that it was located in the centre of the tube, and approximately 5 mm from the bottom. Tube contents took between 2.25 min and 2.5 min to equilibrate to within 0.1 °C of the final temperature. Maximum differences between thermal equilibration times for solutions heat-treated at 50 °C and 90 °C were approximately 15 s. Tubes were then left in the bath for a further 10 min, withdrawn, and cooled rapidly by placing in ice flakes. After cooling, the concentrations of the heat-treated solutions were determined (by A_{280}) so that they could be diluted, with pH 6.7 or pH 7.4 phosphate buffer, or dilute pH 8.1 phosphate/borate buffer (Section 3.2.3.3), to 1.00 mg/mL. Spectroscopic measurements were made on solutions at pH 6.7 and pH 7.4 between 16 hr and 4 days after heat treatment. Except during measurements, protein solutions were stored at either 4 °C or in ice flakes during this period. As outlined in Section 5.1.1, solutions at pH 8.1 were prepared, heat-treated and analysed on the same day.

5.2.1.3. Near UV CD Spectroscopy.

The near UV CD spectra of heat-treated BLG solutions were recorded using the procedure described in Section 3.2.4.2. However, because the spectra of the complete set of heat-treated solutions of a particular variant at a particular pH value were obtained over two consecutive days, the spectrum of the solution recorded last on Day 1 was recorded again first on Day 2 so that data collected on Day 2 could be adjusted relative to those collected on Day 1.

Treatment of Data.

Values for $\Delta\varepsilon_{293}$ determined on Day 2 were adjusted relative to those determined on Day 1 by multiplying by a data adjustment factor. This factor was the ratio of Day 1 to Day 2 $\Delta\varepsilon_{293}$ values in the two spectra acquired from the same sample. Values for $\Delta\varepsilon_{270}$ determined from data collected on Day 2 were also adjusted relative to those determined from data collected on Day 1 by multiplying by the appropriate data adjustment factor determined using $\Delta\varepsilon_{293}$ data. Values for the data adjustment factor ranged from 0.94 to 1.07.

5.2.1.4. Far UV CD Spectroscopy.

The heat-treated BLG solutions used for near UV CD spectroscopy (Section 5.2.1.3) were diluted 6-fold with water prior to far UV CD spectroscopy. Measurements were then made using the 0.5 mm path length cell and the procedure described in Section 3.2.4.3. The complete set of far UV CD measurements for a particular variant at a particular pH value was also made over two consecutive days. Therefore, to permit adjustment of data collected on Day 2 relative to those collected on Day 1, the spectrum of the solution recorded last on Day 1 was recorded again first on Day 2.

Treatment of Data.

Data were treated in a manner similar to that described in Section 5.2.1.3. However, values for $[\theta]_{216}$ and $[\theta]_{205}$, determined from data collected on Day 2 were adjusted relative to those determined from data collected on Day 1 using separate data adjustment factors (i.e. $[\theta]_{216}$(Day 1):$[\theta]_{216}$(Day 2) and $[\theta]_{205}$(Day 1):$[\theta]_{205}$(Day 2)). Values for data adjustment factors for $[\theta]_{216}$ data ranged from 0.83 to 0.95, while those for $[\theta]_{205}$ data ranged from 0.93 to 1.13.

5.2.1.5. Fluorescence Spectroscopy.

The heat-treated BLG solutions on which near UV CD measurements had been made (Section 5.2.1.3) were also used for intrinsic protein and ANS fluorescence studies. All fluorimetric measurements were made in duplicate.

Two fluorimeter cells, each containing 3 mL of heat-treated BLG solution, were stoppered, placed in positions 3 and 4 of the fluorimeter cell holder, and left to equilibrate to 20 °C for 5 min. The cell in position 4 was then excited at 295 nm and the emission spectrum was recorded. Data were acquired using excitation and emission slit widths of 8 nm, at sensitivity 4 or 5, a scan speed of 25 nm/min and a chart speed of 1 cm/min. Measurements were made from 280 nm to 400 nm. The cell placed initially in position 3 was then quickly transferred to position 4 and the emission spectrum was obtained as above. For both samples in each pair, emission spectra were then recorded again, this time using an excitation wavelength of 275 nm, as described above.

Once intrinsic protein fluorescence measurements had been made, a 60 μL volume of 1.41 mM ANS solution was mixed into each 3 mL aliquot of BLG solution (mole ratio ANS:monomeric BLG of 0.5:1). The contents of each cell were mixed thoroughly by inversion and then returned to the cell block of the fluorimeter. After a thermal re-equilibration period of 5 min, the ANS was excited at 370 nm and emission spectra were recorded. Measurements were made using an excitation and emission slit width of 8 nm, at sensitivity 5 or 6, a scan speed of 25 nm/min and a chart speed of 1 cm/min, from 380 nm to 510 nm.

ANS and intrinsic protein fluorescence measurements were also made over two consecutive days. Therefore, the spectrum of the solution recorded last on Day 1 was recorded again first on Day 2.

Treatment of Data: Protein Tryptophan Fluorescence.

Values for emission λ_{max} as well as emission intensities at λ_{max} (I_{Trp}) were measured from chart recordings. All I_{Trp} values measured from spectra recorded at sensitivity 4 were then multiplied by 2.595 (the setting 4:setting 5 emission intensity sensitivity conversion factor, Section 3.2.5.3). In addition, all I_{Trp} data collected on Day 2 were adjusted relative to those collected on Day 1 by multiplying by a data adjustment factor, the ratio of I_{Trp} in the two spectra acquired from the same sample. Values for this data adjustment factor ranged from 0.80 to 0.95.

Treatment of Data: ANS Fluorescence.

Emission intensities (I_{ANS}) and λ_{max} values for the peak near 477 nm were determined as described above. All I_{ANS} data recorded at sensitivity 5 were then multiplied by the setting 5:setting 6 emission intensity sensitivity conversion factor (3.323). Furthermore, all I_{ANS} data collected on Day 2 were adjusted relative to those collected on Day 1 by multiplying by a data adjustment factor calculated as described above using I_{ANS} data. Values for the ANS fluorescence data adjustment factor ranged from 0.92 to 1.00.

5.2.2. CONFIRMATION OF CD AND FLUORESCENCE RESULTS.

Solutions of BLGs A, B and C at pH 6.7 or pH 7.4 were prepared for heat treatment as described in Section 5.2.1.1. However, aliquots of a particular variant solution at a particular pH value were heat-treated at only six different temperatures. These temperatures represent six different positions in plots of I_{ANS} (or $\Delta\varepsilon_{293}$) versus heat treatment temperature (Table 5.2.1).

Table 5.2.1. Selection of β-Lactoglobulin Heat Treatment Temperatures for use in Confirmatory Studies.

Temperature Number	Position on plot of I_{ANS} versus heat treatment temperature (percentage of maximum extent of irreversible spectral change).
1	0 %
2	20 %
3	40 %
4	60 %
5	80 %
6	100 %

All spectroscopic measurements were made as described in Sections 5.2.1.3 - 5.2.1.5 over a period of two days. Near and far UV CD measurements were made on Day 1, and intrinsic protein and ANS fluorescence measurements on Day 2. Data were treated using procedures similar to those described in Sections 5.2.1.3 - 5.2.1.5, but data adjustment factors were not needed.

5.2.3. THIOL AVAILABILITY.

5.2.3.1. Preparation of Protein for Heat Treatment.

Solutions of BLGs A, B or C were dialysed against 4 changes of pH 6.7 or pH 7.4 phosphate buffer, frozen, and then stored at -21 °C until required. When required, a frozen solution was thawed at 4 °C, transferred to a Büchner flask, and carefully degassed using a water pump. The BLG concentration in the degassed solution was then calculated from the measured A_{280} value, after which the solution was diluted to 1.65 mg/mL with phosphate buffer, either pH 6.7 or pH 7.4 as appropriate.

5.2.3.2. Heat Treatment.

Duplicate 3.0 mL volumes of BLG solution prepared as described in Section 5.2.3.1 were delivered into separate 8 mL screw-cap glass vials. Prior to sealing, air was displaced from the headspace of each vial using a stream of dry nitrogen. Pairs of vials were then immersed in the Neslab waterbath and heat-treated for 13.5 min using a procedure similar to that described in Section 5.2.1.2.

Because the solutions used in thiol availability determinations were heat-treated anaerobically, heat-treatment temperatures could not be measured directly. They were, however, estimated by measuring the temperature of a 3.0 mL volume of pH 6.7 phosphate buffer heated at the same waterbath temperatures as protein solutions.

5.2.3.3. Thiol Availability Determinations.

As soon as possible after cooling, 2.85 mL aliquots of each heat-treated BLG solution were delivered into clean 8 mL glass vials. Freshly prepared 7.77 mM DTNB solution (150 μL) was then mixed into each aliquot. Immediately after DTNB solution had been added to the second aliquot, a reference sample was prepared by mixing 2.85 mL phosphate buffer, pH 6.7 or pH 7.4, with 150 μL freshly prepared DTNB solution. Air was then displaced from the headspace of the three vials, as described above, prior to resealing.

Values for A_{412} for solutions heat-treated at pH 6.7 were read, against the corresponding reference sample, using 10 mm path length cells, between 30 min and 2.5 hr after DTNB addition. For solutions heat-treated at pH 7.4, values for A_{412} were read between 30 min and 40 min after DTNB addition. All A_{412} values for a particular set of heat-treated solutions were measured on the same day.

Reduced Glutathione Standard Solutions.

The value for A_{412} following the reaction between DTNB and a reduced glutathione solution which had a thiol concentration equal to that in a 1.65 mg/mL solution of BLG (assuming 2 thiol groups per dimer) was used to estimate the A_{412} which corresponded to 100% BLG thiol exposure.

A 150 μL volume of freshly prepared 2.01 mM reduced glutathione solution was mixed with 2.70 mL phosphate buffer, pH 6.7 or pH 7.4, and 150 μL freshly prepared 7.77 mM DTNB solution in a 3 mL cell. The absorbance at 412 nm was read against a reference sample consisting of 2.85 mL phosphate buffer of the same pH and 150 μL freshly prepared 7.77 mM DTNB solution. Reduced glutathione A_{412} values were measured daily.

Treatment of Data.

Reduced glutathione standard A_{412} values were used to normalise A_{412} values determined from heat-treated BLG solutions. The proportion of the total number of monomeric BLG thiols exposed following heat treatment at temperature *t* was calculated using equation 5.2.3.1, where solution *t* is the BLG solution heat-treated at temperature *t*.

$$\text{Percentage of Total Thiols Exposed in Solution } t = \frac{A_{412}(\text{Solution } t)}{A_{412}(\text{Reduced Glutathione Standard})} \times 100 \qquad (5.2.3.1)$$

5.2.3.4. Urea-induced Thiol Exposure in β-Lactoglobulin.

The extents of thiol exposure in solutions of BLG which occurred during heat treatment in the presence of DTNB were briefly compared to those in solutions of BLG in 5.0 M urea. A volume of BLG B solution at pH 6.7, previously dialysed against pH 6.7 phosphate buffer as described in Section 5.2.1.1, was concentrated to approximately 8 mg/mL using the stirred cell ultrafiltration apparatus described in Section 3.1.2.9. This solution was then diluted to 6.60 mg/mL with pH 6.7 phosphate buffer and a 1.0 mL volume was mixed into a 3.0 mL volume of 6.66 M urea. After a holding time of 10 min, 2.70 mL of this BLG solution was delivered into a spectrophotometer cell and mixed with 300 μL freshly prepared DTNB solution (7.77 mM). The A_{412} of this mixture was then read against a reference sample consisting of 2.70 mL of 5.0 M urea and 300 μL DTNB solution. The concentration of exposed thiols was calculated from the measured A_{412} value. When calculating this concentration, it was assumed that the ε of TNB (thionitrobenzoate, the species which absorbs at 412 nm) in 5.0 urea is equivalent to that in 6 M guanidine hydrochloride (i.e. $\varepsilon = 13\,700\ M^{-1}cm^{-1}$), (Riddles *et al.*, 1979). The A_{412} value for

1.65 mg/mL BLG B in 5.0 M urea was then compared with that obtained from a determination made using a reduced glutathione solution with a thiol concentration equivalent to that of the 1.65 mg/mL BLG solution (Section 5.2.3.3). For this determination, concentrations of TNB were calculated using the ε for aqueous solutions (14 150 $M^{-1}cm^{-1}$), (Riddles *et al.*, 1979).

5.2.4. CONFIRMATION OF THIOL AVAILABILITY RESULTS.

Solutions of BLGs A, B or C at pH 6.7 or pH 7.4 were each heat-treated, in quadruplicate, at six different temperatures and then reacted with DTNB. The extent of thiol exposure in each heat-treated sample was then determined by measuring the A_{412}. Heat treatment temperatures were selected as described in Section 5.2.2.

β-Lactoglobulin solutions were prepared for heat-treatment as described in Section 5.2.3.1. However, after vials had been sealed, they were weighed before being heat-treated as described in Section 5.2.3.2.

As soon as possible after cooling, a 150 μL volume of freshly prepared 7.77 mM DTNB solution was added to each of the four vials and mixed in. A reference sample was prepared immediately after DTNB solution had been added to the four BLG vials. The four BLG vials were then re-weighed and the mass of 150 μL DTNB solution (0.143 g) was subtracted from each. Where appropriate, the mass (up to 20 mg) of water lost during heat treatment was then added to the vials. After mass adjustment, air was displaced from the headspace of the sample and reference vials, which were then re-sealed. Values for A_{412} were then determined as described in Section 5.2.3.3.

Reduced Glutathione Standard Solutions.

The procedure for estimating the A_{412} value corresponding to exposure of 100 % of the thiol groups in a 1.65 mg/mL solution of BLG was identical to that described in Section 5.2.3.3. Similarly, data were treated as described in Section 5.2.3.3.

5.2.5. POLYACRYLAMIDE GEL ELECTROPHORESIS.

5.2.5.1. Heat Treatment.

β-Lactoglobulin solutions were prepared and then heat-treated using a procedure similar to that described in Section 5.2.2. Heat-treated solutions were then divided into three equal volumes, the first for alkaline native-PAGE (Section 5.2.5.2), and the remaining two for SDS-PAGE (Section 5.2.5.3).

5.2.5.2. Alkaline Native-PAGE.

Solutions set aside for alkaline native-PAGE were diluted to BLG concentrations of 0.50 mg/mL (pH 6.7 samples) or 0.75 mg/mL (pH 7.4 samples) with alkaline native-PAGE sample buffer. Aliquots (15 μL and 10 μL in the case of pH 6.7 and pH 7.4 samples respectively) of each diluted sample were then loaded onto 10-lane alkaline native gels. An unheated BLG B standard (15 μL of a 0.5 mg/mL solution or 10 μL of a 0.75 mg/mL solution in the case of pH 6.7 and pH 7.4 samples respectively) was also loaded onto each gel. No sample was loaded in the first or tenth lanes. The complete set of heat-treated BLG A, B and C samples at a particular pH value was accommodated on three gels. Gels were then run, stained, destained, photographed and scanned as described in Section 3.2.1.

5.2.5.3. SDS-PAGE.

All solutions set aside for SDS-PAGE were diluted with SDS-PAGE sample buffer to the concentrations used in Section 5.2.5.2. One sample from each pair was then treated with 2-ME as described in Section 3.2.2.2.

Aliquots (15 μL and 10 μL in the case of pH 6.7 and pH 7.4 samples respectively) of both 2-ME-treated (i.e. disulphide-reduced) and 2-ME-untreated (i.e. disulphide-intact) samples were then loaded onto 10-lane SDS gels. Eight samples were run on each gel: three disulphide-reduced samples, the three corresponding disulphide-intact samples, and two unheated BLG B standards (one disulphide-reduced, the other disulphide-intact). To minimise the diffusion of 2-ME from disulphide-reduced to disulphide-intact samples, gels were loaded as shown in Fig. 5.2.1. The complete set of heat-treated BLG A, B and C samples at a particular pH value, both disulphide-reduced and disulphide-intact, was accommodated on six gels. Gels were run, stained, destained, photographed and scanned as described in Section 3.2.2.

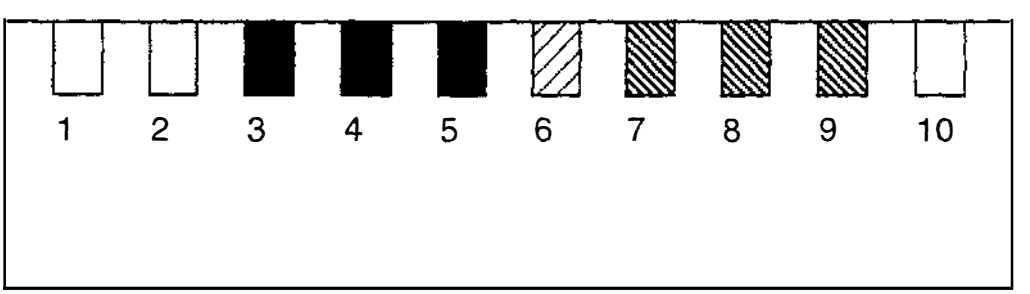


Fig. 5.2.1. Sample loading pattern for SDS gels. The disulphide-reduced BLG B standard ☐ was run in lane 2. Disulphide-reduced samples of heat-treated BLGs A, B and C ■ were run in lanes 3, 4, and 5 respectively. The disulphide-intact BLG B standard ▨ was run in lane 6. Disulphide-intact samples of heat-treated BLGs A, B and C ▧ were run in lanes 7, 8 and 9 respectively.

Samples of BLGs A, B and C at pH 6.7 were heat-treated and then electrophoresed on both alkaline native and SDS gels within a 10 hr period. The samples heat-treated at pH 7.4 were studied in a similar manner, but on a different day.

Concentrations of aggregate and monomeric species on both alkaline native and SDS gels were measured from the scanned images of gels as described in Section 3.2.1.5. Alkaline native-PAGE and SDS-PAGE data treatment protocols are discussed in Section 5.3.9.7.

5.2.6. TIME-DEPENDENT CHANGES IN CONCENTRATIONS OF AGGREGATE SPECIES DURING HEAT TREATMENT.

Both alkaline native-PAGE and disulphide-intact SDS-PAGE were used to study time-dependent changes in the concentrations of BLG monomer and aggregate species during a 2.5 hr heat-treatment period.

5.2.6.1. Heat Treatment.

Frozen solutions of BLGs A, B and C, which had been previously dialysed against 4 changes of pH 6.7 or pH 7.4 phosphate buffer were thawed at 4 °C. Aliquots (2.0 mL) of each were then delivered into 8 mL screw-cap glass vials. After sealing, vials were immersed in the Neslab waterbath which had previously been equilibrated to either 80 °C (pH 6.7 samples) or 74 °C (pH 7.4 samples). At various times after immersion, two 50 µl aliquots were withdrawn from each vial and delivered into clean 1.5 mL eppendorf tubes. Immediately after filling, these tubes were sealed and placed in ice flakes.

After the final aliquots had been withdrawn and cooled, one 50 µL aliquot from each heat-treated sample pair was diluted to a BLG concentration of 0.75 mg/mL with alkaline native-PAGE sample buffer, while the other was diluted to this concentration with SDS-PAGE sample buffer. Solutions of BLGs A, B and C which had not been heat-treated were diluted in a similar manner.

5.2.6.2. Alkaline Native-PAGE and Disulphide-intact SDS-PAGE.

Aliquots (10 µL) of each diluted BLG sample were loaded onto alkaline native and SDS gels. For a particular variant at a particular pH value, all seven heat-treated samples, as well as the sample which had not been heat-treated, were loaded onto the same gel, but never in the first or tenth lanes. Alkaline native and SDS gels were run, stained, destained, photographed and scanned as described in Sections 3.2.1 and 3.2.2.

Samples of BLGs A, B and C at pH 6.7 were heat-treated and electrophoresed, on both alkaline native and SDS gels, on the same day. PAGE data were acquired from pH 7.4 samples in a similar manner, but on a different day. The PAGE data treatment protocols are discussed in Section 5.3.9.8.

5.2.7. TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS.

Species with different M_r values were resolved from aggregates which contained both covalent and non-covalent links using 2D-PAGE (alkaline native-, then disulphide-intact SDS-PAGE). Solutions of BLGs A, B and C at pH 6.7 and pH 7.4 were heat-treated at temperature 5 (Table 5.2.1) on the same day using a procedure similar to that described in Section 5.2.2. These solutions were then stored at 4 °C and gels were run on subsequent days (i.e. pH 6.7 and pH 7.4 samples run the day after, and two days after heat treatment respectively).

In preparation for electrophoresis in the alkaline native dimension, aliquots of heat-treated BLG A, B and C solutions at pH 6.7 and pH 7.4 were diluted, with alkaline native-PAGE sample buffer, to 1.3 mg/mL and 1.95 mg/mL respectively and then loaded onto duplicate alkaline native gels. Both gels were then run as described in Section 3.2.1. At the conclusion of electrophoresis, one of the gels was stained, destained and photographed in the normal way. The other gel was moistened with dilute SDS electrode buffer, and then loosely sandwiched between two glass plates. In preparation for electrophoresis in the SDS dimension, "lanes" containing BLG A, B and C samples were successively sliced from the unstained gel.

For SDS-PAGE, a single "lane" from the unstained alkaline native gel, containing aggregates of either BLG A, B or C, was placed on the edge of a clean glass plate, and oriented so that its long axis was both perpendicular to and equidistant from the two plastic spacers (Fig. 5.2.2). When the "lane" was in position, it was moistened with dilute SDS electrode buffer. After a second glass plate had been placed on top of the gel "lane", the plates were clamped into the casting assembly and were thus mounted vertically with the gel "lane" uppermost. A solution of SDS resolving gel, cooled to 4 °C, was then poured between the two glass plates to a height 1 cm below the bottom of the alkaline native gel "lane". The gel solution was then overlaid with water and care was taken not to wet the gel "lane".

Once the resolving gel had set and the water had been removed, one end of the casting assembly was elevated approximately 2 cm, while keeping the plane of the plate perpendicular to the bench, so that the mounted alkaline native gel "lane" was inclined approximately 20 ° to the horizontal. A solution of SDS stacking gel, cooled to 4 °C, was then carefully poured on top of the resolving gel, around the alkaline native gel "lane", and up to the top of the glass plates. Care was taken to avoid trapping air bubbles on the underside of the alkaline native gel "lane". Loading wells were created in "lanes" 1 and 15 using a comb from which prongs 2 - 14 had been removed and the gel was then left to stand for 45 min. After this time, aliquots (15 μL and 10 μL for pH 6.7 and pH 7.4 sample respectively) of the appropriate original heat-treated BLG sample, diluted with SDS-PAGE sample buffer as described above for PAGE in the alkaline native dimension, were loaded onto the SDS gel in "lanes" 1 and 15. SDS gels prepared in this manner were run one after the other, stained, destained and photographed as described in Section 3.2.2.

After photography, the images of protein-containing "lanes" were sliced from the photograph of the gel which had been stained after alkaline native-PAGE. Each slice was then superimposed onto the image of the now protein-depleted alkaline native-PAGE "lane" in the photograph of the corresponding 2D gel.

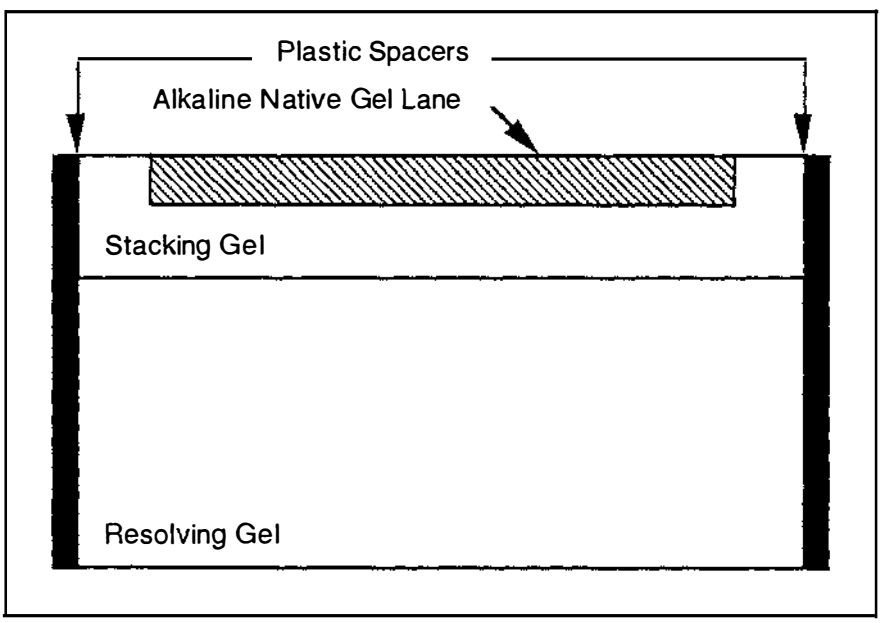


Fig. 5.2.2. Schematic representation of a 2D gel.

5.2.8. FITTING OF EXPERIMENTAL DATA TO 2-STATE THERMAL UNFOLDING MODELS.

5.2.8.1. Near and Far UV CD, ANS Fluorescence and Thiol Availability Data.

The near and far UV CD and ANS emission intensity data (i.e. $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{ANS} respectively) obtained as described in Section 5.2.1 and the thiol availability (i.e. A_{412}) data obtained as described in Section 5.2.3 were fitted to a modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) using the computer program "Enzfitter" (Leatherbarrow, 1987). In this modified model, values for the spectroscopic signal (S_{obs}) were fitted directly using equations 5.2.8.1 and 5.2.8.2 below, rather than fitting the apparent fraction of protein unfolded (F_u), using equation (3) of Luo *et al.* (1995). In equation 5.2.8.1, $(K_{app})/(1+K_{app}) = F_u$.

$$S_{obs} = S_{low}\left(\frac{1}{1+K_{app}}\right) + S_{high}\left(\frac{K_{app}}{1+K_{app}}\right) \qquad (5.2.8.1)$$

K_{app} is the apparent equilibrium constant for irreversible structural change, given by equation 5.2.8.2 (equation (3) of Luo *et al.*, 1995).

$$K_{app} = \frac{\exp\left(-\left[\frac{T_{mid}\Delta S^{o}_{mid}}{T} + \frac{\Delta C_p(T - T_{mid})}{T} - \Delta S^{\circ}_{mid} + \Delta C_p \, ln\left(\frac{T_{mid}}{T}\right)\right]\right)}{R} \qquad (5.2.8.2)$$

Thus, data were fitted using the following parameters: S_{low}, S_{high}, T_{mid}, $\Delta S°_{mid}$ and ΔC_p.

The parameter S_{low} is the value of the spectroscopic signal on the low temperature side of the transition for irreversible structural change (i.e. $\Delta\varepsilon_{293}$(low), $[\theta]_{205}$(low), I_{ANS}(low) or A_{412}(low)). The parameter S_{high} is the value of the spectroscopic signal on the high temperature side of the transition for irreversible structural change (i.e. $\Delta\varepsilon_{293}$(high), $[\theta]_{205}$(high), I_{ANS}(high), or A_{412}(high)). Values for S_{low} and S_{high} were assumed to represent, respectively, that for native BLG and that for the sample in which the proportion of BLG molecules whose structures had been altered irreversibly as a consequence of heat treatment was greatest. The parameter T_{mid} is the midpoint temperature of the transition for irreversible structural change. The parameter $\Delta S°_{mid}$ is the change in entropy at T_{mid} and ΔC_p is the heat capacity difference between the native species and the heat-treated species which gave the signal S_{high}. The variable T represents the temperature at which the BLG solution of interest was heat-treated.

5.2.8.2. Tryptophan Fluorescence Data.

I_{Trp} data (Section 5.2.1.5) were fitted using a modified version of equation 5.2.8.1. Modification of this equation was necessary because the slopes of all plots of I_{Trp} versus heat treatment temperature were negative on the low temperature side of the transition for irreversible structural change. In some instances, the slopes of these plots were also negative on the high temperature side of this transition. Data were fitted using equation 5.2.8.3, in which additional parameters, s_i and s_f, allow for these regions of negative slope. The parameter K_{app} is defined in section 5.2.8.1.

$$S_{obs} = \left[(S_{low} - s_i T)\left(\frac{1}{1+K_{app}}\right)\right] + \left[(S_{high} - s_f T)\left(\frac{K_{app}}{1+K_{app}}\right)\right] \qquad (5.2.8.3)$$

5.2.8.3. Calculation of Approximate Values for ΔG_{app}.

Values for ΔG_{app} for heat-induced irreversible structural change for BLGs A, B and C at pH 6.7 and pH 7.4 were calculated at heat treatment temperatures close to T_{mid}. The computer program "Enzfitter" was used to calculate values for S_{obs} at selected temperatures. These values were then normalised by calculating the values for F_s, the proportion of the total change in spectroscopic signal intensity which occured as a consequence of heat treatment at temperature T. Equations 5.2.8.4 and 5.2.8.5 were used. In these equations, S_N, S_D and S_T are defined as follows. For $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{ANS} and thiol availability data, S_N is the fitted value for the parameter S_{low}. For I_{Trp} data, S_N is the minimum value for I_{Trp} in plots of I_{Trp} versus heat treatment temperature. For $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data, S_D is the fitted value for the parameter S_{high}. For I_{Trp} and I_{ANS} data, S_D is the maximum value for fluorescence emission intensity in plots of I_{Trp} and I_{ANS} versus heat treatment temperature. For thiol availability data, S_D is the average value for S_{high}.

1) For $\Delta\varepsilon_{293}$, $[\theta]_{205}$ data: $$F_s(T) = \frac{S_T - S_N}{S_D - S_N} \qquad (5.2.8.4)$$

2) For I_{Trp}, I_{ANS} and thiol availability data: $$F_s(T) = \frac{S_T - S_D}{S_N - S_D} \qquad (5.2.8.5)$$

Values for F_s were then used to calculate the K_{app}, using equation 5.2.8.6.

$$K_{app} = \frac{F_s}{(1 - F_s)} \qquad (5.2.8.6)$$

From K_{app} values, the apparent change in free energy, ΔG_{app}, was determined using equation 5.2.8.7.

$$\Delta G_{app} = -RT\ln K_{app} \qquad (5.2.8.7)$$

5.3.1. RESULTS AND DISCUSSION: NEAR UV CD.

5.3.1.1. Introduction to Near UV CD Spectroscopy.

Circular dichroism is the difference between the absorption of the left and right components of circularly polarised light by chiral compounds. Bands in the near UV CD spectra of proteins are due to tryptophan, tyrosine and phenylalanine side chains, disulphide bonds and prosthetic groups. For aromatic groups, absorptions involve the transition of an electron from a filled π orbital to an empty higher energy π^* orbital (Strickland, 1974). Because aromatic rings possess several π^* orbitals, a number of transitions occur (Strickland, 1974), which are usually categorised according to the properties of the excited state. However, because the aromatic moieties of proteins possess planes of symmetry and centres of inversion, aromatic CD bands are only observed if aromatic side chains interact in an appropriate manner with nearby groups. For example, when two groups are oriented side by side, an interaction involving two electronic transitions, one in each group, may occur and an increase in CD intensity will result (Strickland, 1974).

The assignment of bands in the near UV CD spectra of proteins is difficult for a number of reasons. Firstly, the intensities and polarities of CD bands are dependent on the conformation which chiral groups assume. Secondly, compared to bands in NMR spectra, those observed in the near UV CD spectra of proteins are usually broad and may represent the superposition or cancellation of the bands of several chiral groups (Strickland, 1974). Nevertheless, the approximate positions and intensities of the tryptophan, phenylalanine, tyrosine, and disulphide bands in the near UV CD spectra of proteins are known. Firstly, the spectrum of a tryptophan side chain fixed in a particular conformation usually exhibits two intense bands with the same sign, 7 nm apart, between approximately 285 nm and 293 nm. Secondly, a pair of small sharp bands with the same sign, separated by approximately 6 nm, and located between 255 nm and 270 nm, is usually observed in the spectrum of a phenylalanine side chain fixed in a particular conformation. Thirdly, for a tyrosine side chain in a fixed conformation, a band is usually observed between 275 nm and 282 nm (Strickland, 1974). Finally, disulphide bonds may exhibit a broad trough centred at approximately 277 nm and a sharp peak at approximately 250 nm in the near UV CD spectra of proteins (Strickland, 1974).

Near UV CD results have been expressed in terms of $\Delta\varepsilon$ (molar circular dichroism) and $[\theta]$ (mean residue ellipticity). $\Delta\varepsilon$ is a measure of the absolute CD of the compound of interest. $[\theta]$ on the other hand is a measure of CD per amino acid residue. Near UV CD should be expressed in terms of $\Delta\varepsilon$ because the number of aromatic side

chains and disulphide bonds in proteins is not directly proportional to M_r (Strickland, 1974). Far UV CD can, however, be expressed as $[\theta]$ because in proteins, the number of amide chromophores is always one less than the number of amino acid residues. Except in the case of Fig. 5.3.1.1, all near UV CD results presented in this section will be expressed in terms of $\Delta\varepsilon$.

5.3.1.2. The Spectra of Unheated β-Lactoglobulins A, B and C.

The spectra of unheated BLGs A, B and C in pH 6.7 phosphate buffer, and also the "baseline" spectrum of this buffer are shown in Fig. 5.3.1.1. The "baseline" spectrum, which did not change appreciably over the period of this study (1994 - 1996), shows that there is little variation in apparent rotation with wavelength arising from the buffer components, the cell or the instrument. The noise level in the spectra of the BLG solutions is high compared to those observed using other spectroscopic techniques and is a reflection of the low signal available from the chiral groups in proteins. Nevertheless, values for rotation in the "baseline" spectrum of phosphate buffer are small compared to those in protein spectra (Fig. 5.3.1.1). The intensity of baseline rotation does, however, increase with decreasing wavelength below approximately 295 nm. For this reason, the phosphate buffer baseline spectrum was subtracted from all protein spectra before $\Delta\varepsilon_{293}$ and $\Delta\varepsilon_{270}$ values were determined.

The BLG spectra in Fig. 5.3.1.1 appear similar to those obtained by Townend *et al.* (1967), Arakawa (1989), Griffin *et al.* (1993), Matsuura and Manning (1994), Creamer (1995), Civera *et al.* (1996) and Iametti *et al.* (1996). Aromatic side chains usually produce strong CD bands when they are packed against other side chains because they are located in chiral environments (Strickland, 1974). Therefore, the considerable intensity of the bands in the near UV CD spectrum of BLG suggests that at least one tryptophan side chain is located in a chiral environment. In the crystal structures of bovine BLG (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998), the side chain of Trp^{19} is packed against the side chains of Thr^{18}, Tyr^{20}, Leu^{46}, Leu^{103} and Arg^{124} (Fig. 2.4.6), while that of Trp^{61} is solvent-exposed (Fig. 2.4.7). Therefore, the 293 nm and 285 nm bands in the spectra of unheated BLGs A, B and C (Fig. 5.3.1.1) may be ascribed to Trp^{19} alone. However, these is no actual proof that this band assignment is correct. For example, the near UV CD spectrum of Mer P, a small protein which does not contain any tryptophan residues, has weak negative CD bands at approximately 287 nm and 293 nm (Aronsson *et al.*, 1997).

Another two sharp bands at 260 nm and 267 nm, probably phenylalanine bands, and small asymmetric peaks centred at 277 nm, probably tyrosine bands, are observed in the spectra in Fig. 5.3.1.1. However, owing to their low intensity, these bands will not be discussed further.

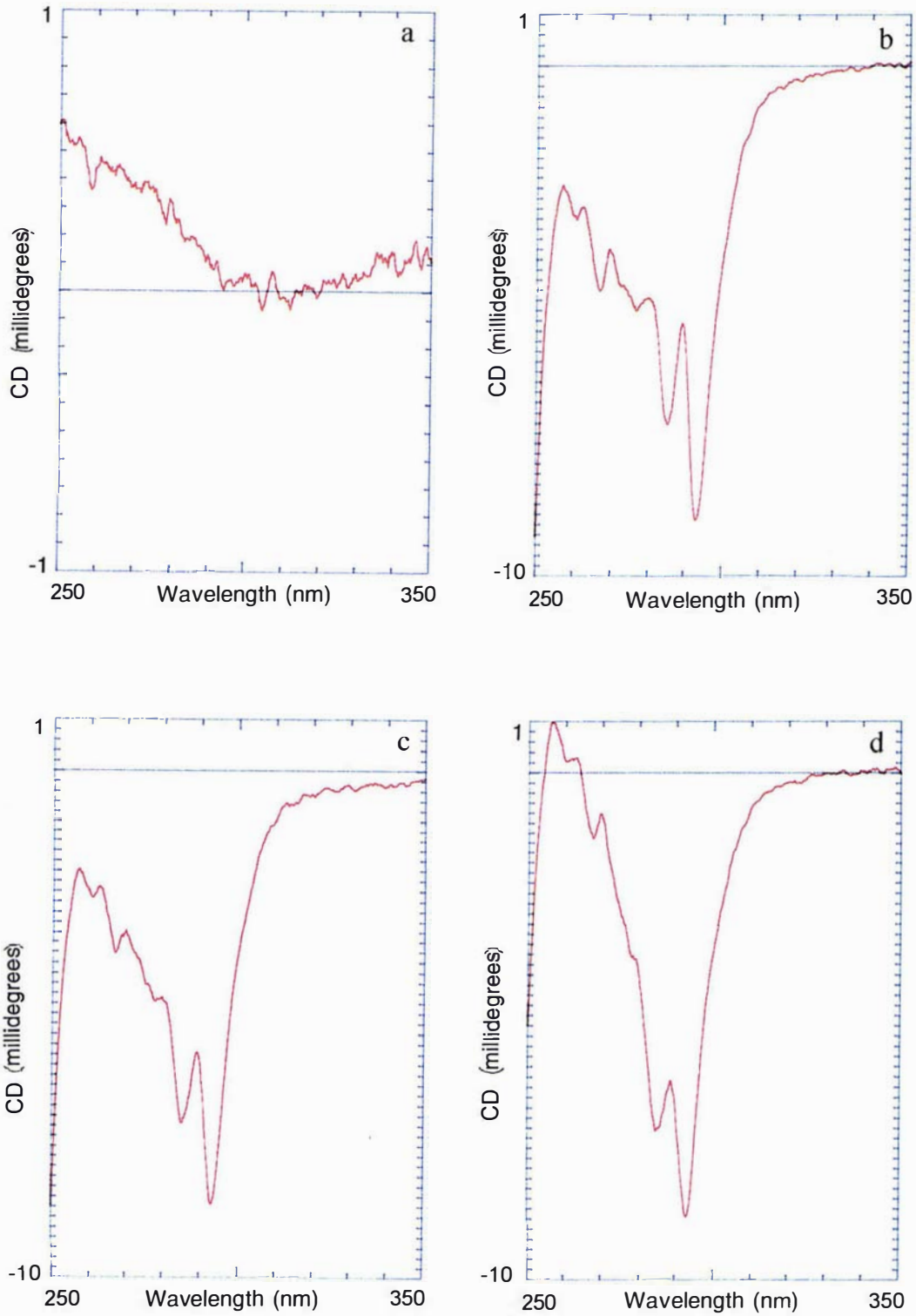
The spectra of unheated BLGs A, B and C at pH 7.4, and the spectrum of unheated BLG A at pH 8.1 (Fig. 5.3.1.2) are similar to those at pH 6.7 (Fig. 5.3.1.1). However, the intensities of the two tryptophan bands in the spectra of pH 7.4 samples are less than those for the corresponding pH 6.7 samples. This trend continues for BLG A at pH 8.1. These shifts may have been caused by a slight expansion of the structures of BLGs A, B and C, which led to a decrease in the chirality of the environment of the side chain of Trp^{19}. For all three variants at pH 7.4, and for BLG A at pH 8.1 in particular, the results in Fig. 5.3.1.2 are therefore consistent with the conclusion drawn by Groves *et al.* (1951), that the rate of BLG A/B denaturation at 25 °C increased with increasing pH in the range 8.0 to 9.5 (Section 2.6.3). Similarly, Casal *et al.* (1988) reported that BLG denatures at room temperature at pH values 8.0 and higher (Section 2.6.3). Thus, although the results in Fig. 5.3.1.2 suggest that the differences in the structure of BLG at pH 6.7 and pH 7.4 are not great, they may be of a similar type to those which lead to denaturation at higher pH values.

5.3.1.3. The Spectra of Heat-treated β-Lactoglobulin B.

The near UV CD spectra of BLG B solutions previously heat-treated at pH 6.7 at various temperatures as described in Section 5.2.1.2, along with the spectrum of the corresponding unheated solution, are shown in Fig. 5.3.1.3. For the solution heat-treated at 56.0 °C, the CD spectrum is similar to that of the corresponding unheated solution. However, as the heat treatment temperature was increased above 56.0 °C, the intensities of both the 285 nm and 293 nm tryptophan CD bands diminished, although there was no discernible wavelength shift (Fig. 5.3.1.3). These results are consistent with those of Matsuura and Manning (1994) and Iametti *et al.* (1996), who also made measurements at room temperature using solutions of BLG A/B previously heat-treated, and found that the intensities of CD bands decrease with increasing heat treatment temperature.

The decrease in the intensity of the tryptophan CD bands after heat treatment (Fig. 5.3.1.3) is indicative of a decrease in the chirality of the environment of Trp^{19}. Therefore, it is likely that the side chain of Trp^{19} is able to assume a number of conformations after heat treatment, observable as a partial cancellation of its CD bands. The results in Fig. 5.3.1.3 also suggest that in BLG solutions, the extent of the decrease in the chirality of the environment of Trp^{19} increases with increasing heat treatment temperature above 56.0 °C.

Fig. 5.3.1.1. The near UV CD spectra of pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) and unheated BLGs A, B and C in this buffer. The spectra are as follows: buffer, panel a; BLG A, panel b; BLG B, panel c; and BLG C, panel d. Protein CD spectra were acquired from 1.0 mg/mL solutions at 20 °C in 10 mm path length cells using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 50 nm/min, using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. In each case the final spectrum is the average of 5 scans of the solution. The baseline spectrum of phosphate buffer has been subtracted from each protein spectrum. Details of the solution preparation protocol are given in Section 5.2.1.1.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CD (millidegrees)
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b
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CD (millidegrees)
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Wavelength (nm)
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c
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CD (millidegrees)
250
Wavelength (nm)
350
d
1
-10
CD (millidegrees)
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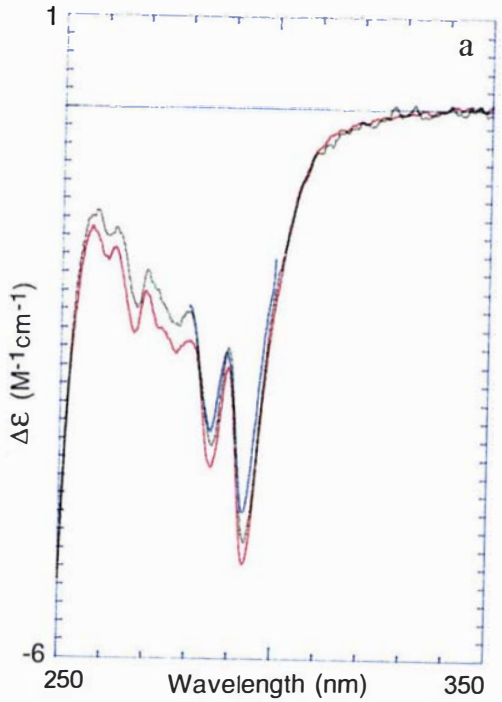
Wavelength (nm)
350

Fig. 5.3.1.2. The near UV CD spectra of unheated BLGs A, B and C at pH 6.7 and pH 7.4 and that of BLG A at pH 8.1. The spectra are as follows: BLG A at pH 6.7, red, panel a; BLG A at pH 7.4, green, panel a; BLG A at pH 8.1, blue, panel a; BLG B at pH 6.7, red, panel b; BLG B at pH 7.4, green, panel b; BLG C at pH 6.7, red, panel c; and BLG C at pH 7.4, green, panel c. The BLG solutions were in pH 6.7 or pH 7.4 phosphate buffers (26 mM, with 68 mM NaCl) or pH 8.1 phosphate/borate buffer (13 mM phosphate, 13 mM borate, with 68 mM NaCl). The spectra were acquired as described in Fig. 5.3.1.1. In each case the final spectrum is the average of 5 scans of the solution. The baseline spectrum of phosphate buffer has been subtracted from each protein spectrum. Details of the solution preparation protocol are given in Section 5.2.1.1.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Wavelength (nm)
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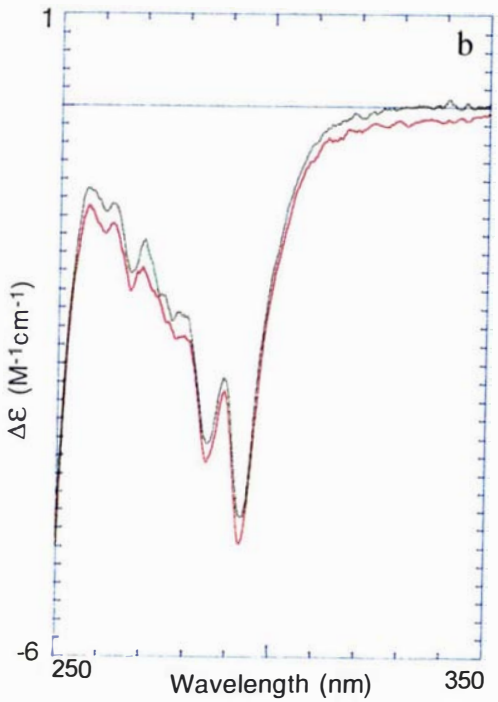
Δε (M-1cm-1)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Wavelength (nm)
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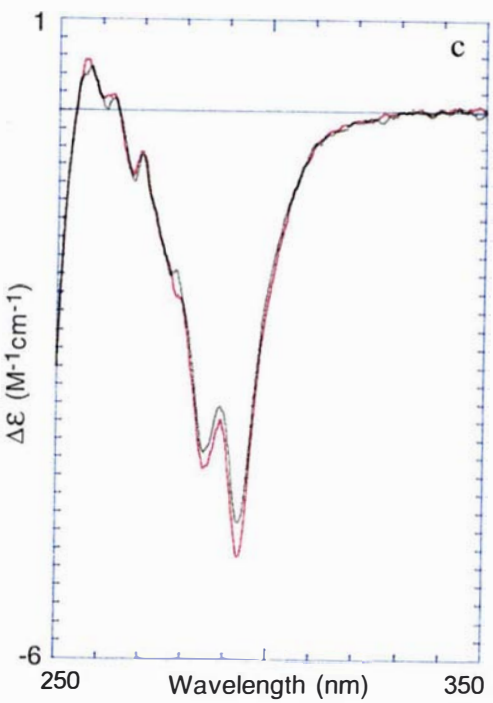
Δε (M-1cm-1)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Wavelength (nm)
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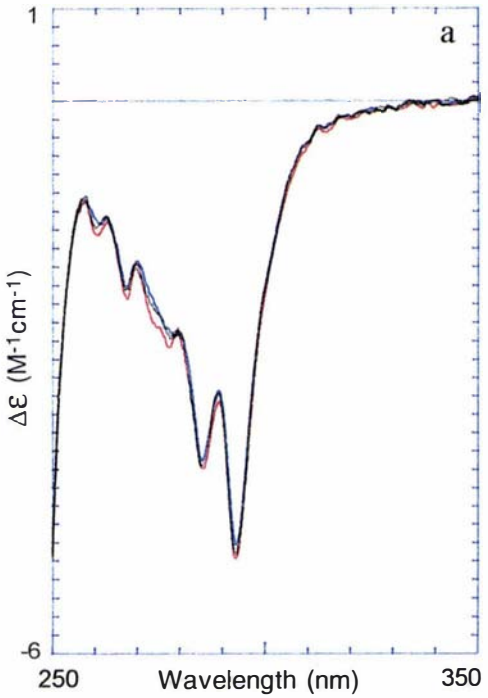
Δε (M-1cm-1)

Fig. 5.3.1.3. The near UV CD spectra of unheated BLG B at pH 6.7 and BLG B previously heat-treated at this pH at different temperatures. The spectra are as follows: Unheated BLG B, red, panel a; BLG B previously heat-treated for 12.5 min at 56.0 °C, green, panel a; 58.0 °C, blue, panel a, and red, panel b; 66.1 °C, green, panel b; 72.0 °C, blue, panel b, and red, panel c; 73.9 °C, green, panel c; 81.8 °C, blue, panel c; and 87.7 °C, purple, panel c. All BLG solutions, in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl), were heat-treated at a concentration of approximately 3 mg/mL and then diluted to 1.00 mg/mL with phosphate buffer in preparation for data collection. The spectra were acquired as described in Fig. 5.3.1.1. In each case the final spectrum is the average of 5 scans of the solution. The baseline spectrum of phosphate buffer has been subtracted from each protein spectrum. Details of the solution preparation and heating protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.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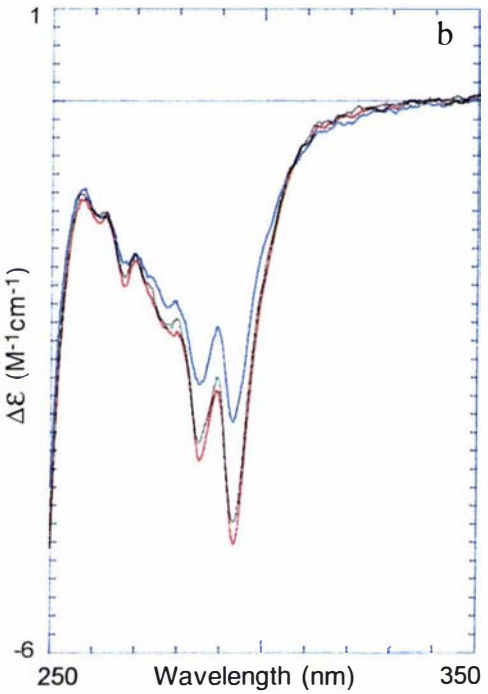
Wavelength (nm)
Δε (M-1cm-1)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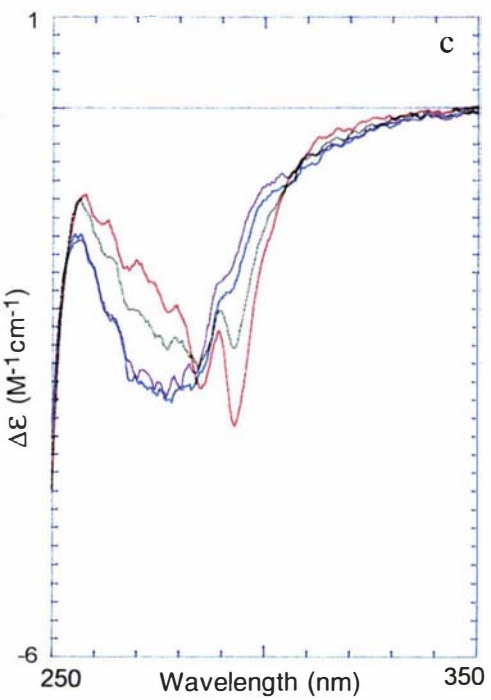
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-6
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Wavelength (nm)
Δε (M-1cm-1)

As heat treatment temperatures were raised, from 72.0 °C to 87.7 °C, the intensity of a broad trough spanning the wavelength interval approximately 257 nm - 295 nm increased (Fig. 5.3.1.3). In the spectra of BLG solutions previously heat-treated at 1.0 mg/mL obtained by Iametti *et al.* (1996), this broad trough is not seen. In contrast, a broad trough spanning the wavelength interval approximately 257 nm - 295 nm was observed by Matsuura and Manning (1994) in the CD spectra of solutions previously heated for 20 min at 90 °C at a BLG concentration of 70 mg/mL. They also reported that the intensity of this trough increased with increasing BLG concentration in the range 1 mg/mL to 100 mg/mL. At concentrations greater than approximately 50 mg/mL, BLG solutions form gels during heat treatment and, as discussed in Section 2.7.1, BLG gels and aggregates are stabilised by intermolecular disulphide bonds. In addition, proteins which contain disulphide bonds may exhibit a broad but often quite intense trough centred at 277 nm (Strickland, 1974). The intensity and λ_{max} of this disulphide trough is, however, affected by the dihedral angle of the disulphide, the C-S-S-C bond angles, and also interactions with neighbouring side chains (Strickland, 1974). For example, when the dihedral angle of a disulphide bond is approximately 90 °, the two $n \rightarrow \sigma^*$ electronic transitions are degenerate and an absorption band is observed (Woody, 1995). Nevertheless, because BLG forms disulphide-linked aggregates when heated (McKenzie, 1971; Griffin *et al.*, 1993; McSwiney *et al.*, 1994a, b; Iametti *et al.*, 1995; Elofsson *et al.*, 1996a, b) and because extents of aggregation increase with increasing BLG concentration in the range 1 mg/mL to 100 mg/mL (Hoffmann *et al.*, 1996), the trough at 277 nm in the spectra of Matsuura and Manning (1994) and therefore that in the spectra in Fig. 5.3.1.3 may be a disulphide CD band. No previous reports on disulphide bands in the near UV CD spectra of heat-treated BLGs appear to exist. The presence of a disulphide band in the spectra of heat-treated BLG solutions therefore suggests that non-native disulphide bonds, which assume different conformations to those in native BLG, are formed as a consequence of heat treatment. If the conformations of non-native disulphide bonds in BLG were similar to those of the native disulphide bonds, then an increase in the intensity of the trough centred at 277 nm should not have been observed.

The spectra of BLG B solutions heat-treated at 87.7 °C at pH 6.7 and at pH 7.4 (Fig. 5.3.1.4) show that the intensity of the broad trough centred at approximately 277 nm is greater at the lower pH. This may indicate that the disulphide bonds formed as a consequence of heat treatment at pH 7.4 are located in less chiral environments than those formed as a consequence of heat treatment at pH 6.7. Alternatively, the difference in the intensities of the disulphide bonds in the spectra of BLG solutions previously

heat-treated at pH 6.7 and pH 7.4 may indicate that the extent of non-native disulphide bond formation is less at the higher pH value. The results in Fig. 5.3.1.4 may therefore be consistent with those of Hoffmann *et al.* (1996), who reported that aggregation rates and aggregate particle size decreased with increasing pH in the range 6.2 - 8.0. This is also consistent with the results of Manderson *et al.* (1997), which will be discussed further in Section 5.3.9.

In the spectrum of the BLG B solution previously heat-treated at pH 6.7 at 87.7 °C, the trough at 285 nm is not visible, while the one at 293 nm appears as a shoulder on the long wavelength side of the putative disulphide trough (Fig. 5.3.1.3c). However, although the observed intensity of the putative disulphide trough increases with increasing heat treatment temperature between 72.0 °C to 87.7 °C, the observed intensity of $\Delta\varepsilon$ at 293 nm (i.e. the difference between the $\Delta\varepsilon$ at 293 nm in BLG spectra and the instrumental baseline $\Delta\varepsilon$, value 0) remains approximately constant. This suggests that the effect on $\Delta\varepsilon$ at 293 nm ($\Delta\varepsilon_{293}$) of the increase in the intensity of the putative disulphide trough with increasing heat treatment temperature was minimal.

5.3.1.4. Quantitative Study: Heat-induced Irreversible Structural Change in β-Lactoglobulin Molecules.

The trends observed in the series of spectra acquired from heat-treated solutions of BLGs A and C at pH 6.7, BLGs A, B and C at pH 7.4 and BLG A at pH 8.1 were similar to those for BLG B at pH 6.7 (Section 5.3.1.3). In particular, the magnitude of heat-induced change in $\Delta\varepsilon$ was greatest at 293 nm. Therefore, extents of heat-induced irreversible structural change in BLG molecules in solutions of the A, B and C variants were determined by measuring changes in $\Delta\varepsilon_{293}$ with heat treatment temperature. Experimental $\Delta\varepsilon_{293}$ data were then fitted using the modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) described in Section 5.2.8.1. Plots of experimental $\Delta\varepsilon_{293}$ data versus heat treatment temperature and the curves obtained by fitting these data to equations 5.2.8.1 and 5.2.8.2 using the computer program "Enzfitter" (Section 5.2.8.1) are shown in Fig. 5.3.1.5. The coincidence of the experimental $\Delta\varepsilon_{293}$ data with the fitted curves shows that the selected model adequately describes heat-induced irreversible structural change in molecules of BLGs A, B and C. Therefore, the fitted value for the parameter T_{mid} (Table 5.3.1.1) for a particular variant at a particular pH was defined as the midpoint temperature of the transition for heat-induced irreversible structural change in the vicinity of Trp^{19}. In Table 5.3.1.1, the errors in the values for T_{mid} are standard errors calculated by "Enzfitter" and are probably underestimates of the true error in the values for this parameter.

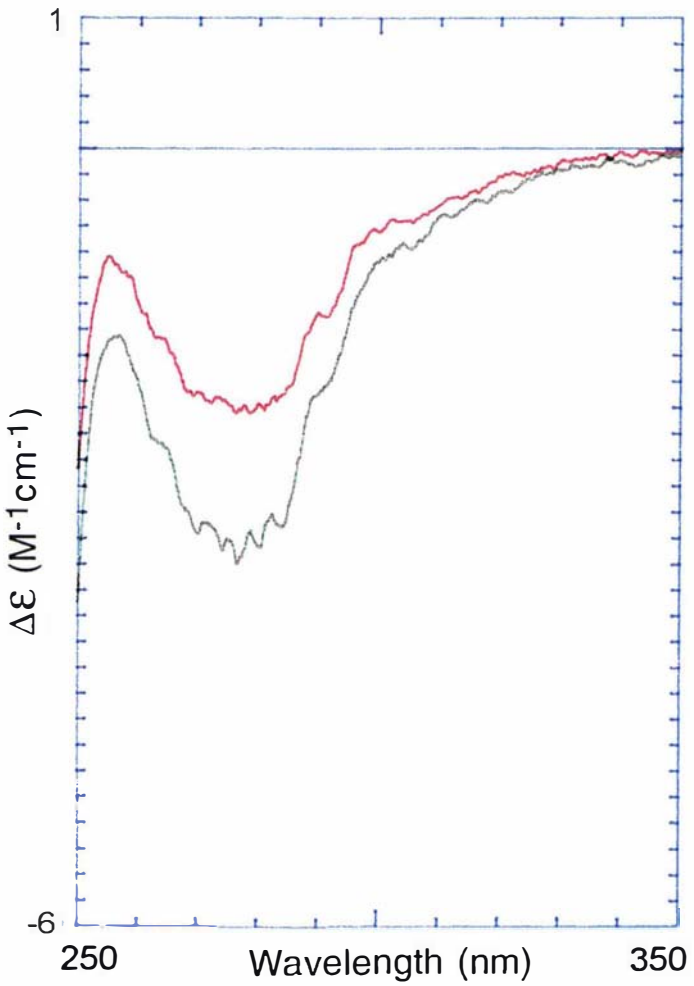


Fig. 5.3.1.4. The near UV CD spectra of BLG B previously heat-treated at pH 6.7 and pH 7.4. The spectra are as follows: BLG B previously heat-treated at pH 6.7, green; and BLG B previously heat-treated at pH 7.4, red. Both BLG solutions were heat-treated for 12.5 min at 87.7 °C in phosphate buffer (26 mM, with 68 mM NaCl) at a concentration of approximately 3 mg/mL and then diluted to 1.00 mg/mL with phosphate buffer prior to data collection. Spectra were acquired as described in Fig. 5.3.1.1. In each case the final spectrum is the average of 5 scans of the solution. The baseline spectrum of phosphate buffer has been subtracted from each protein spectrum. Details of the solution preparation and heating protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.

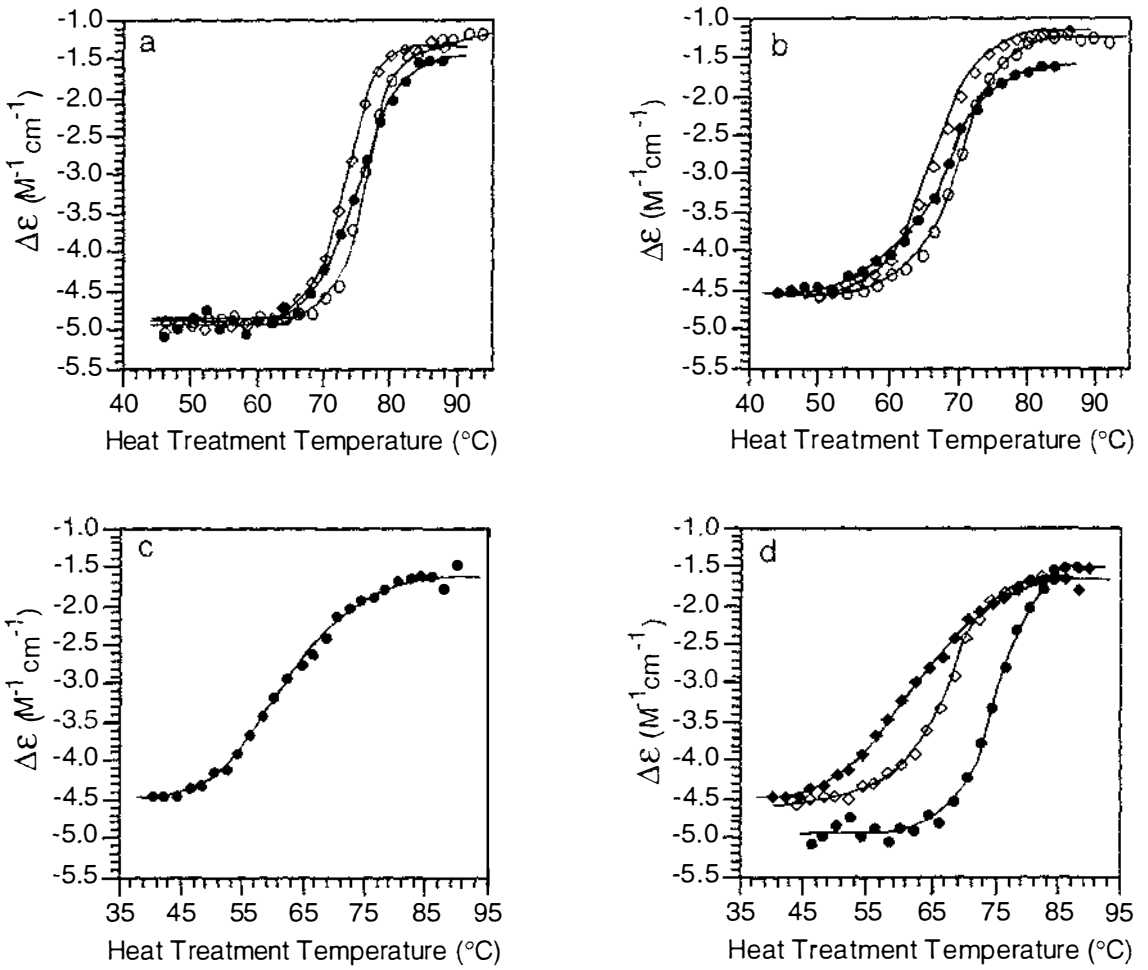


Fig. 5.3.1.5. Effect of heat treatment on $\Delta\varepsilon_{293}$ in the near UV CD spectra of BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1. The plots are as follows: BLGs A (•), B (◇) and C (o) at pH 6.7, panel a; BLGs A, B and C at pH 7.4, panel b; BLG A at pH 8.1, panel c; and in panel d, BLG A at pH 6.7 (•), pH 7.4 (◇) and pH 8.1 (♦). The symbols denote experimental $\Delta\varepsilon_{293}$ data, which were obtained from spectra recorded as described in Fig. 5.3.1.3. The curves were obtained by fitting experimental $\Delta\varepsilon_{293}$ data to equations 5.2.8.1 and 5.2.8.2 as described in Section 5.2.8.1 using the computer program "Enzfitter" (Leatherbarrow, 1987). The values for the fitting parameters are given in Appendix 3.

The plots in Fig. 5.3.1.5 indicate that irreversible structural change in the vicinity of Trp^{19} occurs at temperatures greater than approximately 63 °C at pH 6.7 and approximately 53 °C at pH 7.4. In agreement with these results, the spectra in Fig. 1b of Iametti *et al.* (1996), which were collected under similar conditions to those in Fig. 5.3.1.3, show that irreversible structural change in the vicinity of Trp^{19} occurs at heat treatment temperatures approximately 60 °C and greater.

For all three variants, the plots in Fig. 5.3.1.5 indicate that the average intensity of $\Delta\varepsilon_{293}$ on the low temperature side of the transition for heat-induced irreversible structural change is greater at pH 6.7 than at pH 7.4. This is consistent with the differences in the intensities of the 293 nm tryptophan band in the spectra of unheated BLG solutions at pH 6.7 and pH 7.4 discussed in Section 5.3.1.2, and therefore suggests that for all three variants, the side chain of Trp^{19} is in a less chiral environment at pH 7.4 than at pH 6.7. The plots in Fig. 5.3.1.5 also indicate that for all three variants, the intensities of $\Delta\varepsilon_{293}$ on the high temperature side of the transition for heat-induced irreversible structural change are similar at pH 6.7 and pH 7.4. Thus, for BLGs A, B and C, the maximum extent of change in the intensity of $\Delta\varepsilon_{293}$, and therefore the maximum extent of irreversible structural change in the vicinity of Trp^{19} which occurs as a consequence of heat treatment, is less at pH 7.4 than at pH 6.7.

At both pH 6.7 and pH 7.4, the slopes at T_{mid} of plots of $\Delta\varepsilon_{293}$ versus heat treatment temperature for BLG A are less than those for BLGs B and C (Table 5.3.1.1). This suggests that BLG A responds to heat treatment in a different manner to BLGs B and C. The significance of this will be discussed in Section 8.5.

Values for T_{mid} (Table 5.3.1.1) were used to obtain a quantitative measure of the variation in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change. At pH 6.7, the T_{mid} for BLG B is 1.9 °C lower than that for BLG A, which is 1.3 °C lower than that for BLG C. At pH 7.4, the T_{mid} for BLG B is 0.8 °C lower than that for BLG A, which is 2.5 °C lower than that for BLG C. These T_{mid} values therefore suggest that at both pH 6.7 and pH 7.4, BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change in the vicinity of Trp^{19}.

Table 5.3.1.1. Values for the Midpoint Temperature (T_{mid}) and Slope at T_{mid} for Heat-induced Irreversible Structural Change in the Vicinity of Tryptophan[19] for β-Lactoglobulins A, B and C at pH 6.7 and at pH 7.4 and β-Lactoglobulin A at pH 8.1.

Sample	T_{mid} (°C)	Slope at T_{mid} ($\Delta\varepsilon_{293}$/°C)
pH 6.7, BLG A	74.7 ± 0.3	0.260
pH 6.7, BLG B	72.8 ± 0.1	0.362
pH 6.7, BLG C	76.0 ± 0.1	0.401
pH 7.4, BLG A	67.0 ± 0.2	0.192
pH 7.4, BLG B	66.2 ± 0.1	0.245
pH 7.4, BLG C	69.5 ± 0.1	0.281
pH 8.1, BLG A	62.1 ± 0.9	0.115

Where:

T_{mid} is the midpoint temperature of the transition for heat-induced irreversible structural change in the vicinity of Trp^{19} (i.e. the temperature at which $\Delta\varepsilon_{293}$ is exactly half way between the values for $\Delta\varepsilon_{293}$ on the low and high temperature sides of the transition for heat-induced irreversible structural change in the plots shown in Fig. 5.3.1.5). T_{mid} is one of the parameters used to fit experimental $\Delta\varepsilon_{293}$ data to equations 5.2.8.1 and 5.2.8.2 (Section 5.2.8.1) using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in T_{mid} values are standard errors calculated by "Enzfitter".

The slope at the midpoint temperature was calculated using the fitted $\Delta\varepsilon_{293}$ values in the range T_{mid} ± 0.5 °C.

The T_{mid} values for BLGs B and C at pH 6.7 are, respectively, 6.6 °C and 6.5 °C higher than those at pH 7.4 (Table 5.3.1.1). Additionally, for BLG A, the T_{mid} value at pH 6.7 is 7.7 °C higher than that at pH 7.4, which is 4.9 °C higher than that at pH 8.1 (Table 5.3.1.1 and Fig. 5.3.1.5d). These comparisons therefore indicate that all three variants are more susceptible to heat-induced irreversible structural change in the vicinity of Trp^{19} at pH 7.4 than at pH 6.7, and that this trend continues for BLG A at pH 8.1. This is consistent with the spectral results in Fig. 5.3.1.2, which suggest that the environment of Trp^{19} is slightly less chiral at pH 7.4 and pH 8.1 than at pH 6.7. Therefore, if the structures of BLGs A, B and C are slightly more expanded at pH 7.4 and pH 8.1 than at pH 6.7 (Section 5.3.1.2), then this may explain why these variants are more susceptible to heat-induced irreversible structural change at the two higher pH values.

CD data for BLG at pH 8.1, which could be used to prepare plots of $\Delta\varepsilon_{293}$ versus heat treatment temperature that were sigmoidal, were very difficult to obtain. The number of repeated near UV CD runs needed before the results at pH 8.1 shown in Fig. 5.3.1.5 were obtained was considerably greater than the number needed for the results at pH 6.7 and pH 7.4 (Fig. 5.3.1.5). This was also true for the acquisition of far UV CD and intrinsic protein and ANS fluorescence data from heat-treated solutions of BLG A at pH 8.1 (see later). Thus, although the pH of BLG solutions was not adjusted to 8.1 until the day on which spectroscopic measurements were made (Sections 5.2.1.1 - 5.2.1.3, a maximum of 6 hr between pH increase and data collection), it appears that the effect on results of time-dependent structural change in BLG molecules which occurs at this pH value is appreciable. For this reason, measurements using heat-treated solutions of BLGs B and C at pH 8.1 were not made.

An increase in the susceptibility of BLG to heat-induced structural change with increasing pH has been observed previously (Section 2.6.4). For example, Kella and Kinsella (1988a), reported from measurements made at elevated temperatures that the thermostability of BLG A/B decreased with increasing pH between 6.5 and 7.5 (Section 2.6.4).

5.3.1.5. Thermodynamic Analysis.

In an attempt to identify the nature of the forces responsible for the differences in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change, a thermodynamic analysis of the fitted $\Delta\varepsilon_{293}$ data was made. However, these data were collected under conditions in which the unfolding of BLG is both partial and not reversible. This is also true for the far UV CD, intrinsic protein and ANS fluorescence and thiol availability data presented in subsequent sections of this chapter. During heat treatment at neutral pH, the equilibrium between folded and reversibly unfolded BLG is not established because reversibly unfolded BLG is removed from the system as a consequence of aggregate formation (see introduction to Section 2.6). Therefore, the values for the apparent change in free energy (ΔG_{app}) calculated using data presented in this chapter represent those for heat-induced irreversible structural change in BLG molecules, and not those for reversible unfolding. For example, the $\Delta\varepsilon_{293}$ data presented in this section may be used to calculate the ΔG_{app} for irreversible structural change in the vicinity of Trp^{19}.

In the case of $\Delta\varepsilon_{293}$ data, values for ΔG_{app} for BLGs A, B and C at pH 6.7 and pH 7.4 were determined at 72 °C and 66 °C respectively (Table 5.3.1.2). These temperatures were selected because they are close to the midpoint temperatures for heat-induced irreversible structural change for all three variants, where differences between susceptibilities to heat-induced irreversible structural change are greatest. Furthermore, at temperatures close to T_{mid}, values for ΔG_{app} can be calculated with the greatest accuracy (Hawkes *et al.*, 1984).

Table 5.3.1.2. Values for ΔG_{app} at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for Irreversible Structural Change in the Vicinity of Tryptophan[19] in β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6.7, BLG A	2.362 ± 0.009	1.40*
pH 6.7, BLG B	0.963 ± 0.001	0 (by definition)
pH 6.7, BLG C	5.561 ± 0.007	4.60#
pH 7.4, BLG A	0.726 ± 0.002	0.53*
pH 7.4, BLG B	0.193 ± 0.001	0 (by definition)
pH 7.4, BLG C	3.293 ± 0.001	3.10#

* ΔG_{app}(BLG A)- ΔG_{app}(BLG B).

\# ΔG_{app}(BLG C)- ΔG_{app}(BLG B).

ΔG_{app} is assumed to represent the change in free energy at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for irreversible structural change in the vicinity of Trp^{19}. Values for ΔG_{app} were calculated at these temperatures and pH values using fitted values for $\Delta\varepsilon_{293}$ as described in Section 5.2.8.3. The fitted values for $\Delta\varepsilon_{293}$ were obtained from the fitted curves shown in Fig. 5.3.1.5 using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in ΔG_{app} were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 5.3.1.1).

The values for ΔG_{app} for BLG B are less than those for BLG A, which are less than those for BLG C. Therefore, at pH 6.7 and 72 °C and at pH 7.4 at 66 °C, BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change. This is consistent with the T_{mid} values shown in Table 5.3.1.1. The differences in the ΔG_{app} values for BLGs A, B and C are shown as $\Delta\Delta G_{app}$ values in Table 5.3.1.2. These will be discussed in Section 8.5.

5.3.1.6. Dependence on Heat Treatment Temperature of the Intensity of the Putative Disulphide Trough.

The dependence on heat treatment temperature of the intensity of the broad trough centred at approximately 277 nm, believed to represent the presence of non-native disulphide bonds in molecules of heat-treated BLG (Section 5.3.1.3), was also examined. Although the putative disulphide trough was centred at 277 nm, heat-induced changes in its intensity were followed at 270 nm because the magnitude of spectral change caused by the increase in the depth of this trough was greater at 270 nm than at 277 nm (Fig. 5.3.1.4). Changes in $\Delta\varepsilon_{270}$ with heat treatment temperature are shown in Fig. 5.3.1.6.

For all three variants, the maximum extent of change in $\Delta\varepsilon_{270}$ which occurs as a consequence of heat treatment is less at pH 7.4 than at pH 6.7 (Fig. 5.3.1.6). This suggests that for BLGs A, B and C, either the non-native disulphide bonds formed after heat treatment are located in less chiral environments at pH 7.4 than at pH 6.7, or that the extent of non-native disulphide bond formation, and hence aggregation, is less at pH 7.4 than at pH 6.7. These conclusions were also drawn from the spectral results for solutions of BLG B previously heat-treated at pH 6.7 and pH 7.4 in Fig. 5.3.1.4. The effect of pH on the nature of aggregates formed from BLGs A, B and C will be discussed further in Section 5.3.9.

The plots in Fig. 5.3.1.6 also indicate that at pH 6.7 in particular, the maximum extent of change in $\Delta\varepsilon_{270}$ which occurs as a consequence of heat treatment is less for BLG A than for BLGs B and C. This suggests that the disulphide bonds in aggregates of BLG A are located in less chiral environments than those in aggregates of BLGs B and C, or that aggregate formation in less marked in heat-treated solutions of BLG A than in those of BLGs B and C. Differences in the behaviour of BLG A and BLGs B and C will be discussed further in Section 5.3.9.

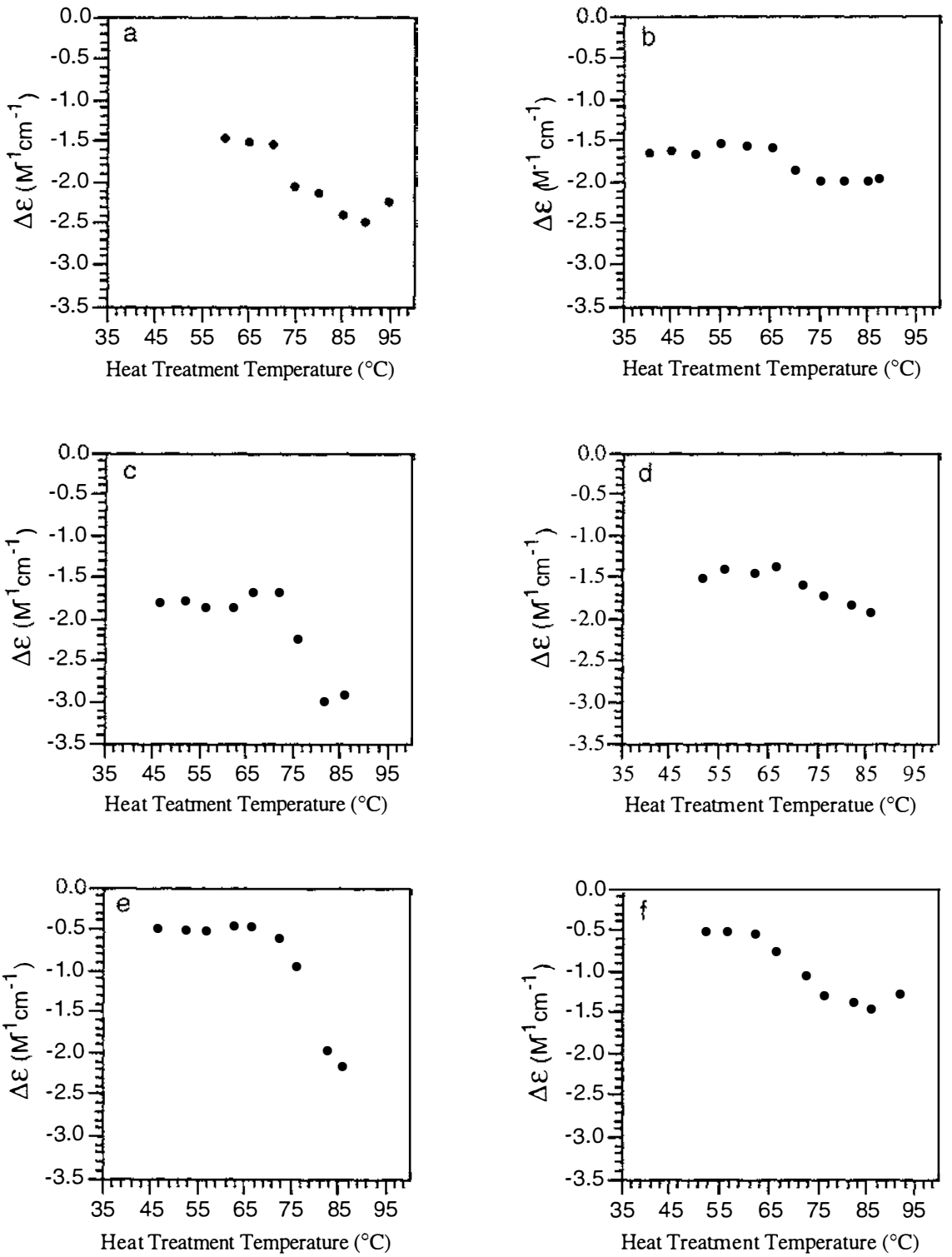


Fig. 5.3.1.6. Effect of heat treatment on $\Delta\varepsilon_{270}$ in the near UV CD spectra of BLGs A, B and C at pH 6.7 and pH 7.4. The plots are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The spectra of heat-treated solutions were recorded as described in Fig. 5.3.1.5.

5.3.1.7. Nature of the Structural Change in β-Lactoglobulin Molecules which Occurs as a Consequence of Heat Treatment.

In the near UV CD spectrum of BLG, decreases in the intensity of $\Delta\varepsilon_{293}$ are assumed to represent structural change in the vicinity of Trp^{19}, while increases in the intensity of $\Delta\varepsilon_{270}$ are assumed to occur as a consequence of the formation of non-native disulphide bonds. Increases in the intensity of $\Delta\varepsilon_{270}$ therefore suggest that structural change in the vicinities of Cys^{121} and the disulphide bonds Cys^{66}-Cys^{160} and Cys^{106}-Cys^{119} occurs as a consequence of heat treatment. Therefore, because these disulphide bonds, Cys^{121} and Trp^{19} are located considerable distances from one another in the structure of native BLG (Fig. 2.4.1), near UV CD results (Sections 5.3.1.3 - 5.3.1.6) suggest that the structure of the entire BLG molecule is altered as a consequence of heat treatment. These results do not, however, indicate whether heat treatment leads to irreversible changes in the amount of secondary structure in molecules of BLGs A, B and C. Changes in secondary structure in BLG molecules which occur as a consequence of heat treatment are considered in the far UV CD study in Section 5.3.2.

5.3.1.8. Comparison of Effects of Urea and Heat on the Structure of β-Lactoglobulin.

Urea-induced changes in the structure of BLG A/B have been examined using near UV CD spectroscopy by Creamer (1995). The near UV CD spectrum of BLG A/B in 7.24 M urea published by Creamer (1995) is completely featureless, suggesting a considerable loss of tertiary structure in the vicinities of the aromatic side chains of this protein. This in turn suggests that the polypeptide chain of BLG is almost totally unfolded in the presence of 7.24 M urea. Indeed, the far UV CD spectra obtained by Creamer (1995) indicate that in 7.24 M urea, BLG possesses negligible amounts of α-helix or β-sheet, suggesting near-complete unfolding.

Conversely, bands are clearly visible in the near UV CD spectra of BLG in solutions previously heat-treated at temperatures 84 °C and above, where the proportions of BLG molecules retained in non-native conformations as a consequence of heat treatment are greatest (Fig. 5.3.1.5). This suggests that some aromatic side chains in molecules of heat-treated BLG, particularly Trp^{19}, are still packed against other side chains in semi-chiral environments, although not as tightly as in the corresponding unheated species. This suggests that BLG does not become completely unfolded as a consequence of heat treatment. Similarly, Lapanje and Poklar (1989) reported that BLG possesses less native structure in the presence of concentrated urea than after heat treatment. Finally, the presence of the broad trough at 277 nm in the spectra of heat-treated solutions shown in Figs 5.3.1.3 and 5.3.1.4 suggests that after heat treatment, at least some of the disulphide bonds of BLG are located in chiral environments (Section 4.3.1.3), and therefore that BLG molecules in heat-treated solutions are not completely unfolded.

5.3.2. RESULTS AND DISCUSSION: FAR UV CD.

5.3.2.1. Introduction to Far UV CD Spectroscopy.

Far UV CD spectroscopy is commonly used to estimate the proportions of α-helix, β-sheet, β-turn and random structure in proteins. Random structure, defined as that which is not α-helix, β-sheet or β-turn, is common in unfolded proteins which possess little tertiary structure (Moore and Fasman, 1993). Each type of protein secondary structure has a particular far UV CD spectrum (Johnson, 1990; Woody, 1995). Spectra characteristic of α-helix, β-sheet, β-turn and random structures are shown in Fig. 5.3.2.1. All of the far UV CD bands of proteins arise from the $n\rightarrow\pi^*$ or $\pi\rightarrow\pi^*$ electronic transitions of the chiral centre adjacent to the amide chromophore (Moore and Fasman, 1993). However, the intensity and λ_{max} of these transitions are dependent on the nature of the macroscopic dipole formed as a consequence of amide alignment, which occurs when polypeptide chains fold into α-helices, β-sheets, β-turns or random structure.

The proportions of each type of secondary structure present in a protein of unknown structure can be determined by comparing the far UV CD spectrum of this protein with those of standards (Fig. 5.3.2.1) derived from proteins whose structures are known. Except in the case of Fig. 5.3.2.2a, all far UV CD results presented in this section will be expressed in terms of [θ], an appropriate measure of CD per amino acid residue (Section 5.3.1.1).

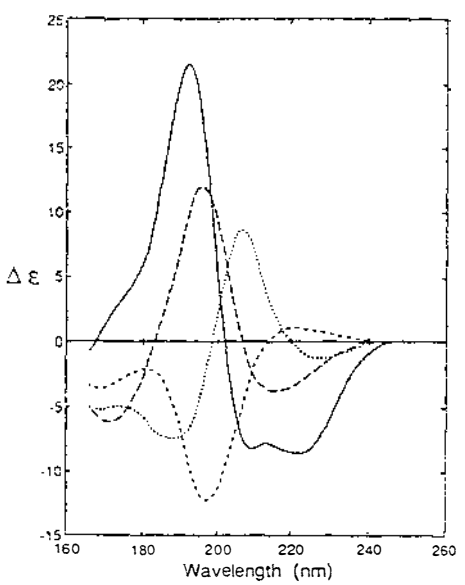


Fig. 5.3.2.1. The far UV CD spectra characteristic of the various types of secondary structures: α-helix, (——), antiparallel β-sheet, (---), β-turn, (····) and "random" coil, (- - -), re-drawn from Brahms and Brahms (1980).

5.3.2.2. The Spectra of Unheated β-Lactoglobulins A, B and C.

The far UV CD spectra of unheated BLGs A, B and C at pH 6.7 and pH 7.4, that of unheated BLG A at pH 8.1, and also the "baseline" spectrum (water) are shown in Fig. 5.3.2.2. In the "baseline" spectrum, which did not change appreciably over the period of this study (1994 - 1996), the intensity of apparent rotation is considerable at wavelengths approximately 210 nm and lower and approximately 228 nm and higher. Therefore, the "baseline" spectrum was always subtracted from protein spectra before values for $[\theta]_{216}$ and $[\theta]_{205}$ were determined.

In the spectra of the BLG solutions, a peak and a trough, each of moderate intensity, are observed at 193 nm and 216 nm respectively. However, the noise level in these CD spectra is high compared with other spectroscopic techniques, reflecting the low signal from the chiral groups in proteins (Section 5.3.1.2).

The BLG spectra in Fig. 5.3.2.2 appear similar to the spectrum of a polypeptide chain which has folded to a structure containing primarily β-sheet (Fig. 5.3.2.1). These spectra also appear similar to those obtained by Townend *et al.* (1967), who calculated that native BLG contains approximately 40 % β-sheet and approximately 10 % α-helix (Section 2.6.2.2). Furthermore, the spectra shown in Fig. 5.3.2.2 are similar to those obtained in the studies of Sawyer *et al.* (1971), Creamer *et al.* (1983), Griffin *et al.* (1993), Matsuura and Manning (1994), Creamer (1995), Molinari *et al.* (1996) and Qi *et al.* (1997).

The intensity of $[\theta]_{205}$ in the spectra of all three variants at pH 7.4 is slightly greater than that in the spectra of all three variants at pH 6.7 (Fig. 5.3.2.2). This trend continues for BLG A at pH 8.1. These results therefore suggest that the amount of random structure in molecules of BLGs A, B and C is greater at the two higher pH values than at pH 6.7. The far UV CD results in Fig. 5.3.2.2, especially those for BLG A at pH 8.1, are consistent with the conclusions drawn by Groves *et al.* (1951) and Casal *et al.* (1988). Groves reported that the rate of BLG denaturation at 25 °C increases with increasing pH in the range 8.0 to 9.5 (Section 2.6.3). Similarly, Casal *et al.* (1988) reported that BLG denatures at room temperature at pH values 8.0 and higher (Section 2.6.3).

5.3.2.3. The Spectra of Heat-treated β-Lactoglobulin B.

The far UV CD spectra of BLG B solutions at pH 6.7 heat-treated as described in Section 5.2.1.2, together with that of the corresponding unheated solution are shown in Fig. 5.3.2.3. The spectra of all solutions heat-treated at temperatures 68.1 °C and below are similar to that of the unheated solution. However, in the spectra of solutions heat-treated at temperatures above 68.1 °C, the band at 216 nm becomes a shoulder on the long wavelength side of a new and more intense negative band centred at approximately 205 nm (Fig. 5.3.2.3). Furthermore, the intensity of the band at approximately 205 nm increases with increasing heat treatment temperature above 68.1 °C. The intensity of $[\theta]_{216}$ also increases with increasing heat treatment temperature above 68.1 °C, but to a lesser extent than that at approximately 205 nm.

The observed increase in the intensity of $[\theta]_{205}$ may also have caused the apparent intensity and λ_{max} of the peak at 193 nm to decrease with increasing heat treatment temperature in the range 66.1 °C to 75.9 °C (Fig. 5.3.2.3). However, above 75.9 °C the intensity of this peak, now centred at 190 nm, increases with increasing heat treatment temperature (Fig. 5.3.2.3). The spectra of BLG B solutions heat-treated at 81.8 °C and 87.7 °C also suggest that the maximum extent of the heat-induced irreversible increase in the intensity of this peak is equivalent to that of the trough at 216 nm (Fig. 5.3.2.3).

The heat-induced increase in the intensity of $[\theta]_{205}$ in the spectra of solutions heat-treated at temperatures 68.1 °C and above (Fig. 5.3.2.3) suggests that the proportion of random structure in BLG molecules increases as a consequence of heat treatment. Furthermore, the similarity in the extents of increase in the intensities of $[\theta]_{216}$ and $[\theta]_{190}$ (Fig. 5.3.2.3) suggests that the amount of β-sheet in molecules of BLG increases as a consequence of heat treatment. However, the spectra in Fig. 5.3.2.3 suggest that the maximum extent of this increase is less marked than that of the increase in random structure.

The spectra in Fig. 5.3.2.3 suggest that the amount of α-helix in BLG molecules does not increase as a consequence of heat treatment. This is because a trough at approximately 222 nm of similar intensity to the one at approximately 205 nm is not observed in these spectra. The opposite is true for the spectra of proteins rich in α-helix (Brahms and Brahms, 1980; Moore and Fasman, 1993, Fig. 5.3.2.1). Furthermore, although the intensity of the peak at approximately 190 nm increases as a consequence of heat treatment, the magnitude of this increase is less than that of the trough at approximately 205 nm. This is also inconsistent with a heat-induced increase in the amount of α-helix in BLG molecules because the intensity of the peak at approximately 190 nm in the spectra of proteins rich in α-helix is appreciably greater than that of the trough at approximately 205 nm.

Fig. 5.3.2.2. The far UV CD spectra of water and unheated BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1. The spectra are as follows: water, red, panel a; BLG B at pH 6.7, green, panel a; BLG A at pH 6.7, red, panel b; BLG A at pH 7.4, green, panel b; BLG A at pH 8.1, blue, panel b; BLG B at pH 6.7, red, panel c; BLG B at pH 7.4, green, panel C; BLG C at pH 6.7, red, panel d; and BLG C at pH 7.4, green, panel d. Protein CD spectra were acquired from 0.17 mg/mL solutions in pH 6.7 or pH 7.4 phosphate buffer (4.3 mM, with 11.3 mM NaCl), or pH 8.1 phosphate/borate buffer (2.1 mM phosphate, 2.1 mM borate, with 11.3 mM NaCl) at 20 °C in 0.5 mm path length cells using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 20 nm/min, using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. The spectrum of water is the average of 15 scans, while the spectra of the BLG solutions are the average of 5 scans. The baseline spectrum of water has been subtracted from each protein spectrum. Details of the solution preparation protocol are given in Section 5.2.1.1.

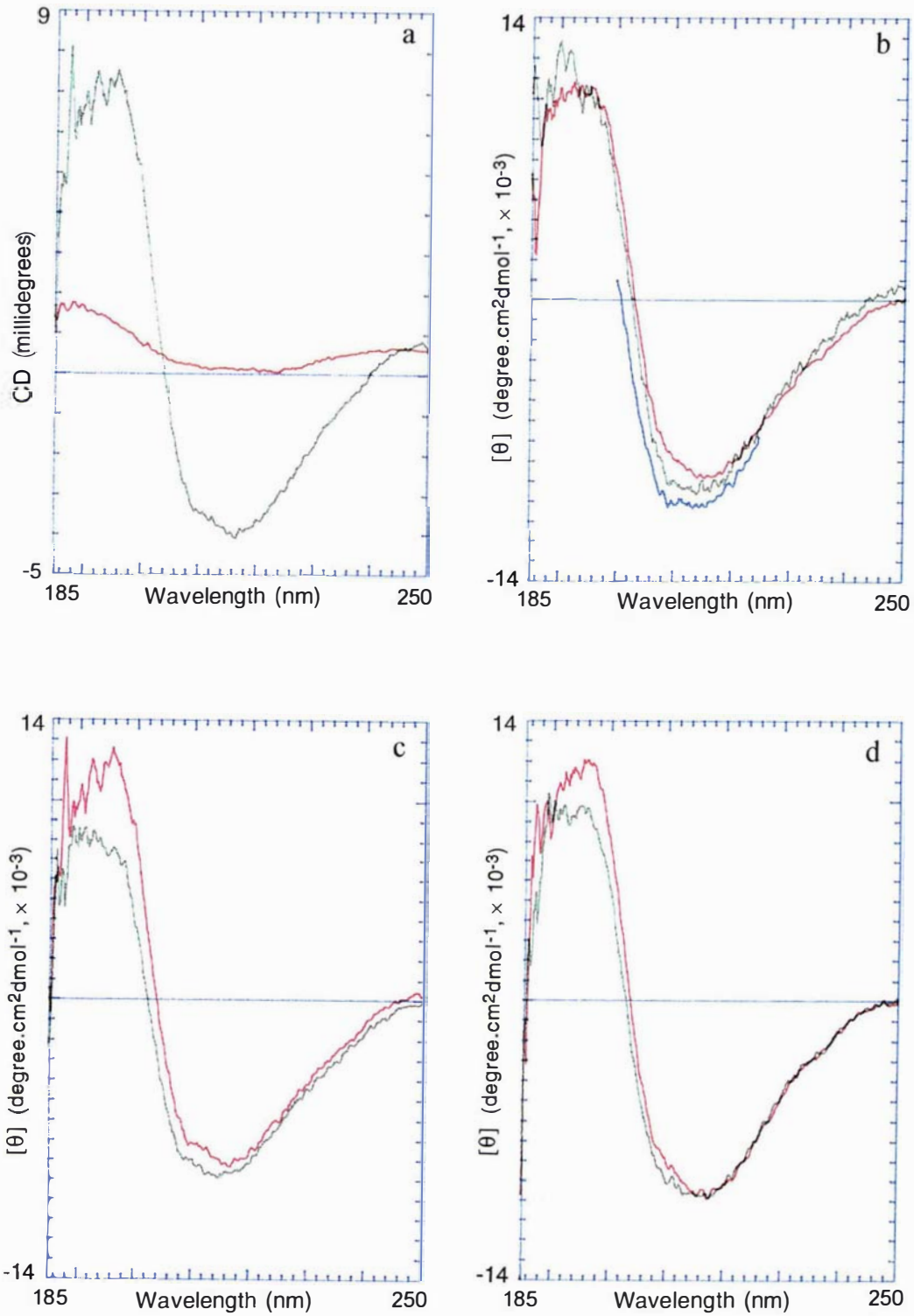
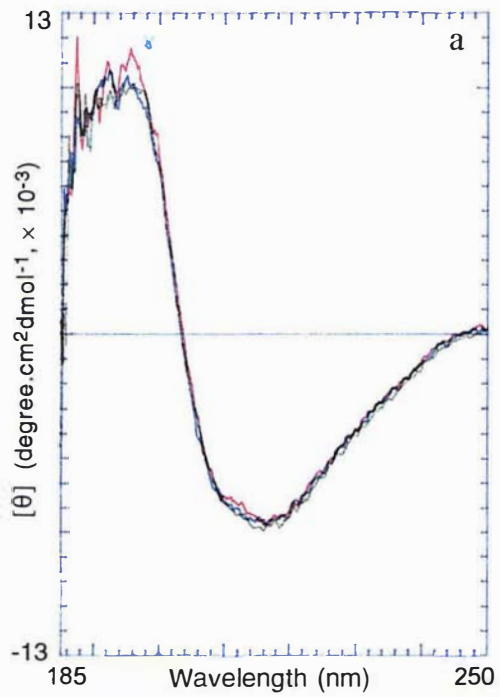
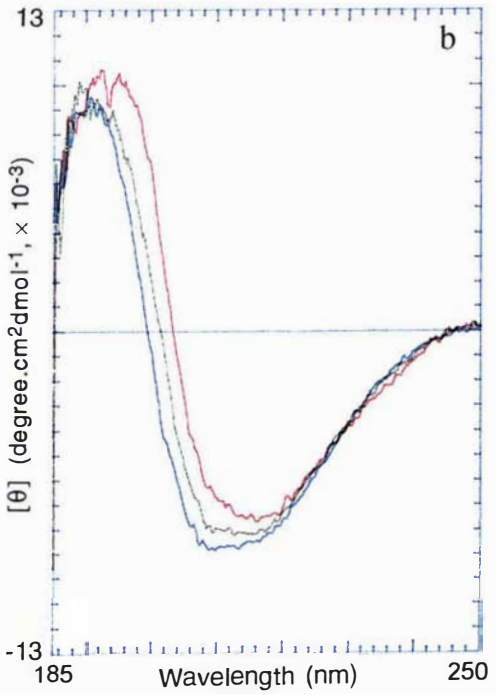
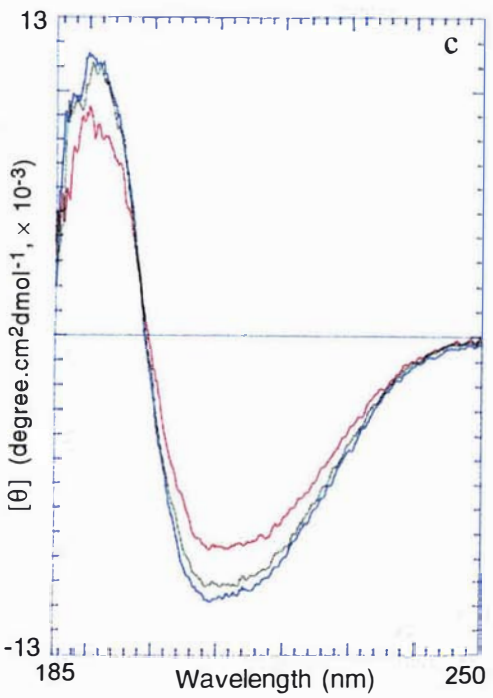

a
9
-5
CD (millidegrees)
185
Wavelength (nm)
250
b
14
-14
[θ] (degree.cm²dmol⁻¹, × 10⁻³)
185
Wavelength (nm)
250
c
14
-14
[θ] (degree.cm²dmol⁻¹, × 10⁻³)
185
Wavelength (nm)
250
d
14
-14
[θ] (degree.cm²dmol⁻¹, × 10⁻³)
185
Wavelength (nm)
250

Fig. 5.3.2.3. The far UV CD spectra of unheated BLG B at pH 6.7 and BLG B previously heat-treated at this pH value at different temperatures. The spectra are as follows: unheated BLG B, red, panel a; BLG B previously heat-treated for 12.5 min at 56.0 °C, green, panel a; 66.1 °C, blue, panel a, and red, panel b; 68.1 °C, green, panel b; 75.9 °C, blue, panel b, and red, panel c; 81.8 °C, green, panel c; and 87.7 °C, blue, panel c. All BLG solutions were heat-treated in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at a concentration of approximately 3 mg/mL. They were then diluted to 1.00 mg/mL with phosphate buffer, and then to 0.17 mg/mL with water in preparation for data collection. Spectra were acquired as described in Fig. 5.3.2.2. Each final spectrum is the average of five scans. The baseline spectrum of water has been subtracted for each protein spectrum. Details of the BLG solution preparation and heating protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.


13
a
[θ] (degree.cm²dmol⁻¹, × 10⁻³)
-13
185
Wavelength (nm)
250


13
b
[θ] (degree.cm²dmol⁻¹, × 10⁻³)
-13
185
Wavelength (nm)
250


13
c
[θ] (degree.cm²dmol⁻¹, × 10⁻³)
-13
185
Wavelength (nm)
250

The spectra in Fig. 5.3.2.3 also suggest that the amount of β-turn in BLG does not increase as a consequence of heat treatment. This is because there is a peak at approximately 205 nm in the spectrum of β-turn (Brahms and Brahms, 1980; Woody, 1995, Fig. 5.3.2.1). In the spectrum of BLG, the intensity of the trough at approximately 205 nm increases as a consequence of heat treatment, which is inconsistent with an increase in the amount of β-turn in BLG molecules.

Tryptophan side chains may also contribute to the far UV CD spectra of proteins (Freskgård *et al.*, 1994), particularly those rich in α-helix (Woody, 1995). Furthermore, the extent and wavelength range of such contributions can vary considerably amongst different proteins (Freskgård *et al.*, 1994). Contributions from disulphide bonds may also be observed in the far UV CD spectra of proteins (Woody, 1995). However, for the reasons discussed above, it is most likely that the results in Fig. 5.3.2.3 indicate that the proportion of random structure and β-sheet structure in BLG molecules increases as a consequence of heat treatment.

An increase in the proportion of random structure in molecules of BLG caused by heat treatment may reflect an irreversible loosening of side chain packing. Furthermore, the results in Fig. 5.3.2.3 indicate that in BLG solutions, the extent of random structure formation, and therefore the extent of the irreversible loosening of side chain packing, increases with increasing heat treatment temperature above approximately 68.1 °C. The far UV CD results in Fig. 5.3.2.3 are therefore consistent with the $\Delta\varepsilon_{293}$ results in Section 5.3.1, which indicate that heat treatment leads to a decrease in the chirality of the environment of Trp^{19}.

The results in Fig. 5.3.2.3 appear inconsistent with those obtained in studies in which far UV CD measurements were made at room temperature at neutral pH using BLG solutions previously heat-treated (Sawyer *et al.*, 1971; Matsuura and Manning, 1994). Sawyer *et al.* (1971) reported that the intensity of $[\theta]_{216}$ in the spectrum of BLG A/B increased approximately 1.8-fold as a consequence of heat treatment at a concentration of 5 mg/mL, and concluded that heat-treated BLG contains more β-sheet than the corresponding unheated species (Section 2.6.2.2). Matsuura and Manning (1994) made far UV CD measurements at room temperature using solutions of BLG A/B previously heat-treated at a concentration of 70 mg/mL. In heat-treating BLG A/B solutions at this concentration, they were able to study spectral changes which occurred as a consequence of gelation (Section 5.3.1.3). Matsuura and Manning (1994) found, for solutions of BLG A/B at this concentration, that heat treatment led to a large increase in the intensity of $[\theta]_{216}$, and concluded that the proportion of β-sheet in molecules of BLG increases as a consequence of gelation. The results in Fig. 5.3.2.3, however, indicate that the intensity of $[\theta]_{216}$ increases only slightly as a consequence of

heat treatment. Furthermore, in contrast to the results in Fig. 5.3.2.3, neither Sawyer *et al.* (1971) nor Matsuura and Manning (1994) reported that the intensity of [θ] at approximately 205 nm increased as a consequence of heat treatment. Because Matsuura and Manning (1994) made measurements using BLG solutions previously heat-treated at 70 mg/mL, a comparison of their results with those in Fig. 5.3.2.3 (acquired from BLG solutions previously heat-treated at approximately 3 mg/mL) may not be appropriate. However, because Sawyer *et al.* (1971) made measurements using BLG solutions previously heat-treated at 5 mg/mL, there is no obvious explanation for the differences between their results and those in Fig. 5.3.2.3.

The results in Fig. 5.3.2.3 are consistent with the results obtained in a number of far UV CD studies made at elevated temperatures (Griffin *et al.*, 1993; Molinari *et al.*, 1996; Qi *et al.*, 1997). In these studies, it was reported that during temperature increase, the intensity of [θ] at approximately 207 nm increases appreciably, while the intensity of [θ] at approximately 216 nm remains approximately constant. On the other hand, Lapanje and Poklar (1989) reported that the intensity of $[\theta]_{216}$ decreases with increasing temperature. To summarise, the results shown in Fig. 5.3.2.3 appear consistent with those obtained in the majority of published far UV CD studies made at elevated temperatures.

5.3.2.4. Quantitative Study: Heat-induced Irreversible Structural Change in β-Lactoglobulin Molecules.

The trends observed in the series of spectra acquired from heat-treated solutions of BLGs A and C at pH 6.7, BLGs A, B and C at pH 7.4 and BLG A at pH 8.1 were similar to those in the series of spectra of heat-treated solutions of BLG B at pH 6.7 (Fig. 5.3.2.3) discussed in Section 5.3.2.3. In particular, the dependence of [θ] on heat treatment temperature was greatest at approximately 205 nm in all seven data sets. For this reason, extents of heat-induced irreversible structural change in BLG molecules in solutions of the A, B and C variants were determined by measuring changes in $[\theta]_{205}$ with heat treatment temperature. Experimental $[\theta]_{205}$ data were then fitted using the modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) described in Section 5.2.8.1. Plots of experimental $[\theta]_{205}$ data versus heat treatment temperature and the curves obtained by fitting these data to equations 5.2.8.1 and 5.2.8.2 using the computer program "Enzfitter" (Section 5.2.8.1) are shown in Fig. 5.3.2.4. The coincidence of the experimental $[\theta]_{205}$ data with the fitted curves shows that the

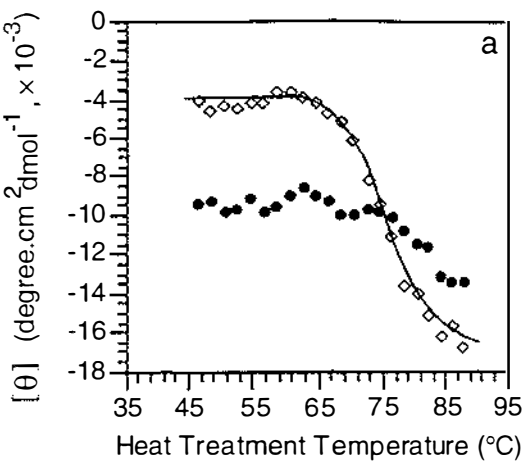
selected model adequately describes heat-induced irreversible structural change in molecules of BLGs A, B and C. Therefore the fitted value for the parameter T_{mid} for a particular variant at a particular pH (Table 5.3.2.1) was assumed to represent the midpoint temperature of the transition for the heat-induced irreversible structural change which leads to an increase in the proportion of random structure in BLG molecules. The errors in the values for T_{mid} (Table 5.3.2.1) are standard errors calculated by "Enzfitter" and are probably underestimates of the true error in the values of this parameter.

Except in the cases of BLG C at pH 7.4 and BLG A at pH 8.1, the plots in Fig. 5.3.2.4 indicate that the intensity of $[\theta]_{216}$ in the far UV CD spectra of BLG solutions increases slightly with increasing heat treatment temperature. This is consistent with the spectral results obtained from previously heat-treated solutions of BLG B at pH 6.7. Therefore, it is concluded that the amount of β-sheet in molecules of BLGs A, B and C increases slightly as a consequence of heat treatment.

Although the plots of experimental $[\theta]_{205}$ data versus heat treatment temperature in Fig. 5.3.2.4 are not particularly sigmoidal, and the extent of scatter of experimental $[\theta]_{205}$ data is higher than that of $\Delta\varepsilon_{293}$ data, the experimental $[\theta]_{205}$ data coincide with the fitted curves. Differences in the extent of scatter of experimental $[\theta]_{205}$ and $\Delta\varepsilon_{293}$ data probably reflect the high signal:noise ratio at approximately 205 nm in far UV CD spectra (Figs 5.3.2.2 and 5.3.2.3). This in turn may be due to the absorption of UV light at wavelengths less than approximately 240 nm by oxygen. Therefore, the intensity of UV light incident on the first PMT plate would have been less in the case of far UV CD measurements compared to that in the case of near UV CD measurements.

At pH 6.7 in particular, the slopes at T_{mid} in the plots of $[\theta]_{205}$ versus heat treatment temperature are greater for BLG A than for BLGs B and C (Table 5.3.2.1), suggesting that BLG A responds to heat treatment in a different manner to BLGs B and C. The significance of this will be discussed in Section 8.5.

Values for T_{mid} were used to obtain a quantitative measure of the differences in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change. The T_{mid} values in Table 5.3.2.1, which were determined from data acquired at room temperature using BLG solutions previously heat-treated, are similar to the value of 75 °C for BLG A at pH 7.0 determined by Griffin *et al.* (1993) from data collected at elevated temperatures. However, they reported that the intensity of $[\theta]_{207}$ increased with increasing temperature until 85 °C, thus making it difficult to identify the T_{mid} value. This is also true for the T_{mid} values determined from the plots shown in Fig. 5.3.2.4.


a
[θ] (degree.cm^{2}dmol^{-1},×10^{-3})
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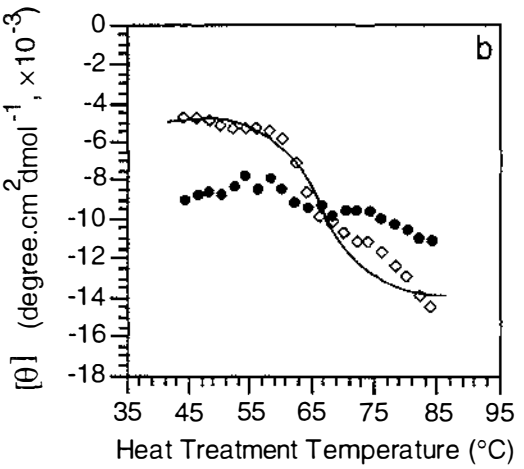
-2
-4
-6
-8
-10
-12
-14
-16
-18
35 45 55 65 75 85 95
Heat Treatment Temperature (°C)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-6
-8
-10
-12
-14
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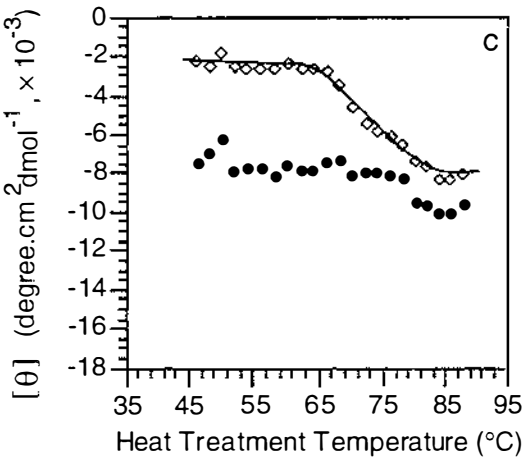
-18
35 45 55 65 75 85 95
Heat Treatment Temperature (°C)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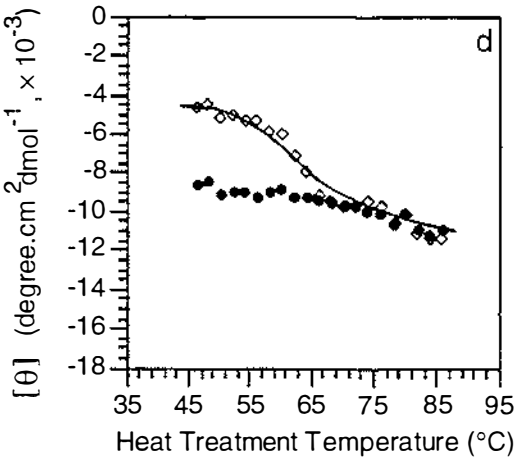
35 45 55 65 75 85 95
Heat Treatment Temperature (°C)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-10
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-14
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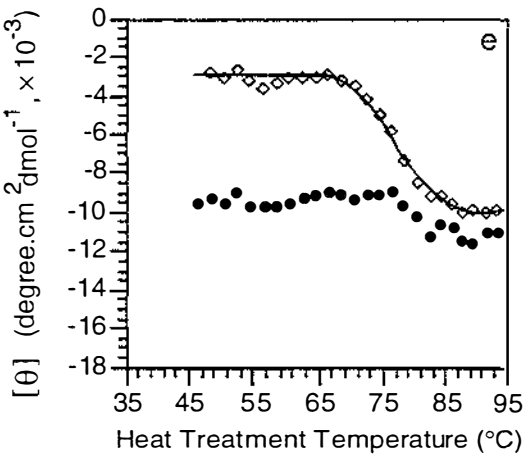
35 45 55 65 75 85 95
Heat Treatment Temperature (°C)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[θ] (degree.cm^{2}dmol^{-1},×10^{-3})
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-6
-8
-10
-12
-14
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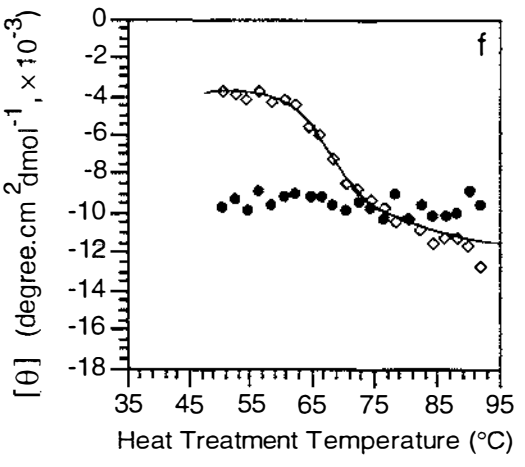
-18
35 45 55 65 75 85 95
Heat Treatment Temperature (°C)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-10
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-14
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35 45 55 65 75 85 95
Heat Treatment Temperature (°C)

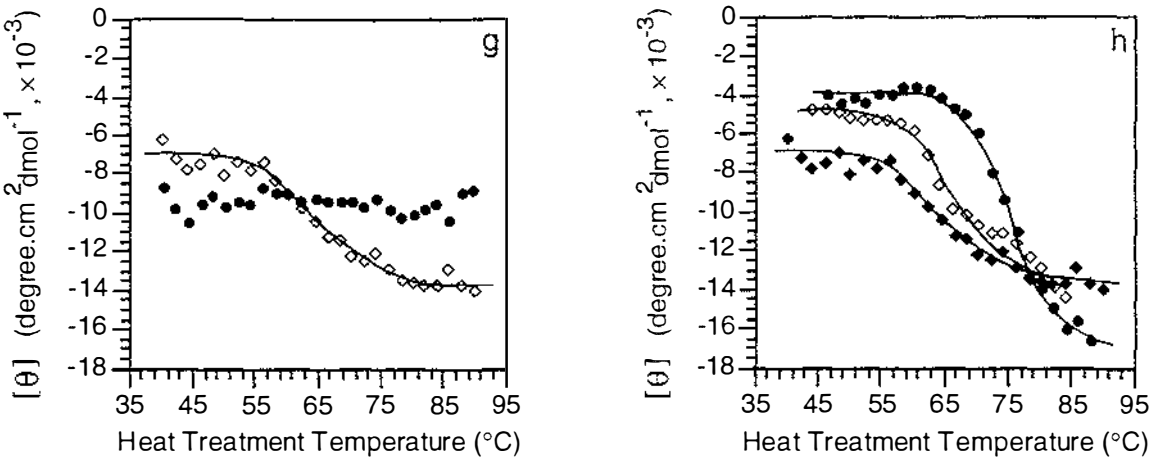


Fig. 5.3.2.4. Effect of heat treatment on $[\theta]_{216}$ and $[\theta]_{205}$ in the far UV CD spectra of BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1. The symbols (•) and (◇) denote experimental $[\theta]_{216}$ and $[\theta]_{205}$ data respectively. The plots are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; BLG C at pH 7.4, panel f; BLG A at pH 8.1, panel g; and in panel h, BLG A at pH 6.7 (•), pH 7.4 (◇) and pH 8.1 (♦). Experimental data were obtained from spectra recorded as described in Fig. 5.3.2.2. The curves were obtained by fitting experimental $[\theta]_{205}$ data to equations 5.2.8.1 and 5.2.8.2 (Section 5.2.8.1) using the computer program "Enzfitter" (Leatherbarrow, 1987). Values for the fitting parameters are given in Appendix 3.

Table 5.3.2.1. Values for the Midpoint Temperature (T_{mid}) and Slope at T_{mid} for the Heat-induced Irreversible Increase in the Proportion of Random Structure in Molecules of β-Lactoglobulins A, B and C at pH 6.7 and at pH 7.4 and β-Lactoglobulin A at pH 8.1.

Sample	T_{mid} (°C)	Slope at T_{mid} ($[\theta]_{205}$/°C)
pH 6.7, BLG A	75.7 ± 0.7	-700
pH 6.7, BLG B	74.8 ± 1.6	-250
pH 6.7, BLG C	77.0 ± 0.1	-446
pH 7.4, BLG A	69.4 ± 0.9	-299
pH 7.4, BLG B	64.6 ± 0.8	-250
pH 7.4, BLG C	70.0 ± 0.4	-181
pH 8.1, BLG A	65.4 ± 0.7	-244

Where:

T_{mid} is the midpoint temperature of the transition for the heat-induced irreversible structural change which leads to an increase in the proportion of random structure in BLG molecules (i.e. the temperature at which $[\theta]_{205}$ is exactly half way between the values for $[\theta]_{205}$ on the low and high temperature sides of the transition for heat-induced irreversible structural change in the plots shown in Fig. 5.3.2.4). T_{mid} is one of the parameters used to fit experimental $[\theta]_{205}$ data to equations 5.2.8.1 and 5.2.8.2 (Section 5.2.8.1) using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in T_{mid} values are standard errors calculated by "Enzfitter".

The slope at the midpoint temperature was calculated using the fitted $[\theta]_{205}$ values in the range T_{mid} ± 0.5 °C.

At pH 6.7 the T_{mid} value for BLG B is 0.9 °C lower than that for BLG A, which is 1.3 °C lower than that for BLG C (Table 5.3.2.1). At pH 7.4 the T_{mid} value for BLG B is 4.8 °C lower than that for BLG A, which is 0.6 °C lower than that for BLG C (Table 5.3.2.1). Thus, the T_{mid} values determined using $[\theta]_{205}$ data suggest that at pH 6.7, the B, A and C variants exhibit the greatest, intermediate and lowest susceptibilities respectively to the heat-induced irreversible structural change which leads to an increase in random structure. Furthermore, these T_{mid} values (Table 5.3.2.1) suggest that at pH 7.4 BLG B exhibits a greater susceptibility to this heat-induced irreversible structural change than BLG A. However, at pH 7.4 the T_{mid} values for

BLGs A and C are equivalent within the limits of the standard errors calculated by "Enzfitter", suggesting that susceptibilities of these variants to heat-induced irreversible random structure increase are equivalent at this pH.

The T_{mid} values shown in Table 5.3.2.1 for BLGs A and B and C at pH 6.7 are, respectively, 6.3 °C, 10.2 °C and 7.0 °C higher than those at pH 7.4. Furthermore, in the case of BLG A, the T_{mid} at pH 7.4 is 4.0 °C higher than that at pH 8.1. These differences suggest that BLGs A, B and C are more susceptible to heat-induced irreversible structural change at pH 7.4 than at pH 6.7 and that this trend continues for BLG A at pH 8.1. This is therefore consistent with the spectral results shown in Fig. 5.3.2.2, which suggest that molecules of BLGs A, B and C possess slightly more random structure at pH 7.4 than at pH 6.7, and that BLG A possesses slightly more random structure at pH 8.1 than at pH 7.4. β-Lactoglobulins A, B and C may therefore be more susceptible to heat-induced irreversible structural change at pH 7.4 and pH 8.1 than at pH 6.7 because their side chains are packed less tightly against each other at the two higher pH values (Section 5.3.2.3).

5.3.2.5. Thermodynamic Analysis.

A thermodynamic analysis of fitted $[\theta]_{205}$ data was also made (Table 5.3.2.2). However, these data were collected under conditions in which the unfolding of BLG is both partial and irreversible (Section 5.3.1.5). Therefore, they may only be used to calculate values for the apparent change in free energy (ΔG_{app}) for the heat-induced irreversible structural change which leads to an increase in the amount of random structure in BLG molecules.

Values for ΔG_{app} for BLGs A, B and C at pH 6.7 were calculated at 72 °C using $[\theta]_{205}$ data because this temperature is close to the T_{mid} for heat-induced irreversible structural change for all three variants at this pH value (Table 5.3.2.1). Furthermore, 72 °C is also that at which ΔG_{app} values were calculated using $\Delta\varepsilon_{293}$ data. Similarly, in the case of $[\theta]_{205}$ data, ΔG_{app} values for BLGs A, B and C at pH 7.4 were calculated at 66 °C because this temperature is close to the T_{mid} for all three variants at this pH value (Table 5.3.2.1) and is also that at which ΔG_{app} values were calculated using $\Delta\varepsilon_{293}$ data.

Table 5.3.2.2. Values for ΔG_{app} at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for the Irreversible Increase in the Proportion of Random Structure in β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6.7, BLG A	3.173 ± 0.029	1.54*
pH 6.7, BLG B	1.638 ± 0.035	0 (by definition)
pH 6.7, BLG C	5.541 ± 0.007	3.90#
pH 7.4, BLG A	2.878 ± 0.040	1.95*
pH 7.4, BLG B	0.926 ± 0.010	0 (by definition)
pH 7.4, BLG C	4.671 ± 0.030	3.75#

* ΔG_{app}(BLG A)- ΔG_{app}(BLG B).

ΔG_{app}(BLG C)- ΔG_{app}(BLG B).

ΔG_{app} is assumed to represent the change in free energy at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for the irreversible structural change which leads to an increase in the proportion of random structure in molecules of BLGs A, B and C. Values for ΔG_{app} were calculated at these temperatures and pH values using fitted values for $[\theta]_{205}$ as described in Section 5.2.8.3. The fitted values for $[\theta]_{205}$ were obtained from the fitted curves shown in Fig. 5.3.2.4 using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in ΔG_{app} were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 5.3.2.1).

The ΔG_{app} values in Table 5.3.2.2 are similar to those in Table 5.3.1.2 and therefore suggest that BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change at 72 °C at pH 6.7 and at 66 °C at pH 7.4. This is consistent with the T_{mid} values shown in Table 5.3.2.1. The differences in the ΔG_{app} values for BLGs A, B and C, which are shown as $\Delta\Delta G_{app}$ values in Table 5.3.2.2, will be discussed further in Section 8.5.

5.3.2.6. Comparison of Effects of Urea and Heat on the Structure of β-Lactoglobulin.

A comparison of the far UV CD results in Sections 5.3.2.3 - 5.3.2.5 with those of Creamer (1995) indicates that the extent of structural change in molecules of BLG caused by urea treatment is greater than that caused by heat treatment, even at temperatures 84 °C and greater, where the proportion of BLG molecules in non-native conformations is greatest (Fig. 5.3.2.4). In the presence of 7.24 M urea at pH 6.7, the far UV CD spectrum of BLG A/B exhibits one band, a trough centred at approximately

200 nm, suggesting a loss of β-sheet structure (Creamer, 1995). Furthermore, because a trough was not observed at 222 nm, Creamer (1995) concluded that in the presence of 7.24 M urea, BLG A/B possess less α-helix than it does in the absence of urea. Creamer (1995) reported that spectral noise below 200 nm, resulting from the use of concentrated urea solutions, made it difficult to determine the shape of the trough centred at approximately 200 nm. This in turn made it difficult to ascertain whether the proportion of random structure in molecules of BLG A/B is greater in the presence of urea than it is in its absence. Nevertheless, the study of Creamer (1995) demonstrates that molecules of BLG A/B possess less secondary structure in the presence of 7.24 M urea than molecules of BLGs A, B and C do after heat treatment.

In contrast to the results of Creamer (1995), Civera *et al.* (1996) reported that at pH 2.1 in the presence of 8 M urea, BLG B possesses an appreciable amount of β-sheet structure. They found that an increase in urea concentration to 8 M led to only a small decrease in the intensity of the negative CD band in the far UV CD spectrum of this variant, but also to a shift in the λ_{min} of this band from approximately 220 nm to approximately 215 nm. In agreement with the results of Civera *et al.* (1996), Kuwajima *et al.* (1996) reported that at pH 3.2 in the presence of 4 M guanidine hydrochloride (Gdn-HCl), BLGs A and B possess an appreciable amount of β-sheet structure. However, the inconsistency between the results of Civera *et al.* (1996) and Kuwajima *et al.* (1996) and those of Creamer (1995) can probably be explained in terms of the much lower pH at which the two former studies were made.

5.3.2.7. Summary of Near and Far UV CD Results.

The near UV CD results in Sections 5.3.1 indicate that after BLG is heat-treated, the environment of Trp^{19} is altered as is the organisation of disulphide bonds. The far UV CD results in Sections 5.3.2.3 - 5.3.2.5 indicate that heat treatment has an irreversible effect on the overall packing of side chains in BLG molecules, but has little effect on the amount of secondary structure in this protein. In the next section, heat-induced structural change in the vicinity of tryptophan side chains is examined further using fluorescence spectroscopy.

5.3.3. RESULTS AND DISCUSSION: INTRINSIC PROTEIN FLUORESCENCE.

5.3.3.1. Introduction to Intrinsic Protein Fluorescence.

Fluorescence is observed when the electrons in excited states in certain compounds return to the ground state by emitting photons. Compounds which fluoresce generally possess delocalised electrons formally present in conjugated double bonds, and may also contain electron donating and electron withdrawing groups. The intensity and λ_{max} of fluorescence emissions are dependent on a number of factors. Firstly, temperature increase leads to a decrease in fluorescence emission intensity (Lakowicz, 1983). Secondly, solvent polarity may affect emission λ_{max}, emission intensity, or both (Lakowicz, 1983). Thirdly, emission intensity decreases when non-fluorescent quenching groups are close to fluorophores, thus allowing electrons in the excited states of the latter to return to the ground state without emitting photons (Lakowicz, 1983). Fluorescence quenching is usually expressed in terms of quantum yield (Q), the ratio of the number of photons emitted to the number absorbed. A Q of 1 indicates that the quenching of the fluorophore in question is negligible, while a Q of 0 indicates that the fluorescence from the fluorophore in question is completely quenched (Lakowicz, 1983). Fourthly, when two fluorophores which emit at different wavelengths are brought close to one another and are fixed in an appropriate orientation, the group which emits at the shorter wavelength may, following excitation, return to the ground state by transferring energy to the group which emits at the longer wavelength. This process, fluorescence quenching by another fluorophore, is known as radiationless (or resonance) energy transfer (RET). When RET occurs, the group which emits at the shorter wavelength does not fluoresce as intensely as it would in the absence of the other fluorophore.

Several fluorophores are found naturally in proteins: tryptophan, tyrosine and phenylalanine side chains. The fluorescence of these side chains is collectively known as intrinsic protein fluorescence. Tryptophan can be excited at wavelengths between approximately 250 nm and 305 nm, and emits between approximately 328 nm and 348 nm (Lakowicz, 1983). Furthermore, emission λ_{max} values increase with increasing solvent polarity (Lakowicz, 1983). For example, the fluorescence emission λ_{max} values for tryptophan in butanol and water are approximately 314 nm and 336 nm respectively. Tryptophan emission intensity, on the other hand, is not particularly sensitive to solvent polarity. The fluorescence emission spectra of native proteins are usually dominated by the contributions from tryptophan side chains (Lakowicz, 1983).

Tyrosine can be excited at wavelengths between approximately 250 nm and 290 nm and emission is usually maximal at about 300 nm, with both λ_{max} and intensity not particularly dependent on solvent polarity (Lakowicz, 1983). Although in aqueous solution the fluorescence quantum yield of tyrosine is greater than that of tryptophan, emission intensity from tyrosine side chains is usually small and often undetectable in native proteins (Lakowicz. 1983). This is due to several phenomena. Firstly, tyrosine emission intensity may be weak because RET from tyrosine to tryptophan is usually very efficient in native proteins. This is because many proteins have diameters comparable to the maximum distance for efficient RET from tyrosine to tryptophan (i.e. the Förster distance, Lakowicz, 1983). Secondly, tyrosine fluorescence can be quenched by uncharged and charged amino and carboxyl groups (Lakowicz, 1983). Thirdly, if tyrosine hydroxyl groups are hydrogen bonded to peptide bonds, then tyrosine fluorescence is not observed (Lakowicz, 1983). Because the wavelength ranges in which tyrosine and tryptophan side chains may be excited overlap only partially, it is possible to selectively excite tryptophan at wavelengths between 295 nm and 305 nm. The quantum yield of phenylalanine in proteins is small, and as a consequence, fluorescence is seldom observed in the presence of tyrosine and tryptophan residues (Lakowicz, 1983). No mention of histidine fluorescence is made by Lakowicz (1983).

Intrinsic protein fluorescence can provide information on the structures of proteins in the vicinities of tyrosine and tryptophan side chains. For example, from the λ_{max} of a tryptophan emission peak, it is possible to ascertain whether tryptophan side chains are solvent-exposed, or are surrounded by other hydrophobic side chains in the core of the protein structure. An estimate of the compactness of a protein structure can also be obtained by exciting the protein at a wavelength less than 295 nm, and comparing the relative intensities of tyrosine and tryptophan emissions. If the protein structure is compact, the majority of tyrosine and tryptophan side chains will be located within the Förster distance (the maximum distance for efficient RET from tyrosine to tryptophan), and the intensity of tyrosine emission will be minimal. Furthermore, by following changes in emission intensity during protein unfolding, it may be possible to determine whether tryptophan side chains were located close to naturally occurring quenching groups (e.g. disulphide bonds) or other fluorophores in the native conformation. Finally, extents of structural change in a protein can be determined by measuring the magnitude of the changes in fluorescence emission intensity and λ_{max}. Intrinsic protein fluorescence was used in the study discussed below to measure extents of irreversible change in the structures of BLGs A, B and C caused by heat treatment.

5.3.3.2. Tryptophan Fluorescence Emission Spectra of Unheated and Heat-treated β-Lactoglobulins.

The tryptophan emission spectra of BLGs A, B and C at pH 6.7, unheated and previously heat-treated at 87.7 °C for 12.5 min and obtained using an excitation wavelength of 295 nm, are shown in Fig. 5.3.3.1. In the spectra of the three unheated solutions, the emission λ_{max} is 332 nm. This value is the same as that reported by Creamer (1995) for native BLG A/B, but is slightly higher than the value of 328 nm reported by Mills (1976), and is consistent with emission from tryptophan side chains that are located in semi-hydrophobic environments (Mills, 1976). Tryptophan emission λ_{max} values are usually lower than 332 nm if the tryptophan side chains of proteins interact with mainly non-polar side chains (Lakowicz, 1983).

The emission peak width at half height ($W_{1/2}$) for the spectra of unheated BLG solutions is approximately 50 nm (Fig. 5.3.3.1), in agreement with the results of Mills (1976) who offered two explanations for this finding. One of these was that the emission intensity from one tryptophan side chain is much less than that from the other. This is consistent with the BLG crystal structures of Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998), in which the side chain of Trp^{19} is partially solvent-inaccessible, while that of Trp^{61} is solvent-exposed and located approximately 6 Å from the disulphide bond Cys^{66}-Cys^{160}. Because disulphide bonds are recognised quenchers of protein tryptophan fluorescence (Cowgill, 1967), it is therefore likely that Trp^{61} fluorescence in native BLG is quenched by the disulphide bond Cys^{66}-Cys^{160}, thus confirming the suggestion of Mills (1976). Cho *et al.* (1994) found that BLG tryptophan fluorescence emission intensity (I_{Trp}) decreased by approximately 80 % when Trp^{19} was replaced with Ala^{19}. This is also consistent with the suggestion put forward by Mills (1976). Quenching of tryptophan fluorescence by a disulphide bond may occur in other proteins. For example, Hennecke *et al.* (1997) have suggested that energy is transferred from Trp^{76} to Phe^{26} and then to the disulphide bond Cys^{30}-Cys^{33} in the oxidised form of the disulphide oxidoreductase DsbA from *Escherichia coli* .

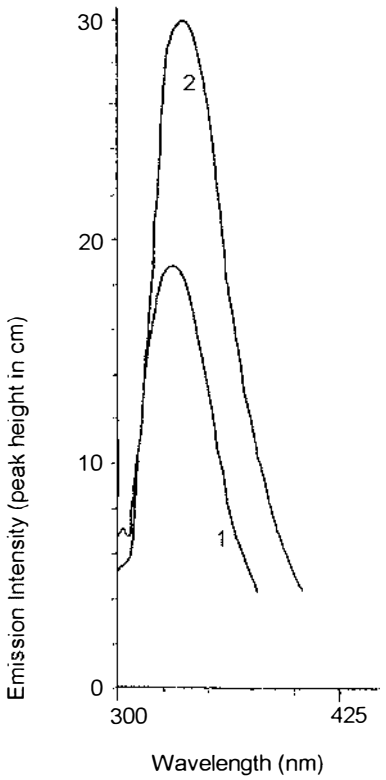
The spectra in Fig. 5.3.3.1 indicate that the tryptophan emission λ_{max} values for BLGs A, B and C at pH 6.7 increase from 332 nm to approximately 339 nm as a consequence of prior heat treatment. These results suggest that at least one tryptophan side chain in these BLGs becomes more solvent-accessible as a consequence of heat-treatment.

Tryptophan emission intensity from BLGs A, B and C increases approximately 1.6-fold as a consequence of heat treatment (Fig. 5.3.3.1). This is not normally observed in proteins because I_{Trp} is not particularly dependent on solvent polarity (Section 5.3.3.1). Therefore, because Trp^{61} fluorescence is probably quenched in native BLG (by the disulphide bond Cys^{66}-Cys^{160}), the heat-induced increase in I_{Trp} for this protein suggests a decrease in the extent of quenching of Trp^{61} fluorescence. This could occur as a consequence of an irreversible increase in the distance between the side chain of Trp^{61} and the disulphide bond Cys^{66}-Cys^{160}, or the loss of the latter. Increases in I_{Trp} may also occur as a consequence of a decrease in the extent of quenching of Trp^{19} fluorescence, but it is not possible to determine whether this is true from the results in this thesis. Irreversible increases in tryptophan fluorescence emission intensity and λ_{max} caused by heat treatment have been reported before for BLG (Mills, 1976; Cairoli *et al.*, 1994; Iametti *et al.*, 1995).

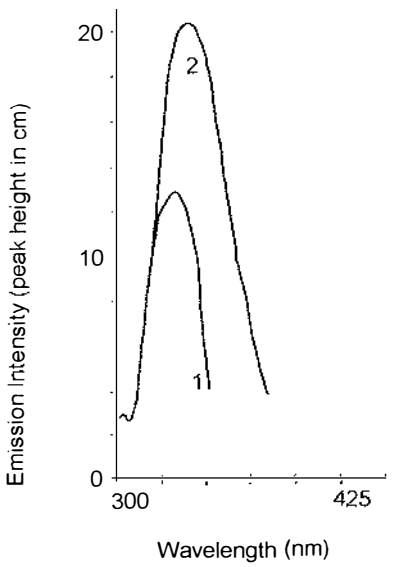
For BLGs A, B and C at pH 7.4, and for BLG A at pH 8.1 (Fig. 5.3.3.2), heat treatment led to increases in I_{Trp} and emission λ_{max} of equivalent magnitude to those described above for solutions at pH 6.7.

Fig. 5.3.3.1. The tryptophan fluorescence emission spectra of unheated and previously heat-treated BLGs A, B and C at pH 6.7. The spectra of unheated BLGs in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) are as follows: BLG A, panel a, spectrum 1; BLG B, panel b, spectrum 1; and BLG C, panel c, spectrum 1. The spectra of BLGs previously heat-treated in pH 6.7 phosphate buffer for 12.5 min at 87.7 °C are as follows: BLG A, panel a, spectrum 2; BLG B, panel b, spectrum 2; and BLG C, panel c, spectrum 2. The spectra were acquired from 1.0 mg/mL solutions at 20 °C in 10 mm path length cells excited at 295 nm using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. Measurements were made using excitation and emission slit widths of 8 nm at a scan speed of 25 nm/min and a chart recorder speed of 1 cm/min. Details of the solution preparation and heat treatment protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.

a


30
20
10
0
2
1
300
425
Emission Intensity (peak height in cm)
Wavelength (nm)

b


20
10
0
2
1
300
425
Emission Intensity (peak height in cm)
Wavelength (nm)

c

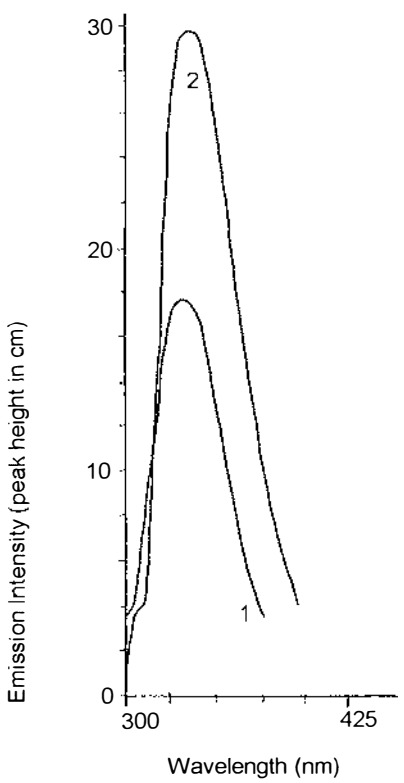
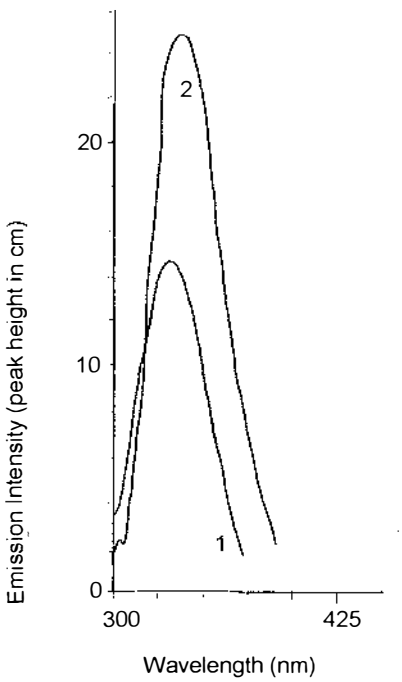
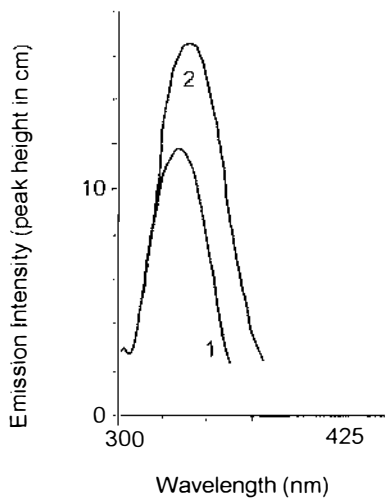

30
20
10
0
2
1
300
425
Emission Intensity (peak height in cm)
Wavelength (nm)

Fig. 5.3.3.2. The tryptophan fluorescence emission spectra of unheated and heat-treated BLGs A, B and C at pH 7.4 and BLG A at pH 8.1. The spectra of unheated BLGs in pH 7.4 phosphate buffer (26 mM, with 68 mM NaCl) and pH 8.1 phosphate/borate buffer (13 mM phosphate, 13 mM borate, with 68 mM NaCl) are as follows: BLG A at pH 7.4, panel a, spectrum 1; BLG B at pH 7.4, panel b, spectrum 1; BLG C at pH 7.4, panel c, spectrum 1; and BLG A at pH 8.1, panel d, spectrum 1. The spectra of BLGs previously heat-treated in pH 7.4 phosphate buffer or pH 8.1 phosphate/borate buffer for 12.5 min at 87.7 °C are as follows: BLG A at pH 7.4, panel a, spectrum 2; BLG B at pH 7.4, panel b, spectrum 2; BLG C at pH 7.4, panel c, spectrum 2; and BLG A at pH 8.1, panel d, spectrum 2. The spectra were acquired from 1.0 mg/mL solutions as described in Fig. 5.3.3.1. Details of the solution preparation and heat treatment protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.

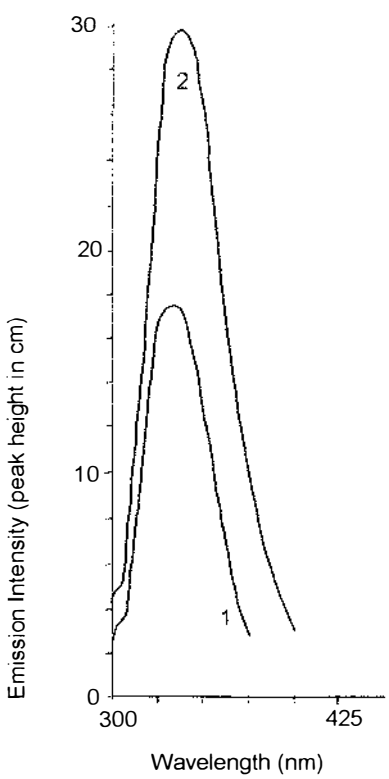
a


Emission Intensity (peak height in cm)
20
10
0
2
1
300
425
Wavelength (nm)

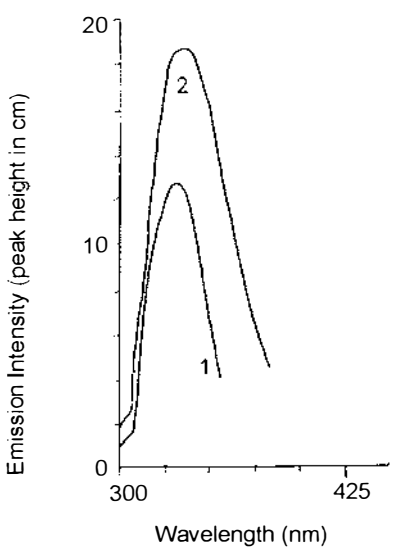
b


Emission Intensity (peak height in cm)
10
0
2
1
300
425
Wavelength (nm)

c


Emission Intensity (peak height in cm)
30
20
10
0
2
1
300
425
Wavelength (nm)

d


Emission Intensity (peak height in cm)
20
10
0
2
1
300
425
Wavelength (nm)

5.3.3.3. Dependence of Tryptophan Emission λ_{max} for β-Lactoglobulins on Heat Treatment Temperature.

For all three variants at both pH 6.7 and pH 7.4, and for BLG A at pH 8.1, tryptophan emission λ_{max} values, and therefore the extent of the solvent-accessibility of one or both of the tryptophan side chains of these variants, increases with increasing heat treatment temperature. This is consistent with the results shown in Figs 5.3.3.1 and 5.3.3.2, which indicate that emission λ_{max} values increase as a consequence of heat treatment.

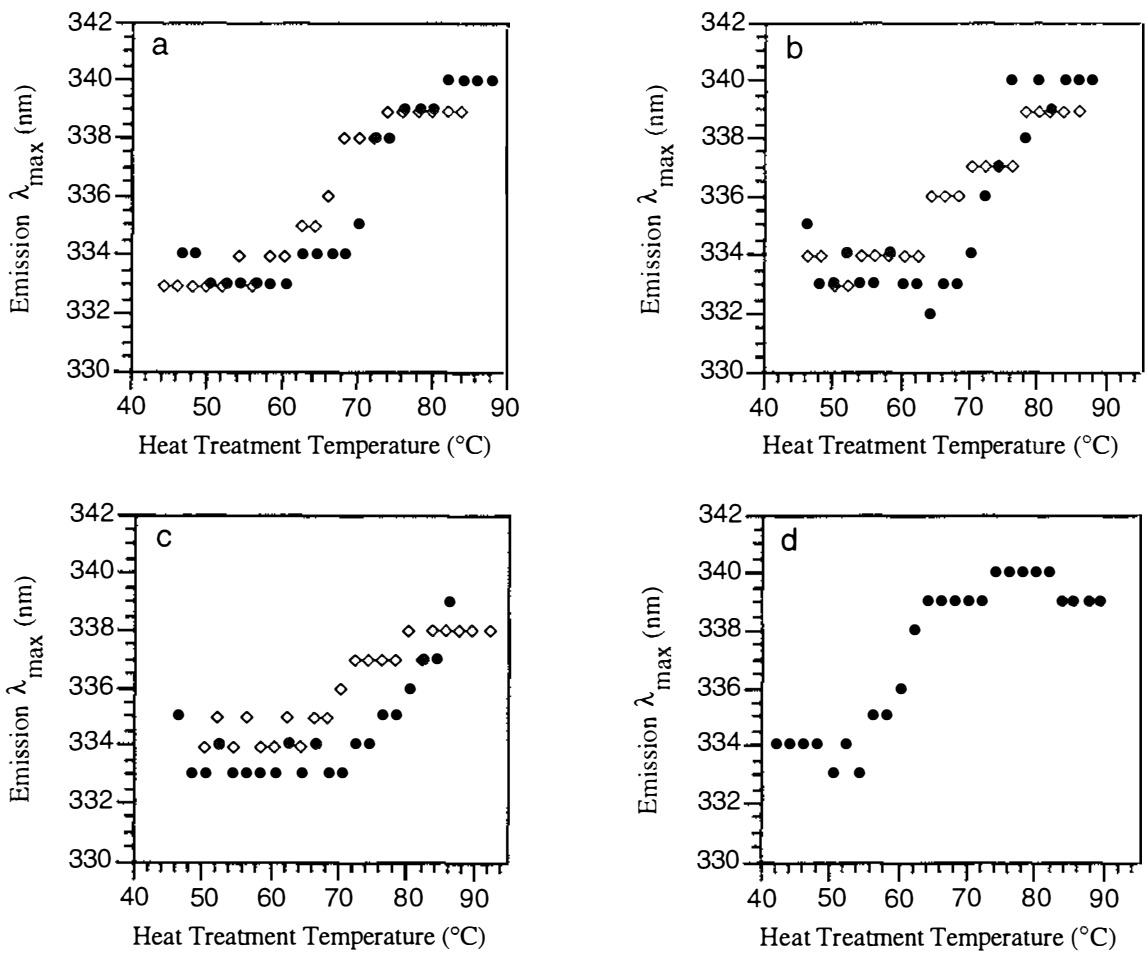


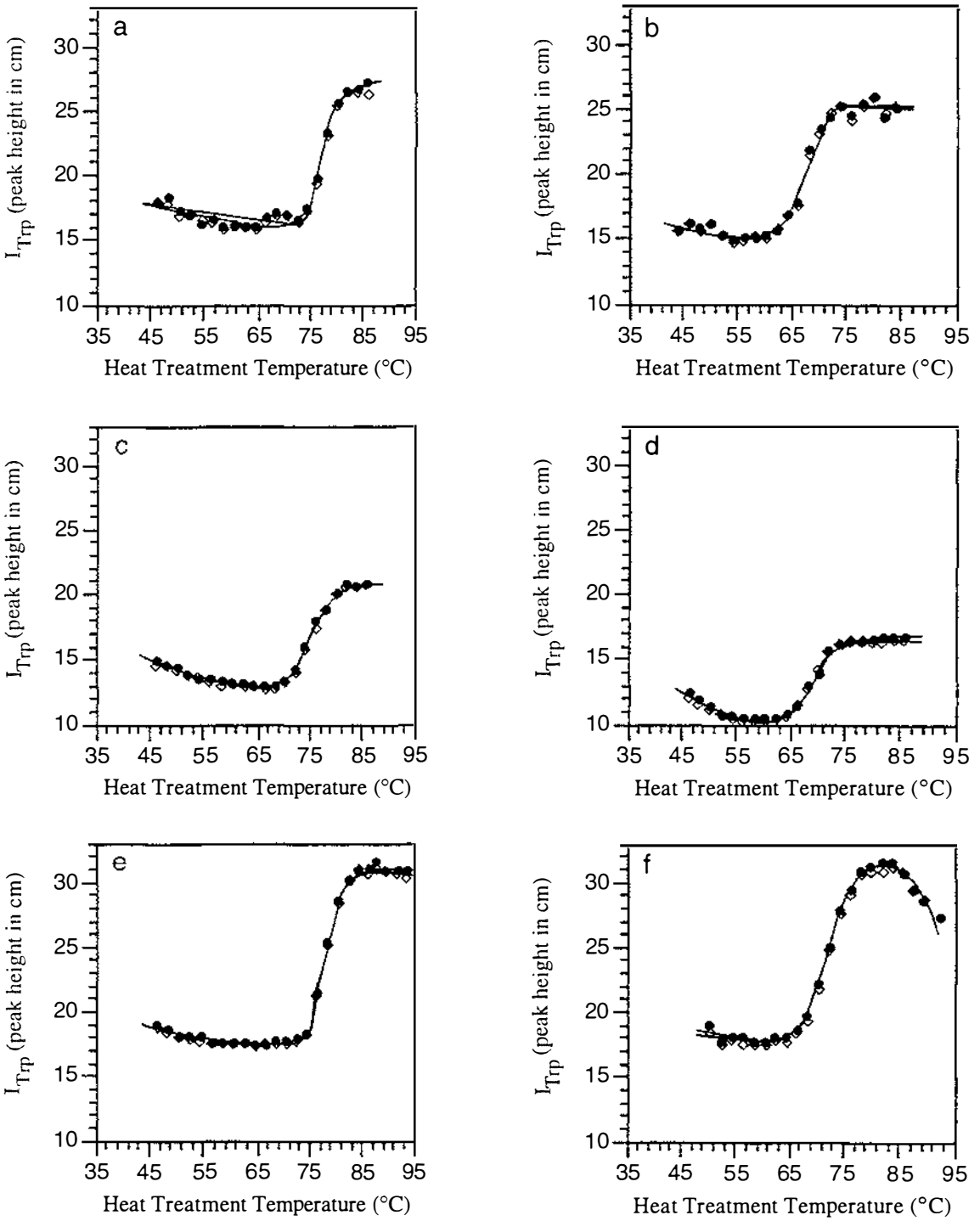
Fig. 5.3.3.3. Effect of heat treatment of solutions of BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1 on tryptophan fluorescence emission λ_{max}. The plots of pH 6.7 (•) and pH 7.4 (◇) data for BLGs A, B and C are shown in panels a, b and c respectively. The plot for BLG A at pH 8.1 is shown in panel d. Values for emission λ_{max} were obtained from spectra recorded in duplicate using an excitation wavelength of 295 nm as described in Fig. 5.3.3.1 (single sets of data plotted for clarity).

The plots in Fig. 5.3.3.3 indicate that for all three variants at pH 6.7, heat treatment at temperatures greater than approximately 68 °C led to an irreversible increase in tryptophan emission λ_{max}. These results are consistent with those of Mills (1976), who found that increases in tryptophan emission λ_{max} were irreversible after heat treatment at temperatures greater than 70 °C. Similarly, Cairoli *et al.* (1994) and Iametti *et al.* (1995) reported that heat-induced increases in tryptophan emission λ_{max} are irreversible after heat treatment at temperatures 65 °C and greater.

5.3.3.4. Quantitative Study of the Dependence of Tryptophan Emission Intensity for β-Lactoglobulins on Heat Treatment Temperature.

The plots presented in Fig. 5.3.3.4, prepared from spectral data acquired using an emission λ_{max} of 295 nm, show the dependence of I_{Trp} for BLGs A, B and C at pH 6.7 and pH 7.4 and for BLG A at pH 8.1 on heat treatment temperature. Although all tryptophan fluorescence measurements were made using the same data collection protocol, these plots (Fig. 5.3.3.4) indicate that I_{Trp} values for the three variants differ. In the case of BLGs A and B, these differences may indicate that the quantum yields of Trp^{19} and Trp^{61} in these variants differ. However, I_{Trp} values for BLG C are probably greater than those for BLGs A and B because the optics of the MPF-2A fluorimeter were cleaned (without the permission of the author) after measurements had been made on the A and B variants, but before they were made on BLG C. No attempt was made to correct for these differences. This is because the major aim of making the fluorescence measurements discussed in this chapter was to identify differences in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change rather than to quantify dissimilarities in tryptophan Q. Nevertheless, to confirm that the differences in I_{Trp} data for BLGs A, B and C reflected differences in the susceptibilities of these variants to heat-induced irreversible structural change, several sets of confirmatory I_{Trp} measurements were made in which the data for all three variants were collected within 8 hours.

For a particular variant at a particular pH value, duplicate I_{Trp} values are similar, especially at temperatures less than approximately 80 °C (Fig. 5.3.3.4). Therefore the error in determining I_{Trp} values is low.


a
b
c
d
e
f
I_{Trp} (peak height in cm)
10
15
20
25
30
35 45 55 65 75 85 95
Heat Treatment Temperature (°C)

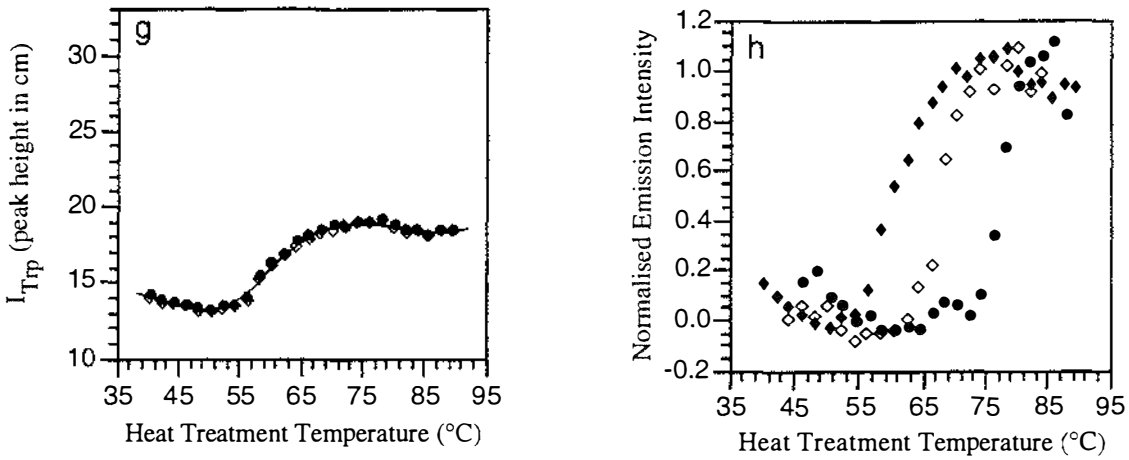


Fig. 5.3.3.4. Effect of heat treatment of solutions of BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1 on tryptophan fluorescence emission intensity (I_{Trp}). The plots of duplicate I_{Trp} data, denoted by the symbols (•) and (◇), are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; BLG C at pH 7.4, panel f; BLG A at pH 8.1, panel g; and in panel h, BLG A at pH 6.7 (•), pH 7.4 (◇) and pH 8.1 (♦). Experimental I_{Trp} data were obtained from spectra recorded as described in Fig. 5.3.3.1. The curves were obtained by fitting experimental I_{Trp} data to equations 5.2.8.2 and 5.2.8.3 as described in Section 5.2.8.2 using the computer program "Enzfitter" (Leatherbarrow, 1987). Values for the fitting parameters are given in Appendix 3. In panel h, experimental and fitted I_{Trp} data were normalised so that the average values for I_{Trp} on the low and high temperature sides of the transition for heat-induced irreversible structural change were 0 and 1 respectively.

In each of the plots in Fig. 5.3.3.4, I_{Trp} decreases with increasing heat treatment temperature between approximately 40 °C and 60 °C, and then increases with increasing heat treatment temperature between approximately 60 °C and 80 °C. In addition, for BLG C at pH 7.4 in particular, I_{Trp} decreases with increasing heat treatment temperature above approximately 80 °C. However, changes in $\Delta\varepsilon_{293}$ with increasing heat treatment temperature were only observed between approximately 60 °C and 80 °C (Fig. 5.3.1.5). Therefore, even though all I_{Trp} data were fitted to a 2-state thermal unfolding model (see below), only the regions of the plots in Fig. 5.3.3.4 in which I_{Trp} increased with increasing heat treatment temperature were interpreted in terms of heat-induced irreversible structural change. The reason why I_{Trp} decreased with increasing heat treatment temperature was not investigated, but may reflect the presence of protein contaminants in BLG solutions.

Experimental I_{Trp} data were fitted to a 2-state thermal unfolding model, which differed from that used to fit $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data. Two additional fitting parameters, which were used to compensate for the two regions of negative slope in the plots in Fig. 5.3.3.4, were incorporated into the model previously used to fit $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data (Section 5.2.8.2). In most instances, the plots of experimental I_{Trp} data and the curves obtained by fitting these data to equations 5.2.8.2 and 5.2.8.3 (Section 5.2.8.2) using the computer program "Enzfitter" (Leatherbarrow, 1987) coincide. This indicates that the selected model adequately describes heat-induced irreversible structural change in molecules of BLGs A, B and C. Therefore, the fitted value for the parameter T_{mid} for a particular variant at a particular pH (Table 5.3.3.1) was assumed to represent the midpoint temperature of the transition for heat-induced irreversible structural change in the vicinities of Trp^{19} and Trp^{61}. The errors for the values for T_{mid} (Table 5.3.3.1) are standard errors calculated by "Enzfitter" and are probably underestimates of the true errors in the values for this parameter.

Values for the slope at T_{mid} determined from normalised plots of I_{Trp} versus heat treatment temperature are shown in Table 5.3.3.1. At pH 6.7, the value for BLG A is greater than that for BLG C, which is greater than that for BLG B. Conversely at pH 7.4, the value for BLG C is less than those for BLGs A and B, which are similar. These orders of slope values are therefore different to that determined from $\Delta\varepsilon_{293}$ data and may reflect differences in the natures of the structural changes which led to increases in the intensity of I_{Trp} and decreases in the intensity of $\Delta\varepsilon_{293}$.

Table 5.3.3.1. Values for the Midpoint Temperature (T_{mid}) and Slope at T_{mid} for Heat-induced Irreversible Structural Change in the Vicinities of Tryptophan^{19} and Tryptophan^{61} in β-Lactoglobulins A, B and C at pH 6.7 and at pH 7.4 and β-Lactoglobulin A at pH 8.1.

Sample	T_{mid} (°C)	Slope at T_{mid} (Normalised I_{Trp}/°C)
pH 6.7, BLG A	77.2 ± 0.3	0.177
	77.7 ± 1.0	0.187
pH 6.7, BLG B	75.4 ± 0.2	0.119
	76.0 ± 1.4	0.112
pH 6.7, BLG C	77.7 ± 0.1	0.150
	77.8 ± 1.3	0.150
pH 7.4, BLG A	67.4 ± 0.4	0.157
	67.4 ± 0.3	0.151
pH 7.4, BLG B	69.2 ± 0.2	0.142
	68.8 ± 0.2	0.143
pH 7.4, BLG C	72.5 ± 0.5	0.113
	72.6 ± 0.4	0.122
pH 8.1, BLG A	60.7 ± 0.2	0.090
	60.7 ± 0.2	0.089

Where:

T_{mid} is the midpoint temperature of the transition for heat-induced irreversible structural change in the vicinities of Trp^{19} and Trp^{61} (i.e. the temperature at which I_{Trp} is exactly half way between the minimum and the maximum values for I_{Trp} in the plots shown in Fig. 5.3.3.4). T_{mid} is one of the parameters used to fit experimental I_{Trp} data to equations 5.2.8.2 and 5.2.8.3 (Section 5.2.8.2) using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in T_{mid} values are standard errors calculated by "Enzfitter".

The slope at the midpoint temperature was calculated using the fitted normalised I_{Trp} values in the range T_{mid} ± 0.5 °C. Sets of fitted I_{Trp} data were normalised so that the average values for I_{Trp} on the low and high temperature sides of the transition for heat-induced irreversible structural change were 0 and 1 respectively.

Values for T_{mid} (Table 5.3.3.1), determined using I_{Trp} data, provide a quantitative measure of the differences between the susceptibilities of BLGs A, B and C at pH 6.7 and pH 7.4, and BLG A at pH 8.1 to heat-induced irreversible structural change. At pH 6.7 the average T_{mid} value for BLG B is 1.8 °C lower than that for BLG A, which is 0.4 °C lower than that for BLG C, The difference for BLGs A and C may, however, be negligible within the limits of experimental error (Table 5.3.3.1). In agreement with the T_{mid} values determined using $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data, those determined using I_{Trp} data therefore suggest that at pH 6.7, BLG B is more susceptible to heat-induced irreversible structural change than BLG A. At pH 7.4 the average T_{mid} value for BLG A is 1.6 °C lower than that for BLG B, which is 3.6 °C lower than that for BLG C (Table 5.3.3.1). Therefore, in contrast to I_{Trp} results at pH 6.7 and $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ results at both pH 6.7 and pH 7.4, I_{Trp} results at pH 7.4 indicate that BLGs A, B and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change.

For BLGs A, B and C, the average T_{mid} values at pH 6.7 are, respectively, 10.1 °C, 6.7 °C and 5.2 °C higher than those at pH 7.4 (Table 5.3.3.1). Furthermore, the average T_{mid} value for BLG A at pH 7.4 is 6.7 °C higher than that at pH 8.1. These comparisons indicate that all three variants are more susceptible to heat-induced irreversible structural change in the vicinities of Trp^{19} and Trp^{61} at pH 7.4 than at pH 6.7, and that this trend continues for BLG A at pH 8.1.

5.3.3.5. Thermodynamic Analysis.

Values for the ΔG_{app} for the irreversible structural change in BLG molecules that leads to an increase in I_{Trp} are shown in Table 5.3.3.2. As with all ΔG_{app} values presented in this chapter, those in Table 5.3.3.2 do not represent the ΔG_{app} for the reversible unfolding of BLG because I_{Trp} data were collected under conditions where the unfolding of this protein is both partial and irreversible (Section 5.3.1.5). However, it is likely that the values in Table 5.3.3.2 represent those for the ΔG_{app} for heat-induced irreversible structural change in the vicinities of Trp^{19} and Trp^{61}. Values for ΔG_{app} for BLGs A, B and C at pH 6.7 were calculated at 75 °C because this temperature is close to the T_{mid} values for all three variants at this pH value (Table 5.3.3.1). For the same reason, values for ΔG_{app} for all three variants were calculated at 67 °C at pH 7.4. At pH 6.7 and pH 7.4 , the temperatures 72 °C and 66 °C respectively, those at which ΔG_{app} values were calculated using $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data, are below the transition for irreversible change in I_{Trp}.

Table 5.3.3.2. Values for ΔG_{app} at 75 °C at pH 6.7 and at 67 °C at pH 7.4 for Irreversible Structural Change in the Vicinities of Tryptophan^{19} and Tryptophan^{61} in β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6.7, BLG A	3.994 ± 0.030	3.93*
pH 6.7, BLG B	0.062 ± 0.001	0 (by definition)
pH 6.7, BLG C	5.669 ± 0.050	5.61#
pH 7.4, BLG A	0.871 ± 0.050	-1.61*
pH 7.4, BLG B	2.482 ± 0.007	0 (by definition)
pH 7.4, BLG C	6.885 ± 0.040	4.40#

* ΔG_{app}(BLG A)- ΔG_{app}(BLG B).

ΔG_{app}(BLG C)- ΔG_{app}(BLG B).

ΔG_{app} is assumed to represent the change in free energy at 75 °C at pH 6.7 and at 67 °C at pH 7.4 for irreversible structural change in the vicinities of Trp^{19} and Trp^{61}. Values for ΔG_{app} were calculated at these temperatures and pH values using fitted values for I_{Trp} as described in Section 5.2.8.3. The fitted values for I_{Trp} were obtained from the fitted curves shown in Fig. 5.3.3.4 using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in ΔG_{app} were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 5.3.3.1).

At pH 6.7 the value for ΔG_{app} for BLG B is less than that for BLG A, which is less than that for BLG C. Thus, in agreement with the T_{mid} values (Table 5.3.3.1), this indicates that at pH 6.7 BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change. At pH 7.4 the ΔG_{app} value for BLG A is less than that for BLG B, which is less than that for BLG C. Therefore, at this pH the A, B and C variants exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change, in agreement with the T_{mid} values shown in Table 5.3.3.1. The differences in the values for ΔG_{app} for BLGs A, B and C, shown as $\Delta\Delta G_{app}$values in Table 5.3.3.2, will be discussed in Section 8.5.

5.3.3.6. Comparison of the Temperature Ranges over which Irreversible Increases in Tryptophan Emission λ_{max} and Intensity were Observed.

In most instances, the temperature ranges over which changes in tryptophan fluorescence emission λ_{max} (Fig. 5.3.3.3) and intensity (Fig. 5.3.3.4) were observed coincide (reasonably) well. For example, in the case of BLG B at pH 6.7, increases in both λ_{max} and I_{Trp} occur as a consequence of heat treatment at temperatures greater than 68 °C. This suggests that the structural changes which lead to an irreversible increase in the solvent-accessibilities of the environments of the side chains of Trp^{19} and Trp^{61} occur at similar heat treatment temperatures to those which lead to a decrease in the extent of tryptophan fluorescence quenching.

5.3.3.7. Radiationless Energy Transfer from Tyrosine to Tryptophan.

Intrinsic protein fluorescence measurements on heat-treated solutions of BLGs A, B and C at pH 6.7 and pH 7.4, and BLG A at pH 8.1 were also made using an excitation wavelength of 275 nm. As discussed in Section 5.3.3.1, the tyrosine and tryptophan side chains of proteins can both be excited at this wavelength. The emission spectra of unheated solutions of BLGs A, B and C at pH 6.7 recorded using an excitation wavelength of 275 nm, and also those of solutions that had been previously heat-treated at 87.7 °C for 12.5 min, are shown in Fig. 5.3.3.6.

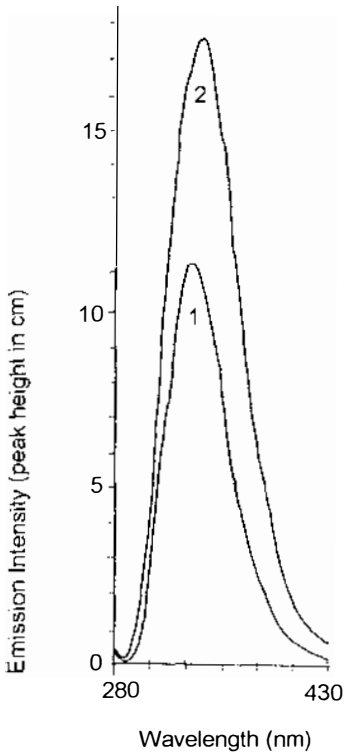
None of the spectra of the unheated BLG solutions at pH 6.7 (Fig. 5.3.3.6) or at pH 7.4 and pH 8.1 (spectra not shown) exhibit a shoulder at approximately 300 nm on the low wavelength side of the tryptophan emission peak. This suggests that the intensity of tyrosine emission is negligible compared to that of tryptophan emission. This in turn suggests that tyrosine side chains in excited states return to the ground state by transferring energy in a non-radiative manner (radiationless energy transfer, RET) to either one or both tryptophan side chains. The occurrence of RET from tyrosine to tryptophan requires that tyrosine and tryptophan side chains be within approximately 14 nm of one another (Section 5.3.3.1), as observed in the crystal structures (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998). Thus, it is likely that the BLG molecules in the unheated solutions used in the measurements discussed in this section were native.

Shoulders are not observed on the low wavelength side of the tryptophan emission peak in the spectra of the heat-treated solutions of BLGs A, B and C at pH 6.7 (Fig. 5.3.3.6), or at pH 7.4 and BLG A at pH 8.1 (spectra not shown). Therefore, irreversible increases in the distances between tyrosine and tryptophan side chains caused by heat treatment are likely to be negligible. This in turn suggests that the molecules of BLGs A, B and C in heat-treated solutions are only partially unfolded. The far UV CD results in Section 5.3.2 are consistent with this suggestion because they indicate that molecules of both unheated and heat-treated BLGs A, B and C possess appreciable amounts of secondary structure.

The intrinsic protein fluorescence results of Creamer (1995) suggest that BLG A/B possesses less structure in the presence of 9.48 M urea than do BLGs A, B and C after heat treatment. Creamer (1995) found that when the fluorescence emission spectrum of BLG A/B was recorded in the presence of 9.48 M urea using an excitation wavelength of 275 nm, a pronounced shoulder was observed at 305 nm on the low wavelength side of the tryptophan emission peak. In the fluorescence spectra obtained by Creamer (1995), the tyrosine emission shoulder is particularly well defined because the emission λ_{max} of the tryptophan emission peak is located at approximately 350 nm. Creamer (1995) interpreted his results as indicating that the efficiency of RET from tyrosine to tryptophan side chains is appreciably less in the presence of 9.48 M urea than in its absence. These results suggest that the distances between at least some tyrosine and tryptophan side chains in molecules of BLG A/B are greater than the Förster distance for tyrosine-tryptophan transfer (i.e. 14 Å, Section 5.3.3.1) in the presence of 9.48 M urea. Thus, the extent of BLG unfolding in the presence of 9.48 M urea is greater than that resulting from heat treatment. Inefficient RET from tyrosine to tryptophan may also indicate that in the presence of 9.48 M urea, at least some of these side chains are not fixed in a particular conformation (Section 5.3.3.1).

Fig. 5.3.3.6. The fluorescence emission spectra of unheated and heat-treated BLGs A, B and C at pH 6.7 obtained using an excitation wavelength of 275 nm. The spectra of unheated BLGs in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) are as follows: BLG A, panel a, spectrum 1; BLG B, panel b, spectrum 1; and BLG C, panel c, spectrum 1. The spectra of BLGs previously heat-treated in pH 6.7 phosphate buffer for 12.5 min at 87.7 °C are displayed as follows: BLG A, panel a, spectrum 2; BLG B, panel b, spectrum 2; and BLG C, panel c, spectrum 2. The spectra were acquired from 1.0 mg/mL solutions at 20 °C in 10 mm path length cells excited at 275 nm using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. Measurements were made using excitation and emission slit widths of 8 nm at a scan speed of 25 nm/min and a chart recorder speed of 1 cm/min. Details of the solution preparation and heat treatment protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.

a


Emission Intensity (peak height in cm)
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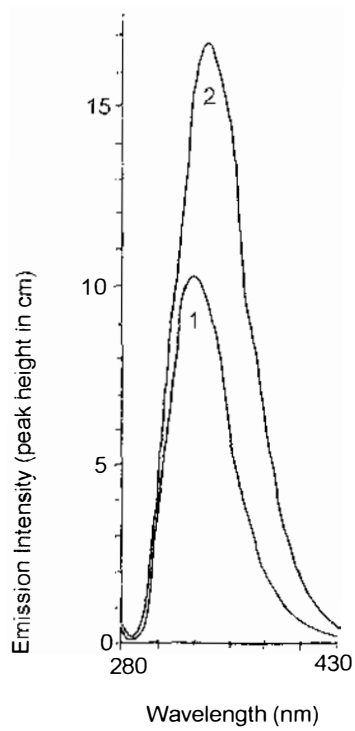
5
10
15
1
2
280
430
Wavelength (nm)

b


Emission Intensity (peak height in cm)
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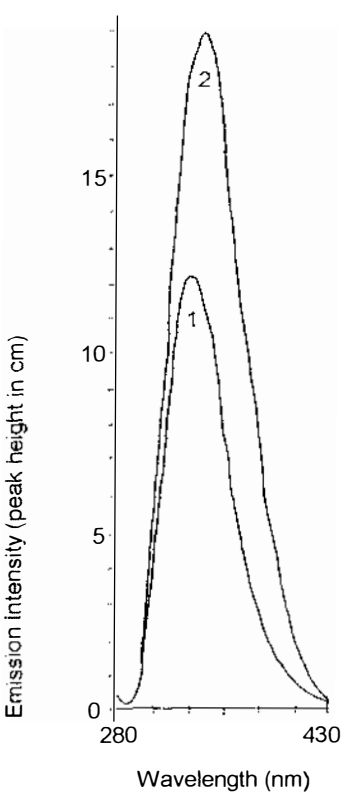
5
10
15
1
2
280
430
Wavelength (nm)

c


Emission intensity (peak height in cm)
0
5
10
15
1
2
280
430
Wavelength (nm)

5.3.3.8. Nature of the Structural Change in β-Lactoglobulin Molecules which Occurs as a Consequence of Heat-treatment.

The tryptophan fluorescence emission spectrum of unheated BLG is dominated by the contribution from Trp^{19}, which is located in a semi-hydrophobic environment. In native BLG it is likely that Trp^{61} fluorescence is quenched by the disulphide bond Cys^{66}-Cys^{160}. Heat treatment probably leads to an irreversible change in the environments of both Trp^{19} and Trp^{61}. Increases in tryptophan emission λ_{max} suggest that the solvent-accessibility of one or both tryptophan side chains increases as a consequence of heat treatment. Increases in I_{Trp} caused by heat treatment probably reflect either an increase in the distance between the side chain of Trp^{61} and the disulphide bond Cys^{66}-Cys^{160} or the loss of the latter. The $\Delta\varepsilon_{270}$ and $\Delta\varepsilon_{277}$ results in Section 5.3.1 suggest that non-native disulphide bonds are formed in BLG molecules as a consequence of heat treatment. Therefore, the increases in I_{Trp} discussed in Sections 5.3.3.2 and 5.3.3.4 may reflect the loss of the disulphide bond Cys^{66}-Cys^{160}, which would occur as non-native disulphide bonds are formed.

5.3.3.9. Summary.

The tryptophan fluorescence results above confirm that the environment of Trp^{19} is altered irreversibly as a consequence of heat treatment and indicate that this is also true for Trp^{61}. In the next section, the effect of heat treatment on the solvent-accessible hydrophobic regions of the BLG structure capable of binding the fluorescent probe ANS will be examined.

5.3.4. RESULTS AND DISCUSSION: ANS FLUORESCENCE.

5.3.4.1. Introduction to ANS Fluorescence.

Protein structure and structural change can also be studied by measuring the fluorescence from bound probes. For example, the hydrophobic fluorescent probe ANS binds specifically to a wide range of proteins (Slavík, 1982; Lakowicz, 1983), including BLG (Creamer, 1995). This probe has been frequently used to follow changes in protein structure (Slavík, 1982) and is particularly useful for such studies because emission intensities and emission λ_{max} values increase and decrease respectively with decreasing solvent polarity. For example, emission intensity from aqueous ANS is negligible (quantum yield (Q) = 0.004) and the emission λ_{max} is approximately 515 nm (Stryer, 1965). The negligible emission intensity from aqueous ANS is usually ignored, thus simplifying the interpretation of experimental data (Lakowicz, 1983). On the other hand, in pure ethanol emission intensity is markedly greater (Q = 0.37), and is maximal at 468 nm (Stryer, 1965). Furthermore, emission intensity from ANS is maximal when its two aromatic rings are coplanar, allowing electrons to pass freely between donor and acceptor groups (i.e. the amine and sulphonate groups respectively, Stryer, 1965). ANS fluorescence emission intensity results can therefore provide information pertaining to the structures of the hydrophobic ligand binding sites of proteins. In the study discussed below, changes in BLG structure which occurred as a consequence of heat treatment were examined by following changes in the emission intensity and the emission λ_{max} from molecules of bound ANS.

5.3.4.2. ANS Titrations.

Before the effect of heat treatment of BLG on the fluorescence from bound ANS was studied, the quantity of ANS capable of binding to BLG in unheated and heat-treated solutions was examined by titrating ANS into solutions of BLG. Plots of ANS fluorescence emission intensity (I_{ANS}) versus mole ratio ANS:monomeric BLG are shown in Fig. 5.3.4.1.

For unheated BLG B (panel a), I_{ANS} increases continuously with increasing mole ratio ANS:monomeric BLG at least as far as 1.4, although there is a slight change in slope at a mole ratio ANS:monomeric BLG of approximately 1.0. These results suggest that unheated BLG binds one equivalent of ANS, but binding is not particularly strong. This is consistent with the results of Laligant *et al.* (1991). The positive slope above a mole ratio ANS:monomeric BLG of 1.0 (Fig. 5.3.4.1a) suggests that a molar excess of ANS greater than 1 is needed to ensure that all of the BLG molecules in unheated solutions are complexed with ANS. Cairoli *et al.* (1994) reported that unheated monomeric BLG A/B binds 0.5 equivalents of ANS, while Mills and Creamer (1975) found, by extrapolation, that at pH 6.5 and at pH 2.0, monomeric BLG A binds 0.78 and 0.74 equivalents of ANS respectively.

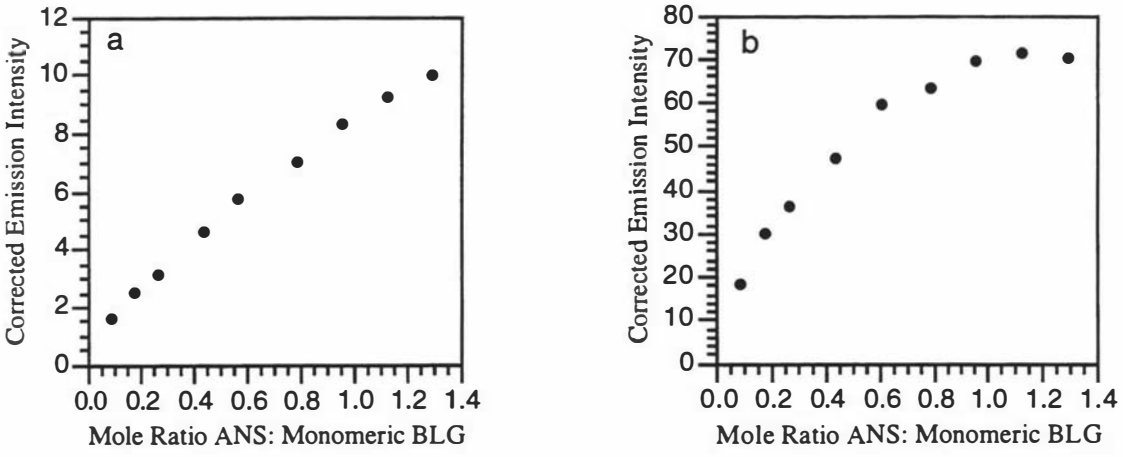


Fig. 5.3.4.1. Titration with ANS of unheated and previously heat-treated BLG B at pH 6.7. The plots for unheated BLG B in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) and BLG B previously heat-treated in this buffer for 12.5 min at 80 °C are shown in panels a and b respectively. Emission spectra were acquired from 1.0 mg/mL solutions of BLG (initial volume of 3.0 mL) excited at 370 nm using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. Measurements were made at 20 °C and a scan speed of 25 nm/min, using excitation and emission band widths of 8 nm. ANS (1.41 mM) was added in 20 μL aliquots. Emission intensities were corrected for the absorption of both incident and emitted light by ANS (inner filter effects) as described in Section 4.2.2.3.

In the plot prepared using data acquired from heat-treated BLG B (Fig. 5.3.4.1b), a break is observed at a mole ratio ANS:monomeric BLG of approximately 0.6, although corrected I_{ANS} varies continuously with increasing mole ratio ANS:monomeric BLG at least as far as 1.4. This plot therefore suggests that previously heat-treated BLG binds between 0.6 and 1.0 equivalents of ANS more strongly than the corresponding unheated species. The ANS fluorescence measurements discussed in subsequent sections were made at a mole ratio ANS:monomeric BLG of 0.5, to keep the absorption of incident and emitted light by ANS to a minimum, while maintaining a reasonable intensity of emission from the bound probe.

5.3.4.3. The Fluorescence Emission Spectra of ANS Bound to Unheated and Heat-treated β-Lactoglobulins.

The emission spectra of ANS bound to BLGs A, B and C at 6.7, either unheated or previously heat-treated at 82 °C for 12.5 min, are shown in Fig. 5.3.4.2. The intensity of emissions from aqueous ANS was negligible (data not shown).

For all three variants, the emission λ_{max} values for ANS bound to unheated BLG (i.e. typically 479 nm) are higher than those for ANS bound to heat-treated BLG (i.e. typically 467 nm, Fig. 5.3.4.2). This suggests that the hydrophobicity of the ANS binding site of heat-treated BLG is greater than that of the ANS binding site of the corresponding unheated species (Section 5.3.4.1).

Although the λ_{max} of fluorescence emission from ANS decreases from approximately 515 nm to approximately 467 nm upon binding to heat-treated BLG, the magnitude of this decrease (48 nm) is not particularly large. For example, when ANS binds in the haem pocket of apomyoglobin, the emission λ_{max} value decreases by 61 nm (Stryer, 1965). This indicates that the ANS binding site of heat-treated BLG is considerably less hydrophobic than the haem pocket of myoglobin.

The spectra in Fig. 5.3.4.2 also indicate that for all three variants at pH 6.7, the intensity of emission from ANS bound to heat-treated BLG is greater than that from ANS bound to unheated BLG. These results can be interpreted in two ways: either heat-treated BLG has more ANS binding sites than the corresponding unheated species, or the ANS binding site of heat-treated BLG is different to that of the corresponding unheated species. However, a detailed binding study was outside the scope of this study. Therefore, from I_{ANS} results alone it is not possible to determine the exact nature of the structural change in BLG molecules which leads to the observed increases in fluorescence emission intensity from bound ANS. The I_{ANS} results in Fig. 5.3.4.2 and the remaining I_{ANS} results in this section can therefore only be interpreted in terms of a heat-induced irreversible structural change which leads to an increase in intensity of emissions from ANS bound to BLG.

For BLGs A, B and C at pH 7.4, similar trends to those described above were observed (Fig. 5.3.4.3). However, for all three variants, the intensity of emission from ANS bound to unheated BLG at pH 6.7 was less than that from ANS bound to unheated BLG at pH 7.4 (Fig. 5.3.4.4). This trend continued for BLG A at pH 8.1. These results therefore suggest that the ANS binding site of unheated BLGs A, B and C at pH 6.7 differs to that of unheated BLGs A, B and C at pH 7.4, which differs to that of BLG A at pH 8.1.

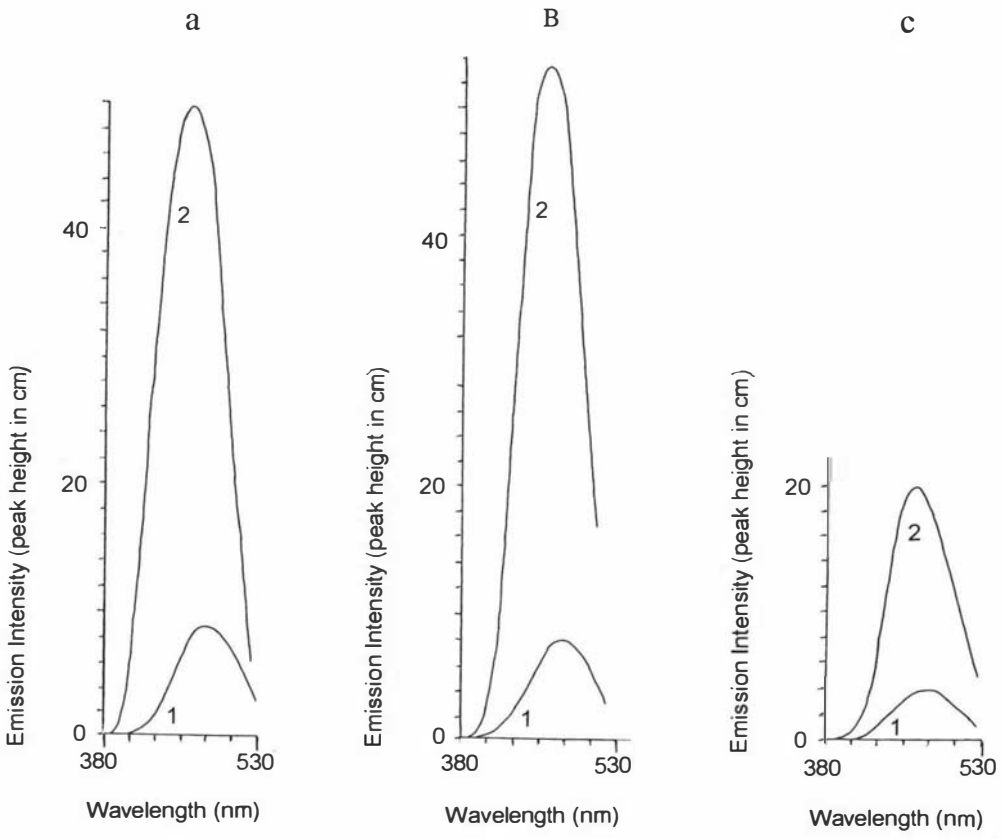


Fig. 5.3.4.2. The fluorescence emission spectra of ANS bound to unheated and previously heat-treated BLGs A, B and C at pH 6.7. The spectra for unheated BLGs in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) are as follows: BLG A, panel a, spectrum 1; BLG B, panel b, spectrum 1; and BLG C, panel c, spectrum 1. The spectra for BLGs previously heat-treated in pH 6.7 phosphate buffer for 12.5 min at 82 °C are as follows: BLG A, panel a, spectrum 2; BLG B, panel b, spectrum 2; and BLG C, panel c, spectrum 2. The spectra were acquired from mixtures of 1.0 mg/mL BLG and 28 μM ANS solutions (i.e. mole ratio ANS:monomeric BLG of 0.5:1) at 20 °C in 10 mm path length cells excited at 370 nm using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. Measurements were made using excitation and emission slit widths of 8 nm at a scan speed of 25 nm/min and a chart recorder speed of 1 cm/min. Details of the solution preparation and heat treatment protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.

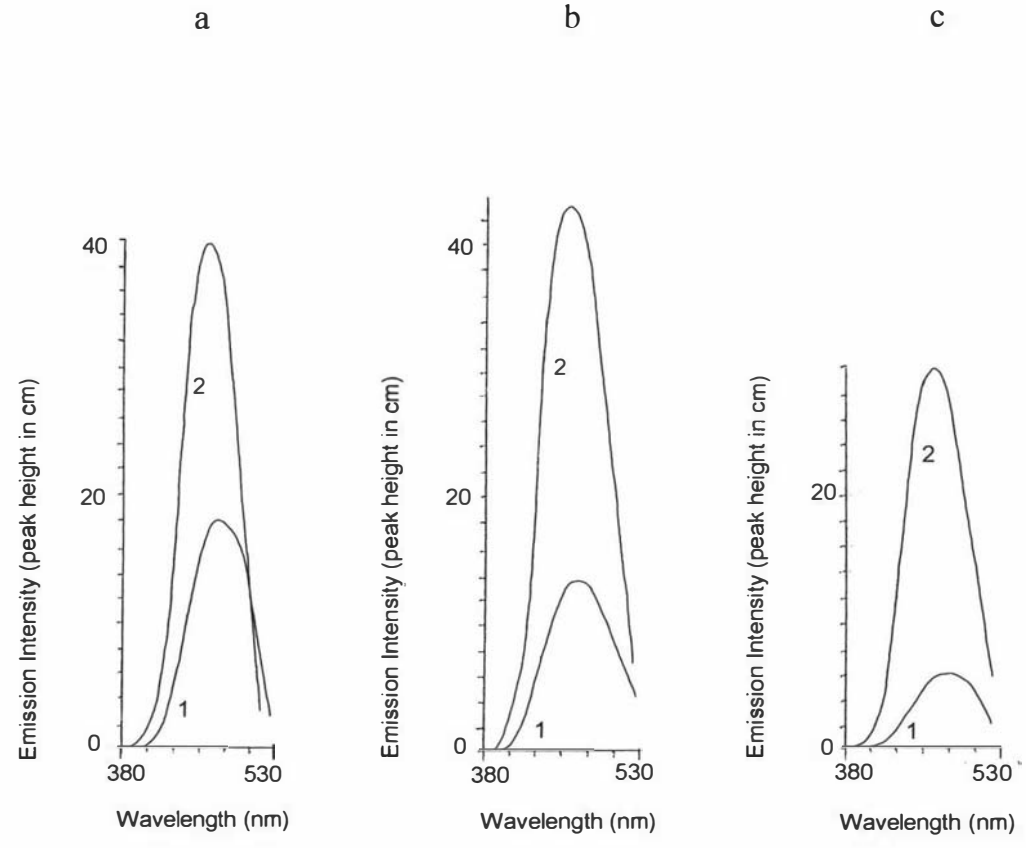
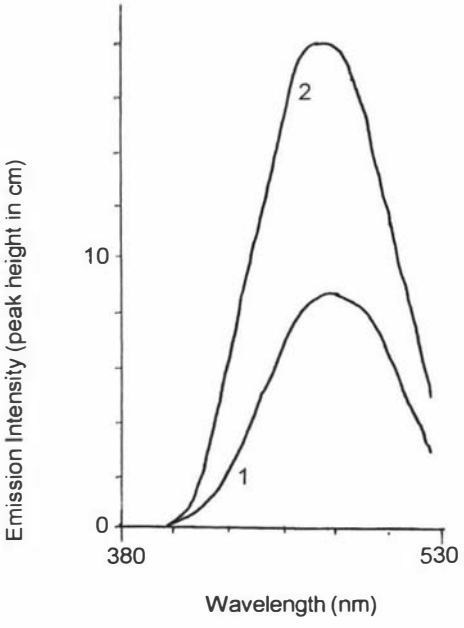


Fig. 5.3.4.3. The fluorescence emission spectra of ANS bound to unheated and previously heat-treated BLGs A, B and C at pH 7.4 and BLG A at pH 8.1. The spectra for unheated BLGs in pH 7.4 phosphate buffer (26 mM, with 68 mM NaCl) and pH 8.1 phosphate/borate buffer (13 mM phosphate, 13 mM borate, with 68 mM NaCl) are as follows: BLG A at pH 7.4, panel a, spectrum 1; BLG B at pH 7.4, panel b, spectrum 1; BLG C at pH 7.4, panel c, spectrum 1; and BLG A at pH 8.1, panel d, spectrum 1. The spectra of BLGs previously heat-treated in pH 7.4 phosphate or pH 8.1 phosphate/borate buffers for 12.5 min at 82 °C are as follows: BLG A at pH 7.4 (panel a, spectrum 2), BLG B at pH 7.4 (panel b, spectrum 2), BLG C at pH 7.4 (panel c, spectrum 2) and BLG A at pH 8.1 (panel d, spectrum 2). The spectra were acquired from mixtures of 1.0 mg/mL BLG and 28 μM ANS solutions (i.e. mole ratio ANS:monomeric BLG of 0.5:1) as described in Fig. 5.3.4.2. Details of the solution preparation and heat treatment protocols are given in Sections 5.2.1.1 and 5.2.1.2 respectively.

Fig. 5.3.4.4. The fluorescence emission spectra of ANS bound to unheated BLGs A, B and C at pH 6.7 and pH 7.4. The spectra are as follows: BLG A at pH 6.7, panel a, spectrum 1; BLG A at pH 7.4, panel a, spectrum 2; BLG B at pH 6.7, panel b, spectrum 1; BLG B at pH 7.4, panel b, spectrum 2; BLG C at pH 6.7, panel c, spectrum 1; and BLG C at pH 7.4, panel c, spectrum 2. All BLG solutions were in either pH 6.7 or pH 7.4 phosphate buffer (26 mM, with 68 mM NaCl). The spectra were acquired from mixtures of 1.0 mg/mL BLG and 28 μM ANS solutions (i.e. mole ratio ANS:monomeric BLG of 0.5:1) as described in Fig. 5.3.4.2. Details of the solution preparation protocol are given in Section 5.2.1.1.

a


Emission Intensity (peak height in cm)
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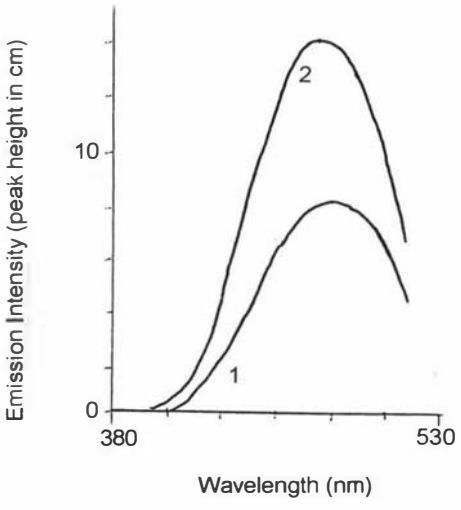
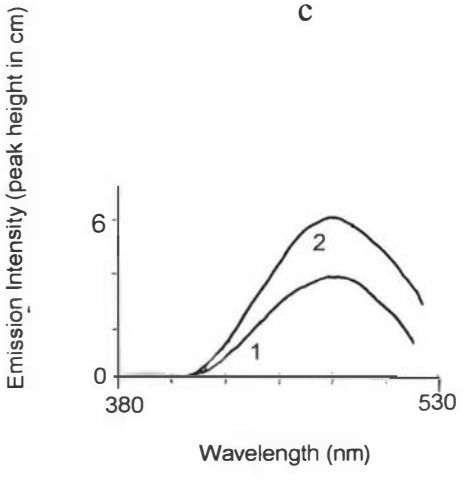
0
2
1
380
530
Wavelength (nm)

b


Emission Intensity (peak height in cm)
10
0
2
1
380
530
Wavelength (nm)

c


Emission Intensity (peak height in cm)
6
0
2
1
380
530
Wavelength (nm)

5.3.4.4. Dependence of Emission λ_{max} for ANS Bound to β-Lactoglobulins on Heat Treatment Temperature.

For all three variants at both pH 6.7 and pH 7.4 and for BLG A at pH 8.1, ANS emission λ_{max} values decrease with increasing heat treatment temperature (Fig. 5.3.4.5). This indicates that as heat treatment temperatures are raised, the ANS binding site of BLG becomes increasingly hydrophobic (Section 5.3.4.1). The plots in Fig. 5.3.4.5 also suggest that the heat-induced increase in ANS binding site hydrophobicity occurs at lower heat treatment temperatures at pH 7.4 than at pH 6.7, suggesting that molecules of BLGs A, B and C are more susceptible to heat-induced irreversible structural change at the higher pH value.

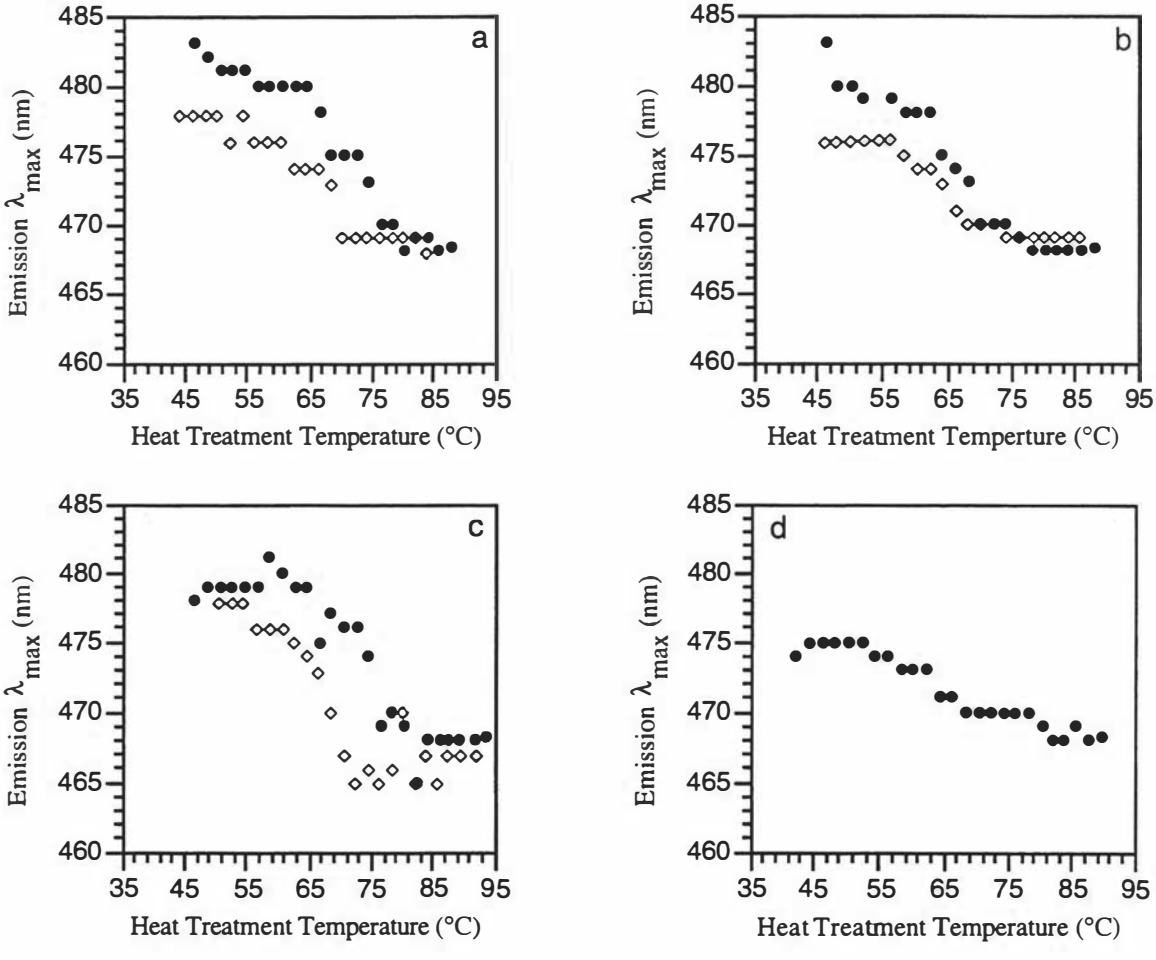


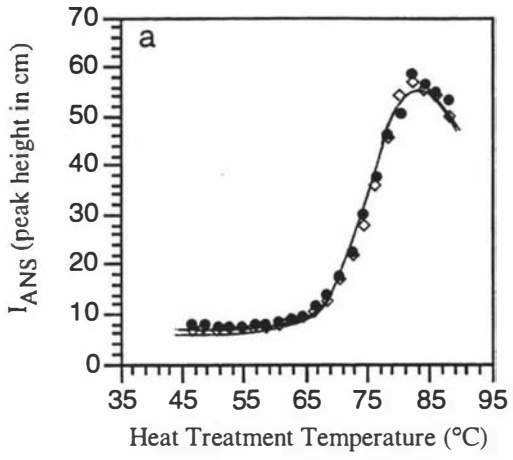
Fig. 5.3.4.5. Effect of heat treatment of solutions of BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1 on the λ_{max} of emission from bound ANS. The plots of pH 6.7 (•) and pH 7.4 (◇) data for BLGs A, B and C are shown in panels a, b and c respectively. The plot for BLG A at pH 8.1 is shown in panel d. Values for emission λ_{max} were obtained from spectra recorded in duplicate as described in Fig. 5.3.4.2 (single sets of data plotted for clarity).

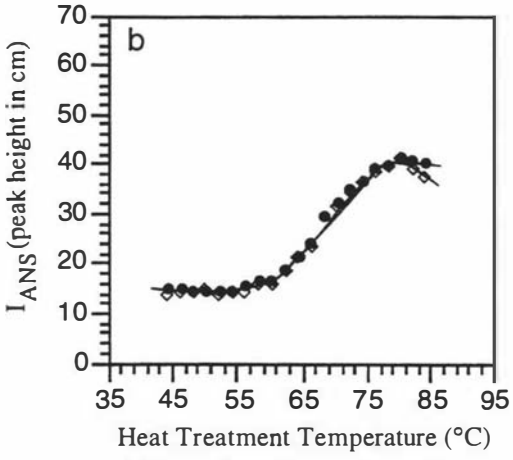
5.3.4.5. Quantitative Study: Emission Intensity Values for ANS Bound to Heat-treated β-Lactoglobulins.

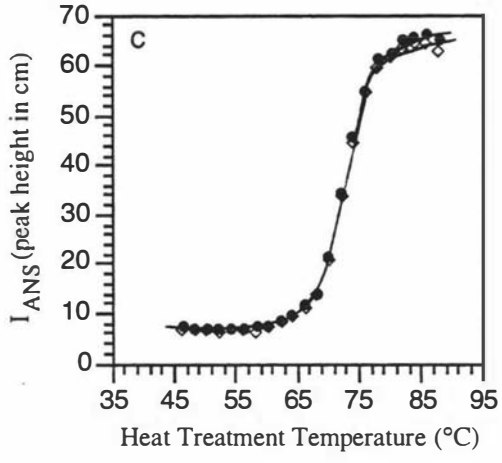
The plots presented in Fig. 5.3.4.6 show the dependence of emission intensity from ANS bound to BLG A at pH 6.7, pH 7.4 and pH 8.1 and BLGs B and C at pH 6.7 and pH 7.4 on protein heat treatment temperature. Although all ANS fluorescence measurements were made using the same data collection protocol, emission intensities from ANS bound to BLGs A, B and C vary. This may indicate that trace amounts of fatty acids were present in the purified solutions of BLG and were competing for hydrophobic binding sites with ANS. However, because the purpose of making ANS fluorescence measurements was to identify differences in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change, this variation was not investigated further, and no attempt was made to correct for it. Nevertheless, to confirm that the differences in I_{ANS} data for BLGs A, B and C reflected differences in the susceptibilities of these variants to heat-induced irreversible structural change, several series of confirmatory I_{ANS} measurements were made in which the data for all three variants were collected within 8 hours.

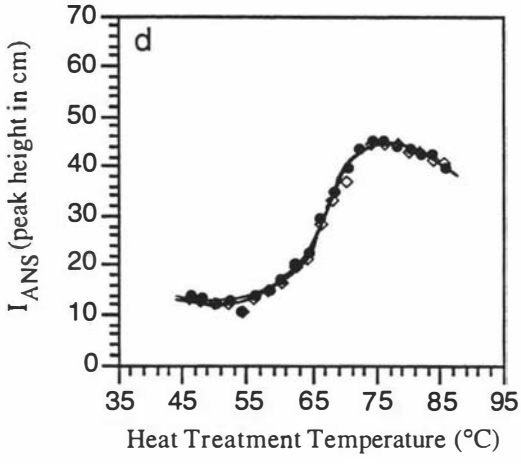
The plots in Fig. 5.3.4.6 also indicate that duplicate ANS emission intensity data for a particular variant at a particular pH value coincide extremely well, except in the case of solutions previously heat-treated at temperatures approximately 80 °C and higher. This indicates that the error in determining I_{ANS} values is low.

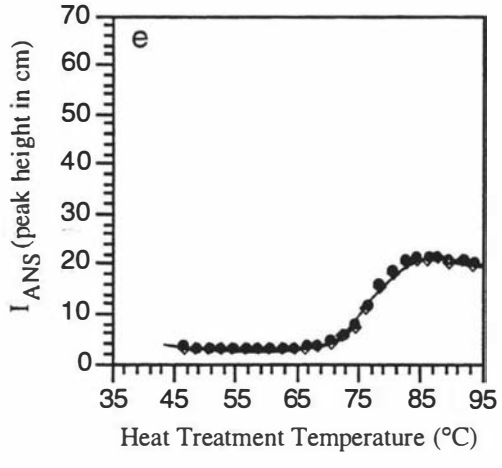
Some of the plots in Fig. 5.3.4.6 exhibit negative slope above approximately 75 °C or 80 °C. Therefore, experimental I_{ANS} data were fitted to the 2-state thermal unfolding model to which I_{Trp} data were fitted. In this model, two additional parameters, which compensate for regions of negative slope in plots of experimental fluorescence emission intensity data versus heat treatment temperature, were incorporated into the model previously used to fit $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data (Section 5.2.8.2). The plots of experimental I_{ANS} data and the curves obtained by fitting these data to equations 5.2.8.2 and 5.2.8.3 (Section 5.2.8.2) using the computer program "Enzfitter" (Leatherbarrow, 1987) coincide. This indicates that the selected model adequately describes heat-induced structural change in molecules of BLGs A, B and C. Therefore, it was assumed that the fitted value for the parameter T_{mid} for a particular variant at a particular pH (Table 5.3.4.1) represents the midpoint temperature of the transition for heat-induced irreversible structural change as determined by following changes in emission intensity from bound ANS. The errors for the values for T_{mid} are standard errors calculated by "Enzfitter" and are probably underestimates of the errors in the values for this parameter.

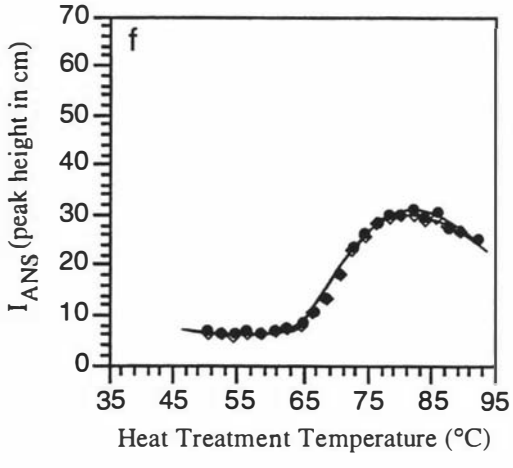

a
I_{ANS} (peak height in cm)
Heat Treatment Temperature (°C)


b
I_{ANS} (peak height in cm)
Heat Treatment Temperature (°C)


c
I_{ANS} (peak height in cm)
Heat Treatment Temperature (°C)


d
I_{ANS} (peak height in cm)
Heat Treatment Temperature (°C)


e
I_{ANS} (peak height in cm)
Heat Treatment Temperature (°C)


f
I_{ANS} (peak height in cm)
Heat Treatment Temperature (°C)

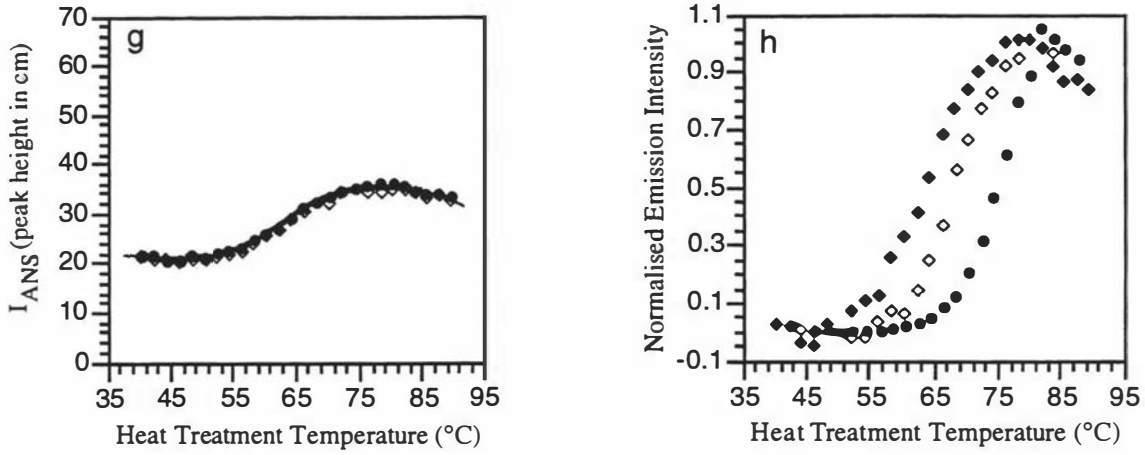


Fig. 5.3.4.6. Effect of heat treatment of solutions of BLGs A, B and C at pH 6.7 and pH 7.4 and BLG A at pH 8.1 on emission intensity from bound ANS. The plots of duplicate I_{ANS} data, denoted by the symbols (•) and (◇), are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; BLG C at pH 7.4, panel f; BLG A at pH 8.1, panel g; and in panel h, BLG A at pH 6.7 (•), pH 7.4 (◇) and pH 8.1 (♦). Experimental I_{ANS} data were obtained from spectra recorded as described in Fig. 5.3.4.2. The curves were obtained by fitting experimental I_{ANS} data to equations 5.2.8.2 and 5.2.8.3 as described in Section 5.2.8.2 using the computer program "Enzfitter" (Leatherbarrow, 1987). Values for the fitting parameters are given in Appendix 3. In panel h, experimental and fitted I_{ANS} data were normalised so that the average values for I_{ANS} on the low and high temperature sides of the transition for heat-induced irreversible structural change were 0 and 1 respectively.

The plots in Fig. 5.3.4.6 indicate that for all three variants, the maximum extent of the increase in I_{ANS}, and therefore the maximum extent of heat-induced irreversible structural change in BLG molecules, is greater at pH 6.7 than at pH 7.4. This trend continues for BLG A at pH 8.1. These results may reflect the spectral results shown in Fig. 5.3.4.3, which indicate that for all three variants, the intensity of emission from ANS bound to unheated BLG is greater at pH 7.4 than at pH 6.7. Furthermore, for BLGs A and B, the maximum extent of the increase in I_{ANS} which occurs as a consequence of heat treatment is less at pH 7.4 than at pH 6.7 because for these variants the values for I_{ANS} on the high temperature side of the transition for heat-induced irreversible structural change are lower at pH 7.4 than at pH 6.7.

The plots in Fig. 5.3.4.6 indicate that the transition for heat-induced irreversible structural change, as determined by following changes in emission intensity from bound ANS, occurs over a temperature range of approximately 23 °C at pH 6.7, pH 7.4 and pH 8.1. This temperature range is similar to that for the transition for heat-induced irreversible structural change in the vicinity of Trp^{19} as determined by following changes in $\Delta\varepsilon_{293}$ (Section 5.3.1.4).

At both pH 6.7 and pH 7.4, the slopes at T_{mid} of the plots of I_{ANS} versus heat treatment temperature in Fig. 5.3.4.6 are slightly greater for BLGs B and C than for BLG A (Table 5.3.4.1). This suggests that BLG A responds to heat treatment in a different manner to BLGs B and C, and the significance of this will be discussed in Section 8.5.

The T_{mid} values in Table 5.3.4.1 provide a quantitative measure of the differences in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change. At pH 6.7 the average T_{mid} value for BLG B is 2.2 °C lower than that for BLG A, which is 1.7 °C lower than that for BLG C. Similarly, at pH 7.4 the average T_{mid} value for BLG B is 2.4 °C lower than that for BLG A, which is 2.2 °C lower than that for BLG C (Table 5.3.4.1). Thus the I_{ANS} results indicate that BLGs B, A and C exhibited the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change at both pH 6.7 and pH 7.4. Furthermore, the average T_{mid} values for BLGs A, B and C are all between 6 °C and 7 °C lower at pH 7.4 than at pH 6.7, suggesting that all three variants are more susceptible to heat-induced irreversible structural change at the higher pH value. At pH 8.1, the average T_{mid} value for BLG A, 67.3 °C, is 0.4 °C lower than that at pH 7.4 (Table 5.3.4.1). This indicates that BLG A is more susceptible to heat-induced irreversible structural change at pH 8.1 than at pH 7.4.

Table 5.3.4.1. Values for the Midpoint Temperature (T_{mid}) and Slope at T_{mid} for the Heat-induced Irreversible Structural Change which Leads to an Increase in the Emission Intensity from Bound ANS for β-Lactoglobulins A, B and C at pH 6.7 and at pH 7.4 and β-Lactoglobulin A at pH 8.1.

Sample	T_{mid} (°C)	Slope at T_{mid} (Normalised I_{ANS}/°C)
pH 6.7, BLG A	74.6 ± 0.2	0.090
	74.7 ± 0.2	0.099
pH 6.7, BLG B	72.5 ± 0.0	0.107
	72.4 ± 0.0	0.109
pH 6.7, BLG C	76.3 ± 0.1	0.117
	76.3 ±0.1	0.116
pH 7.4, BLG A	67.9 ± 0.1	0.074
	67.4 ± 0.0	0.76
pH 7.4, BLG B	65.2 ± 0.4	0.099
	65.4 ± 0.1	0.37
pH 7.4, BLG C	69.9 ± 0.2	0.112
	69.8 ± 0.1	0.105
pH 8.1, BLG A	68.7 ± 0.3	0.049
	65.8 ± 1.2	0.049

Where:

T_{mid} is the midpoint temperature of the transition for heat-induced irreversible structural change as determined by following changes in emission intensity from bound ANS (i.e. the temperature at which I_{ANS} is exactly half way between the minimum and the maximum values for I_{ANS} in the plots shown in Fig. 5.3.4.6). T_{mid} is one of the parameters used to fit experimental I_{ANS} data to equations 5.2.8.2 and 5.2.8.3 (Section 5.2.8.2) using the computer program "Enzfitter" (Leatherbarrow , 1987). The errors in T_{mid} values are standard errors calculated by "Enzfitter".

The slope at the midpoint temperature was calculated using the fitted normalised I_{ANS} values in the range T_{mid} ± 0.5 °C. Sets of fitted I_{ANS} data were normalised so that the average values for I_{ANS} on the low and high temperature sides of the transition for heat-induced irreversible structural change were 0 and 1 respectively.

5.3.4.6. Thermodynamic Analysis.

Values for the ΔG_{app} for heat-induced irreversible structural change in molecules of BLGs A, B and C at pH 6.7 at 72 °C and at pH 7.4 at 66 °C were calculated using I_{ANS} data (Table 5.3.4.2). As with the ΔG_{app} values calculated in other sections of this chapter, those obtained using I_{ANS} data do not represent the ΔG_{app} for reversible unfolding because these data were collected under conditions where the unfolding of BLG is both partial and irreversible (Section 5.3.1.5). Nevertheless, the values in Table 5.3.4.2 probably represent those for the ΔG_{app} for the heat-induced irreversible structural change in BLG molecules which leads to an increase in the intensity of fluorescence emission from bound ANS. The values for ΔG_{app} for BLGs A, B and C were calculated at 72 °C and at 66 °C because these temperatures are close to the T_{mid} of the transition for heat-induced irreversible structural change for all three variants at pH 6.7 and at pH 7.4 respectively (Table 5.3.4.1). These temperatures are also those at which ΔG_{app} values were calculated using $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data.

Table 5.3.4.2. Values for ΔG_{app} at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for the Irreversible Structural Change which Leads to an Increase in the Emission Intensity from Bound ANS for β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6.7, BLG A	2.884 ± 0.008	2.41*
pH 6.7, BLG B	0.473 ± 0.001	0 (by definition)
pH 6.7, BLG C	5.406 ± 0.007	4.93#
pH 7.4, BLG A	1.575 ± 0.001	2.44*
pH 7.4, BLG B	-0.863 ± 0.003	0 (by definition)
pH 7.4, BLG C	4.144 ± 0.009	5.01#

* ΔG_{app}(BLG A)- ΔG_{app}(BLG B)

\# ΔG_{app}(BLG C)- ΔG_{app}(BLG B).

ΔG_{app} is assumed to represent the change in free energy at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for the irreversible structural change in BLG molecules which leads to an increase in the intensity of fluorescence emission from bound ANS. Values for ΔG_{app} were calculated at these temperatures and pH values using fitted values for I_{ANS} as described in Section 5.2.8.3. The fitted values for I_{ANS} were obtained from the fitted curves shown in Fig. 5.3.4.6 using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in ΔG_{app} were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 5.3.4.1).

The values for ΔG_{app} in Table 5.3.4.2 are similar to those determined using $\Delta\varepsilon_{293}$ and $[\theta]_{205}$ data (Tables 5.3.1.2 and 5.3.2.2 respectively) and therefore indicate that BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change. This is consistent with the values for T_{mid} determined using I_{ANS} data (Table 5.3.4.1). The differences in the values for ΔG_{app} for BLGs A, B and C, as determined using I_{ANS} data, are shown as $\Delta\Delta G_{app}$ values in Table 5.3.4.2 and the significance of these differences will be discussed in Section 8.5.

5.3.4.7. Nature of the Structural Change in Molecules of β-Lactoglobulin which Occurs as a Consequence of Heat Treatment.

Iametti and co-workers (Cairoli *et al.*, 1994; Iametti *et al.*, 1995) have used emission intensity from bound ANS to study heat-induced structural change in molecules of BLG A/B. They found that after rapid temperature increase to values below 70 °C - 75 °C, I_{ANS} increased over periods of up to approximately 2 min. In addition, the extent of this increase became more pronounced as heating temperatures were raised in this range. After rapid temperature increase to values greater than 70 °C - 75 °C, Iametti and co-workers found that I_{ANS} first increased, and then decreased; the extent and rate of the initial emission intensity increase and the subsequent decrease became more pronounced as temperatures were increased. Iametti and co-workers suggested that the increase in I_{ANS} observed at all heating temperatures reflects "protein swelling", while the subsequent decrease observed at higher temperatures reflects a collapse of the swollen species. They suggested that during the collapse phase, the hydrophobic groups which had become exposed as a consequence of temperature increase were re-buried, producing a species with a structure that is, presumably, more compact than that of the "heat swollen" species. Iametti and co-workers also suggested that solvent-exposed hydrophobic surfaces could become buried during aggregate formation, a process which leads to a decrease in the solvent-accessibility of the hydrophobic surfaces which became exposed in the early stages of heat treatment. The nature of aggregates of BLG is discussed in Section 2.7.

Iametti and co-workers also reported that at room temperature, molecules of BLG in both unheated solutions and in solutions previously heat-treated appear to bind an equivalent amount of ANS. However, the plot in Fig. 3 of Cairoli *et al.* (1994) indicates that the number of ANS binding sites in molecules of BLG decreases slightly with increasing heat treatment temperature above approximately 75 °C. Nevertheless, from their results Iametti and co-workers concluded that "protein swelling", postulated to occur at elevated temperatures, is reversible when BLG solutions are cooled to room temperature. This suggests that a net increase in the solvent-accessible hydrophobic surface of BLG does not occur as a consequence of heat treatment (Cairoli *et al.*, 1994). However, the hydrophobic surfaces of molecules of heat-treated BLG may not necessarily be the same as those of the corresponding native species (Cairoli *et al.*, 1994).

The conclusions drawn by Iametti and co-workers suggest that irreversible increases in the emission intensity from ANS bound to BLG caused by heat treatment (Figs 5.3.4.2 and 5.3.4.3) must reflect a difference in the ANS binding site of unheated and heat-treated BLG species. This difference could arise from two structural changes.

Firstly, because ANS emission intensity increases with decreasing solvent polarity (Lakowicz, 1983), the observed increases in I_{ANS} discussed in Sections 5.3.4.3, 5.3.4.5 and 5.3.4.6 may indicate that the ANS binding site of heat-treated BLG is more hydrophobic than that of the corresponding unheated species. This suggestion is consistent with the observed decrease in the emission λ_{max} from bound ANS which occurs as a consequence of heat treatment (Fig. 5.3.4.5). However, because the temperature ranges over which decreases in ANS emission λ_{max} and increases in I_{ANS} are observed do not coincide particularly well (Figs 5.3.4.5 and 5.3.4.6 respectively), the increases in I_{ANS} may occur as a consequence of the other structural change also.

Secondly, ANS emission intensity is maximal when the two rings of this molecule are co-planar (Section 5.3.4.1). Therefore, the observed increases in I_{ANS} may also indicate that ANS is forced to assume a more planar conformation in the binding site of heat-treated BLG compared to that when bound to the corresponding unheated species.

Although it appears likely that heat-induced irreversible increases in I_{ANS} occur as a consequence of the above two structural changes, further studies are required to determine exactly how these structural changes arise. For this reason, the I_{ANS} results in Sections 5.3.4.3, 5.3.4.5 and 5.3.4.6 were only interpreted in terms of a heat-induced irreversible structural change which leads to an increase in the intensity of emission from ANS bound to BLG.

The "hydrophobic collapse" phenomenon, which Iametti and co-workers suggest occurs concurrently with heat-induced aggregate formation, may also be reflected in ANS fluorescence results obtained from measurements made at room temperature using BLG solutions previously heat-treated. As discussed above, the results of Cairoli *et al.* (1994) suggest that the number of ANS binding sites in molecules of BLG previously heat-treated appears to decrease slightly with increasing heat treatment temperature above approximately 75 °C. Similarly, I_{ANS} decreases with increasing heat treatment temperature above 75 °C - 80 °C in some of the plots in Fig. 5.3.4.6. These decreases may therefore indicate that as the extent of aggregate formation increases with increasing heat treatment temperature, the number of hydrophobic sites on BLG molecules capable of binding ANS decreases.

The suggestion that I_{ANS} results are affected by aggregate formation is consistent with $\Delta\varepsilon_{270}$, $\Delta\varepsilon_{277}$ (near UV CD) and I_{Trp} (tryptophan fluorescence) results. $\Delta\varepsilon_{270}$ and $\Delta\varepsilon_{277}$ results suggest that non-native disulphide bonds in BLG molecules are formed as a consequence of heat treatment (Sections 5.3.1.3 and 5.3.1.6), while I_{Trp} results suggest that heat treatment leads to the loss of the disulphide bond Cys^{66}-Cys^{160} (Section 5.3.3.8). Both of these events probably occur as a consequence of the formation of disulphide-linked aggregates.

5.3.4.8. Urea-induced Unfolding of β-Lactoglobulin.

The results of Creamer (1995) indicate that the extent of structural change in BLG A/B in the presence of urea is greater than that in BLGs A, B and C which occurs as a consequence of heat treatment (Fig. 5.3.4.6). Creamer (1995) found that when the concentration of urea in solutions of BLG A/B was gradually increased from 0 M to 8.1 M, ANS emission intensity first increased slightly[1], and then decreased to approximately zero (i.e. that observed in the absence of BLG). He concluded that the integrity of the ANS binding site of BLG was lost in the presence of 8.1 M urea. In contrast, the results in Fig. 5.3.4.6 indicate that heat-treated BLG species are able to bind ANS, and that emission intensity is greater than that from ANS bound to the corresponding unheated species. This indicates that heat-treated BLG species are folded in such a way that emission intensity from bound ANS is greater than that from ANS bound to the corresponding unheated species.

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[1] Creamer (1995) suggested that the small increase in emission intensity from bound ANS which was observed as the urea concentration of BLG solutions was increased from 0 M to 3.4 M reflects dimer dissociation. Kella and Kinsella (1988b) found that as the urea concentration of BLG solutions is increased in this range, dimers dissociate to monomers. Therefore, ANS may bind more readily to monomeric BLG than to the corresponding dimeric species (Creamer, 1995).

5.3.4.9. Summary.

The ANS fluorescence results in the above sections indicate that solvent-exposed hydrophobic regions of the BLG structure capable of binding ANS are altered as a consequence of heat treatment. Along with $\Delta\varepsilon_{270}$, $\Delta\varepsilon_{277}$ and I_{Trp} results, they also suggest that aggregates of BLG are formed as a consequence of heat treatment. Aggregate formation is believed to occur when the thiol group of BLG becomes solvent-exposed (Section 2.7) and heat-induced thiol group exposure will be examined in the next section.

5.3.5. RESULTS AND DISCUSSION: THIOL AVAILABILITY.

5.3.5.1. Thiol Availability Measurement Protocol.

Heat-induced irreversible structural change in molecules of BLGs A, B and C at pH 6.7 and pH 7.4 was also studied by determining extents of the solvent-exposure of the thiol group of these variants. Determinations were made by treating previously heat-treated BLG solutions with the thiol reagent DTNB, which reacts with thiol groups as shown in Fig. 5.3.5.1.

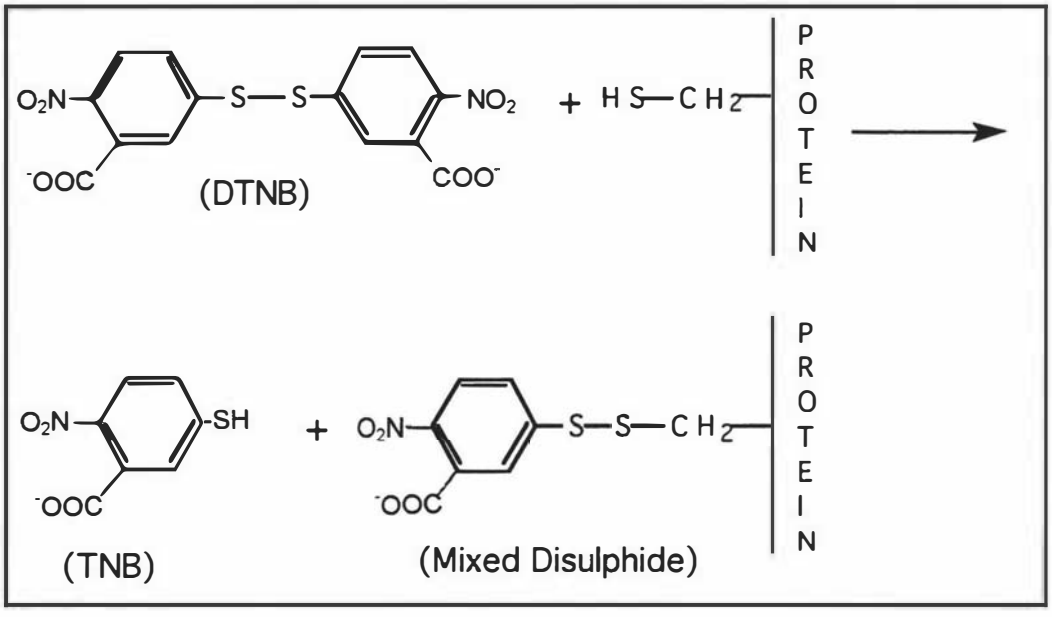


Fig. 5.3.5.1. The reaction of DTNB with protein thiol groups.

This reaction leads to the formation of a mixed disulphide and the liberation of thionitrobenzoate (TNB), which absorbs at 412 nm (ε = 14,150 M^{-1} cm^{-1}, Riddles *et al.*, 1979). Thus, concentrations of protein thiol groups available for reaction with DTNB can be calculated from A_{412} values. Furthermore, if the concentration of protein thiol groups is known, then the proportion of thiol groups available for reaction with DTNB can be determined. Prior to the commencement of the thiol availability study discussed in this section, the results from several preliminary and confirmatory experiments were required to ensure that extents of thiol exposure in heat-treated solutions of BLG could be determined reliably from measured A_{412} values. The results from these preliminary and confirmatory experiments are presented and discussed in Appendix 2.

5.3.5.2. Extents of Thiol Exposure in Unheated Solutions of β-Lactoglobulins.

When unheated BLGs A, B and C at pH 6.7 were reacted with DTNB, A_{412} values of 0.015, 0.009 and 0.012 respectively were obtained. However, when DTNB was mixed into a solution of reduced glutathione with a thiol concentration equivalent to those of the unheated BLG solutions, an A_{412} value of 1.32 was obtained. This indicates that prior to heat treatment at pH 6.7 approximately 1 % of the molecules of BLGs A, B and C possessed a solvent-accessible thiol group. These results are therefore consistent with the crystal structures (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998), in which the thiol group of Cys^{121} is sandwiched between the exterior of the calyx and the α-helix, and is thus unavailable for reaction with DTNB (Section 2.4.2).

The reaction of DTNB with unheated BLGs A, B and C at pH 7.4 gave final A_{412} values of 0.069, 0.059 and 0.052 respectively. At this pH the A_{412} value of 1.26 was obtained when DTNB was mixed into a solution of reduced glutathione with a thiol concentration equivalent to those of the unheated BLG solutions. This indicates that at pH 7.4 approximately 5 % of the molecules of BLGs A, B and C in the unheated solutions possessed a solvent-exposed thiol group. Therefore the proportion of molecules of BLGs A, B and C in unheated solutions which possess a solvent-exposed thiol group is greater at pH 7.4 than at pH 6.7. This in turn suggests that the structures of these variants in the vicinity of Cys^{121} are more open at pH 7.4 than at 6.7.

Although DTNB is hydrolysed more rapidly at pH 7.4 than at pH 6.7 (Riddles *et al.*, 1979), it is unlikely that this is the reason why the A_{412} values for BLGs A, B and C at pH 7.4 are higher than those at pH 6.7. This is because all BLG thiol availability determinations were made against a reference sample, consisting of pH 6.7 or pH 7.4 phosphate buffer and DTNB, prepared at the same time as the BLG samples were prepared.

The thiol availability results discussed above are consistent with the near UV CD results (Fig. 5.3.1.2), which suggested that the structures of unheated BLGs A, B and C in the vicinity of Trp^{19} are slightly more expanded at pH 7.4 than at pH 6.7 (Section 5.3.1.2). Similarly, the far UV CD spectra in Fig. 5.3.2.2, which suggest that the structures of unheated BLGs A, B and C contain more random structure at pH 7.4 than at pH 6.7, are consistent with the thiol availability results discussed above.

5.3.5.3. Extents of Thiol Exposure in Heat-treated Solutions of β-Lactoglobulins.

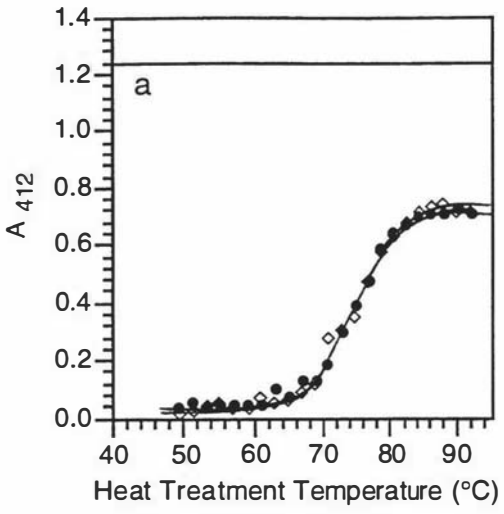
When BLGs A, B and C at pH 6.7 previously heat-treated at 80 °C for 13.5 min were reacted with DTNB, A_{412} values rapidly increased to 0.639, 0.796 and 0.687 respectively. These rapid and substantial increases in A_{412} indicate that the proportions of molecules of BLGs A, B and C which possess a thiol group available for reaction with DTNB are greater in heat-treated solutions than in unheated solutions. This in turn suggests that the thiol group of BLG becomes permanently solvent-exposed as a consequence of heat treatment, this being consistent with heat-induced irreversible structural change.

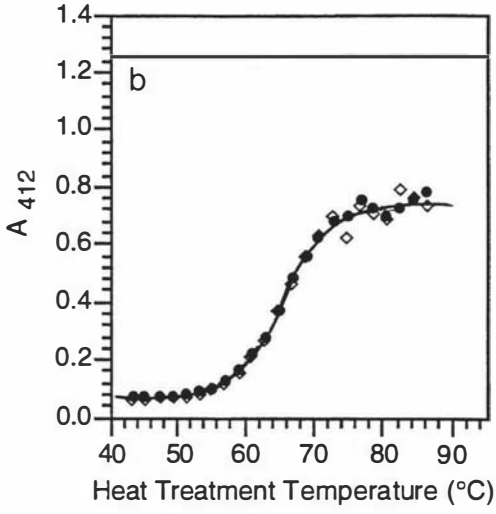
The thiol availability results for heat-treated BLG may indicate that the α-helix, which shields Cys^{121} from DTNB in native BLG (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998), is irreversibly peeled from the exterior of the BLG calyx as a consequence of heat treatment. Alternatively, these thiol availability results may indicate that the α-helix of BLG is lost as a consequence of heat treatment, in agreement with the results of Qi *et al.* (1997), which indicate that the α-helix of BLG is lost during temperature increase from 60 °C to 70 °C. The thiol group of BLG is unlikely to become solvent-exposed as a consequence of dimer dissociation, as suggested by Iametti *et al.* (1996), because the side chain of Cys^{121} is a considerable distance from the dimer interface in the crystal structures of Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998), (Section 2.4.3).

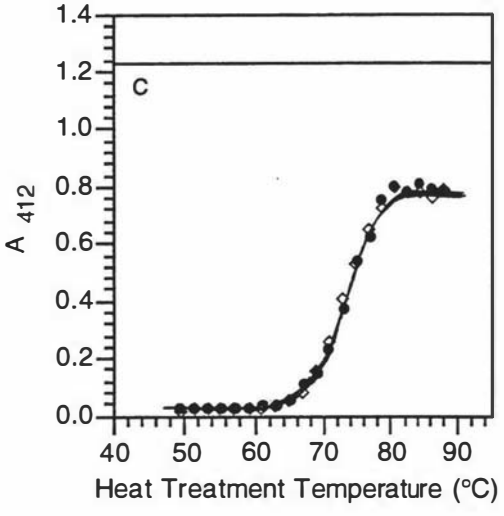
5.3.5.4. Quantitative Study of Extents of Thiol Exposure in Heat-treated β-Lactoglobulin Solutions.

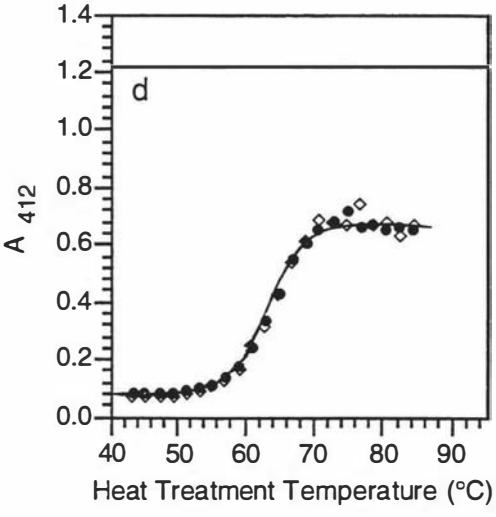
Thiol availability measurements were made on solutions of BLGs A, B and C at pH 6.7 and pH 7.4 previously heat-treated at various temperatures and the results are shown as plots of A_{412} versus heat treatment temperature in Fig. 5.3.5.2. The horizontal lines at the tops of these plots denote the A_{412} values obtained after DTNB was added to solutions of reduced glutathione with thiol concentrations equivalent to those of the unheated BLG solutions. These A_{412} values were assumed to represent those obtained after DTNB was added to BLG solutions in which 100 % of the BLG molecules possess a solvent-exposed thiol group.

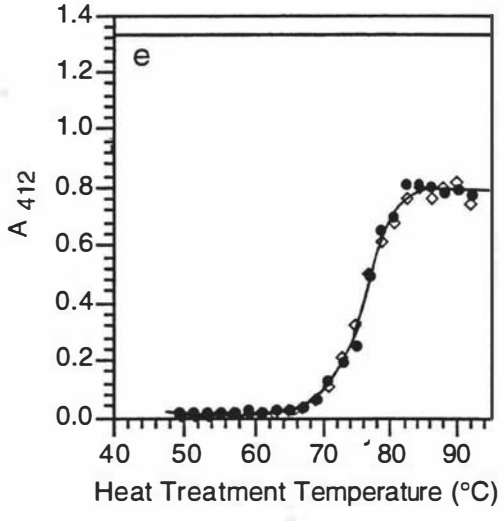
Fig. 5.3.5.2. Effect of heat treatment of solutions of BLGs A, B and C at pH 6.7 and pH 7.4 on the availability of the thiol group for reaction with DTNB. The plots are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. Paired symbols denote duplicate A_{412} data obtained by reacting heat-treated BLG solutions with DTNB and reading the A_{412} against a reference sample consisting of buffer and DTNB (Section 5.2.3). The horizontal lines at the tops of the plots denote the A_{412} values obtained after DTNB was added to solutions of reduced glutathione with thiol concentrations equivalent to those of the unheated BLG solutions. These A_{412} values were assumed to represent those obtained after DTNB was added to BLG solutions in which 100 % of the BLG molecules possessed a solvent-exposed thiol group. The curves were obtained by fitting experimental A_{412} data to equations 5.2.8.1 and 5.2.8.2 as described in Section 5.2.8.1 using the computer program "Enzfitter" (Leatherbarrow, 1987). Values for the fitting parameters are given in Appendix 3. Details of the solution preparation and heat treatment protocols are given in Sections 5.2.3.1 and 5.2.3.2 respectively.

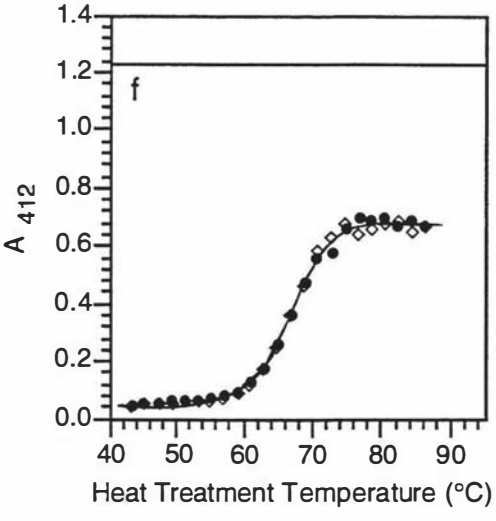

a
A 412
Heat Treatment Temperature (°C)


b
A 412
Heat Treatment Temperature (°C)


c
A 412
Heat Treatment Temperature (°C)


d
A 412
Heat Treatment Temperature (°C)


e
A 412
Heat Treatment Temperature (°C)


f
A 412
Heat Treatment Temperature (°C)

The plots in Fig. 5.3.5.2 indicate that for all three variants, duplicate experimental thiol availability data coincide, except in the case of data acquired from solutions previously heat-treated at temperatures higher than 80 °C at pH 6.7 and 75 °C at pH 7.4. This suggests that thiol availability data acquired from solutions heat-treated at temperatures higher than 80 °C at pH 6.7 and 75 °C at pH 7.4 is less reproducible than those acquired from BLG solutions heat-treated at lower temperatures. The significance of this will be discussed in Sections 5.3.5.7 and 5.3.8.

Thiol availability data were fitted to equations 5.2.8.1 and 5.2.8.2 (Section 5.2.8.1) which are based on the 2-state thermal unfolding model of Luo *et al.* (1995). The experimental data agree with the fitted curves (Fig. 5.3.5.2), showing that the selected model adequately describes the heat-induced structural change in BLG molecules which leads to thiol group exposure. Therefore, the fitted values for the parameter A_{412}(low) shown in Table 5.3.5.1 represent the A_{412} values obtained after DTNB had been mixed into solutions of unheated BLG in which the thiol group in the majority of BLG molecules was solvent-inaccessible. Furthermore, the fitted values for the parameter A_{412}(high) shown in Table 5.3.5.1 represent the A_{412} values obtained after DTNB was mixed into solutions of heat-treated BLG in which the proportion of molecules which possessed a permanently solvent-exposed thiol group was greatest. Values for A_{412}(low) and A_{412}(high) are also expressed as %Ex(low) and %Ex(high) respectively (Table 5.3.5.1). The errors in the values for the parameters in Table 5.3.5.1 are probably underestimates of the actual errors.

The values for %Ex(low) for BLGs A, B and C at pH 6.7 (Table 5.3.5.1) are similar. This suggests that in unheated solutions at pH 6.7, the proportions of molecules of BLGs A, B and C which posses a solvent-exposed thiol group are equivalent, and therefore that the structures of these variants in the vicinity of Cys^{121} are similar. However, for all three variants, values for %Ex(low) are less at pH 6.7 than at pH 7.4 (Table 5.3.5.1), in agreement with the thiol availability results obtained for unheated BLGs A, B and C at pH 6.7 and pH 7.4 (Section 5.3.5.2).

Table 5.3.5.1. Fitted Values for the Parameters A_{412}(low) and A_{412}(high) and the Derived Values %Ex(low) and %Ex(high) for β-Lactoglobulins A, B and C at pH 6.7 and pH 7.4.

Sample	A_{412}(low)	%Ex(low)	A_{412}(high)	%Ex(high)
pH 6.7, BLG A	0.014 ± 0.077	1.28 ± 7.04	0.738 ± 0.014	67.27 ± 1.28
	0.037 ± 0.032	3.37 ± 2.90	0.714 ± 0.007	65.11 ± 0.65
pH 6.7, BLG B	0.022 ± 0.007	1.77 ± 0.57	0.794 ± 0.007	63.87 ± 0.64
	0.007 ± 0.018	0.56 ± 1.44	0.800 ± 0.007	64.63 ± 0.65
pH 6.7, BLG C	0.014 ± 0.007	1.06 ± 0.53	0.794 ± 0.090	60.15 ± 0.60
	0.014 ± 0.008	1.06 ± 0.60	0.800 ± 0.006	60.61 ± 0.61
pH 7.4, BLG A	0.073 ± 0.014	5.78 ± 1.10	0.800 ± 0.006	59.43 ± 0.59
	0.067 ± 0.009	5.31 ± 0.69	0.794 ± 0.090	59.03 ± 0.65
pH 7.4, BLG B	0.059 ± 0.018	4.83 ± 1.45	0.687 ± 0.008	56.21 ± 0.67
	0.059 ± 0.012	4.83 ± 0.97	0.669 ± 0.005	54.75 ± 0.55
pH 7.4, BLG C	0.049 ± 0.019	3.81 ± 1.48	0.674 ± 0.005	52.45 ± 0.52
	0.056 ± 0.007	4.36 ± 0.57	0.685 ± 0.007	53.31 ± 0.53

Where:

A_{412}(low) is the A_{412} value obtained after DTNB had been mixed into a solution of unheated BLG.

%Ex(low) is the percentage of BLG molecules in unheated solutions which possess a solvent-exposed thiol group.

A_{412}(high) is the A_{412} value obtained after DTNB had been mixed into a solution of heat-treated BLG in which the proportion of permanently solvent-exposed thiol groups was greatest.

%Ex(high) is the maximum percentage of BLG molecules in heat-treated solutions which possess a solvent-exposed thiol group. A_{412}(low) and A_{412}(high) are two of the parameters used to fit experimental thiol availability data (i.e. values for A_{412}) as described in Fig. 5.3.5.2. The errors in A_{412}(low) and A_{412}(high) values are standard errors calculated by the computer program "Enzfitter" (Leatherbarrow, 1987). Values for %Ex(low) and %Ex(high) were calculated by dividing values for A_{412}(low) and A_{412}(high) by the appropriate A_{412} value obtained after a solution of reduced glutathione with a thiol concentration equivalent to those of the unheated BLG solutions had been mixed with DTNB. The errors in %Ex(low) and %Ex(high) values were calculated from the percentage errors in the values for A_{412}(low) and A_{412}(high).

The values for %Ex(high) for BLGs A, B and C at pH 6.7 indicate that a maximum of 60 % - 65 % of the protein molecules possess a solvent-exposed thiol group after heat treatment. In contrast, the results obtained from measurements made as described in Section 5.2.3.4 indicate that at pH 6.7 the thiol group in close to 100 % of BLG B molecules is available for reaction with DTNB in the presence of 5.0 M urea. A solution of BLG B in 5.0 M urea and a solution of reduced glutathione with a thiol concentration equivalent to that of the BLG solution gave A_{412} values of 1.075 and 1.138 respectively. These A_{412} values indicate that 97.0 % and 99.5 % of the total number of thiol groups were available for reaction with DTNB in the BLG B and reduced glutathione solutions respectively. Thus, with respect to thiol group exposure caused by heat treatment, the values for %Ex(high) in Table 5.3.5.1 may indicate that for between 35 % - 40 % of BLG molecules, the thiol group is solvent-exposed at elevated temperatures but becomes solvent-inaccessible during the subsequent cooling period. This could occur as a consequence of re-folding. The values for %Ex(high) may also indicate that, although almost all of the thiol groups in solutions of BLGs A, B and C become solvent-exposed during heat treatment, between 35 % and 40 % were unavailable for reaction with DTNB because they had been oxidised to disulphides. This suggestion is consistent with the results of Watanabe and Klostermeyer (1976), which indicate that thiol groups in solutions of BLG A are oxidised during heat treatment. Furthermore, in the case of the results in Table 5.3.5.1, the maximum proportions of thiol groups which remain exposed after heat treatment (i.e. %Ex(high)) appear similar to those shown in Fig. 1 of Watanabe and Klostermeyer (1976).

If the results in Table 5.3.5.1 indicate that BLG thiol groups are oxidised to disulphides during heat treatment, then they may be consistent with $\Delta\varepsilon_{270}$ and $\Delta\varepsilon_{277}$ results, which suggest that non-native disulphide bonds form as a consequence of heat treatment (Section 5.3.1.3).

The values for %Ex(high) in Table 5.3.5.1 indicate that in solutions of BLGs A, B and C at pH 7.4, a maximum of between 52 % and 60% of molecules possess a solvent-exposed thiol group after heat treatment. Thus, for all three variants, the values for %Ex(high) determined using pH 7.4 thiol availability data are less than those determined using pH 6.7 thiol availability data (Table 5.3.5.1). These differences may be due to differences in the stability of aqueous TNB at pH 6.7 and pH 7.4. The results in Section A2.2.5 of Appendix 2 suggest that at pH 7.4 at a given time after addition of DTNB to a previously heat-treated BLG solution, the concentration of TNB is always less than the concentration of solvent-exposed thiol groups present prior to the addition of DTNB. In contrast, the results in Section A2.2.3 of Appendix 2 indicate that at pH 6.7, the concentration of TNB is equivalent to the concentration of solvent-exposed thiol groups present in BLG solutions prior to the addition of DTNB.

5.3.5.5. Midpoint Temperatures for the Heat-induced Structural Change that leads to Irreversible Thiol Group Exposure.

The fitted values for the parameter T_{mid} (Table 5.3.5.2) were used to obtain a quantitative measure of the differences in the susceptibilities of BLGs A, B and C at pH 6.7 and pH 7.4 to the heat-induced irreversible structural change which led to thiol group exposure. T_{mid} was assumed to represent the midpoint temperature of the transition for this irreversible structural change. At pH 6.7 the average T_{mid} value for BLG B is 1.4 °C lower than that for BLG A, which is 1.3 °C lower than that for BLG C. Similarly, at pH 7.4 the average T_{mid} value for BLG B is 1.9 °C lower than that for BLG A, which is 1.4 °C lower than that for BLG C. Therefore, in agreement with T_{mid} values determined using $\Delta\varepsilon_{293}$, $[\theta]_{205}$, and I_{ANS} data (Tables 5.3.1.1, 5.3.2.1 and 5.3.4.1 respectively), those determined using thiol availability data suggest that at both pH 6.7 and pH 7.4, BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change.

For BLGs A, B and C the T_{mid} values at pH 7.4 are approximately 9 °C lower than those at pH 6.7 (Table 5.3.5.2). This suggests that all three variants are more susceptible to heat-induced irreversible structural change at pH 7.4 than at pH 6.7.

At pH 6.7 in particular, the slopes at T_{mid} of the plots of A_{412} versus heat treatment temperature are less for BLG A than for BLGs B and C (Table 5.3.5.2). This suggests that BLG A responds to heat treatment in a different manner to BLGs B and C. The significance of this will be discussed in Section 8.5.

Because the results in Fig. 5.3.5.2 indicate that heat treatment of BLG solutions leads to irreversible thiol group exposure, it is likely that the BLG solutions used in thiol availability measurements contained aggregate species. Nevertheless, aggregation is unlikely to affect the fitting of thiol availability data to the selected 2-state thermal unfolding model because these data describe BLG molecules in only 2 states: those which possess a solvent-inaccessible thiol group and those which possess a solvent-accessible thiol group. A study of aggregate formation in BLG solutions heat-treated under similar conditions to those used to prepare the BLG solutions used in CD, fluorescence and thiol availability studies (Sections 5.3.1, 5.3.2, 5.3.3, 5.3.4 and 5.3.5) is presented in Section 5.3.9.

Table 5.3.5.2. Values for the Midpoint Temperature (T_{mid}) and Slope at T_{mid} for the Heat-induced Irreversible Structural Change in β-Lactoglobulins A, B and C at pH 6.7 and at pH 7.4 which leads to the Solvent-exposure of the Thiol Group.

Sample	T_{mid} (°C)	Slope at T_{mid} (A_{412}/°C)
pH 6.7, BLG A	74.2 ± 0.8	0.05
	74.3 ± 0.3	0.05
pH 6.7, BLG B	72.6 ± 0.1	0.08
	73.0 ± 0.2	0.07
pH 6.7, BLG C	75.4 ± 0.1	0.09
	75.8 ± 0.1	0.08
pH 7.4, BLG A	65.4 ± 0.4	0.05
	65.3 ± 0.2	0.05
pH 7.4, BLG B	63.6 ± 0.3	0.06
	63.4 ± 0.2	0.06
pH 7.4, BLG C	66.6 ± 0.2	0.05
	66.8 ± 0.2	0.06

Where:

T_{mid} is the midpoint temperature of the transition for the heat-induced irreversible structural change which leads to thiol group exposure (i.e. the temperature at which the value for A_{412} is exactly half way between A_{412}(low) and A_{412}(high)). T_{mid} is one of the parameters used to fit thiol availability data as described in Fig. 5.3.5.2. The errors in T_{mid} are standard errors calculated by "Enzfitter".

The slope at the midpoint temperature was calculated using the fitted A_{412} values in the range T_{mid} ± 0.5 °C.

5.3.5.6. Thermodynamic Analysis.

Values for ΔG_{app} at 72 °C and pH 6.7 and at 66 °C and pH 7.4 were calculated using thiol availability data (Table 5.3.5.3). These estimates do not, however, represent those for the reversible unfolding of BLG because thiol availability data were collected under conditions where the unfolding of BLG is neither complete nor reversible. This is because non-native BLG is removed from the equilibrium with the corresponding folded species as a consequence of aggregate formation (Section 5.3.1.5) and as a consequence of the reaction with DTNB (equation 5.3.5.1) after heat treatment. In equation 5.3.5.1, BLG and BLG^{SH} are the species which possess a solvent-inaccessible and solvent-accessible thiol group respectively and BLG-S-S-TNB is the mixed disulphide formed from the reaction of BLG with DTNB.

$$BLG \underset{-k_1}{\overset{k_1}{\rightleftharpoons}} BLG^{SH} \xrightarrow{k_2} BLG\text{-}S\text{-}S\text{-}TNB^- \qquad (5.3.5.1)$$

Nevertheless, it is likely that the values in Table 5.3.5.3 represent the ΔG_{app} for heat-induced irreversible thiol group exposure in BLG molecules. Values for ΔG_{app} were calculated at 72 °C and at 66 °C because these temperatures are close to the T_{mid} of the transition for heat-induced thiol group exposure for all three variants at pH 6.7 and pH 7.4 respectively (Table 5.3.5.2). Additionally, these are the temperatures at which estimates of ΔG_{app} were calculated using $\Delta\varepsilon_{293}$, $[\theta]_{205}$ and I_{ANS} data.

Table 5.3.5.3. Values for ΔG_{app} at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for Irreversible Thiol Group Exposure in β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6.7, BLG A	2.054 ± 0.020	1.16*
pH 6.7, BLG B	0.899 ± 0.002	0 (by definition)
pH 6.7, BLG C	4.296 ± 0.006	3.40#
pH 7.4, BLG A	1.929 ± 0.009	1.43*
pH 7.4, BLG B	0.498 ± 0.002	0 (by definition)
pH 7.4, BLG C	3.712 ± 0.010	3.21#

* ΔG_{app}(BLG A)- ΔG_{app}(BLG B)

ΔG_{app}(BLG C)- ΔG_{app}(BLG B).

ΔG_{app} is assumed to represent the change in free energy at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for the irreversible structural change in BLG molecules which leads to the permanent solvent-exposure of the thiol group. Values for ΔG_{app} were calculated at these temperatures and pH values using fitted values for A_{412} as described in Section 5.2.8.3. The fitted values for A_{412} were obtained from the fitted curves shown in Fig. 5.3.5.2 using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in ΔG_{app} values were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 5.3.5.2).

At both pH 6.7 and pH 7.4, the ΔG_{app} values for BLG B are less than those for BLG A, which are less than those for BLG C. Thus, in agreement with the T_{mid} values in Table 5.3.5.2, the ΔG_{app} values in Table 5.3.5.3 indicate that at both pH 6.7 and pH 7.4, BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change. In Table 5.3.5.3, differences in ΔG_{app} values for the A, B and C variants are shown as $\Delta\Delta G_{app}$ values and the significance or these differences will be discussed in Section 8.5.

5.3.5.7. Extent of Scatter of A_{412} Data Obtained from β-Lactoglobulin Solutions Previously Heat-treated at High Temperatures.

The plots in Fig. 5.3.5.2 indicate that the scatter of A_{412} values obtained from BLG solutions previously heat-treated at temperatures greater than approximately 80 °C at pH 6.7 and 75 °C at pH 7.4 was greater than that of A_{412} values for solutions previously heat-treated at lower temperatures. This scatter may reflect either variations in extents of loss of water from different vials during heat treatment at the higher temperatures, or differences in extents of leakage of air into vials, the latter leading to differences in extents of thiol group oxidation during heat treatment. These effects may be related to how well the vials in which BLG solutions were heat-treated could be sealed. Evaporation of water during heat treatment could not have affected the results obtained in CD and fluorescence measurements because heat-treated BLG solutions were diluted to 1.00 mg/mL before all measurements were made. In contrast, because the BLG solutions used in thiol availability measurements were reacted with DTNB as soon as possible after heat treatment, and because the complete set of data for a particular variant at a particular pH value were collected on the same day, these solutions could not be diluted to equivalent concentrations before measurements were made. Thiol availability data were acquired in this manner because the results in Section A2.2.4 of Appendix 2 indicate that an appreciable decrease in the concentration of solvent-exposed BLG thiol groups occurs within hours of heat treatment.

In an attempt to minimise extents of water loss from BLG solutions during heat treatment, the thiol availability assay procedure was modified. Confirmatory thiol availability measurements were made using this modified procedure, and the results obtained (Section 5.3.8) are compared with those obtained using the original assay procedure (Sections 5.3.5.3 - 5.3.5.5).

5.3.6. COMPARISON OF T_{mid} VALUES DETERMINED USING $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS}, AND THIOL AVAILABILITY DATA.

All of the T_{mid} values determined from the data presented in Sections 5.3.1, 5.3.2, 5.3.3, 5.3.4 and 5.3.5 are shown in Table 5.3.6.1. With the exception of those determined using I_{Trp} data, all of the T_{mid} values in this table suggest that at both pH 6.7 and pH 7.4, BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change. Also, for a particular variant at pH 6.7 the T_{mid} values determined using $\Delta\varepsilon_{293}$, I_{ANS} and thiol availability data agree. This suggests that at pH 6.7, irreversible structural change in the vicinity of Trp^{19}, the irreversible structural change leading to changes in emission intensity from bound ANS and the irreversible structural change which leads to the solvent-exposure of the thiol group all occur as a consequence of heat treatment at similar temperatures. The values for T_{mid} determined using $[\theta]_{205}$ data (Table 5.3.6.1) may also agree with those determined using $\Delta\varepsilon_{293}$, I_{ANS} and thiol availability data, but the scatter of $[\theta]_{205}$ data is greater than that of $\Delta\varepsilon_{293}$, I_{ANS} and thiol availability data and therefore the values for T_{mid} determined using $[\theta]_{205}$ data are the least accurate.

For BLGs A, B and C at pH 6.7, the values for T_{mid} determined using I_{Trp} data are approximately 2 °C higher than those determined using $\Delta\varepsilon_{293}$, I_{ANS} and thiol availability data (Table 5.3.6.1). However, changes in I_{Trp} may occur as a consequence of the loss of the disulphide bond Cys^{66}-Cys^{160} rather than tertiary and secondary structure change (Sections 5.3.3.2 and 5.3.3.8). The loss of this disulphide bond may in turn occur during the formation of non-native disulphide bonds, and therefore during aggregate formation. A comparison of the T_{mid} values determined using I_{Trp} data and the other data in this chapter may therefore be inappropriate.

At pH 7.4, as at pH 6.7, the T_{mid} values determined using $\Delta\varepsilon_{293}$ and I_{ANS} are similar, while those determined using I_{Trp} data are approximately 2 °C higher. However, at pH 7.4, the T_{mid} values determined using thiol availability data are approximately 2 °C lower than those determined using $\Delta\varepsilon_{293}$ and I_{ANS} data and possibly $[\theta]_{205}$ data. Therefore, because the T_{mid} values determined using $\Delta\varepsilon_{293}$, I_{ANS} and thiol availability data agree at pH 6.7 (see above), the difference between T_{mid} values determined using thiol availability data and those determined using $\Delta\varepsilon_{293}$, I_{ANS}, I_{Trp} and possibly $[\theta]_{205}$ data is greater at pH 7.4 than at pH 6.7. This suggests that the irreversible structural change which leads to thiol group exposure exhibits a greater dependence on pH than those which lead to changes in $\Delta\varepsilon_{293}$, I_{ANS}, I_{Trp} and $[\theta]_{205}$.

Table 5.3.6.1. Comparison of Midpoint Temperature (T_{mid}) Values Determined using $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and Thiol Availability Data.

Sample	T_{mid} (°C)				
	$\Delta\varepsilon_{293}$	$[\theta]_{205}$	I_{Trp}	I_{ANS}	Thiol Availability
pH 6.7, BLG A	74.7 ± 0.3	75.7 ± 0.7	77.4 ± 0.7	74.7 ± 0.2	74.3 ± 0.8
pH 6.7, BLG B	72.8 ± 0.1	74.8 ± 1.6	75.7 ± 0.8	72.5 ± 0.1	72.8 ± 0.2
pH 6.7, BLG C	76.0 ± 0.1	77.0 ± 0.1	77.7 ± 0.7	76.3 ± 0.1	75.6 ± 0.1
pH 7.4, BLG A	67.0 ± 0.2	69.4 ± 0.9	67.4 ± 0.4	67.7 ± 0.1	65.4 ± 0.4
pH 7.4, BLG B	66.2 ± 0.1	64.6 ± 0.8	69.0 ± 0.2	65.3 ± 0.4	63.5 ± 0.3
pH 7.4, BLG C	69.5 ± 0.1	70.0 ± 0.4	72.5 ± 0.5	69.9 ± 0.2	66.7 ± 0.2
pH 8.1, BLG A	62.1 ± 0.9	65.4 ± 0.7	60.7 ± 0.2	67.3 ± 1.2	-

Where:

T_{mid} is the midpoint temperature of the transition for heat-induced irreversible structural change (Tables 5.3.1.1, 5.3.2.1, 5.3.3.1, 5.3.4.1 and 5.3.5.2). In the cases of I_{Trp}, I_{ANS} and A_{412} data, the average of the two T_{mid} values for a particular variant at a particular pH value and the corresponding average standard error are shown above. The T_{mid} values were determined as described in the captions of the tables listed immediately above.

5.3.7. CONFIRMATORY STUDIES: CD AND FLUORIMETRY.

Additional near and far UV CD, tryptophan and ANS fluorescence measurements were made to confirm the correctness of the orders of the susceptibilities to heat-induced irreversible structural change of BLGs A, B and C as determined using near UV CD (Section 5.3.1.4), far UV CD (Section 5.3.2.4), tryptophan fluorescence (Section 5.3.3.4) and ANS fluorescence (Section 5.3.4.5) results. Confirmatory measurements were made using series of BLG solutions previously heat-treated at only six different temperatures (Table 5.3.7.1) so that complete sets of data acquired using a particular technique could be collected within 8 hours. The heat treatment temperatures for each variant at each pH were chosen using earlier $\Delta\varepsilon_{293}$ (Fig. 5.3.1.5) and I_{ANS} (Fig. 5.3.4.6) results and correspond to approximately 0 %, 20 %, 40 % ,60 %, 80 % and 100 % of the total observed change in signal intensity. The selection of the six heat treatment temperatures for BLG B at pH 6.7 is illustrated in Fig. 5.3.7.1.

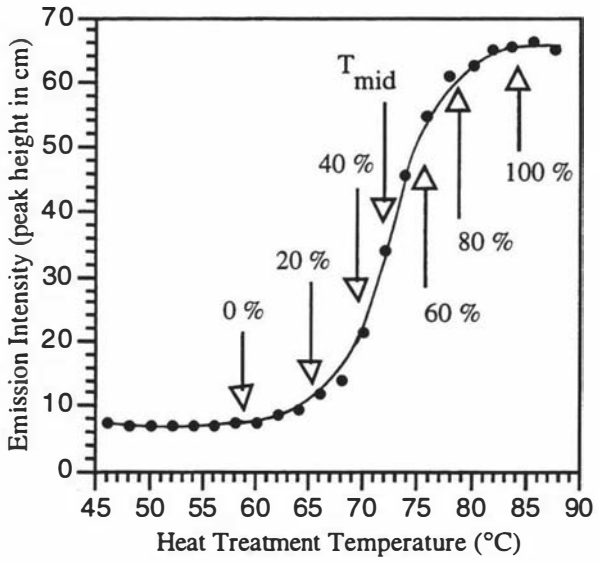


Fig. 5.3.7.1. Use of plots of I_{ANS} versus heat treatment temperature to select the heat treatment temperatures for BLG B at pH 6.7 for use in confirmatory studies. The arrows denote the temperatures which correspond to 0 %, 20 %, 40 %, 60 %, 80 % and 100 % of the maximum extent of heat-induced irreversible spectral change.

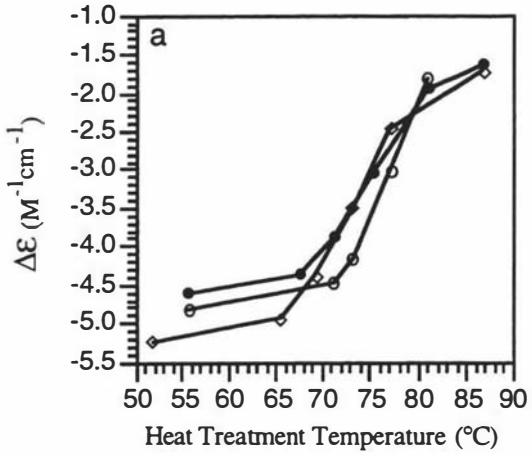
Table 5.3.7.1. Heat Treatment Temperatures for β-Lactoglobulin Samples used in Confirmatory Studies.

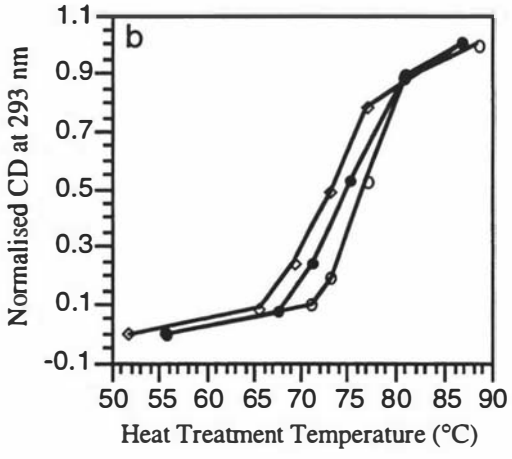
	Temperature (°C)		
	BLG A	BLG B	BLG C
pH 6.7	56	52	56
	68	66	72
	72	70	74
	76	74	78
	82	78	82
	88	88	90
pH 7.4	50	50	56
	60	58	64
	66	64	68
	70	68	72
	74	72	76
	80	78	82

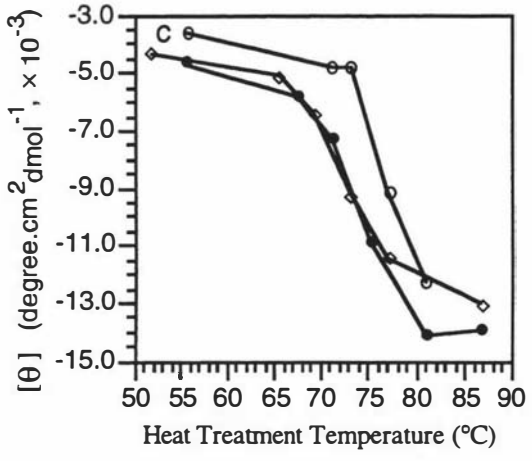
Plots of raw and normalised $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp} and I_{ANS} values versus heat treatment temperature, prepared using data from the first of the two sets of confirmatory measurements made on BLGs A, B and C at pH 6.7, are shown in Fig. 5.3.7.2. However, from results obtained in this way, differences in the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change are more difficult to identify. This is because the relative positions of the plots of raw data for BLGs A, B and C in Fig. 5.3.7.2 (used to judge relative differences in susceptibilities to heat-induced irreversible structural change) are affected by dissimilarities in the intensities of the signals from these variants. To compensate for these dissimilarities in signal intensity, plots of normalised data are also shown in Fig. 5.3.7.2.

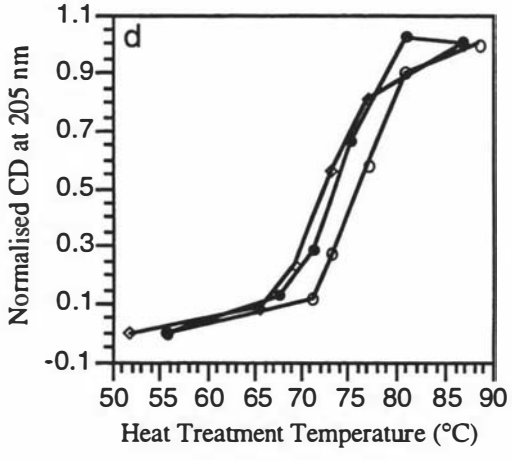
The plots of normalised $\Delta\varepsilon_{293}$, $[\theta]_{205}$ and I_{ANS} versus heat treatment temperature all suggest that BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities to heat-induced irreversible structural change respectively. However, the plots of normalised I_{Trp} data suggest that although BLG C is most resistant to heat-induced irreversible structural change, the susceptibilities of BLGs A and B to heat-induced irreversible structural change are equivalent. The results obtained in the second series of confirmatory experiments made at pH 6.7 are shown in Fig. 5.3.7.3, and except in the case of the I_{Trp} results, appear consistent with those shown in Fig. 5.3.7.2. All of the results obtained in the series of confirmatory experiments made at pH 7.4 (Fig. 5.3.7.4) suggest that BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change.

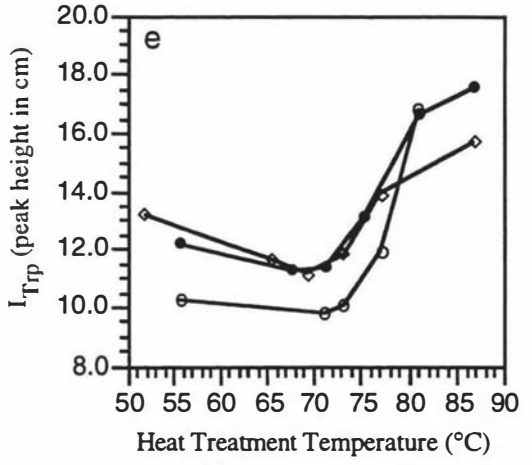
Within the limits of experimental error, the majority of the results obtained in the three sets of confirmatory measurements are consistent with the near and far UV CD and ANS fluorescence results of Sections 5.3.1.4, 5.3.2.4 and 5.3.4.5 respectively. Therefore, the conclusion that BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change is confirmed. Furthermore, the two sets of pH 6.7 confirmatory measurements were made using two different batches of purified BLGs A, B and C, neither of which had been used in the original sets of spectroscopic measurements (Section 5.1.2). Likewise, pH 7.4 confirmatory experiments were made using a different batch of purified BLGs A, B and C to that used in the original sets of spectroscopic measurements. Obtaining similar results from different batches of purified BLG therefore increases the likelihood that BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change.

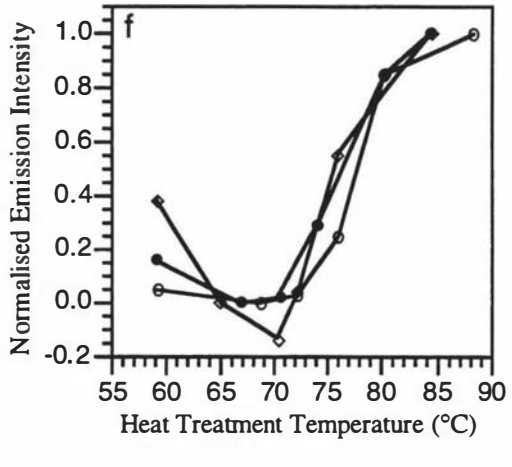

a
$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
50 55 60 65 70 75 80 85 90
Heat Treatment Temperature (°C)


b
Normalised CD at 293 nm
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
50 55 60 65 70 75 80 85 90
Heat Treatment Temperature (°C)


c
[θ] (degree.cm^{2}dmol^{-1}, x 10^{-3})
-3.0
-5.0
-7.0
-9.0
-11.0
-13.0
-15.0
50 55 60 65 70 75 80 85 90
Heat Treatment Temperature (°C)


d
Normalised CD at 205 nm
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
50 55 60 65 70 75 80 85 90
Heat Treatment Temperature (°C)


e
I_{Trp} (peak height in cm)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
50 55 60 65 70 75 80 85 90
Heat Treatment Temperature (°C)


f
Normalised Emission Intensity
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
55 60 65 70 75 80 85 90
Heat Treatment Temperature (°C)

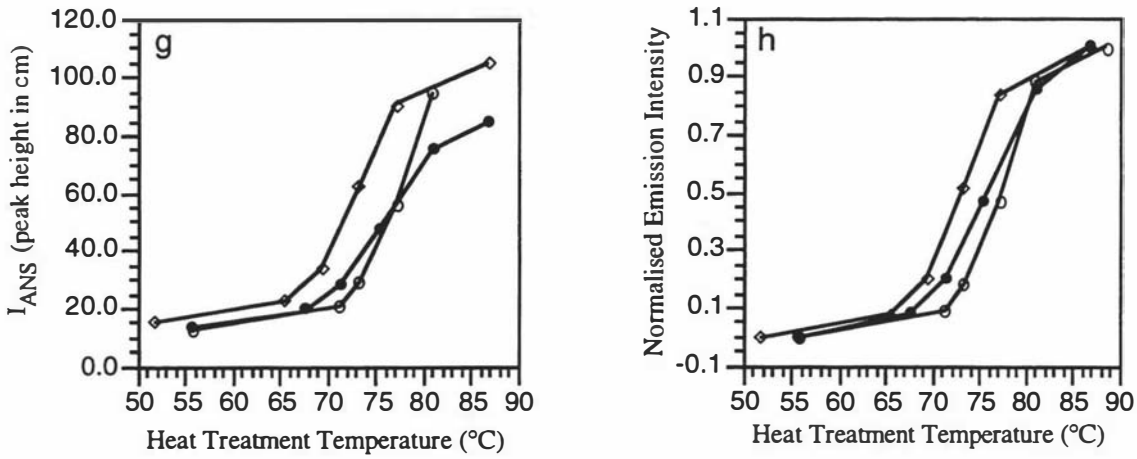
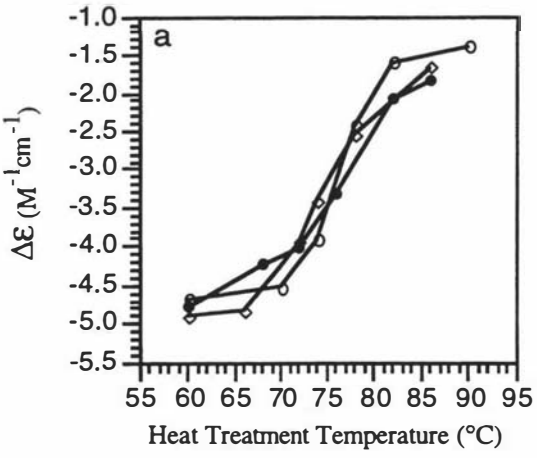
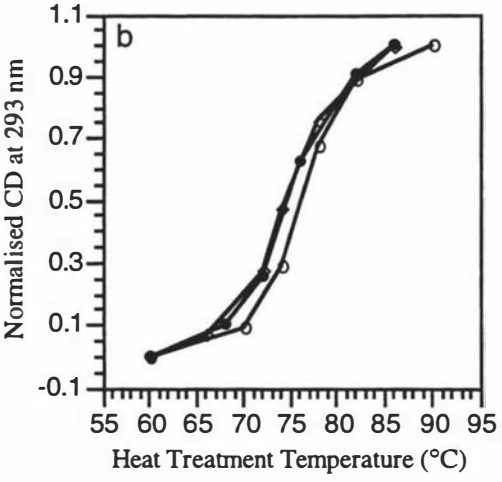
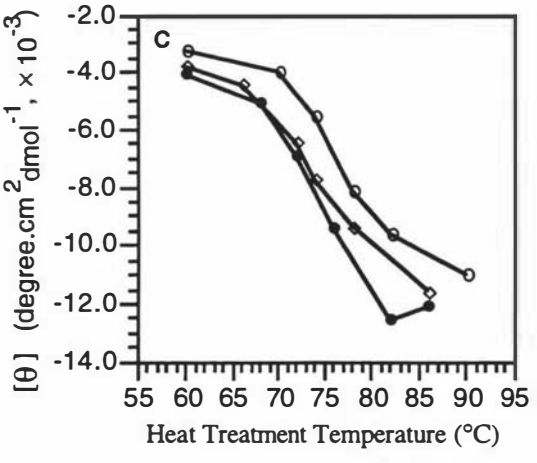
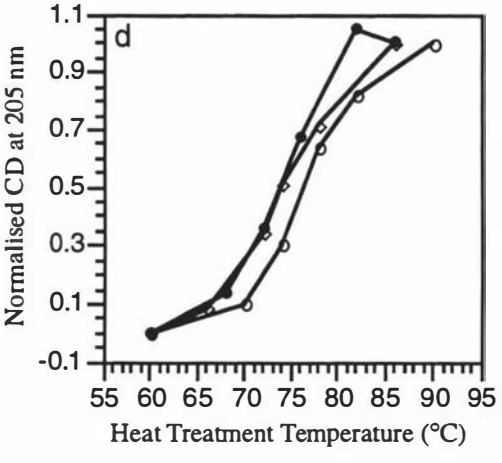


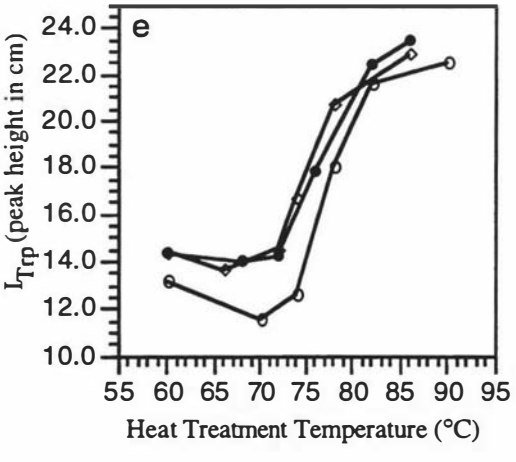
Fig. 5.3.7.2. Confirmatory measurements at pH 6.7, set 1: Effect of heat treatment of solutions of BLGs A, B and C on $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp} and I_{ANS}. The plots for BLGs A (•), B (◇) and C (○), prepared from data acquired using a particular technique, are shown in the same panel. The panels are as follows: raw $\Delta\varepsilon_{293}$, panel a; normalised $\Delta\varepsilon_{293}$, panel b; raw $[\theta]_{205}$, panel c; normalised $[\theta]_{205}$, panel d; raw I_{Trp}, panel e; normalised I_{Trp}, panel f; raw I_{ANS}, panel g; and normalised I_{ANS}, panel h. Sets of experimental data were normalised so that the minimum and maximum in each were 0 and 1 respectively.

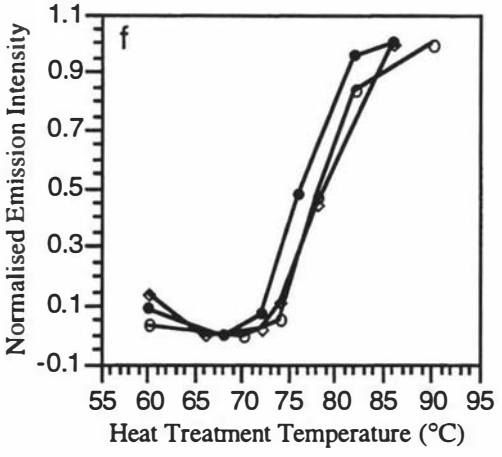

a
$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
55 60 65 70 75 80 85 90 95
Heat Treatment Temperature (°C)


b
Normalised CD at 293 nm
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
55 60 65 70 75 80 85 90 95
Heat Treatment Temperature (°C)


c
[θ] (degree.cm^{2}dmol^{-1}, x 10^{-3})
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
55 60 65 70 75 80 85 90 95
Heat Treatment Temperature (°C)


d
Normalised CD at 205 nm
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
55 60 65 70 75 80 85 90 95
Heat Treatment Temperature (°C)


e
I_{Trp} (peak height in cm)
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
55 60 65 70 75 80 85 90 95
Heat Treatment Temperature (°C)


f
Normalised Emission Intensity
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
55 60 65 70 75 80 85 90 95
Heat Treatment Temperature (°C)

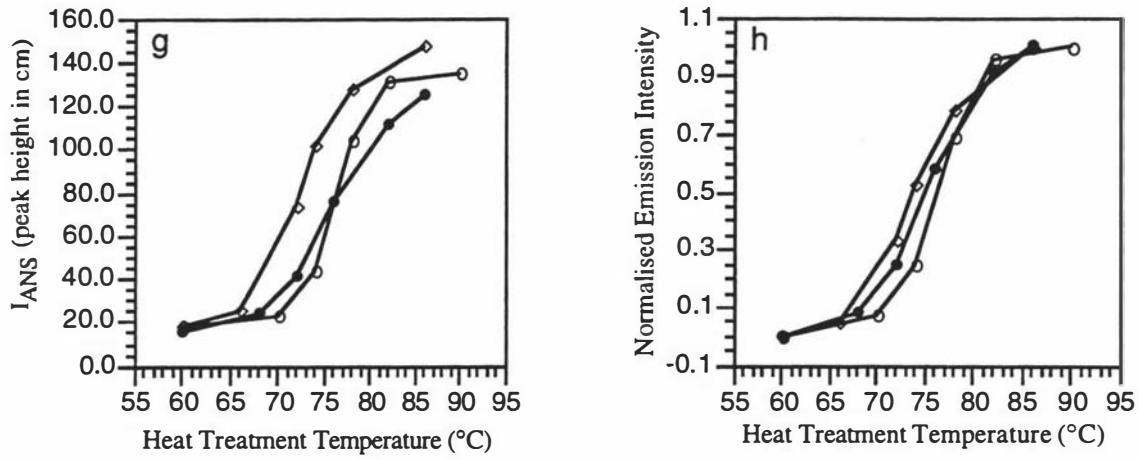
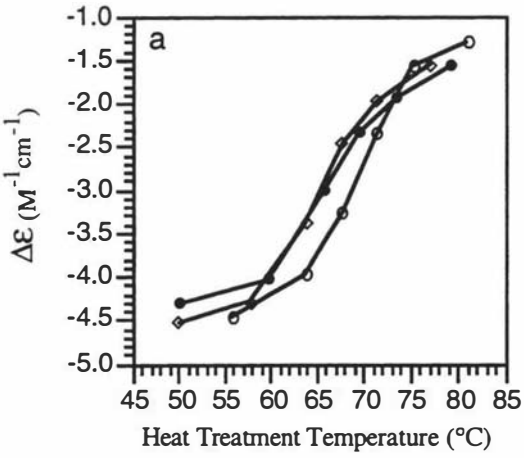
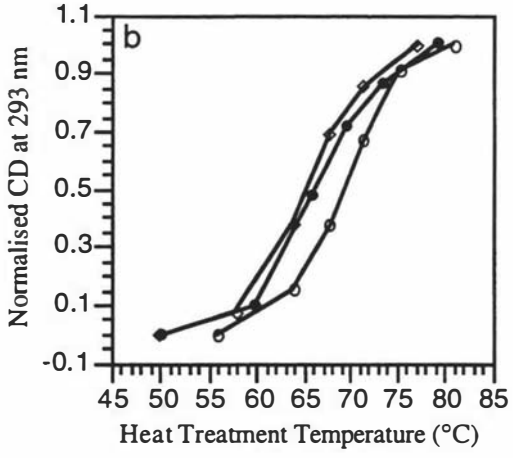
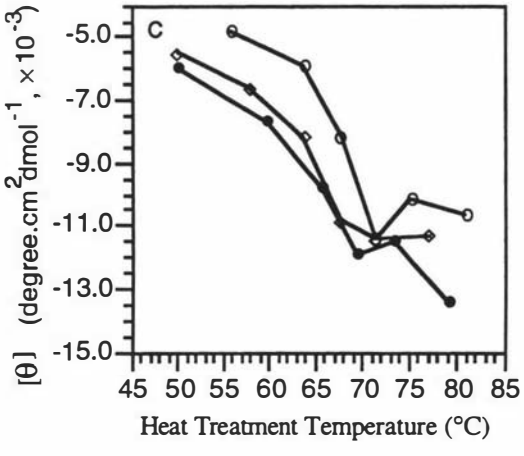
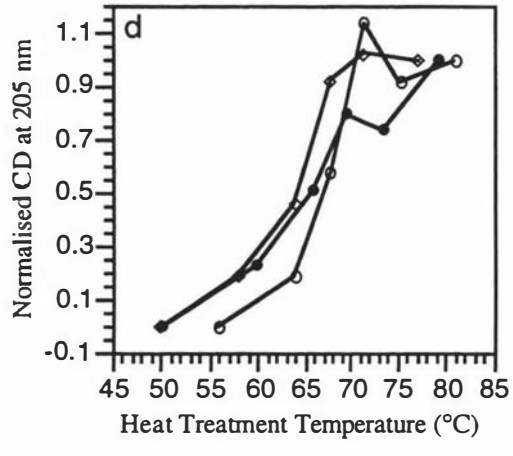


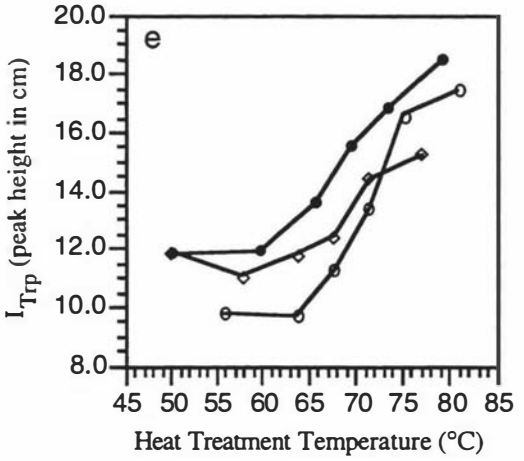
Fig. 5.3.7.3. Confirmatory measurements at pH 6.7, set 2: Effect of heat treatment of solutions of BLGs A, B and C on $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp} and I_{ANS}. The plots for BLGs A (•), B (◇) and C (o), prepared from data acquired using a particular technique, are shown in the same panel. The panels are as follows: raw $\Delta\varepsilon_{293}$, panel a; normalised $\Delta\varepsilon_{293}$, panel b; raw $[\theta]_{205}$, panel c; normalised $[\theta]_{205}$, panel d; raw I_{Trp}, panel e; normalised I_{Trp}, panel f; raw I_{ANS}, panel g; and normalised I_{ANS}, panel h. Sets of experimental data were normalised so that the minimum and maximum in each were 0 and 1 respectively.

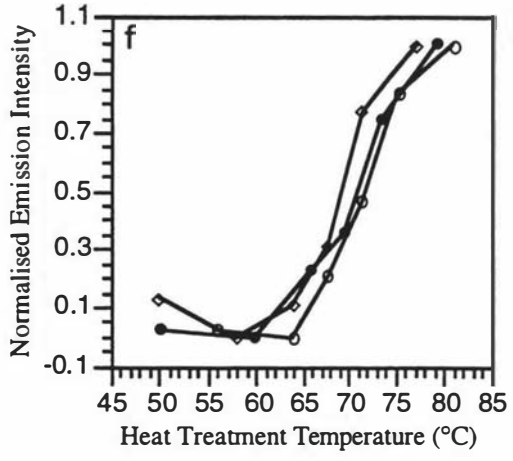

a
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
Δε (M-1cm-1)
45 50 55 60 65 70 75 80 85
Heat Treatment Temperature (°C)


b
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Normalised CD at 293 nm
45 50 55 60 65 70 75 80 85
Heat Treatment Temperature (°C)


c
-5.0
-7.0
-9.0
-11.0
-13.0
-15.0
[θ] (degree.cm2dmol-1, ×10-3)
45 50 55 60 65 70 75 80 85
Heat Treatment Temperature (°C)


d
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Normalised CD at 205 nm
45 50 55 60 65 70 75 80 85
Heat Treatment Temperature (°C)


e
20.0
18.0
16.0
14.0
12.0
10.0
8.0
ITrp (peak height in cm)
45 50 55 60 65 70 75 80 85
Heat Treatment Temperature (°C)


f
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Normalised Emission Intensity
45 50 55 60 65 70 75 80 85
Heat Treatment Temperature (°C)

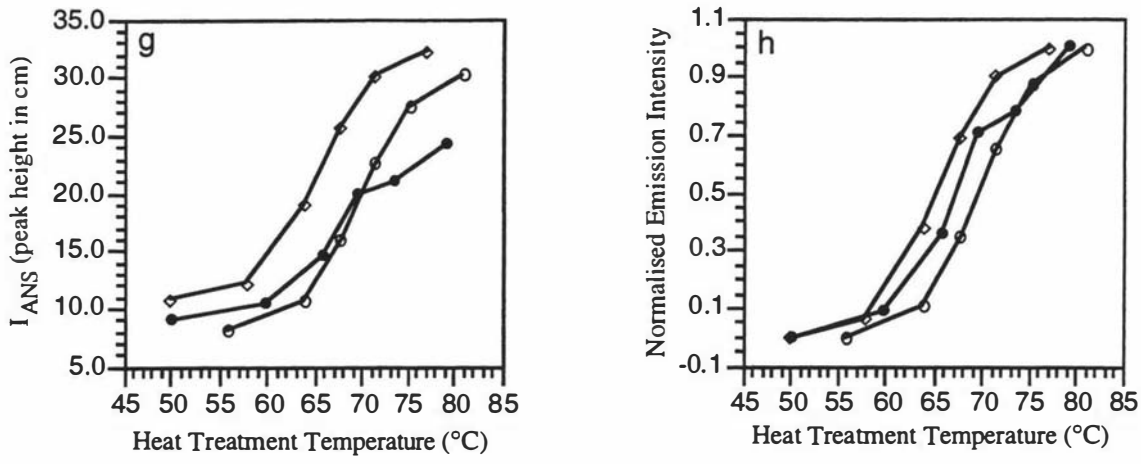


Fig. 5.3.7.4. Confirmatory measurements at pH 7.4: Effect of heat treatment of solutions of BLGs A, B and C on $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp} and I_{ANS}. The plots for BLGs A (•), B (◇) and C (o), prepared from data acquired using a particular technique, are shown in the same panel. The panels are as follows: raw $\Delta\varepsilon_{293}$, panel a; normalised $\Delta\varepsilon_{293}$, panel b; raw $[\theta]_{205}$, panel c; normalised $[\theta]_{205}$, panel d; raw I_{Trp}, panel e; normalised I_{Trp}, panel f; raw I_{ANS}, panel g; and normalised I_{ANS}, panel h. Sets of experimental data were normalised so that the minimum and maximum in each were 0 and 1 respectively.

The T_{mid} values summarised in Table 5.3.6.1 appear to fall within the same temperature ranges as those of the transition for heat-induced irreversible structural change in the corresponding plots of confirmatory data (Figs 5.3.7.2 - 5.3.7.4). For example, in the case of both the original and the confirmatory sets of $\Delta\varepsilon_{293}$ and I_{ANS} data at pH 6.7, the T_{mid} values (Table 5.3.6.1) and the transition region in the plots of confirmatory data fall within the temperature range 72 °C - 77 °C. These similarities further validate spectroscopic results.

5.3.8. CONFIRMATORY STUDIES: THIOL AVAILABILITY.

Additional thiol availability measurements were made primarily to confirm that the order of the susceptibilities of BLGs A, B and C to heat-induced irreversible structural change determined from the results in Fig. 5.3.5.2 is correct. In addition, confirmatory thiol availability measurements were made in quadruplicate to obtain an estimate of the experimental error associated with the measurement of thiol availabilities. Furthermore, as discussed in Section 5.3.5.7, the original procedure for measuring thiol availability (Section 5.2.3) was modified in an attempt to reduce the scatter in A_{412} values obtained from solutions of BLG previously heat-treated at high temperatures.

Plots of both raw and normalised A_{412} data versus heat treatment temperature are shown in Fig. 5.3.8.1 and suggest that, at both pH 6.7 and pH 7.4, BLGs B, A and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced irreversible structural change. Furthermore, although both the original and the confirmatory thiol availability measurements were made using the same batch of purified BLGs A, B and C, all thiol availability results are consistent with the results obtained in both the original and the confirmatory CD and fluorescence studies, thus validating thiol availability results.

The T_{mid} values determined from the original thiol availability data (summarised in Table 5.3.6.1) fall within the same temperature ranges as those of the transition for heat-induced irreversible structural change in the plots of either pH 6.7 or pH 7.4 confirmatory data (Fig. 5.3.8.1). This further validates thiol availability results.

Experimental Error.

The means and standard errors of the quadruplicate A_{412} values for solutions of BLGs A, B and C at pH 6.7 and pH 7.4 heat-treated at temperatures 1 - 6 (Table 5.3.7.1) are shown in Table 5.3.8.1. For BLG solutions that had been heat-treated at the five lowest temperatures, the standard errors in the mean are no greater than 0.005. At temperatures close to T_{mid} a change in A_{412} (y axis) of 0.005 occurs over a temperature range of approximately $\pm$ 0.1 °C (x axis). This suggests that the T_{mid} values shown in Table 5.3.5.2 are accurate to within 0.1 °C, in agreement with most of the standard errors calculated by "Enzfitter". Thus, the results obtained in the confirmatory thiol availability measurements show that the differences in the T_{mid} values for BLGs A, B and C discussed in Section 5.3.5.5 (approximately 1.5 °C) are outside the limits of experimental error. This in turn validates the conclusion that BLGs A, B and C differ in their susceptibilities to the heat-induced irreversible structural change.

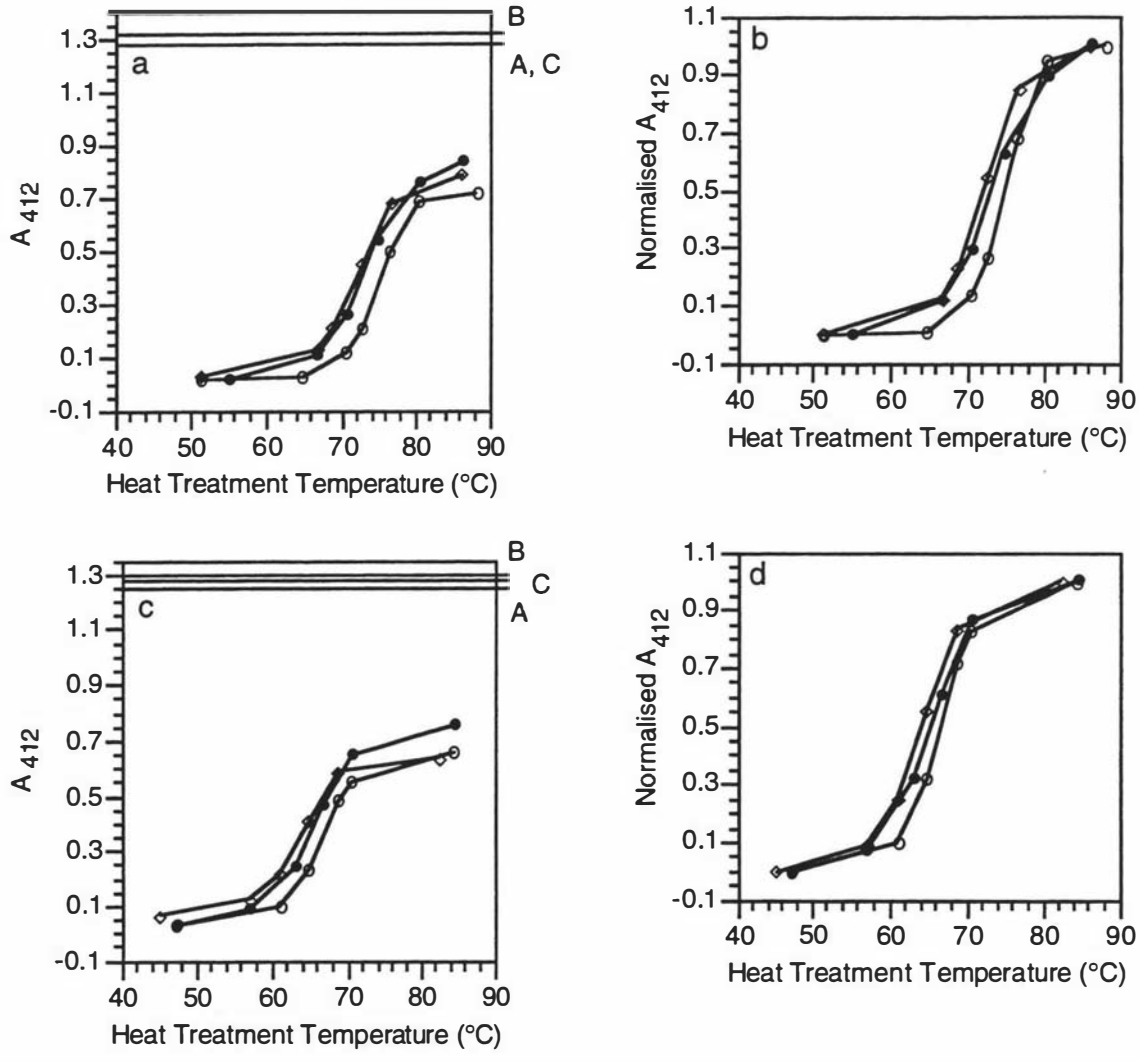


Fig. 5.3.8.1. Thiol availability confirmatory measurements: Effect of heat treatment of solutions of BLGs A, B and C at pH 6.7 and pH 7.4 on the availability of the thiol group for reaction with DTNB. The plots for BLGs A (•), B (◇) and C (o) prepared using data acquired at a particular pH are shown in the same panel. The panels are as follows: raw pH 6.7, panel a; normalised pH 6.7, panel b; raw pH 7.4, panel c; and normalised pH 7.4, panel d. In the plots of raw data, each point is the mean of four thiol availability determinations. An explanation of the horizontal lines at the tops of plots of raw data is given in Fig. 5.3.5.2. The line for each variant is denoted by an A, B or C. Heat treatment temperatures were selected as described in Section 5.3.7. Sets of experimental thiol availability data were normalised so that the minimum and maximum values in each were 0 and 1 respectively.

Table 5.3.8.1. The Mean A_{412} Values and Their Standard Errors Obtained in Confirmatory Thiol Availability Measurements.

	Average A_{412} for solutions heated at temperatures 1 - 6					
Sample	1	2	3	4	5	6
pH 6.7, BLG A	0.014 ± 0.0004	0.104 ± 0.002	0.270 ± 0.005	0.545 ± 0.004	0.762 ± 0.004	0.831 ± 0.004
pH 6.7, BLG B	0.031 ± 0.0008	0.136 ± 0.004	0.206 ± 0.0004	0.448 ± 0.004	0.691 ± 0.005	0.782 ± 0.005
pH 6.7, BLG C	0.019 ± 0.002	0.023 ± 0.0002	0.111 ± 0.003	0.204 ± 0.003	0.692 ± 0.005	0.763 ± 0.013
pH 7.4, BLG A	0.035 ± 0.0005	0.088 ± 0.001	0.258 ± 0.005	0.470 ± 0.003	0.636 ± 0.006	0.724 ± 0.010
pH 7.4, BLG B	0.066 ± 0.0005	0.124 ± 0.002	0.221 ± 0.001	0.419 ± 0.003	0.594 ± 0.004	0.645 ± 0.016
pH 7.4, BLG C	0.032 ± 0.0005	0.099 ± 0.001	0.230 ± 0.004	0.473 ± 0.005	0.565 ± 0.005	0.642 ± 0.014

* Temperatures 1 - 6 are listed in Table 5.3.7.1.

Effectiveness of Changes to the Thiol Availability Assay Protocol.

Evaporation of water from BLG sample vials during heat treatment was believed to have contributed to the observed scatter of A_{412} values obtained from solutions at pH 6.7 and pH 7.4 that had been heat-treated at higher temperatures (Section 5.3.5.7). Therefore, the weight of water equivalent to that lost during heat treatment was added to each BLG solution before A_{412} values were read. However, the standard error in the mean of quadruplicate A_{412} values is appreciably greater for BLG solutions heat-treated at temperature 6 than for those heat-treated at temperatures 1 - 5. This suggests that the procedure used to correct for solvent evaporation had no appreciable effect on the extent of scatter of measured A_{412} values. Therefore, it appears that solvent evaporation was not responsible for the observed scatter in duplicate and quadruplicate A_{412} values. Differences in extents of leakage of air into vials during heat treatment, and therefore, differences in the extents of thiol group oxidation may instead explain why this phenomenon was observed.

5.3.9. RESULTS AND DISCUSSION: AGGREGATION OF β-LACTOGLOBULINS.

5.3.9.1. Introduction.

As part of one of the major research aims (Section 2.9) PAGE was used to separate and to compare the concentrations of the different non-native BLG species present in previously heat-treated solutions. In the first of two major series of PAGE studies solutions of a particular BLG variant were heat-treated for the same time at different temperatures; the aim was to investigate how the relative concentrations of aggregate species vary with heat treatment temperature. In the second series of PAGE studies solutions of a particular BLG variant were heat-treated at the same temperature for different times, to confirm the suggestion of McSwiney *et al.* (1994b) that non-covalently-linked aggregates of BLG exist as reaction intermediates on the aggregation pathway of this protein.

The first series of PAGE measurements were made using solutions of each variant at each pH value previously heat-treated at the six different temperatures used in the confirmatory experiments (Section 5.3.7). In PAGE studies the temperatures which correspond to 0 %, 20 %, 40 %, 60 %, 80 % and 100 % of the total observed change in spectroscopic signal intensity will be referred to as T1, T2, T3, T4, T5 and T6 respectively. By heat-treating BLG solutions in this manner differences in gel band intensities reflect dissimilarities in the aggregation behaviour of BLGs A, B and C and not differences in their thermostabilities.

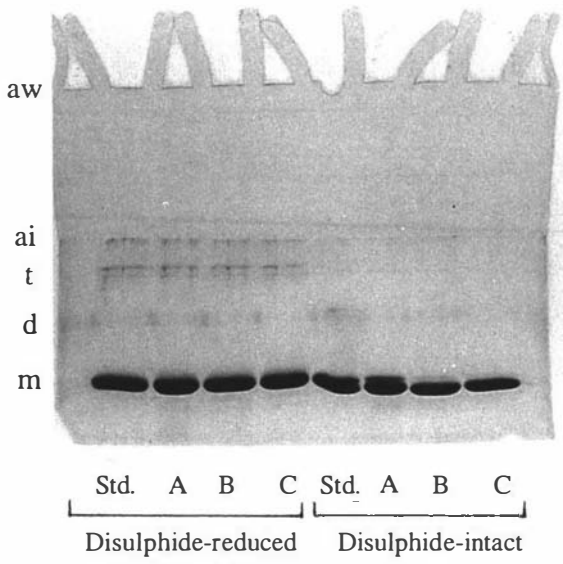
The intensities of most of the bands on the gels presented in the PAGE studies discussed below were measured using laser densitometry. However, the majority of PAGE results will be discussed qualitatively.

5.3.9.2. SDS-PAGE: β-Lactoglobulins Heat-treated at pH 6.7.

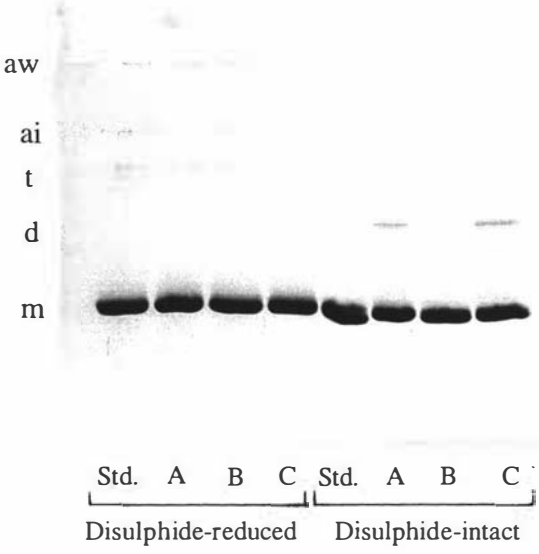
5.3.9.2.1. Disulphide-reduced SDS-PAGE.

There is only one band in each lane in the disulphide-reduced portions of gels 1 - 6 (Fig. 5.3.9.1), with a mobility corresponding to an M_r of about 23 500 (Fig. 5.3.9.2) and therefore to monomeric BLG (M_r = 18 400). The M_r estimated from Fig. 5.3.9.2 is not particularly accurate because this figure was prepared using Rf values for reduced proteins, while the Rf for BLG is that of the non-reduced species. The intensity of the monomeric band does not appear to vary appreciably with heat treatment temperature, suggesting that the proportion of BLG aggregates held together by covalent bonds other than disulphide bonds was negligible.

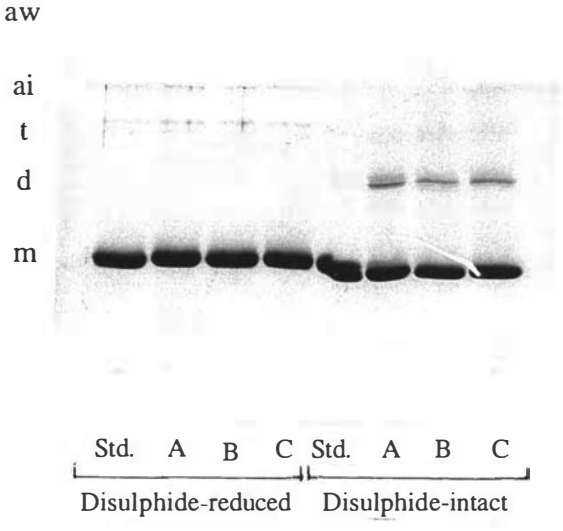
gel 1



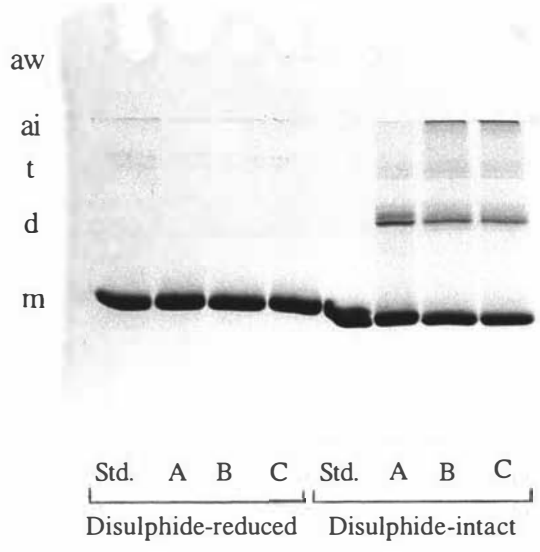
gel 2



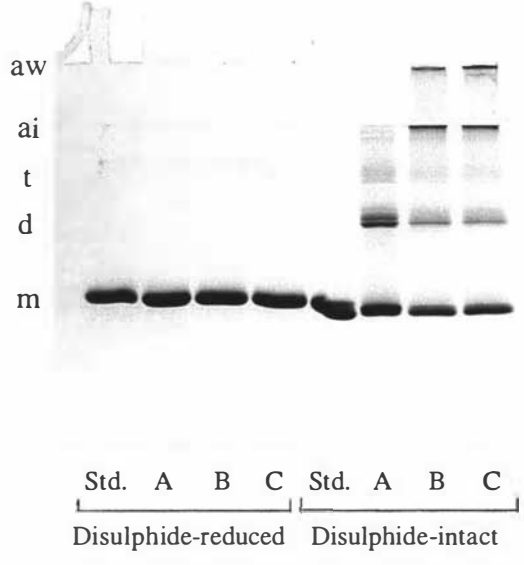
gel 3



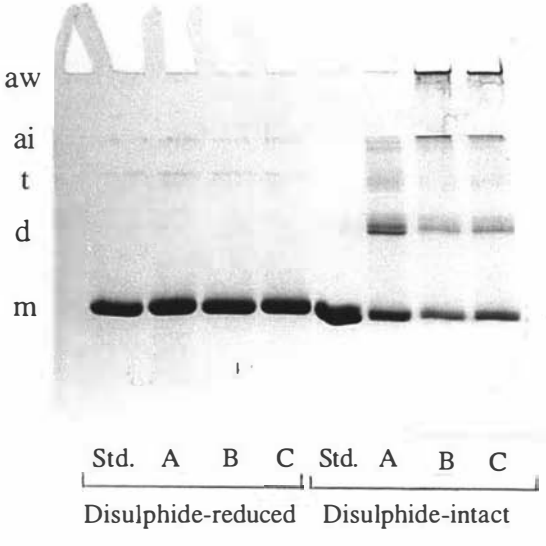
gel 4



gel 5



gel 6



gel 7

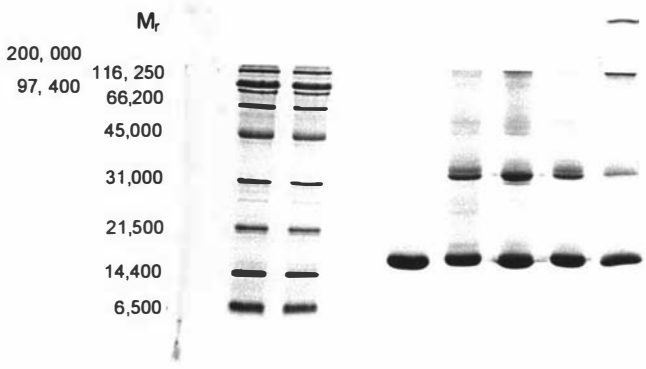


Fig. 5.3.9.1. SDS-PAGE electropherograms of samples of BLGs A, B and C heat-treated at pH 6.7 at various temperatures. The BLG solutions were heat-treated at the same temperatures as those used in the confirmatory experiments (Section 5.3.7). The nomenclature of the heat treatment temperatures is discussed in the introductory portion of Section 5.3.9.1. All of the BLG samples heat-treated at the same temperature number are shown on the same gel. The gels are as follows: T1 samples, gel 1; T2 samples, gel 2; T3 samples, gel 3; T4 samples, gel 4; T5 samples, gel 5; and T6 samples, gel 6. On gels 1 - 6 lanes contain, from left to right:

1) the disulphide-reduced unheated BLG B standard
2) disulphide-reduced BLG A
3) disulphide-reduced BLG B
4) disulphide-reduced BLG C
5) the disulphide-intact unheated BLG B standard
6) disulphide-intact BLG A
7) disulphide-intact BLG B
8) disulphide-intact BLG C.

On gel 7 lanes contain, from left to right:

1) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)
2) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)
3) blank
4) the disulphide-intact unheated BLG B standard
5) disulphide-intact BLG A heat-treated at pH 7.4 at T5
6) disulphide-intact BLG B heat-treated at pH 7.4 at T5
7) disulphide-intact BLG A heat-treated at pH 6.7 at T5
8) disulphide-intact BLG B heat-treated at pH 6.7 at T5.

Details of the sample preparation and heating protocols are given in Section 5.2.5.1. The PAGE protocol is described in Section 5.2.5.3. The labels ***m***, ***d***, ***t***, ***ai*** and ***aw*** are discussed in the text on page 275.

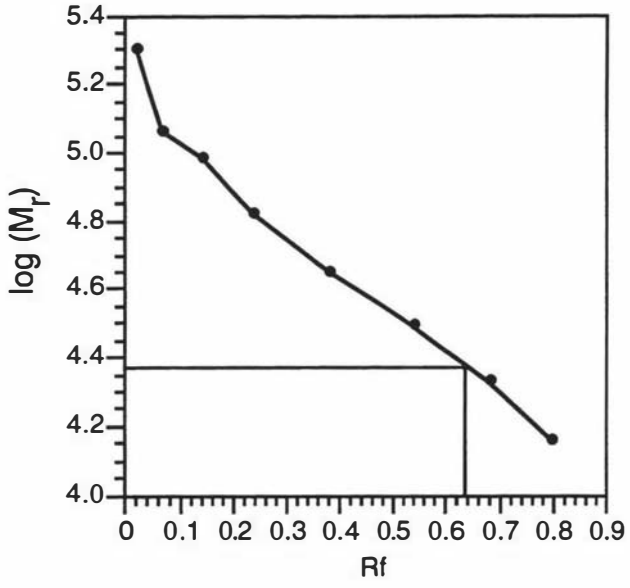


Fig. 5.3.9.2. Dependence of M_r on electrophoretic mobility on 2-ME-free SDS gels for the series of disulphide-reduced proteins shown in gel 7 in Fig. 5.3.9.1. For disulphide-intact samples of heat-treated BLG, the band in the region *m* of the gels shown in Fig. 5.3.9.1 exhibits an Rf value of 0.64.

5.3.9.2.2. Disulphide-intact SDS-PAGE.

In the lanes of gels 1 - 6 (Fig. 5.3.9.1) in which disulphide-intact samples were run, only one band, that of monomeric BLG, is observed for the T1 samples. However, in the region where monomeric BLG runs (region *m*, Fig. 5.3.9.1) in the lanes containing the T5 and T6 samples of BLGs B and C, two bands with similar mobilities are observed, confirming the report of Brittan (1997). For all three variants a number of bands with mobilities less than those of the monomeric BLGs are present in the lanes containing disulphide-intact T5 and T6 samples (Fig. 5.3.9.1.). In region *d* either three or four bands of similar mobility which correspond to protein species with M_r values of about 36 000 are observed, suggesting that several disulphide-linked dimeric BLG species are formed as a consequence of heat treatment. In region *t* of the gels in Fig. 5.3.9.1 at least four faint bands with mobilities which correspond to species with M_r values between 45 000 and 66 200 are observed. These bands probably represent trimeric BLG species, confirming the reports of Cairoli *et al.* (1994) and Iametti *et al.* (1996). Another two intense, very sharp and homogenous bands are present in the regions *ai* (the stacking/resolving gel interface) and *aw* (the bottom of sample loading wells) in lanes in which T5 and T6 samples were run. These bands were assumed to represent two populations of disulphide-linked aggregates with M_r values greater than 200 000 (the M_r cut-off of the resolving gel) and 500 000 (the M_r cut-off of the stacking gel) respectively. All aggregate species with M_r values greater than approximately 200 000 are subsequently referred to as large aggregates.

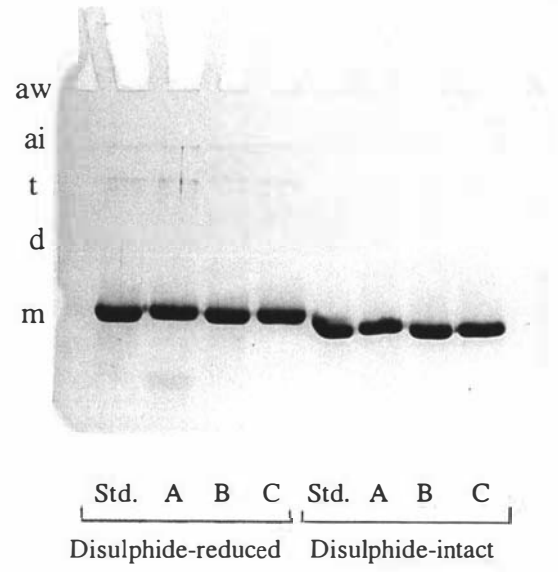
For BLGs A, B and C the intensities of the monomeric bands in lanes containing disulphide-intact samples decrease with increasing heat treatment temperature (Fig. 5.3.9.1). In contrast, the intensities of most of the corresponding non-monomeric bands increase with increasing heat treatment temperature. This indicates that the extent of conversion of monomeric BLG to disulphide-linked aggregates increases with increasing heat treatment temperature. These results are therefore consistent with those of Iametti *et al.* (1995), who used disulphide-intact SDS-PAGE and gel permeation chromatography to show that extents of disulphide cross-linking in BLG aggregates are affected by heat treatment temperature and protein concentration. The PAGE results in Fig. 5.3.9.1 are also consistent with those of Hoffmann *et al.* (1996), who, using *in situ* dynamic light scattering, found that aggregate particle size increases with increasing solution temperature between 61.5 °C and 70.0 °C for heat treatment times less than approximately 1 hr.

The gels in Fig. 5.3.9.1 show that the distribution of BLGs A, B and C amongst the various aggregate species differs. In lanes containing T5 and T6 samples, the intensities of the bands of disulphide-linked dimers and trimers (i.e. bands in the regions *d* and *t* respectively) are greater for BLG A than for BLGs B and C. However, the intensities of the bands of large aggregates (i.e. bands in the regions *ai* and *aw*) are negligible for heat-treated solutions of BLG A. These two comparisons therefore suggest that large disulphide-linked aggregates are formed more slowly from native BLG A than from native BLGs B and C. In the region *aw* of the disulphide-intact portion of the gel for T5 samples, the band for BLG C is slightly more intense than that for BLG B. This suggests that BLG C forms large disulphide-linked aggregates slightly more rapidly than BLG B.

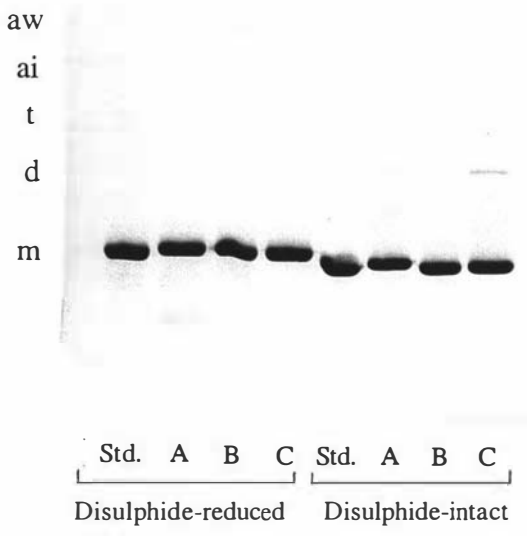
5.3.9.3. SDS-PAGE: β-Lactoglobulins Heat-treated at pH 7.4.

SDS gels for samples of BLGs A, B and C heat-treated at pH 7.4, both disulphide-reduced and disulphide-intact, are shown in Fig. 5.3.9.3. For all three variants, the SDS-PAGE results obtained at pH 7.4 are similar to those obtained at pH 6.7. However, the intensities of the bands in the region *ai* are less in lanes containing T5 and T6 samples at pH 7.4 than those in lanes containing T5 and T6 samples at pH 6.7 (Fig. 5.3.9.1). This suggests that in T5 and T6 samples, concentrations of large aggregates are less at pH 7.4 than at pH 6.7. Therefore, it appears that these aggregates are formed less readily during heat treatment at pH 7.4 (Fig. 5.3.9.3) than during heat treatment at pH 6.7 (Fig. 5.3.9.1). Similarly, Hoffmann *et al.* (1996) reported that both aggregate particle size and rates of aggregate formation in unbuffered 50 mg/mL solutions of BLG A/B at 68.5 °C decrease with increasing pH between 6.2 and 8.0.

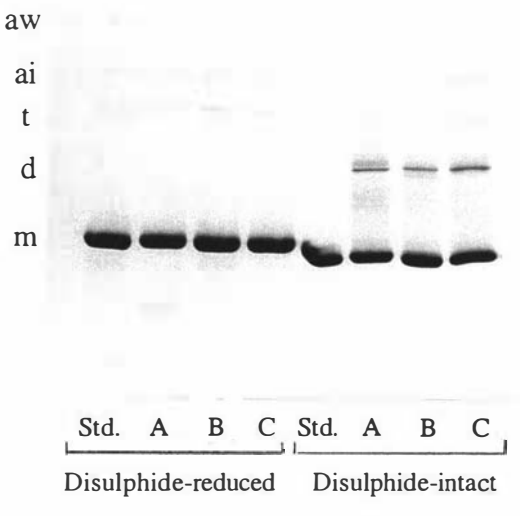
gel 1



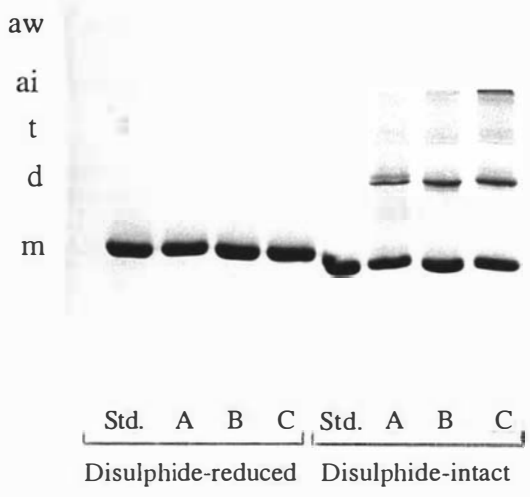
gel 2



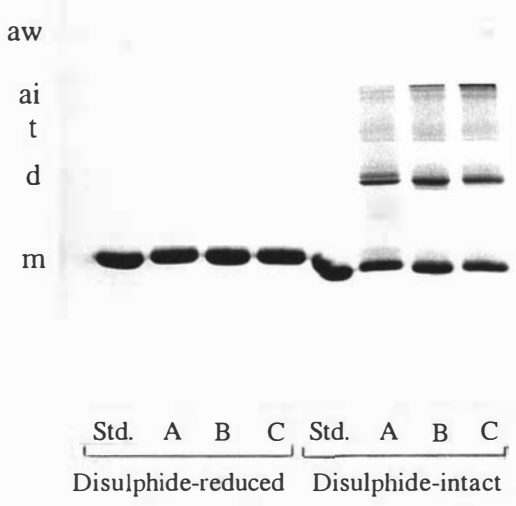
gel 3



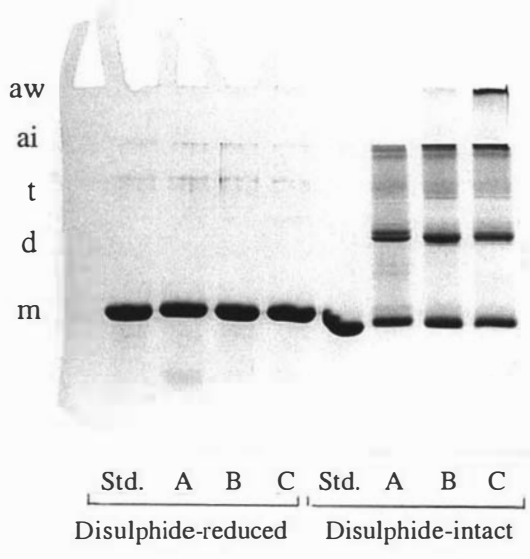
gel 4



gel 5



gel 6



gel 7

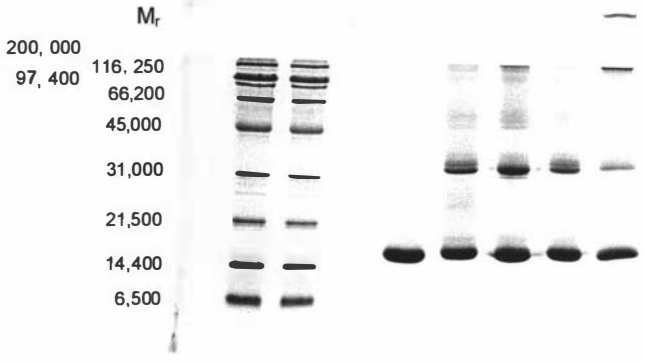


Fig. 5.3.9.3. SDS-PAGE electropherograms of samples of BLGs A, B and C heat-treated at pH 7.4 at various temperatures. The BLG solutions were heat-treated at the same temperatures as those used in the confirmatory experiments (Section 5.3.7). The nomenclature of the heat treatment temperatures is discussed in Section 5.3.9.1. All of the BLG samples heat-treated at the sample temperature number are shown on the same gel. The gels are as follows: T1 samples, gel 1; T2 samples, gel 2; T3 samples, gel 3; T4 samples, gel 4; T5 samples, gel 5; and T6 samples, gel 6. On gels 1 - 6 lanes contain, from left to right:

1) the disulphide-reduced unheated BLG B standard
2) disulphide-reduced BLG A
3) disulphide-reduced BLG B
4) disulphide-reduced BLG C
5) the disulphide-intact unheated BLG B standard
6) disulphide-intact BLG A
7) disulphide-intact BLG B
8) disulphide-intact BLG C.

On gel 7 lanes contain, from left to right:

1) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)
2) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)
3) blank
4) the disulphide-intact unheated BLG B standard
5) disulphide-intact BLG A heat-treated at pH 7.4 at T5
6) disulphide-intact BLG B heat-treated at pH 7.4 at T5
7) disulphide-intact BLG A heat-treated at pH 6.7 at T5
8) disulphide-intact BLG B heat-treated at pH 6.7 at T5.

Details of the sample preparation and heating protocols are given in Section 5.2.5.1. The PAGE protocol is described in Section 5.2.5.3.

The differences in the distribution of BLGs A and B among the various aggregate species in heat-treated solutions are less at pH 7.4 (Fig. 5.3.9.3) than at pH 6.7 (Fig. 5.3.9.1). For T6 samples of BLGs A and B at pH 7.4, no band of appreciable intensity in the region *aw* is observed in the disulphide-intact portions of SDS gels (Fig. 5.3.9.3). Similarly, for both BLGs A and B heat-treated under these conditions, the intensities of the bands in the region *ai* of the disulphide-intact portions of SDS gels are equivalent. Conversely, for T6 samples at pH 6.7, a band in the region *aw* is clearly visible for BLG B but not for BLG A (Fig. 5.3.9.1).

Differences in the distribution of BLGs B and C amongst the various aggregate species are greater at pH 7.4 (Fig. 5.3.9.3) than at pH 6.7 (Fig. 5.3.9.1). For T4 - T6 samples at pH 7.4, the intensities of the bands in the regions *ai* and *aw* (or both) of the disulphide-intact portions of SDS gels are greater for BLG C than for BLG B. At pH 6.7, however, only a slight difference in the intensities of the band in the region *aw* was observed for the T5 samples of BLGs B and C (Fig. 5.3.9.1).

The disulphide-intact SDS-PAGE results (Figs 5.3.9.1 and 5.3.9.3) appear consistent with the thiol availability results discussed in Section 5.3.5.4. The latter indicate that the thiol group in no more than 65 % and 55 % of BLG molecules in solutions at pH 6.7 and pH 7.4 respectively is available for reaction with DTNB after heat treatment (Fig. 5.3.5.2, Table 5.3.5.1). From these results, it was concluded that at least some of the thiol groups of BLG had been oxidised to disulphides during heat treatment. This is consistent with aggregate formation. The disulphide-intact SDS-PAGE results may also be consistent with the $\Delta\varepsilon_{277}$ and $\Delta\varepsilon_{270}$ results discussed in Sections 5.3.1.3 and 5.3.1.6, which suggest that non-native disulphide bonds in molecules of BLGs A, B and C are formed as a consequence of heat treatment.

5.3.9.4. Alkaline Native-PAGE: β-Lactoglobulins Heat-treated at pH 6.7.

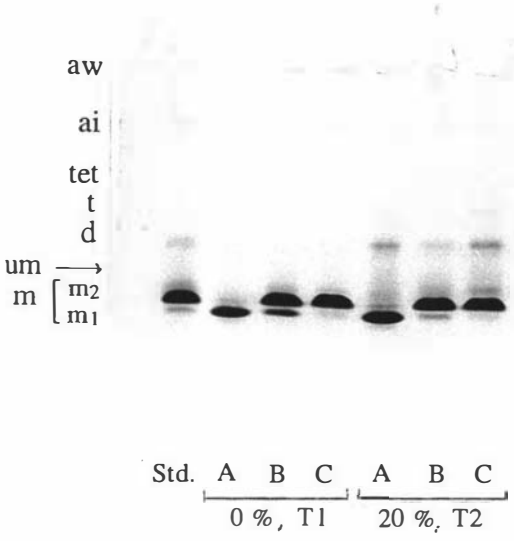
The alkaline native gels for heat-treated samples of BLGs A, B and C at pH 6.7 are shown in Fig. 5.3.9.4. For the T1 sample of BLG A the only band observed has a similar mobility to those of the bands for the unheated BLG B standard, and is thus likely to be "native-like" monomeric BLG A. The BLG in these bands is neither dimeric nor native because at pH 8.8, at which alkaline native gels are run, native dimers cannot form and BLG denatures (Groves *et al.*, 1951; Casal *et al.*, 1988).

Fig. 5.3.9.4. Alkaline native-PAGE electropherograms of samples of BLGs A, B and C heat-treated at pH 6.7 at various temperatures. The BLG solutions were heat-treated at the same temperatures as those used in the confirmatory experiments (Section 5.3.7). The nomenclature of the heat treatment temperatures is discussed in Section 5.3.9.1. All of the T1 and T2 samples are on gel 1, all of the T3 and T4 samples are on gel 2 and all of the T5 and T6 samples are on gel 3. On each gel lanes contain, from left to right:

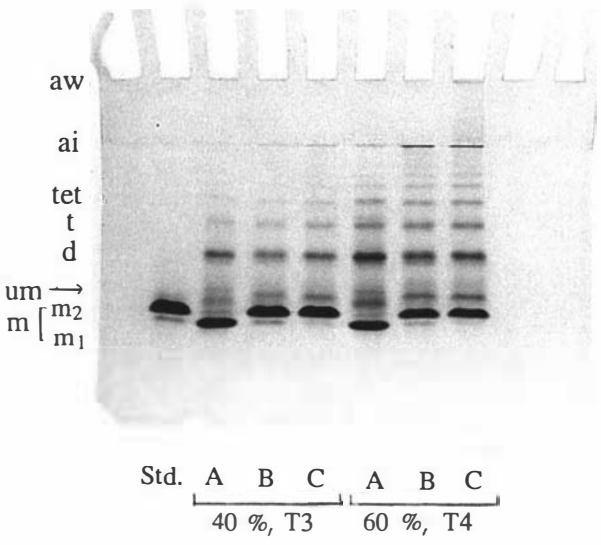
1) The unheated BLG B standard
2) BLG A heat-treated at the lower temperature
3) BLG B heat-treated at the lower temperature
4) BLG C heat-treated at the lower temperature
5) BLG A heat-treated at the higher temperature
6) BLG B heat-treated at the higher temperature
7) BLG C heat-treated at the higher temperature

Details of the sample preparation and heating protocols are given in Section 5.2.5.1. The PAGE protocol is described in Section 5.2.5.2.

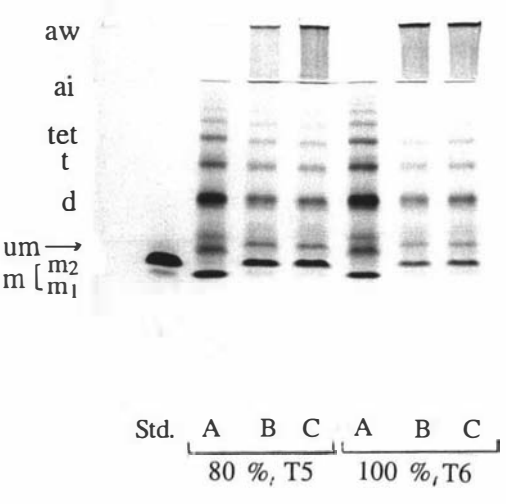
gel 1



gel 2



gel 3



More intense heat treatment of BLG A gave rise to a number of bands and diffuse regions of stained protein with lower mobilities than that of "native-like" protein (Fig. 5.3.9.4). In the case of the T5 sample, the bands in the resolving gel are likely to represent, in order of decreasing mobility, "native-like" monomer (*m*), "unfolded" monomer (*um*), dimer (*d*), trimer (*t*), tetramer (tet) etc. The diffuse regions of stained protein are likely to represent aggregate species with $M_{r,apparent}$ values intermediate between those in the bands. No reports discussing the presence of "unfolded" monomeric BLG species (those which have mobilities intermediate between those of "native-like" monomers and dimers) in heat-treated solutions of BLG have been found. For BLG A, a band is also observed in the region *ai* (Fig. 5.3.9.4) and it represents a population of aggregates with M_r values greater than approximately 200 000 (i.e. large aggregates).

The lanes containing the unheated BLG B standard and the T1 sample of this variant show two bands: that with a mobility slightly less than that of BLG A is likely to be BLG B, while the fainter band with a higher mobility is likely to be a lactose adduct of this variant. Such adducts have been reported by Morgan *et al.* (1997).

Samples of BLG B previously heat-treated under more severe conditions gave a similar pattern of non-"native-like" bands to that described above for BLG A, but there was less material in the resolving gel for BLG B. Furthermore, bands in both the regions *ai* and *aw* and a diffuse region of stained material between these bands is observed for BLG B. The band in the region *aw* represents a population of aggregate species with M_r values greater than approximately 500 000, while the diffuse region of stained material represents aggregate species with M_r values between 200 000 and 500 000.

Heat-treated samples of BLG C gave a similar pattern of bands to BLG B. However, the concentration of the putative lactose adduct in the T1 sample of BLG C was negligible.

For all three variants, the intensities of monomer bands, and therefore the concentration of "native-like" monomeric species in heat-treated solutions, decrease with increasing heat treatment temperature between approximately 50 °C and 90 °C (i.e. T1 - T6, Fig. 5.3.9.4). However, for all three variants, the intensities of the bands in the region *ai* (and also *aw* for BLGs B and C), and therefore the concentrations of large aggregates, increase with increasing heat treatment temperature in this range. Thus, in agreement with the disulphide-intact SDS-PAGE results (Figs 5.3.9.1), the alkaline native-PAGE results (Fig. 5.3.9.4) indicate that the extent of conversion of monomeric BLG to aggregate species increases with increasing heat treatment temperature.

The alkaline native-PAGE results (Fig. 5.3.9.4) also indicate that the intensities of the bands of non-"native-like" BLG species with M_r values less than approximately 200 000 increase with increasing heat treatment temperature between approximately 50 °C and 80 °C (i.e. T1 - T5), but decrease between approximately 80 °C and 90 °C (i.e. T5 - T6). This suggests that these species are converted to large aggregates during heat treatment under severe conditions. Therefore, non-"native-like" species with M_r values less than approximately 200 000 may exist as reaction intermediates during heat treatment.

Alkaline native-PAGE results (Fig. 5.3.9.4) show that the distribution of BLGs A, B and C amongst the various aggregate species differs. In lanes containing T5 samples, the intensities of the bands of non-"native-like" species with M_r values less than approximately 200 000 are greatest for BLG A. However, in these lanes, the intensities of the bands of large aggregates are greatest for BLGs B and C. This suggests that large aggregates are formed more slowly from native BLG A than from native BLGs B and C. The disulphide-intact SDS-PAGE results discussed in Section 5.3.9.2.2 suggest that the same is true for large disulphide-linked aggregates.

In lanes containing T4 and T5 samples, the intensities of bands in the regions *ai* and *aw* are slightly greater for BLG C than for BLG B. This suggests that large aggregates are formed slightly more rapidly from BLG C than from BLG B.

5.3.9.5. Alkaline Native-PAGE: β-Lactoglobulins Heat-treated at pH 7.4.

Most of the alkaline native-PAGE results obtained at pH 7.4 (Fig. 5.3.9.5) are similar to those obtained at pH 6.7 (Fig. 5.3.9.4), except that bands are not observed in the region *aw* on the gels of samples heat-treated at pH 7.4. This indicates that the largest aggregates formed as a consequence of heat treatment at pH 6.7 are not present in BLG solutions heat-treated at pH 7.4. Furthermore, the intensities of the bands of non-"native-like" BLG species with M_r values less than approximately 200 000 are greater in solutions heat-treated at pH 7.4 (Fig. 5.3.9.5) than in those heat-treated at pH 6.7 (Fig. 5.3.9.4). These two comparisons suggest that large aggregates are formed less readily during heat treatment at pH 7.4 than during heat treatment at pH 6.7.

Multiple dimeric bands for BLGs A, B and C are observed on the gels of samples heat-treated at pH 7.4 (Fig. 5.3.9.5). This suggests that several dimeric BLG species with different hydrodynamic sizes are formed as a consequence of heat treatment at this pH.

The differences in the distribution of BLGs A and BLGs B and C amongst the various aggregate species in heat-treated solutions are less at pH 7.4 (Fig. 5.3.9.5) than at pH 6.7 (Fig. 5.3.9.4). For pH 7.4 samples, the intensities of all bands in lanes containing T5 and T6 samples of BLGs A, B and C are similar, but bands are not observed in the region *aw*. However, for pH 6.7 samples, bands in the region *aw* are observed in lanes containing T5 and T6 samples of BLGs B and C. This is consistent with the comparison of the disulphide-intact SDS-PAGE results for BLGs A, B and C at pH 6.7 and pH 7.4 discussed in Section 5.3.9.3.

At pH 7.4, BLGs B and C are distributed amongst the various aggregate species in a slightly different manner. For T4 and T5 samples, the intensity of the band in the region *ai* is slightly greater for BLG C than for BLG B. This is consistent with the comparison of disulphide-intact SDS-PAGE results for BLGs B and C at pH 7.4 (Section 5.3.9.3).

The "smearing" phenomenon observed on alkaline native gels may be analogous to the multiple band phenomenon observed in the disulphide-intact portions of SDS gels because both suggest that stable partially "unfolded" monomeric and polymeric BLG species are formed as a consequence of heat treatment. However, smearing is likely to be indicative of a greater number of such species than multiple banding. A comparison of disulphide-intact SDS-PAGE and alkaline native-PAGE results therefore indicates that the majority of partially unfolded BLG species, present as both "monomers" and in aggregates, are stabilised in only a non-covalent manner.

5.3.9.6. Two-dimensional-PAGE.

The gels in Figs 5.3.9.4 and 5.3.9.5 suggest that heat-treated solutions of BLGs A, B and C contain an "unfolded" monomeric species. If this is true, then the "unfolded" monomers resolved by alkaline native-PAGE should run as monomers on disulphide-intact SDS gels. To confirm this, aggregates in heat-treated samples of BLGs A, B and C were resolved using 2D- (alkaline native- then disulphide intact SDS-) PAGE (Fig. 5.3.9.6).

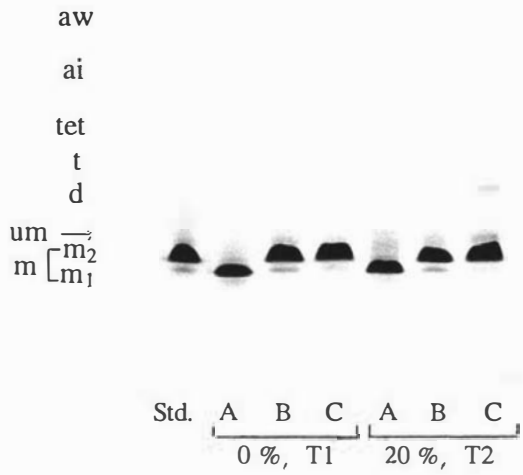
For most of the bands resolved in the alkaline native dimension (those in regions *m*, *d*, *t* etc. in Fig. 5.3.9.6), a band with a similar relative mobility is resolved in the disulphide-intact SDS dimension (those in regions *m'*, *d'*, *t'* etc. in Fig. 5.3.9.6). Thus, by comparing the relative mobilities of the bands of the M_r standards shown in Figs 5.3.9.1 and 5.3.9.3 with those of the bands in the regions *m'*, *d'*, *t'* etc. it is possible to estimate the M_r values of the species in the bands in the regions *m*, *d* and *t* etc. in the native dimension of the 2D gels shown in Fig. 5.3.9.6. The bands in the regions *m*, *d* and *t* are therefore likely to be those of monomers, dimers and trimers respectively, confirming the assignments made in Section 5.3.9.4.

Fig. 5.3.9.5. Alkaline native-PAGE electropherograms of samples of BLGs A, B and C heat-treated at pH 7.4 at various temperatures. The BLG solutions were heat-treated at the same temperatures as those used in the confirmatory experiments (Section 5.3.7). The nomenclature of the heating temperatures is discussed in Section 5.3.9.1. All of the T1 and T2 samples are on gel 1, all of the T3 and T4 samples are on gel 2 and all of the T5 and T6 samples are on gel 3. On each gel lanes contain, from left to right:

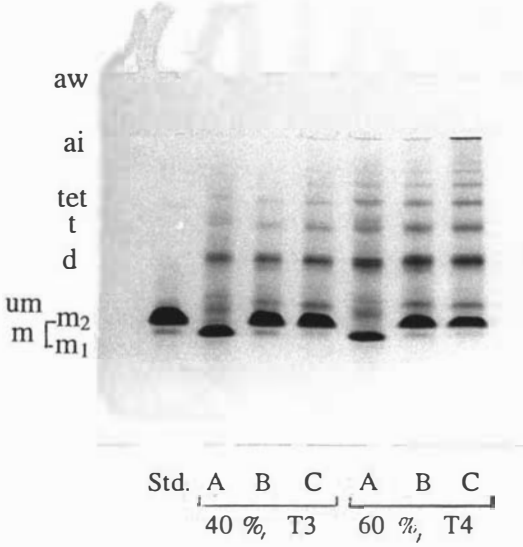
1) The unheated BLG B standard
2) BLG A heat-treated at the lower temperature
3) BLG B heat-treated at the lower temperature
4) BLG C heat-treated at the lower temperature
5) BLG A heat-treated at the higher temperature
6) BLG B heat-treated at the higher temperature
7) BLG C heat-treated at the higher temperature

Details of the sample preparation and heating protocols are given in Section 5.2.5.1. The PAGE protocol is described in Section 5.2.5.2.

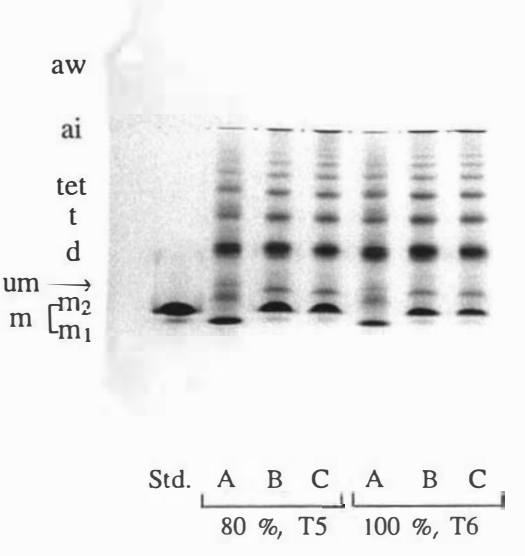
gel 1



gel 2



gel 3

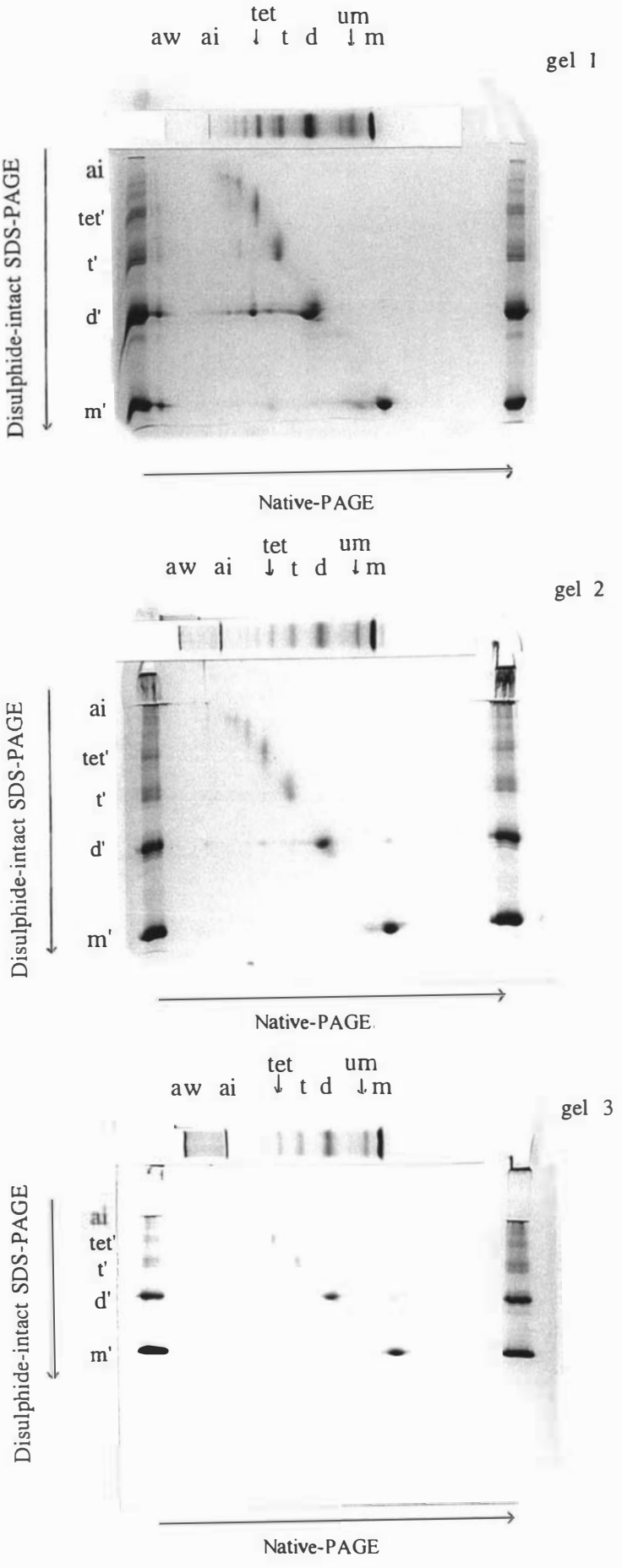


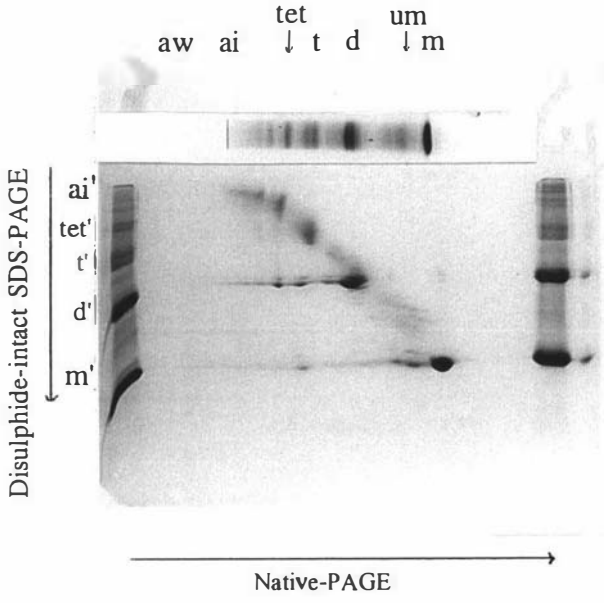
Most of the material present in the region *um* in the alkaline native-PAGE dimension runs in the region *m'* in the SDS-PAGE dimension (Fig. 5.3.9.6). This confirms that partially "unfolded" monomeric BLG species are formed as a consequence of heat treatment. However, for BLG A at pH 7.4 not all of the species present in the "unfolded" monomer bands resolved by alkaline native-PAGE have mobilities in the SDS dimension as great as that of the monomeric species (Fig. 5.3.9.6). This suggests that in solutions of BLG A previously heat-treated at pH 7.4, an appreciable proportion of the "unfolded" monomers are stabilised by non-native intramolecular disulphide bonds.

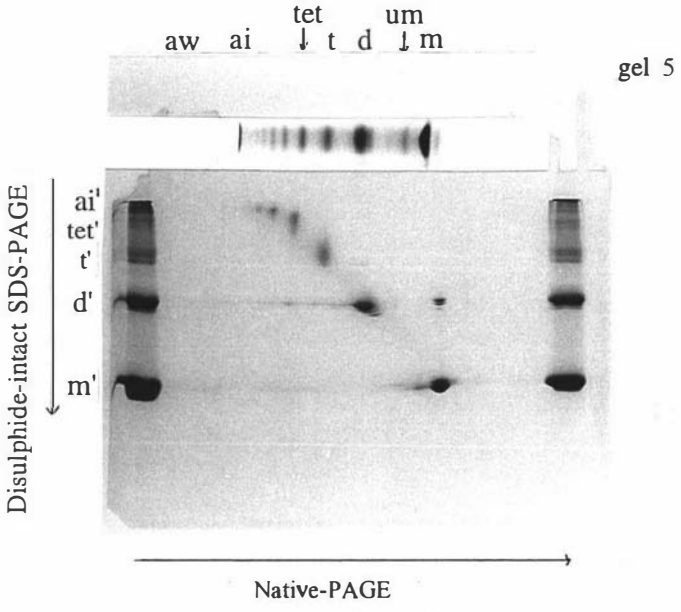
Material in the regions *m'* and *d'* is observed in most of the "lanes" of the SDS-PAGE dimension of the 2D gels in Fig. 5.3.9.6, suggesting that the majority of BLG aggregate species resolved by alkaline native-PAGE contain monomers and disulphide-linked dimers associated in a non-covalent manner. Therefore, it is likely that the larger aggregates present in heat-treated BLG solutions are formed from both monomeric and disulphide-linked dimeric intermediates. This confirms the results of McSwiney *et al.* (1994a, b), that both disulphide-linked and non-covalently-linked aggregates of BLG are formed as a consequence of heat treatment. In the SDS dimension of the 2D gels for BLG A, another two very faint bands are observed in the regions *t'* and *tet'*, suggesting that some aggregate species of this variant contain disulphide-linked trimers and tetramers associated in a non-covalent manner. These two bands are not observed on the 2D gels for BLGs B and C.

At both pH 6.7 and pH 7.4, the intensities of bands, especially those of disulphide-linked dimers (region *d'*) resolved in the SDS dimension of 2D gels, are greater for BLG A than for BLGs B and C (Fig. 5.3.9.6). This suggests that the proportion of aggregates stabilised in predominantly a non-covalent manner is greater for BLG A than for BLGs B and C.

In most of the 2D gels (Fig. 5.3.9.6) bands in the region *m* resolved by alkaline native-PAGE give rise to bands in both the regions *m'* and *d'* in the SDS-PAGE dimension. Because disulphide-linked dimers should have been separated from monomers during PAGE in the alkaline native dimension, this result may indicate that these dimers were formed after alkaline native-PAGE but before SDS-PAGE. This in turn suggests that disulphide-linked dimers are readily formed at pH 8.8, that at which alkaline native gels were run. Similarly, McKenzie (1971) reported that aggregation occurs when BLG is diluted into buffers at the pH values commonly used in electrophoretic studies (i.e. higher than 8). Because non-covalently-linked aggregates dissociate in the presence of SDS, it is unlikely that monomers were able to form dimers during PAGE in the SDS dimension.


tet
um
aw ai ↓ t d ↓ m
gel 1
Disulphide-intact SDS-PAGE
ai
tet'
t'
d'
m'
Native-PAGE
tet
um
aw ai ↓ t d ↓ m
gel 2
Disulphide-intact SDS-PAGE
ai
tet'
t'
d'
m'
Native-PAGE
tet
um
aw ai ↓ t d ↓ m
gel 3
Disulphide-intact SDS-PAGE
ai
tet'
t'
d'
m'
Native-PAGE


tet
um
aw ai ↓ t d ↓ m
gel 4
Disulphide-intact SDS-PAGE
ai'
tet'
t'
d'
m'
Native-PAGE


tet
um
aw ai ↓ t d ↓ m
gel 5
Disulphide-intact SDS-PAGE
ai'
tet'
t'
d'
m'
Native-PAGE

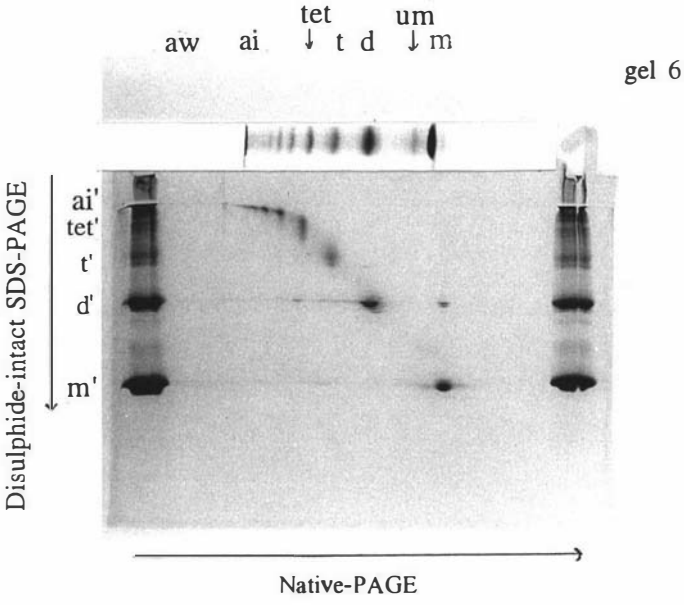

tet
um
aw ai ↓ t d ↓ m
gel 6
Disulphide-intact SDS-PAGE
ai'
tet'
t'
d'
m'
Native-PAGE

Fig. 5.3.9.6. 2D- (alkaline native- then disulphide-intact-SDS-) PAGE electropherograms of heat-treated samples of BLGs A, B and C at pH 6.7 and pH 7.4. At pH 6.7 BLGs A, gel 1; B, gel 2; and C, gel 3; were heat-treated at 82 °C, 78 °C and 82 °C respectively for 12.5 min, while at pH 7.4 BLGs A, gel 4; B, gel 5; and C, gel 6; were heat-treated at 74 °C, 72 °C and 76 °C respectively for the same time. The electrophoretic origin is in the top left-hand corner of each gel. For PAGE in the alkaline native dimension (across the top of the gel) the electrophoretic mobility of species increases from left to right. For PAGE in the disulphide-intact SDS dimension (the remainder of the gel) the electrophoretic mobility of species increases from top to bottom. The band patterns on the extreme left-hand and right-hand ends of each gel are those of the appropriate heat-treated BLG sample electrophoresed in only the SDS dimension. The 2D-PAGE protocol is described in Section 5.2.7.

5.3.9.7. Quantitative Analysis of PAGE Results.

Changes in the concentrations of monomeric, "unfolded" monomeric and dimeric species of BLGs A, B and C with heat treatment temperature were examined quantitatively by measuring the intensities of the appropriate bands on the gels shown in Figs 5.3.9.1 and 5.3.9.3 - 5.3.9.5 using laser densitometry.

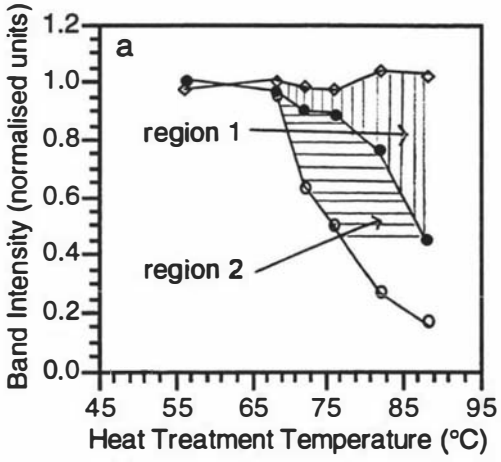
The band intensities of the unheated BLG B standard run on each SDS and alkaline native gel differ, suggesting appreciable systematic differences between band intensities on different gels. These differences were assumed to arise from several factors, including the type of gel, PAGE running conditions and variation in densitometer performance, and they were minimised using the following normalisation protocol. The band intensities on alkaline native gels and in the disulphide-intact and disulphide-reduced portions of SDS gels were treated as three subsets. Within each of these there were six sub-subsets for data collected at the two pH values and for the three variants. Band intensities in different sub-subsets of alkaline native-PAGE and disulphide-reduced SDS-PAGE results were normalised relative to one another using the band intensities for the unheated BLG B standard; after normalisation the band intensities for the standard were equivalent. This approach assumes that the magnitude of systematic error between all of the band intensities in pairs of sub-subsets was equivalent to the ratio of band intensity values for the unheated BLG B standard.

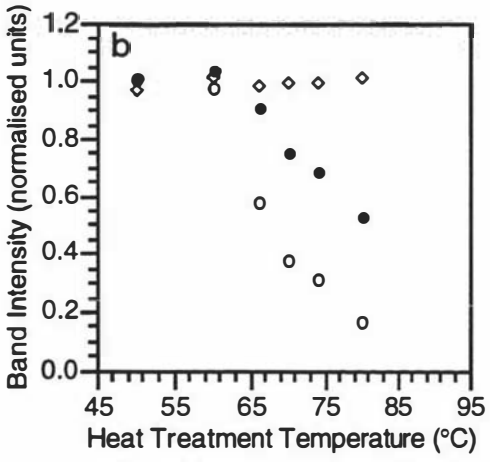
Disulphide-intact SDS-PAGE band intensities were normalised relative to other band intensities in a similar manner, except that the monomer band for the T1 sample was used as the standard because it was slightly more intense than that of the disulphide-intact unheated BLG B standard.

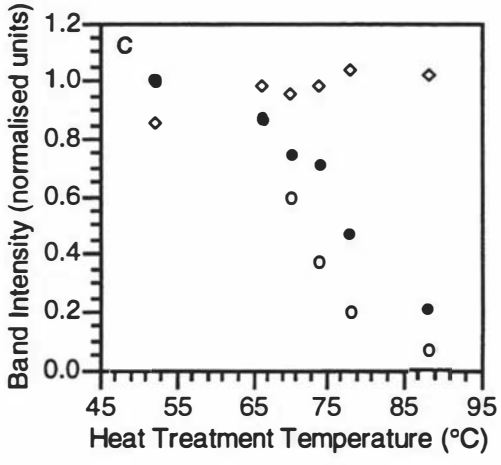
5.3.9.7.1. Results.

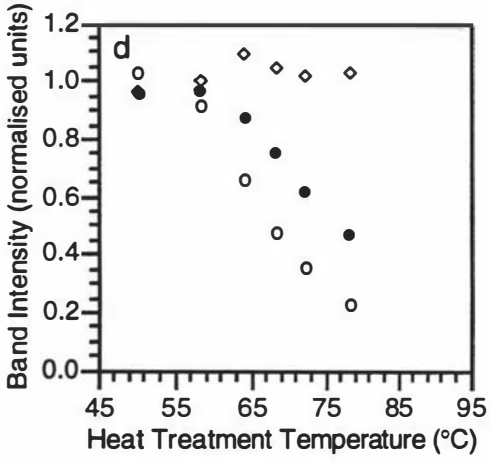
The normalised values for the intensity of the monomeric BLG band in the lanes of SDS gels containing disulphide-reduced samples do not vary appreciably with heat treatment temperature (Fig. 5.3.9.7), confirming that the proportion of covalently-linked aggregates that were not held together by disulphide bonds was negligible in all of the heat-treated samples prepared for use in the present PAGE study.

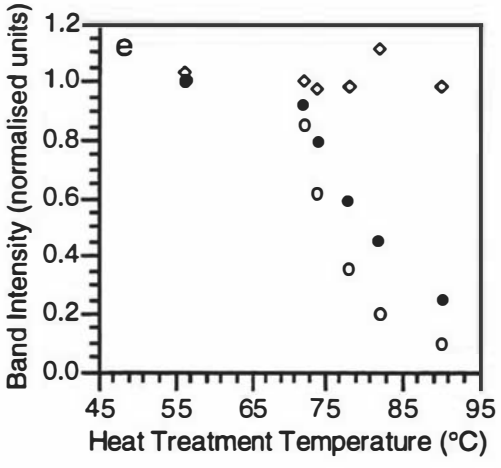
Fig. 5.3.9.7. Effect of heat treatment temperature on the intensity of the monomeric BLG band on polyacrylamide gels for BLGs A, B and C at pH 6.7 and pH 7.4. The band intensities in the disulphide-reduced (◇) and disulphide-intact (●) portions of SDS gels and on alkaline native gels (o) for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. Monomer band intensities were determined from the scanned images of the gels shown in Figs 5.3.9.1 and 5.3.9.3 - 5.3.9.5 using the software package "ImageQuant" as described in Section 3.1.2.8. Band intensity values were normalised as described in the introductory portion of Section 5.3.9.7.

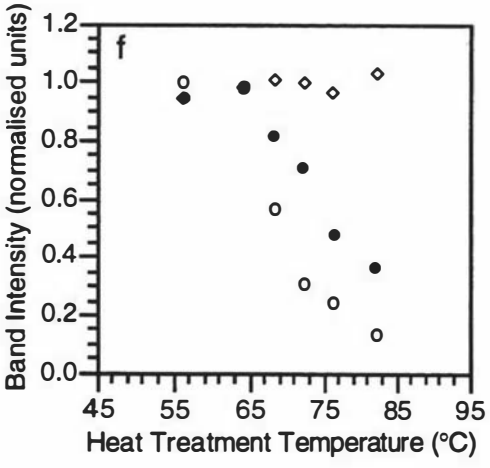

a
region 1
region 2
Band Intensity (normalised units)
Heat Treatment Temperature (°C)


b
Band Intensity (normalised units)
Heat Treatment Temperature (°C)


c
Band Intensity (normalised units)
Heat Treatment Temperature (°C)


d
Band Intensity (normalised units)
Heat Treatment Temperature (°C)


e
Band Intensity (normalised units)
Heat Treatment Temperature (°C)


f
Band Intensity (normalised units)
Heat Treatment Temperature (°C)

Decreases in monomer band intensity with increasing heat treatment temperature are more marked on alkaline native gels than in the disulphide-intact portions of SDS gels (Fig. 5.3.9.7). This suggests that native BLG forms two classes of aggregate species during heat treatment: one which can be studied using both disulphide-intact SDS-PAGE and alkaline native-PAGE, and another which can be studied using only alkaline native-PAGE. Therefore, the alkaline native-PAGE results probably reflect the formation of both non-covalently-linked and disulphide-linked aggregates, while the disulphide-intact SDS-PAGE results reflect only the formation of disulphide-linked aggregates (see Fig. 2.7.3). The difference between the monomer band intensity values determined from the disulphide-intact portions of SDS gels and from alkaline native gels therefore corresponds to the concentration of non-covalently-linked aggregates and "unfolded" monomers in heat-treated BLG samples (see Fig. 2.7.3). Furthermore, the difference in band intensity values determined from the disulphide-reduced and the disulphide-intact portions of SDS gels corresponds to the concentration of disulphide-linked aggregates in heat-treated BLG samples (see Fig. 2.7.3). The use of different PAGE techniques to study the formation of disulphide-linked and non-covalently-linked aggregates has recently been described by McSwiney *et al.* (1994a, b), Gezimati *et al.* (1997) and Havea *et al.* (1998).

At both pH 6.7 and pH 7.4, the difference between monomer band intensities in the disulphide-reduced and disulphide-intact portions of SDS gels (region 1 in panel a of Fig. 5.3.9.7), and therefore the concentration of disulphide-linked aggregates, increases with increasing heat treatment temperature. Similarly, the difference between monomer band intensities on alkaline native gels and in the disulphide-intact portions of SDS gels (region 2 in panel a of Fig. 5.3.9.7), and therefore the concentration of non-covalently-linked aggregates, increases with increasing heat treatment temperature. However, this increase is only observed between T1 and T5 (up to approximately 80 °C) at pH 6.7; the concentration of these aggregate species is slightly less in solutions previously heat-treated at T6 (approximately 90 °C) than in those previously heat-treated at T5. Non-covalently-linked aggregates of BLG may therefore be unstable at higher temperatures at pH 6.7.

At both pH 6.7 and pH 7.4, the proportion of aggregates stabilised in only a non-covalent manner is greater for BLG A than for BLGs B and C (Fig. 5.3.9.7). Furthermore, the magnitude of this difference is greater at pH 6.7 than at pH 7.4. Finally, the results in Fig. 5.3.9.7 indicate that at pH 6.7 in particular, the decrease in

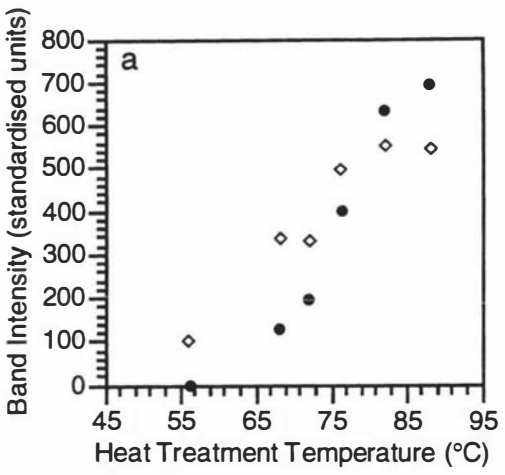
monomer band intensity with increasing heat treatment temperature on both alkaline native gels and in the disulphide-intact portions of SDS gels is less marked for BLG A than for BLGs B and C. This suggests that monomers and "unfolded" monomers of BLG A are incorporated into aggregate structures more slowly than those of BLGs B and C.

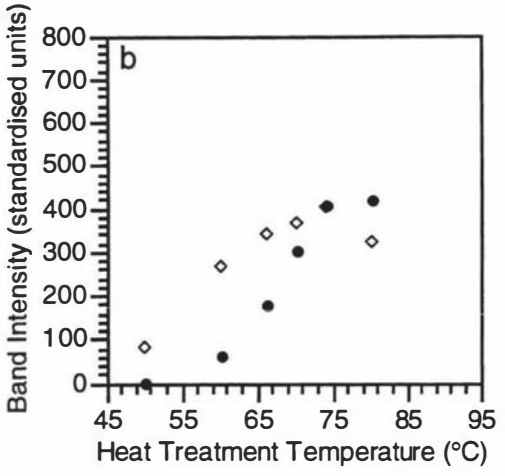
Plots of "unfolded" monomer and dimer band intensity versus heat treatment temperature are shown in Fig. 5.3.9.8. Dimeric and "unfolded" monomeric BLGs are defined as those species which migrate to regions *d* and *um* respectively on alkaline native gels (Section 5.3.9.4). The concentration of "unfolded" monomers was assumed to correspond to the total intensity in the region *um* of the gels shown in Figs 5.3.9.4 and 5.3.9.5.

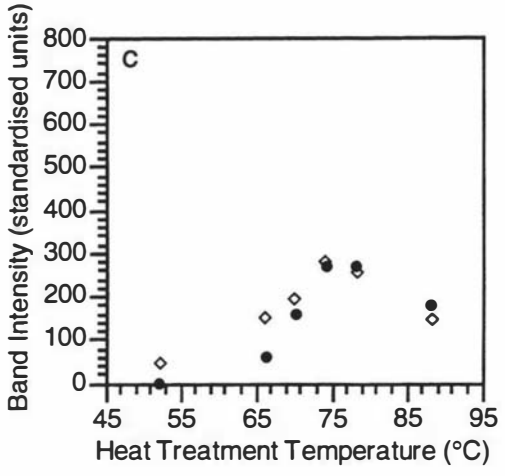
Except for BLG C at pH 7.4, concentrations of "unfolded" monomers are greater than those of dimers in samples heat-treated at lower temperatures (T1 - T3 samples, plus T4 samples in some instances, Fig. 5.3.9.8). This suggests that in most instances during heat treatment at lower temperatures "unfolded" monomers are more stable than dimers. In contrast, except for BLGs B and C at pH 6.7, concentrations of dimers are greater than those of "unfolded" monomers in samples heat-treated at higher temperatures (T5 - T6 samples, plus T4 samples in some instances). This suggests that for BLG A at both pH 6.7 and pH 7.4 and for BLGs B and C at pH 7.4, dimers are more stable than "unfolded" monomers during heat treatment at higher temperatures. For BLGs B and C at pH 6.7, concentrations of "unfolded" monomers and dimers are equivalent in T5 and T6 samples, suggesting that these species are equally stable during heat treatment at higher temperatures.

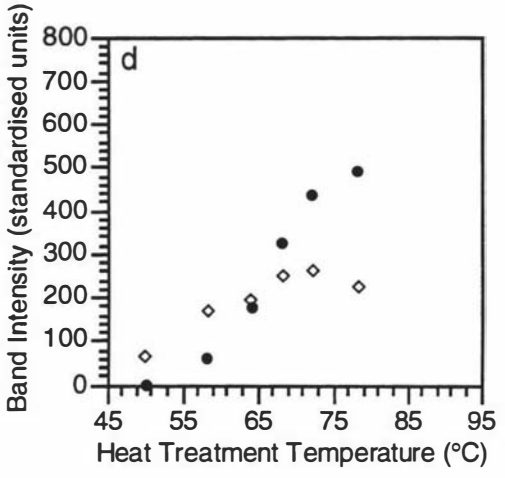
At both pH 6.7 and pH 7.4, concentrations of dimers and "unfolded" monomers are higher in samples of BLG A than in those of BLGs B and C (Fig. 5.3.9.8). This is also true for trimers and tetramers (data not shown). Thus, in agreement with the results discussed in Sections 5.3.9.3 and 5.3.9.5, those in this section suggest that large aggregates are formed more slowly from BLG A that from BLGs B and C.

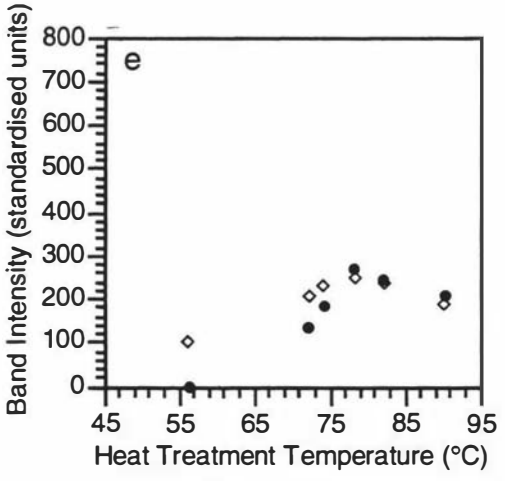
Fig. 5.3.9.8. Effect of heat treatment temperature on the intensity of the dimeric and "unfolded" monomeric BLG bands on alkaline native gels for BLGs A, B and C at pH 6.7 and pH 7.4. The intensities of the dimer (●) and "unfolded" monomer (◇) bands for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f). Band intensities were determined from the scanned images of the gels shown in Figs 5.3.9.4 and 5.3.9.5 using the software package "ImageQuant" as described in Section 3.1.2.8. Band intensity values were normalised as described in the introductory portion of Section 5.3.9.7.

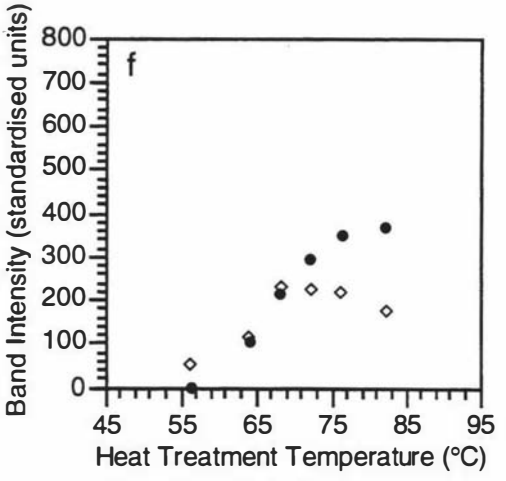

a
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
45 55 65 75 85 95
Heat Treatment Temperature (°C)


b
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
45 55 65 75 85 95
Heat Treatment Temperature (°C)


c
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
45 55 65 75 85 95
Heat Treatment Temperature (°C)


d
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
45 55 65 75 85 95
Heat Treatment Temperature (°C)


e
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
45 55 65 75 85 95
Heat Treatment Temperature (°C)


f
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
45 55 65 75 85 95
Heat Treatment Temperature (°C)

5.3.9.8. Time-course PAGE.

Time-course PAGE results were used primarily to confirm that non-covalently-linked aggregates are reaction intermediates on the BLG aggregation pathway. To examine time-dependent changes in the concentrations of these species during heat treatment the intensities of the monomer band on both the disulphide-intact SDS and alkaline native gels shown in Figs 5.3.9.9 - 5.3.9.12 were measured and compared in a similar manner to that described in Section 5.3.9.7. Band intensities on time-course gels were normalised relative to one another using the band intensity values for the unheated BLG samples. Plots of monomer band intensity versus heat treatment time are shown in Fig. 5.3.9.13.

The decrease in the intensity of the monomeric BLG band with increasing heat treatment time (Fig. 5.3.9.13) is more marked on alkaline native gels than on disulphide-intact SDS gels, confirming the reports of McSwiney *et al.* (1994a, b). Thus, for the reasons given in Section 5.3.9.7.1, time-dependent decreases in monomeric BLG band intensity on alkaline native gels reflect the formation of "unfolded" monomers and non-covalently-linked and disulphide-linked aggregates. Decreases in monomeric BLG band intensity on disulphide-intact SDS gels reflect the formation of disulphide-linked aggregates only.

The magnitude of the difference between monomer band intensities on disulphide-intact SDS and alkaline native gels (Fig. 5.3.9.13) decreases with increasing heat treatment time after 10 min. For the reasons given in Section 5.3.9.7.1, this suggests that concentrations of "unfolded" monomers and aggregate species stabilised in only a non-covalent manner decrease with increasing heat treatment time after 10 min. Therefore, because the plots in Fig. 5.3.9.13 indicate that these species are not present in unheated BLG solutions, it is likely that they exist as reaction intermediates during heat treatment. The SDS gels in Figs 5.3.9.9 and 5.3.9.10 indicate that the concentrations of non-monomeric BLG species with M_r values less than approximately 200 000 also decrease with increasing heat treatment time after 10 min. In the case of the alkaline native gels (Figs 5.3.9.11 and 5.3.9.12), this is true for all non-"native-like" species with M_r values less than approximately 200 000 (Section 5.3.9.4 for definition). Therefore, because such species are not present in unheated BLG solutions (Figs 5.3.9.9 - 5.3.9.12), it appears that they also exist as reaction intermediates during heat treatment. In contrast the concentrations of large aggregates increase continuously with increasing heat treatment time (Figs 5.3.9.9 - 5.3.9.12). Therefore, because non-"native-like" species with M_r values less than approximately 200 000 appear to exist as reaction intermediates during heat treatment, it is likely that large disulphide-linked aggregates are the end product of the BLG aggregation pathway, in agreement with the BLG aggregation models discussed in Section 2.7.

The present time-course PAGE results indicate that the aggregation behaviour of BLG A differs from that of BLGs B and C. For BLG samples previously heat-treated for times of 120 min and less the differences between the intensities of monomer bands on alkaline native and disulphide-intact SDS gels are greater for BLG A than for BLGs B and C (Fig. 5.3.9.13). This suggests that in solutions heat-treated for such periods at both pH 6.7 and pH 7.4, concentrations of "unfolded" monomers and aggregate species stabilised in only a non-covalent manner are higher for BLG A than for BLGs B and C. Therefore, it appears that "unfolded" monomers and non-covalently-linked aggregates of BLG A are more stable than those of BLGs B and C. Similarly, the plots in Fig. 5.3.9.7 indicate that, in solutions heat-treated for 12.5 min at temperatures between approximately 50 °C and 80 °C at pH 6.7 in particular, concentrations of aggregate species stabilised in only a non-covalent manner are higher for BLG A than for BLGs B and C.

In the gels for BLG samples heat-treated at pH 6.7 in particular (Figs 5.3.9.9 and 5.3.9.11), the intensities of all bands corresponding to species with M_r values less than approximately 200 000 decrease more gradually with increasing heat treatment time for BLG A than for BLGs B and C. This suggests that during heat treatment, species with M_r values less than approximately 200 000 are more stable in solutions of BLG A than those of BLGs B and C. Time-course PAGE results are therefore consistent with the PAGE results in Figs 5.3.9.1 and 5.3.9.3 - 5.3.9.5, which suggest that, after heat treatment at temperatures between 50 °C and 90 °C for a period of 12.5 min, concentrations of species with M_r values less than approximately 200 000 are higher in solutions of BLG A than in those of BLGs B and C.

Time-dependent changes in the concentrations of BLG aggregate species are affected by pH (Figs 5.3.9.9 - 5.3.9.12). For all three variants, the time-dependent decrease in the intensities of bands in the resolving portion of alkaline native gels and disulphide-intact SDS gels is less marked at pH 7.4 than at pH 6.7. This indicates that, for all three variants, concentrations of species with M_r values less than approximately 200 000 decrease more slowly during heat treatment at pH 7.4 than at pH 6.7. Therefore, it is likely that these aggregates are more stable at the higher pH. Similarly, in solutions of BLGs A, B and C heat-treated at temperatures between approximately 50 °C and 90 °C for 12.5 min, concentrations of species with M_r values less than approximately 200 000 are greater at pH 7.4 than at pH 6.7 (Figs 5.3.9.1 and 5.3.9.3 - 5.3.9.5).

Fig. 5.3.9.9. Disulphide-intact SDS-PAGE electropherograms of samples of BLGs A, B and C previously heat-treated for various times at 80 °C at pH 6.7. The complete set of heat-treated samples for a particular variant is shown on the same gel, as follows: BLG A, gel 1; BLG B, gel 2; and BLG C, gel 3. On gels 1 - 3 lanes contain, from left to right, the BLG sample heat-treated for 0, 10, 20, 30, 60, 90, 120 and 150 min. On gel 4 lanes contain, from left to right:

1) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)

2) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)

3) blank

4) the disulphide-intact unheated BLG B standard

5) disulphide-intact BLG A heat-treated at pH 7.4 at 74 °C

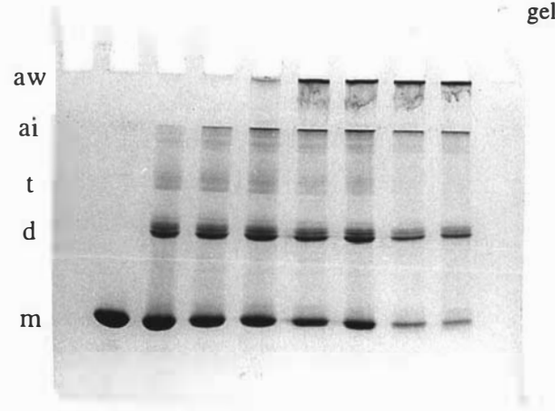
6) disulphide-intact BLG B heat-treated at pH 7.4 at 72 °C

7) disulphide-intact BLG A heat-treated at pH 6.7 at 82 °C

8) disulphide-intact BLG B heat-treated at pH 6.7 at 78 °C

Details of the sample preparation and heating protocol are given in Section 5.2.6.1. The PAGE protocol is described in Section 5.2.6.2.

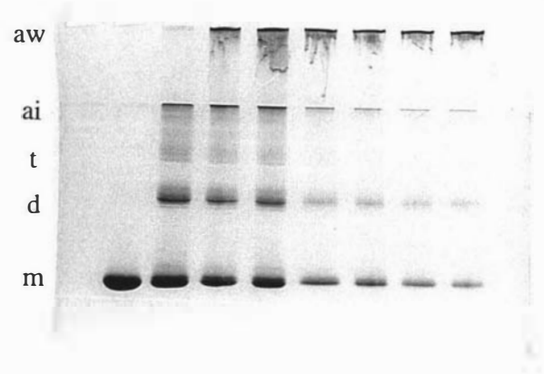
gel 1



0 10 20 30 60 90 120 150

Heat Treatment Time (min)

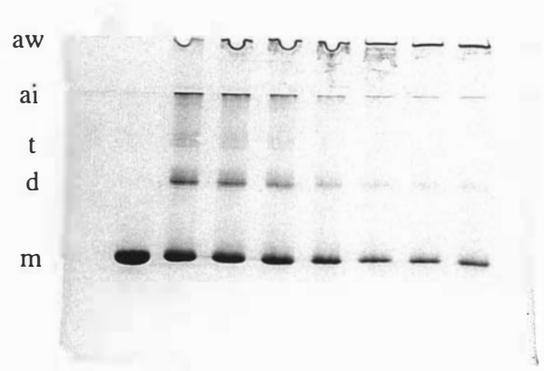
gel 2



0 10 20 30 60 90 120 150

Heat Treatment Time (min)

gel 3



0 10 20 30 60 90 120 150

Heat Treatment Time (min)

gel 4

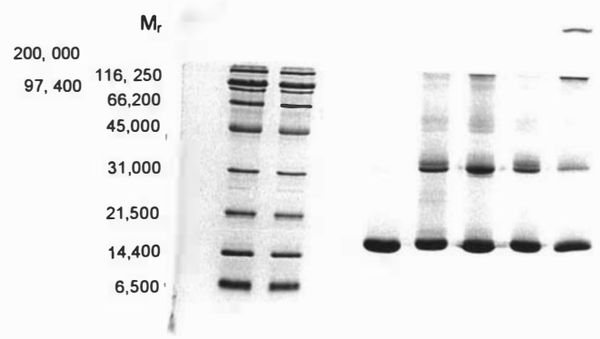


Fig. 5.3.9.10. Disulphide-intact SDS-PAGE electropherograms of samples of BLGs A, B and C previously heat-treated for various times at 74 °C at pH 7.4. The complete set of heat-treated samples for a particular variant is shown on the same gel, as follows: BLG A, gel 1; BLG B, gel 2; and BLG C, gel 3. On gels 1 - 3 lanes contain, from left to right, the BLG sample heat-treated for 0, 10, 20, 30, 60, 90, 120 and 150 min. On gel 4 lanes contain, from left to right:

1) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)

2) M_r markers (treated with 2-mercaptoethanol, Bio-Rad)

3) blank

4) the disulphide-intact unheated BLG B standard

5) disulphide-intact BLG A heat-treated at pH 7.4 at 74 °C

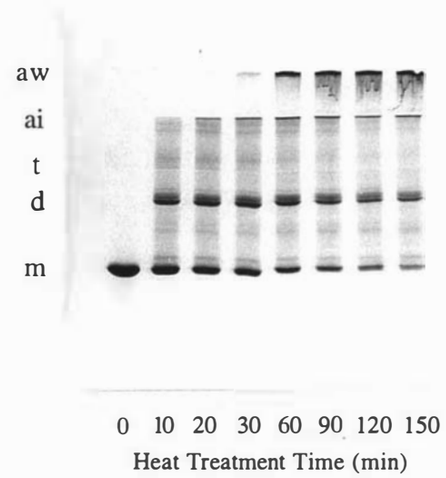
6) disulphide-intact BLG B heat-treated at pH 7.4 at 72 °C

7) disulphide-intact BLG A heat-treated at pH 6.7 at 82 °C

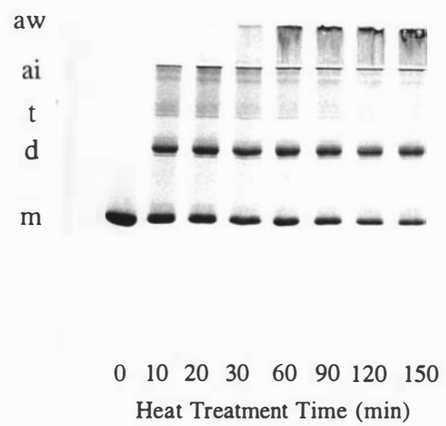
8) disulphide-intact BLG B heat-treated at pH 6.7 at 78 °C

Details of the sample preparation and heating protocol are given in Section 5.2.6.1. The PAGE protocol is described in Section 5.2.6.2.

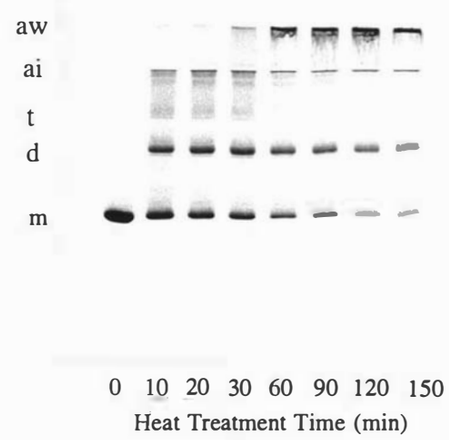
gel 1



gel 2



gel 3



gel 4

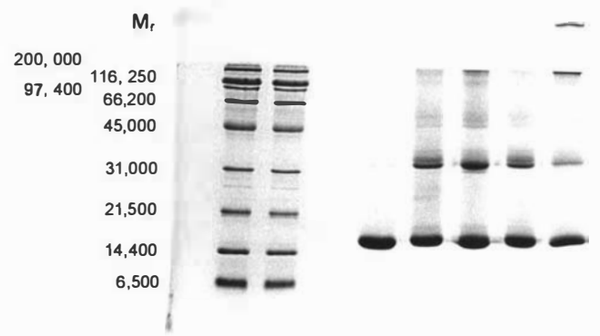
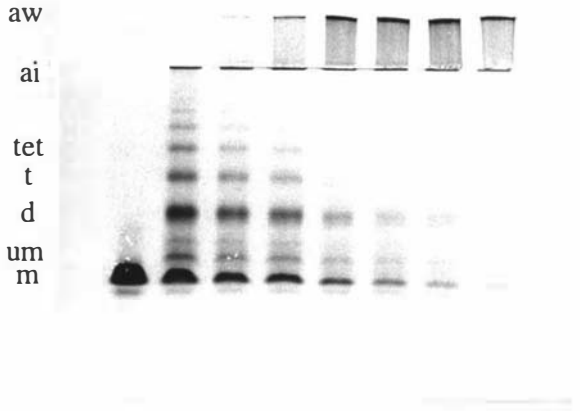


Fig. 5.3.9.11. Alkaline native-PAGE electropherograms of samples of BLGs A, B and C previously heat-treated for various times at 80 °C at pH 6.7. The complete set of heat-treated samples for a particular variant is shown in the same gel, as follows: BLG A, gel 1; BLG B, gel 2; and BLG C, gel 3. On each gel lanes contain, from left to right, the BLG sample heat-treated for 0, 10, 20, 30, 60, 90, 120 and 150 min. Details of the sample preparation and heating protocol are given in Section 5.2.6.1. The PAGE protocol is described in Section 5.2.6.2.

gel 1

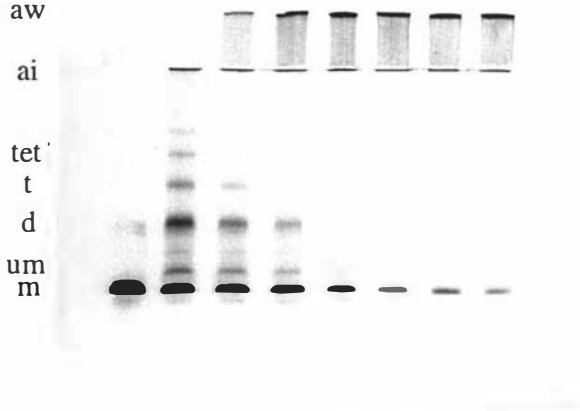
0 10 20 30 60 90 120 150
Heat Treatment Time (min)

gel 2



0 10 20 30 60 90 120 150
Heat Treatment Time (min)

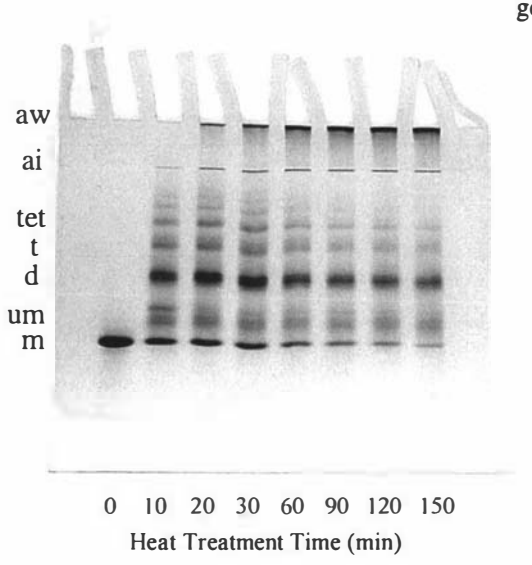
gel 3



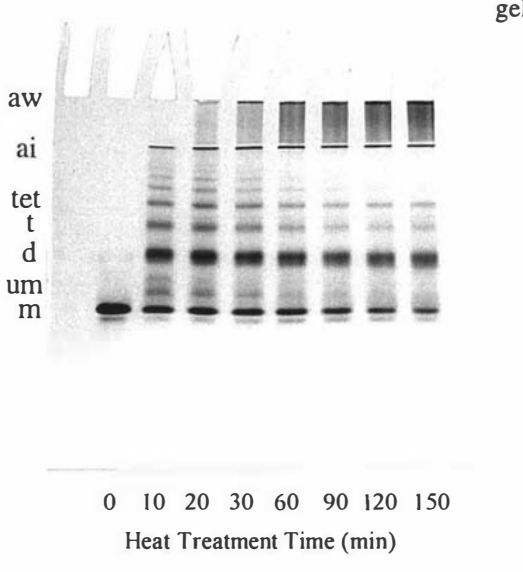
0 10 20 30 60 90 120 150
Heat Treatment Time (min)

Fig. 5.3.9.12. Alkaline native-PAGE electropherograms of samples of BLGs A, B and C previously heat-treated for various times at 74 °C at pH 7.4. The complete set of heat-treated samples for a particular variant is shown in the same gel, as follows: BLG A, gel 1; BLG B, gel 2; and BLG C, gel 3. On each gel lanes contain, from left to right, the BLG sample heat-treated for 0, 10, 20, 30, 60, 90, 120 and 150 min. Details of the sample preparation and heating protocol are given in Section 5.2.6.1. The PAGE protocol is described in Section 5.2.6.2.

gel 1



gel 2



gel 3

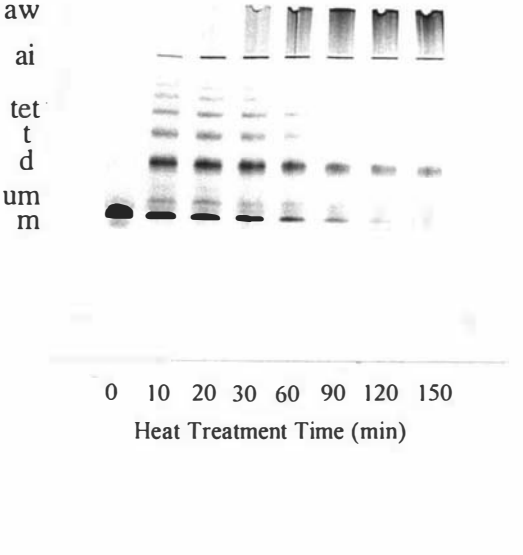
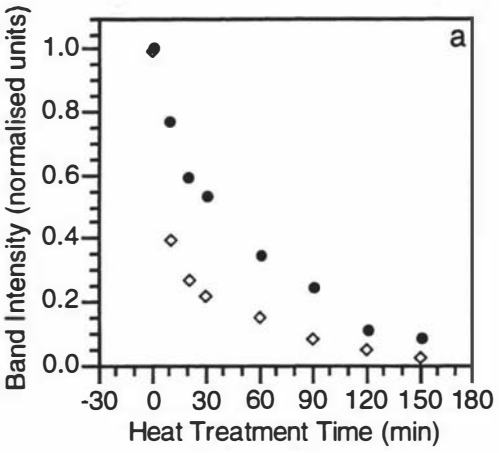
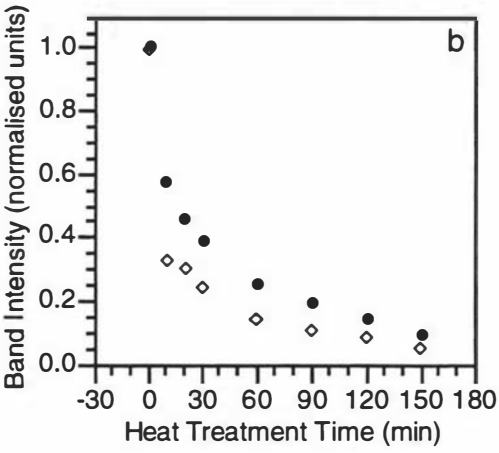
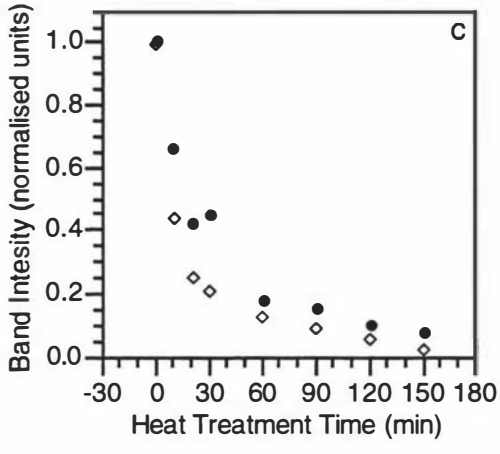
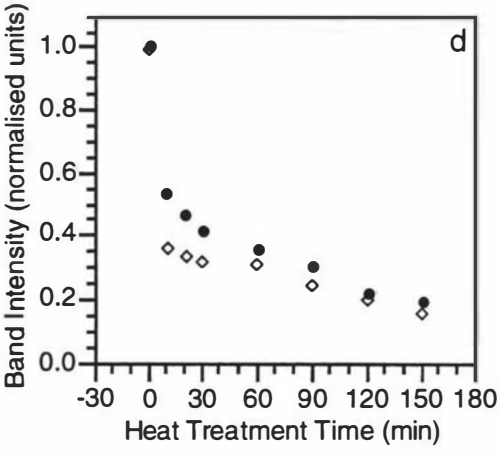


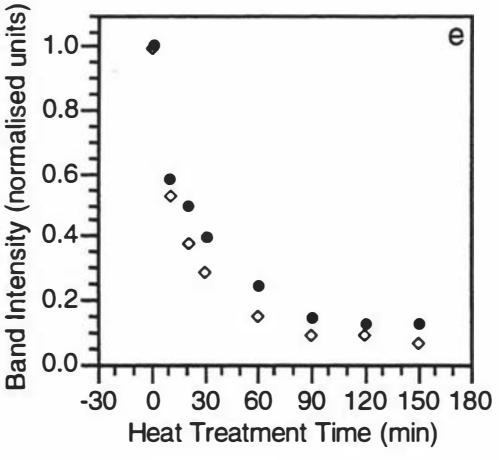
Fig. 5.3.9.13. Effect of heat treatment time on the intensity of the monomeric BLG band for BLGs A, B and C at pH 6.7 and pH 7.4 on disulphide-intact SDS and alkaline native gels. The disulphide-intact (•) and alkaline native (◇) band intensities for a particular variant at a particular pH value are shown in the same panel. At zero time, the symbols (•) and (◇) are superimposed. The panels are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. At pH 6.7 samples of BLGs A, B and C were heat-treated at 80 °C. At pH 7.4 samples of BLGs A, B and C were heat-treated at 74 °C. Monomer band intensities were determined from the scanned images of the gels shown in Figs 5.3.9.9 - 5.3.9.12 using the software package "ImageQuant" as described in Section 3.1.2.8. Band intensity values were normalised as described in the introductory portions of Sections 5.3.9.7 and 5.3.9.8.

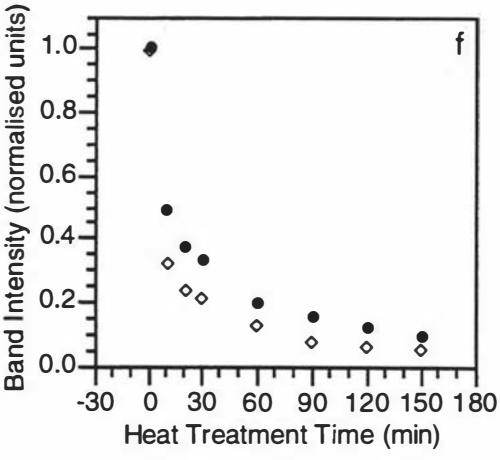

a
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


b
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


c
Band Intesity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)

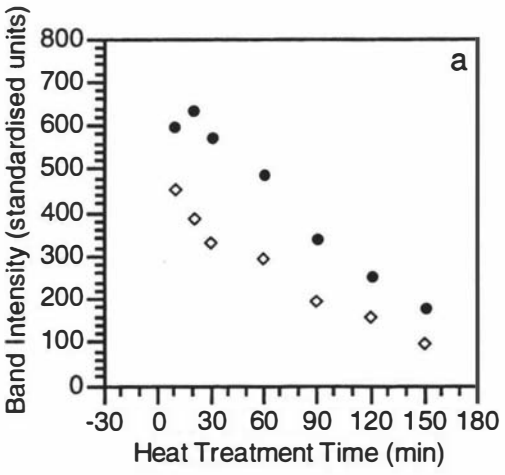

d
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)

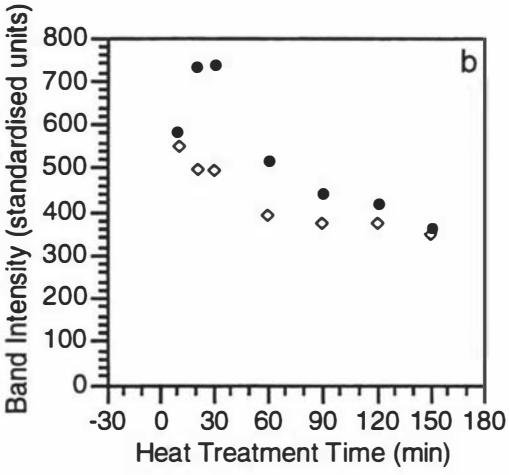

e
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)

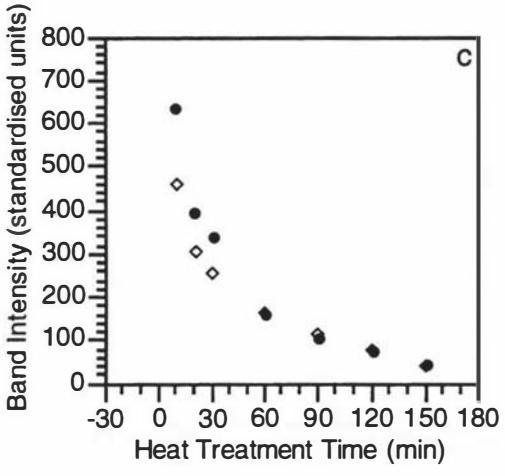

f
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)

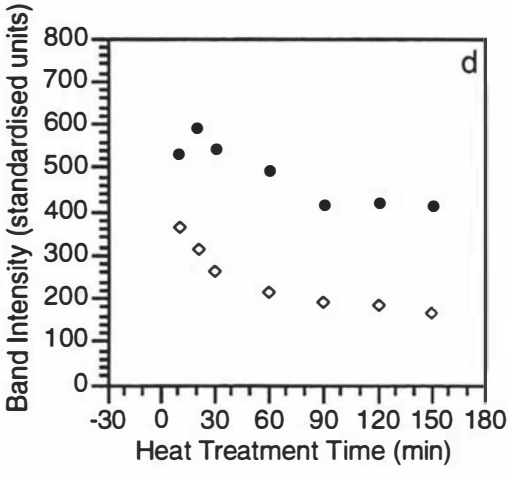
Plots of standardised dimeric and "unfolded" monomeric BLG band intensity versus heat treatment time (Fig. 5.3.9.14) indicate that concentrations of both dimers and "unfolded" monomers decrease with increasing heat treatment time after 10 min. Thus, because these species are not present in unheated samples (Figs 5.3.9.9 - 5.3.9.12), the plots in Fig. 5.3.9.14 confirm that they exist as reaction intermediates during heat treatment. Additionally, except in the case of BLG C at pH 6.7, concentrations of dimers are greater than those of "unfolded" monomers in samples heat-treated for times up to 150 min (Fig. 5.3.9.14). This suggests that, in most instances, dimers are more stable than "unfolded" monomers during heat treatment at 80 °C at pH 6.7 and at 74 °C at pH 7.4, in agreement with most of the results for T5 samples in Fig. 5.3.9.8. Time-course PAGE results also indicate that dimers are more stable than trimers during heat treatment at 80 °C at pH 6.7 and at 74 °C at pH 7.4 for times up to 150 min (data not shown).

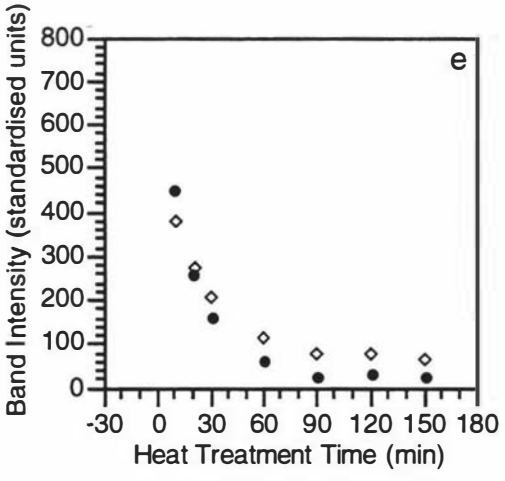
Fig. 5.3.9.14. Effect of heat treatment time on the intensity of the dimeric and "unfolded" monomeric BLG bands on alkaline native gels for BLGs A, B and C at pH 6.7 and pH 7.4. The intensities of the dimer (•) and "unfolded" monomer (◇) bands for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. At pH 6.7 samples of BLGs A, B and C were heat-treated at 80 °C. At pH 7.4 samples of BLGs A, B and C were heat-treated at 74 °C. Band intensities were determined from the scanned images of the gels shown in Figs 5.3.9.11 and 5.3.9.12 using the software package "ImageQuant" as described in Section 3.1.2.8. Band intensity values were standardised as described in the introductory portions of Sections 5.3.9.7 and 5.3.9.8.

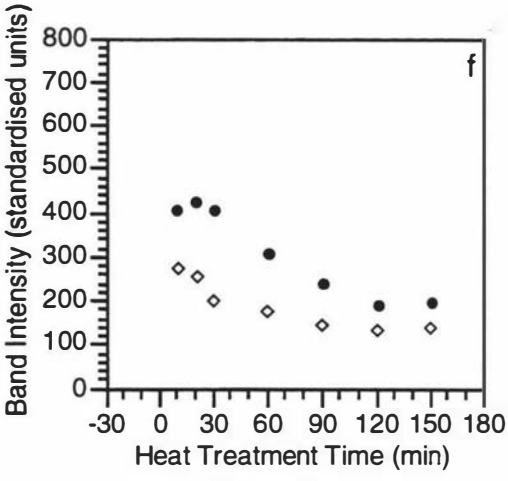

a
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


b
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


c
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


d
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


e
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)


f
Band Intensity (standardised units)
800
700
600
500
400
300
200
100
0
-30 0 30 60 90 120 150 180
Heat Treatment Time (min)

5.3.9.9. Effect of Variant Type on the Aggregation of β-Lactoglobulin.

The PAGE results in the preceding sections suggest that heat-treated BLG A is distributed amongst the various non-"native-like" and aggregate species in a different manner to BLGs B and C.

1) One-dimensional alkaline native-PAGE and 1D disulphide-intact SDS-PAGE results (Figs 5.3.9.1 and 5.3.9.3 - 5.3.9.5) suggest that, after heat treatment at temperatures between 50 °C and 90 °C for 12.5 min, concentrations of non-"native-like" species with M_r values less than approximately 200 000 are higher in solutions of BLG A than in those of BLGs B and C, while the opposite was true for large aggregates. This suggests that large aggregates are formed more slowly from BLG A than from BLGs B and C. A similar conclusion was drawn from the time-course PAGE results in Figs 5.3.9.9 - 5.3.9.12. The results in the preceding sections are consistent with those of McSwiney *et al.* (1994b), which show that concentrations of monomeric species are higher for BLG A than for BLG B after heat treatment for periods longer than approximately 13.3 min.

2) The plots in Fig. 5.3.9.7 suggest that concentrations of species stabilised in only a non-covalent manner in heat-treated solutions are higher for BLG A than for BLGs B and C. This suggests that aggregates of BLG A containing only non-covalent links are more stable than those of BLGs B and C.

3) The plots in Fig. 5.3.9.8 indicate that concentrations of "unfolded" monomers and dimers in heat-treated solutions are higher for BLG A than for BLGs B and C.

4) Time-course PAGE results (Figs 5.3.9.9 - 5.3.9.12) indicate that concentrations of species with M_r values less than approximately 200 000 decrease more gradually with increasing heat treatment time for BLG A than for BLGs B and C.

The amino acid substitution at position 64, where glycine in BLGs B and C is replaced by aspartate in BLG A, may explain the differences between the aggregation behaviour of these variants. This will be discussed in Section 8.5.

The PAGE results in the preceding sections suggest that in heat-treated solutions, concentrations of large aggregates are higher for BLG C than for BLG B. This behaviour probably reflects the amino acid substitution at position 59, where glutamine in BLG B is replaced by histidine in BLG C, and will be discussed further in Section 8.5.

5.3.10. CORRELATION OF SPECTROSCOPIC AND PAGE RESULTS.

5.3.10.1. Correlation of $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp} I_{ANS}, Thiol Availability and PAGE Results.

Each set of $\Delta\varepsilon_{293}$ (Section 5.3.1), $[\theta]_{205}$ (Section 5.3.2), I_{Trp} (Section 5.3.3), I_{ANS} (Section 5.3.4) and thiol availability (Section 5.3.5) data can be adequately explained in terms of a 2-state process. However, the PAGE results in Section 5.3.9 indicate that a large number of BLG aggregate species are formed as a consequence of heat treatment. Therefore, with respect to $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and thiol availability (subsequently referred to as spectroscopic) results, although state 1 is likely to be native BLG, state 2 could be any one of the many non-"native-like" species identified by PAGE. Because aggregates were present in the solutions on which spectroscopic measurements were made, the results presented in previous sections may reflect aggregation as well as changes in tertiary and secondary structure in BLG molecules. To determine whether or not this is true, the correlation of spectroscopic data with quantitative PAGE data (monomer band intensities) was examined. If PAGE and spectroscopic data both describe the heat-induced loss of native BLG, then linear correlations should exist. The correlations are presented as plots of normalised monomer band intensity (both alkaline native-PAGE and disulphide-intact SDS-PAGE results) versus normalised $\Delta\varepsilon_{293}$ (Fig. 5.3.10.1), $[\theta]_{205}$ (Fig. 5.3.10.2), I_{Trp} (Fig. 5.3.10.3), I_{ANS} (Fig. 5.3.10.4) and A_{412} (Fig. 5.3.10.5).

In most of the plots in Figs 5.3.10.1 - 5.3.10.5, normalised alkaline native-PAGE monomer band intensity values decrease with increasing normalised spectroscopic signal intensity in an approximately linear manner. Except in the case of I_{Trp} data, correlation coefficients range from 0.890 to 0.984 (Tables 5.3.10.1, 5.3.10.2, 5.3.10.4 and 5.3.10.5). The correlation coefficients for I_{Trp} data with alkaline native-PAGE data range from 0.752 to 0.792 (Table 5.3.10.3). All of the correlations are significant at a 95 % confidence level (Tables 5.3.10.1 - 5.3.10.5). These correlations therefore suggest the following:

1) Only the species which run as monomers on alkaline native gels exhibit a trough at 293 nm in their near UV CD spectrum.

2) As the proportion of random structure in BLG molecules increases (as shown by an increase in the intensity of $[\theta]_{205}$) the species which run as monomers on alkaline native gels are lost.

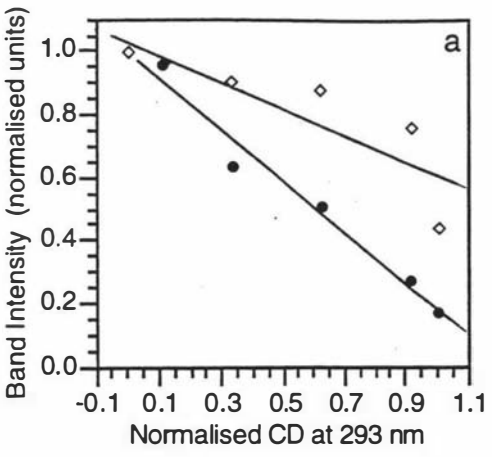
3) Only "native-like" BLG species have a disulphide bond between Cys^{66} and Cys^{160}.

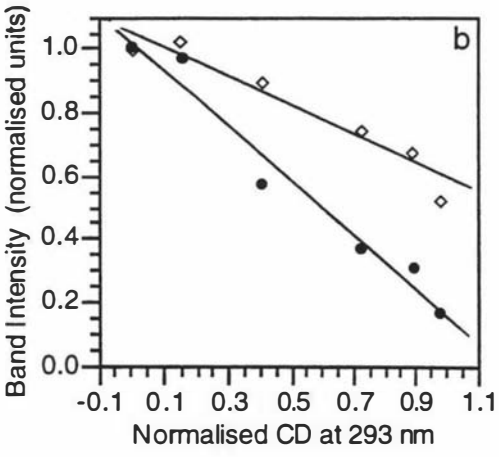
4) The structure of the ANS binding site of BLG changes as the species which run as monomers on alkaline native gels are lost.

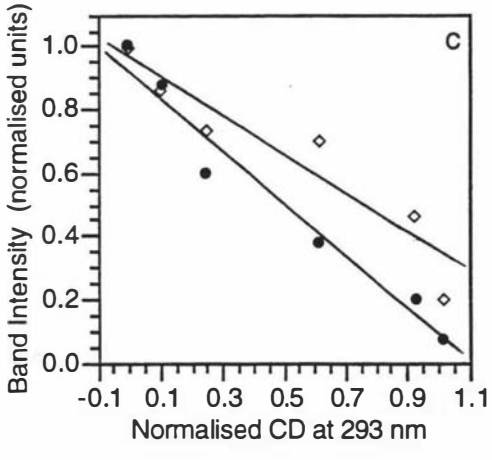
5) The thiol group is solvent-inaccessible only in "native-like" BLG species.

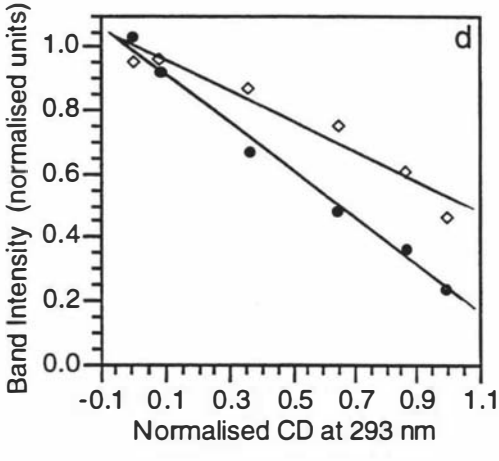
Thus, spectroscopic results appear to reflect the loss of "native-like" BLG and not the formation of aggregates.

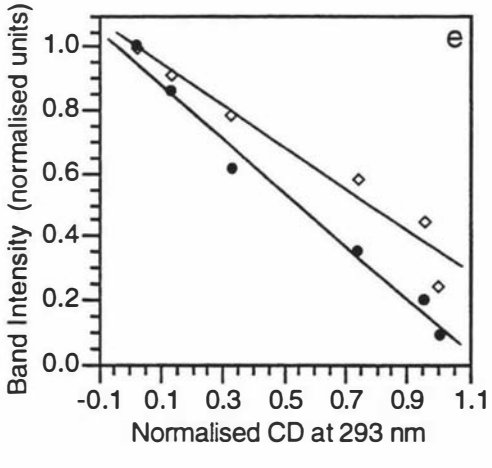
Fig. 5.3.10.1. Plots of normalised $\Delta\varepsilon_{293}$ versus normalised alkaline native-PAGE monomer band intensity and versus normalised disulphide-intact SDS-PAGE monomer band intensity for BLGs A, B and C at pH 6.7 and pH 7.4. The plots for alkaline native-PAGE (•) and SDS-PAGE (◇) data for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The normalised $\Delta\varepsilon_{293}$ value 0.0 represents that for a sample of unheated BLG, while the value 1.0 represents that for the sample in which the proportion of BLG molecules whose structures had been altered irreversibly as a consequence of heat treatment is greatest. Normalised monomer band intensity values of 0.0 and 1.0 represent those for samples of unheated BLG and the BLG sample heat-treated at the highest temperature used in the PAGE study. The temperatures at which the BLG samples were heat-treated (Table 5.3.6.1) increase from left to right.


a
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 293 nm


b
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 293 nm


c
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 293 nm


d
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 293 nm


e
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 293 nm

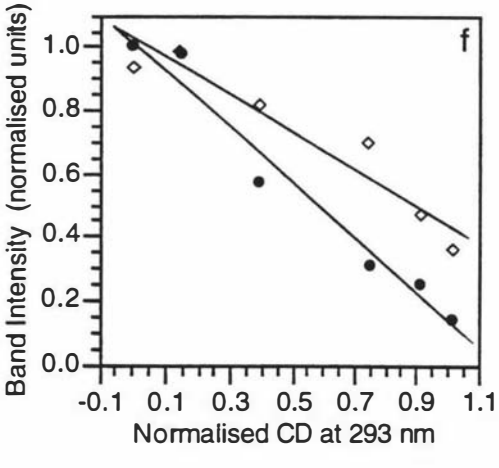
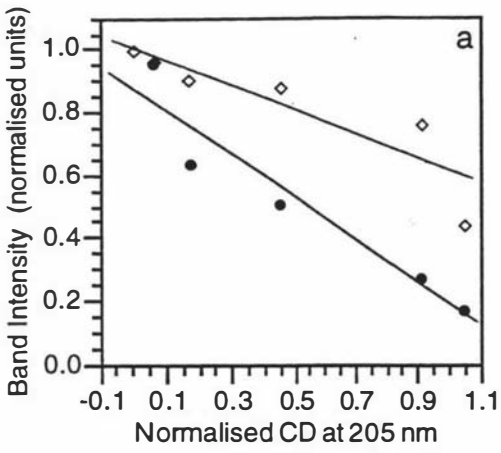
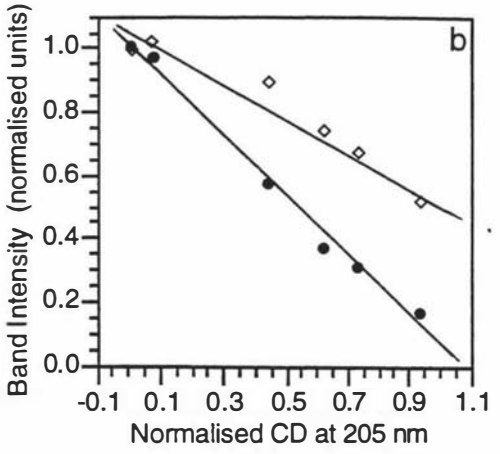
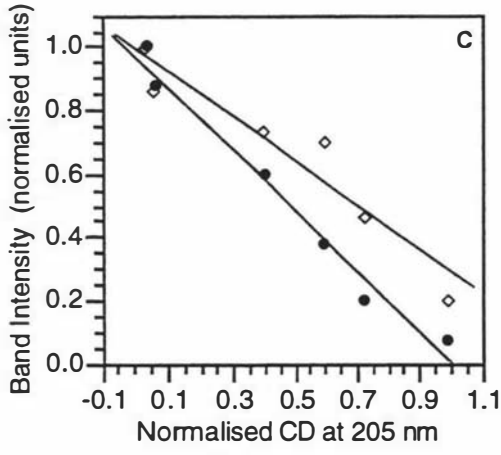
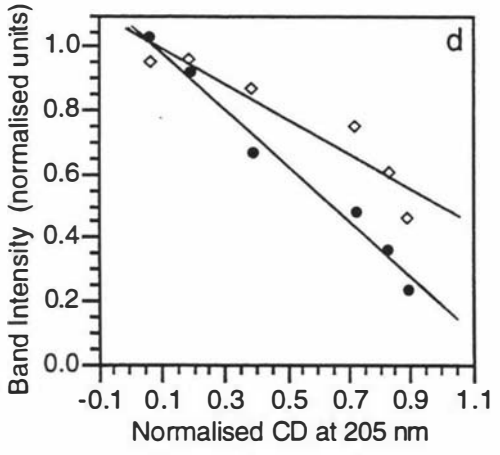

f
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 293 nm

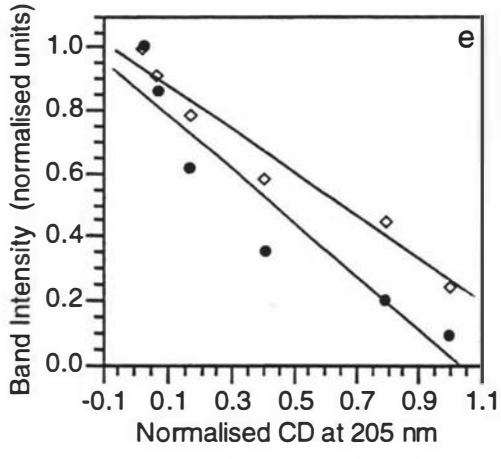
Fig. 5.3.10.2. Plots of normalised $[\theta]_{205}$ versus normalised alkaline native-PAGE monomer band intensity and versus normalised disulphide-intact SDS-PAGE monomer band intensity for BLGs A, B and C at pH 6.7 and pH 7.4. The plots for alkaline native-PAGE (•) and SDS-PAGE (◇) data for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The data normalisation procedures are the same as those described in Fig. 5.3.10.1. The temperatures at which the BLG samples were heat-treated (Table 5.3.6.1) increase from left to right.


a
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 205 nm


b
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 205 nm


c
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 205 nm


d
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 205 nm


e
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 205 nm

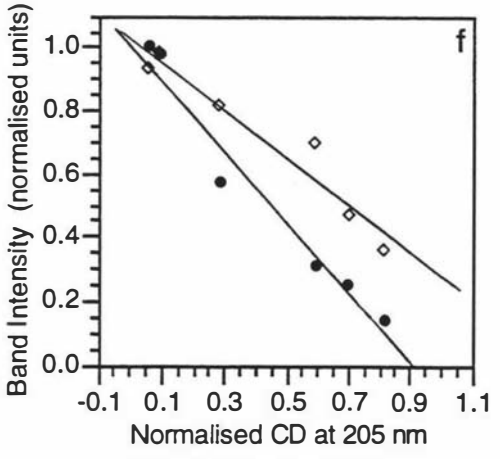
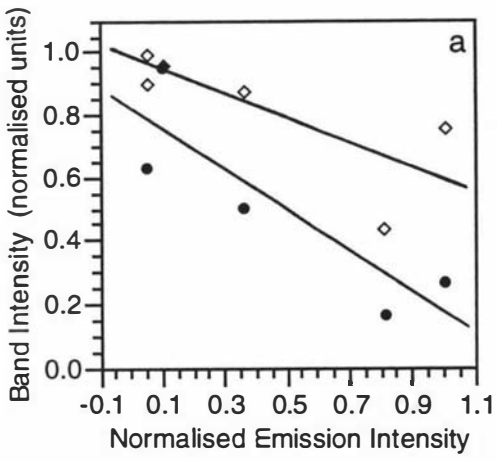
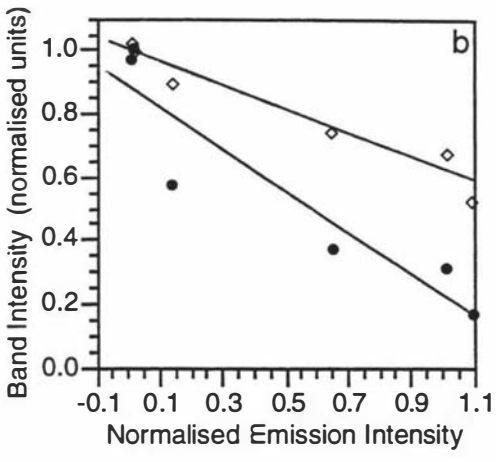
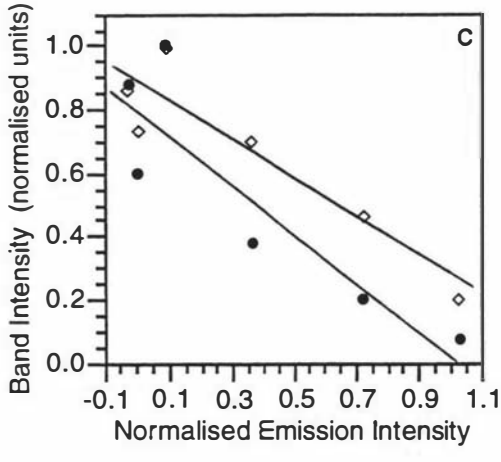
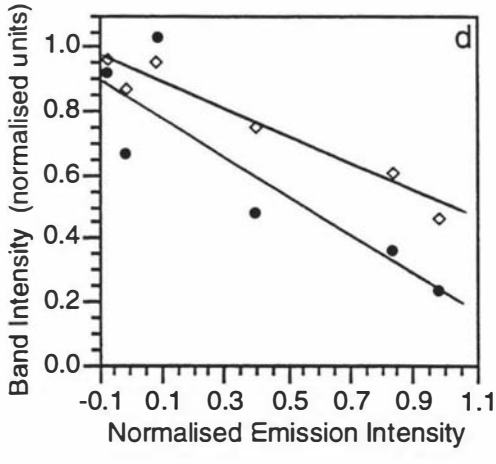

f
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised CD at 205 nm

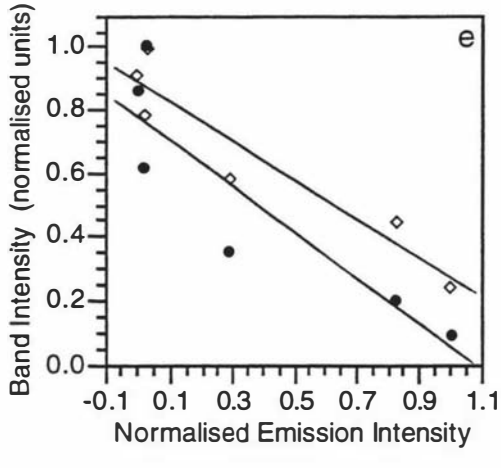
Fig. 5.3.10.3. Plots of normalised I_{Trp} versus normalised alkaline native-PAGE monomer band intensity and versus normalised disulphide-intact SDS-PAGE monomer band intensity for BLGs A, B and C at pH 6.7 and pH 7.4. The plots for alkaline native-PAGE (•) and SDS-PAGE (◇) data for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The data normalisation procedures are the same as those described in Fig. 5.3.10.1. The temperatures at which the BLG samples were heat-treated (Table 5.3.6.1) increase from left to right.


a
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Emission Intensity


b
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Emission Intensity


c
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Emission Intensity


d
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Emission Intensity


e
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Emission Intensity

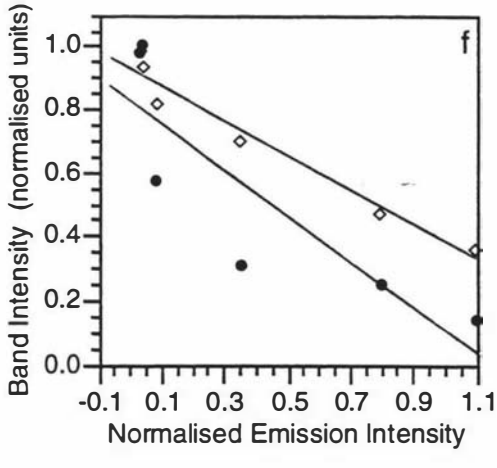
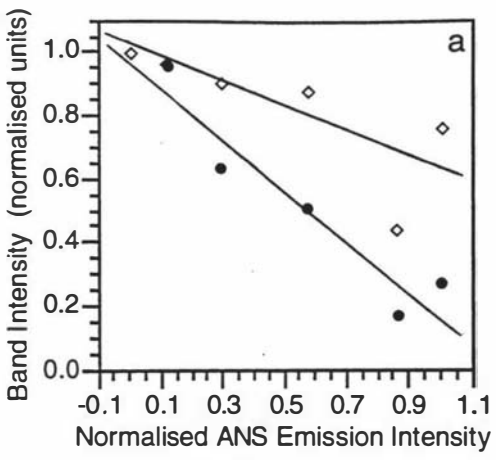
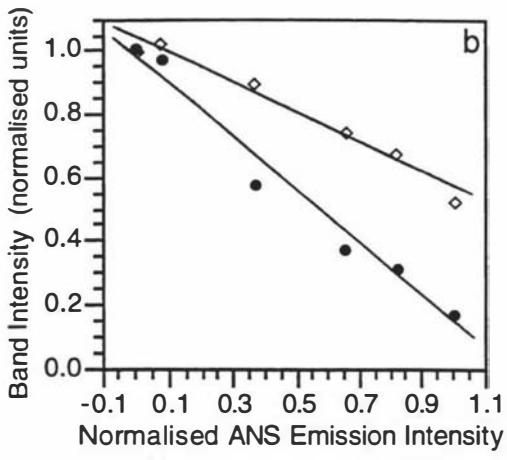
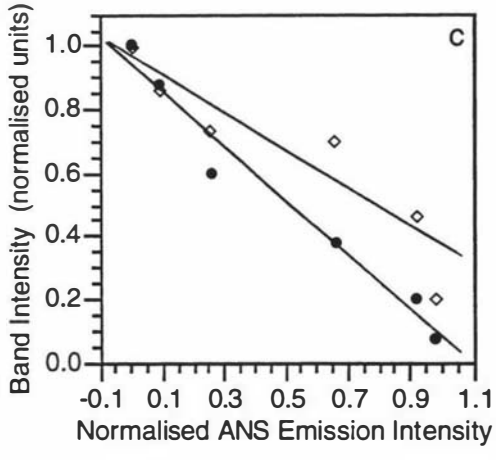
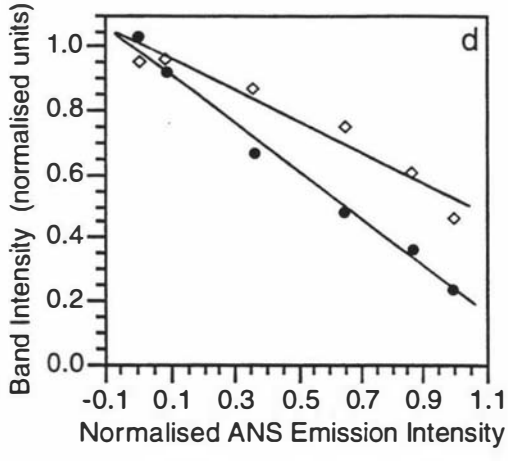

f
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Emission Intensity

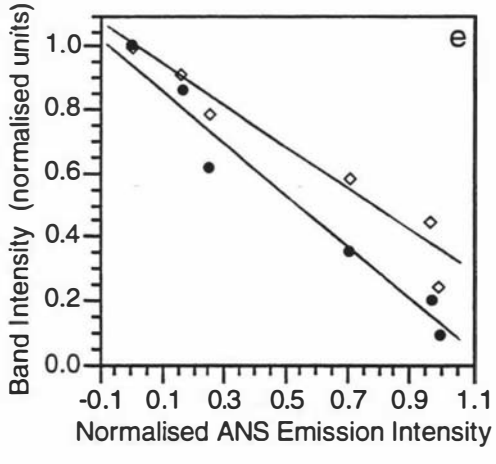
Fig. 5.3.10.4. Plots of normalised I_{ANS} versus normalised alkaline native-PAGE monomer band intensity and versus normalised disulphide-intact SDS-PAGE monomer band intensity for BLGs A, B and C at pH 6.7 and pH 7.4. The plots for alkaline native-PAGE (•) and SDS-PAGE (◇) data for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The data normalisation procedures are the same as those described in Fig. 5.3.10.1. The temperatures at which the BLG samples were heat-treated (Table 5.3.6.1) increase from left to right.


a
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised ANS Emission Intensity


b
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised ANS Emission Intensity


c
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised ANS Emission Intensity


d
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised ANS Emission Intensity


e
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised ANS Emission Intensity

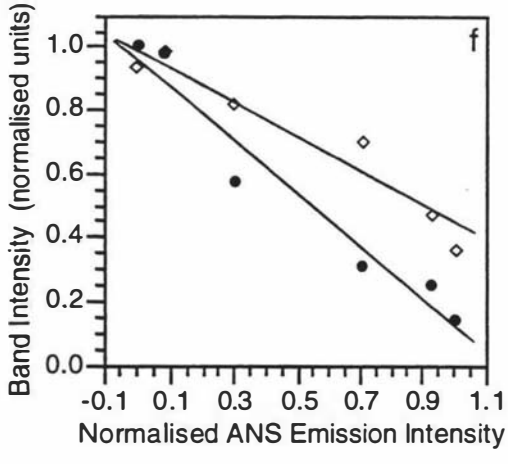
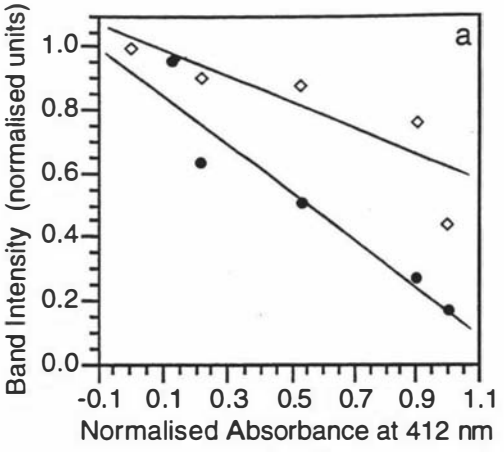
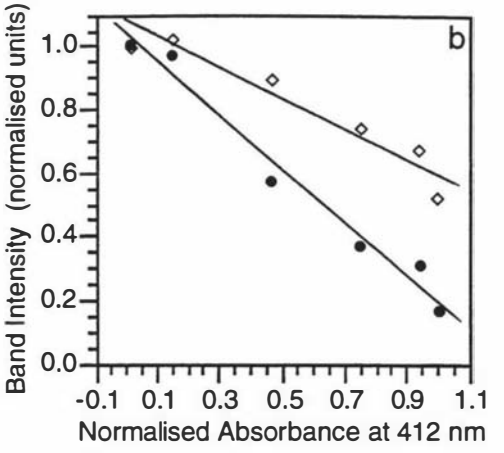
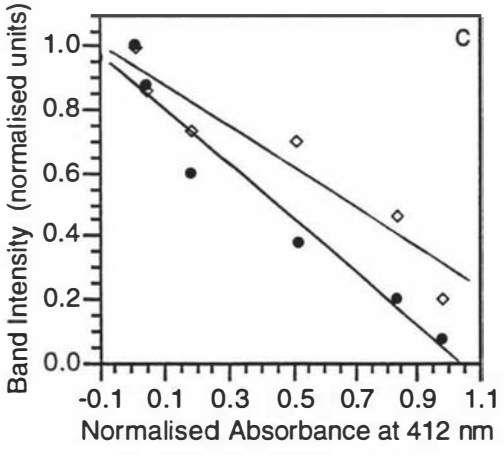
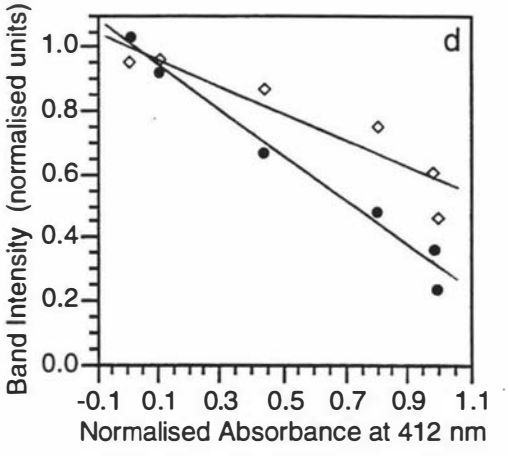

f
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised ANS Emission Intensity

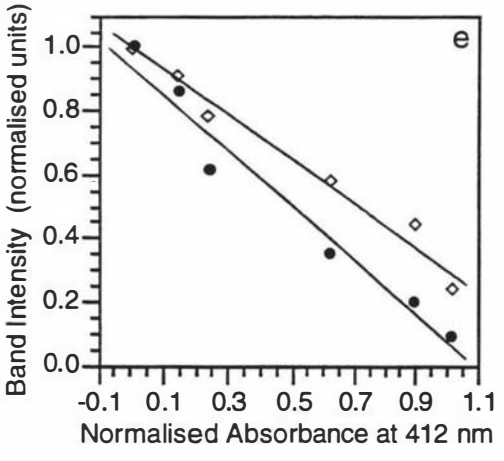
Fig. 5.3.10.5. Plots of normalised A_{412} versus normalised alkaline native-PAGE monomer band intensity and versus normalised disulphide-intact SDS-PAGE monomer band intensity for BLGs A, B and C at pH 6.7 and pH 7.4. The plots for alkaline native-PAGE (•) and SDS-PAGE (◇) data for a particular variant at a particular pH are shown in the same panel, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The data normalisation procedures are the same as those described in Fig. 5.3.10.1. The temperatures at which the BLG samples were heat-treated (Table 5.3.6.1) increase from left to right.


a
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Absorbance at 412 nm


b
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Absorbance at 412 nm


c
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Absorbance at 412 nm


d
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Absorbance at 412 nm


e
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Absorbance at 412 nm

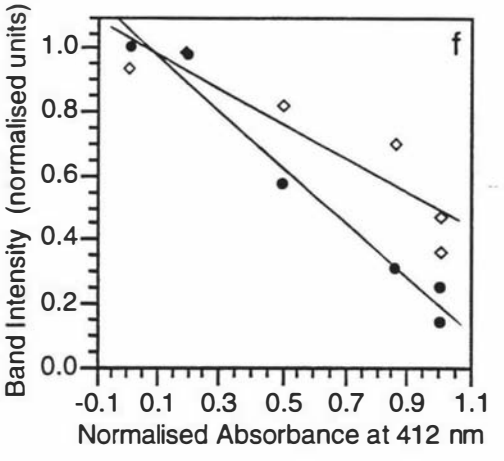

f
Band Intensity (normalised units)
1.0
0.8
0.6
0.4
0.2
0.0
-0.1 0.1 0.3 0.5 0.7 0.9 1.1
Normalised Absorbance at 412 nm

Table 5.3.10.1. Correlation of Normalised $\Delta\varepsilon_{293}$ Data with Normalised Alkaline Native-PAGE and Normalised Disulphide-intact SDS-PAGE Monomer Band Intensity Data.

Sample	Alkaline Native-PAGE		SDS-PAGE		*t* value*	
	Correlation Coefficient (ρ)	Slope of plot	Correlation Coefficient (ρ)	Slope of plot	Alkaline Native-PAGE	SDS-PAGE
pH 6.7, BLG A	0.969	-0.810	0.735	-0.421	7.88	2.17
pH 6.7, BLG B	0.963	-0.841	0.891	-0.626	7.19	3.93
pH 6.7, BLG C	0.984	-0.849	0.954	-0.662	11.04	6.37
pH 7.4, BLG A	0.967	-0.864	0.932	-0.474	7.60	5.13
pH 7.4, BLG B	0.988	-0.766	0.947	-0.475	12.63	5.88
pH 7.4, BLG C	0.967	-0.885	0.915	-0.574	7.60	4.54

* Calculated using the equation below, where ρ is the correlation coefficient and n is the number of data points. A value for *t* greater than 0.792 shows that the correlation is significant at a 95 % confidence level (Walker and Lev, 1969).

$$t = \rho\sqrt{\frac{n - 2}{1 - \rho^2}}$$

Table 5.3.10.2. Correlation of Normalised $[\theta]_{205}$ Data with Normalised Alkaline Native-PAGE and Normalised Disulphide-intact SDS-PAGE Monomer Band Intensity Data.

Sample	Alkaline Native-PAGE		SDS-PAGE		*t* value*	
	Correlation Coefficient (ρ)	Slope of plot	Correlation Coefficient (ρ)	Slope of plot	Alkaline Native-PAGE	SDS-PAGE
pH 6.7, BLG A	0.910	-0.677	0.809	-0.412	4.38	2.76
pH 6.7, BLG B	0.978	-0.970	0.916	-0.762	9.33	4.56
pH 6.7, BLG C	0.890	-0.851	0.960	-0.700	3.90	6.83
pH 7.4, BLG A	0.991	-0.951	0.951	-0.521	14.45	6.13
pH 7.4, BLG B	0.979	-0.897	0.883	-0.540	9.51	3.76
pH 7.4, BLG C	0.961	-1.148	0.935	-0.756	6.95	5.29

* see caption of Table 5.3.10.1.

Table 5.3.10.3. Correlation of Normalised I_{Trp} Data with Normalised Alkaline Native-PAGE and Normalised Disulphide-intact SDS-PAGE Monomer Band Intensity Data.

Sample	Alkaline Native-PAGE		SDS-PAGE		*t* value*	
	Correlation Coefficient (ρ)	Slope of plot	Correlation Coefficient (ρ)	Slope of plot	Alkaline Native-PAGE	SDS-PAGE
pH 6.7, BLG A	0.752	-0.635	0.588	-0.377	2.28	1.45
pH 6.7, BLG B	0.791	-0.767	0.867	-0.621	2.59	3.47
pH 6.7, BLG C	0.792	-0.729	0.885	-0.611	2.60	3.80
pH 7.4, BLG A	0.857	-0.653	0.943	-0.383	3.33	5.68
pH 7.4, BLG B	0.781	-0.620	0.940	-0.431	2.50	5.51
pH 7.4, BLG C	0.731	-0.712	0.956	-0.543	2.14	6.52

* see caption of Table 5.3.10.1.

Table 5.3.10.4. Correlation of Normalised I_{ANS} Data with Normalised Alkaline Native-PAGE and Normalised Disulphide-intact SDS-PAGE Monomer Band Intensity Data.

Sample	Alkaline Native-PAGE		SDS-PAGE		*t* value*	
	Correlation Coefficient (ρ)	Slope of plot	Correlation Coefficient (ρ)	Slope of plot	Alkaline Native-PAGE	SDS-PAGE
pH 6.7, BLG A	0.899	-0.712	0.603	-0.391	4.11	1.51
pH 6.7, BLG B	0.967	-0.856	0.857	-0.624	7.61	3.33
pH 6.7, BLG C	0.964	-0.833	0.944	-0.653	7.24	5.73
pH 7.4, BLG A	0.972	-0.854	0.973	-0.477	8.29	8.37
pH 7.4, BLG B	0.988	-0.766	0.947	-0.475	12.63	5.88
pH 7.4, BLG C	0.948	-0.840	0.936	-0.557	5.94	5.34

* see caption of Table 5.3.10.1.

Table 5.3.10.5. Correlation of Normalised Thiol Availability Data with Normalised Alkaline Native-PAGE and Normalised Disulphide-intact SDS-PAGE Monomer Band Intensity Data.

Sample	Alkaline Native-PAGE		SDS-PAGE		*t* value*	
	Correlation Coefficient (ρ)	Slope of plot	Correlation Coefficient (ρ)	Slope of plot	Alkaline Native-PAGE	SDS-PAGE
pH 6.7, BLG A	0.912	-0.758	0.775	-0.431	4.44	2.45
pH 6.7, BLG B	0.942	-0.867	0.711	-0.659	5.63	4.41
pH 6.7, BLG C	0.962	-0.858	0.975	-0.684	7.07	8.85
pH 7.4, BLG A	0.976	-0.841	0.920	-0.456	8.94	4.69
pH 7.4, BLG B	0.977	-0.715	0.868	-0.427	9.25	3.49
pH 7.4, BLG C	0.975	-0.862	0.847	-0.537	8.85	3.19

* see caption of Table 5.3.10.1.

Normalised disulphide-intact SDS-PAGE monomer band intensity data do not correlate as well with normalised spectroscopic data as do normalised alkaline native-PAGE monomer band intensity data (Tables 5.3.10.1 - 5.3.10.5). This probably reflects the change in slope at normalised $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and A_{412} values (subsequently referred to as *x* axis values) of approximately 0.9 in the plots prepared using disulphide-intact SDS-PAGE data (Figs 5.3.10.1 - 5.3.10.5). Furthermore, the slopes of the plots prepared using disulphide-intact SDS-PAGE data are less than those of the plots prepared using alkaline native-PAGE data at *x* axis values less than approximately 0.9. However, the opposite is true at *x* axis values greater than approximately 0.9.

The lower slopes of the plots of SDS-PAGE data between *x* axis values of 0.0 and 0.9 can be explained in terms of differences in how band intensities on alkaline native and disulphide-intact SDS gels vary with heat treatment temperature. On disulphide-intact SDS gels the monomeric BLG band contains "native-like" monomers (i.e. folded monomers which probably exist as dimers at neutral pH in the absence of SDS), "unfolded" monomers and possible some monomeric material which associates in aggregates in only a non-covalent manner. However, on alkaline native gels the monomeric BLG band contains only "native-like" monomers. Thus, fewer BLG molecules run in bands with mobilities less than that of the monomeric species on disulphide-intact SDS gels than on alkaline native gels, thus explaining why monomer

band intensities are always greater on disulphide-intact SDS gels than on alkaline native gels. Therefore, differences in the nature of species resolved by alkaline native-PAGE and disulphide-intact SDS-PAGE explain why the apparent decrease in normalised monomer band intensity with increasing x axis values between 0.0 and 0.9 is less marked in the plots prepared using normalised disulphide-intact SDS-PAGE data than in those prepared using normalised alkaline native-PAGE data (Figs 5.3.10.1 - 5.3.10.5).

The reason for the change in slope at x axis values of approximately 0.9 in the plots prepared using spectroscopic and SDS-PAGE data (Figs 5.3.10.1 - 5.3.10.5) can be deduced from the plots in Fig. 5.3.9.7, the disulphide-intact SDS gels in Figs 5.3.9.1 and 5.3.9.3 and the alkaline native gels in Figs 5.3.9.4 and 5.3.9.5. The alkaline native gels indicate that concentrations of both "native-like" monomeric and "unfolded" monomeric BLG species decrease with increasing heat treatment temperature between approximately 80 °C and 90 °C. Therefore, alkaline native-PAGE results suggest that the decrease in monomer band intensity with increasing heat treatment temperature between approximately 80 °C and 90 °C observed in the disulphide-intact portions of SDS gels reflects the loss of both "native-like" monomeric and "unfolded" monomeric BLG species. Decreases in monomeric BLG band intensity with increasing heat treatment temperature in this range should therefore be more marked in the disulphide-intact portions of SDS gels than on alkaline native gels. The plots in Fig. 5.3.9.7 indicate that this assumption is correct. This in turn explains why the magnitude of the slopes of the plots shown in Figs 5.3.10.1 - 5.3.10.5 prepared using spectroscopic and SDS-PAGE data increase at an x axis value of about 0.9.

The plots in Figs 5.3.10.1 - 5.3.10.5 show the linear correlation between monomeric BLG band intensity results and spectroscopic results. Therefore, it appears that only the species which run as monomers on alkaline native gels ("native-like" BLG species) give near and far UV CD spectra and tryptophan and ANS fluorescence emission spectra similar to that of native BLG, and possesses a solvent-inaccessible thiol group. For these reasons, it is likely that the presence of aggregate species in heat-treated BLG solutions does not affect the interpretation of $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and thiol availability results in terms of the process which leads to heat-induced irreversible changes in tertiary and secondary structure in BLG molecules. However, it is not possible to ascertain from the results discussed in this section whether there is more than one "native-like" BLG species which runs as a monomer on alkaline native gels, and whether more than one of these species exhibits spectral characteristics similar to those of native BLG. Nevertheless, it is concluded that the majority of spectroscopic results (Sections 5.3.1, 5.3.2, 5.3.4 and 5.3.5) reflect heat-induced irreversible changes in tertiary and secondary structure in BLG molecules.

5.3.10.2. Comparison of $\Delta\varepsilon_{270}$ and PAGE Results.

The trends in the $\Delta\varepsilon_{270}$ data in Section 5.3.1.6 pertaining to the heat-induced formation of non-native disulphide bonds are consistent with the PAGE results in Section 5.3.9. For BLGs A, B and C the extent of change in $\Delta\varepsilon_{270}$ which occurs as a consequence of heat treatment at pH 6.7 is greater than that at pH 7.4. In Section 5.3.1.6 it was suggested that the extent of non-native disulphide bond formation, and therefore aggregation, is less marked at the higher pH. This is consistent with the PAGE results in Sections 5.3.9.2 and 5.3.9.3, which suggest that large (M_r greater than approximately 200 000) disulphide-linked aggregates of all three variants are formed less readily during heat treatment at pH 7.4 than at pH 6.7.

The $\Delta\varepsilon_{270}$ results in Section 5.3.1.6 also indicate that at both pH 6.7 and pH 7.4 the extent of change in $\Delta\varepsilon_{270}$ which occurs as a consequence of heat treatment is less for BLG A than for BLGs B and C. This suggests that the formation of non-native disulphide bonds, and therefore the formation of disulphide-linked aggregates, occurs less readily in solutions of BLG A than in those of BLGs B and C. Similarly, the PAGE results in Sections 5.3.9.2 - 5.3.9.5 suggest that large disulphide-linked aggregates are formed more slowly from BLG A than from BLGs B and C. Furthermore, the plots in Figs 5.3.9.7 suggest that concentrations of non-covalently-linked aggregates are higher in heat-treated solutions of BLG A than in those of BLGs B and C.

Chapter 6.

TIME-DEPENDENT CHANGES IN THE STRUCTURES OF β-LACTOGLOBULINS AT ELEVATED TEMPERATURES.

6.1. INTRODUCTION.

To complement the results in Chapter 5, which describe heat-induced irreversible structural change in molecules of BLGs A, B and C and also aggregate formation in solutions of these variants, the kinetics of heat-induced structural change was examined. Time-dependent changes in $\Delta\varepsilon_{293}$ (i.e. near UV tryptophan CD), tryptophan emission intensity (I_{Trp}), and increases in A_{412} which occur as a consequence of the reaction of DTNB with the thiol group of these variants were followed.

6.2. METHODS.

6.2.1. PREPARATION OF PROTEIN.

Frozen solutions of BLGs A, B and C were thawed, dialysed and filtered (Section 3.1.2.9) and protein concentrations were then calculated from measured A_{280} values (Section 4.2.1.7). Protein concentrations ranged from 2.65 mg/mL to 4.17 mg/mL. However, stock solutions with concentrations of at least 25 mg/mL were required for the measurements made as described below and therefore dialysed solutions of each variant at each pH value were concentrated at 20 °C using the stirred cell ultrafiltration apparatus described in Section 3.1.2.9. Final concentrations, calculated from measured A_{280} values, ranged from 32.4 mg/mL to 46.6 mg/mL. These concentrated solutions were stored at -21 °C.

6.2.2. EXPOSURE OF THE THIOL GROUP OF β-LACTOGLOBULIN TO SOLVENT AT ELEVATED TEMPERATURES.

6.2.2.1. Protocol for Following the Reaction of the Thiol Group of β-Lactoglobulin with DTNB at Elevated Temperatures.

The reaction of the thiol group of BLG with DTNB at elevated temperatures (thiol availability assays) was studied using the stock solutions of BLGs A, B and C prepared as described in Section 6.2.1. Prior to use, these stock solutions were diluted to the concentrations required for assays, 29.5 mg/mL and 33.2 mg/mL for measurements made at pH 6.7 and pH 7.4 respectively, using phosphate buffer of the same pH.

In preparation for assays at pH 6.7, the waterbath was set at 68.5 °C, the temperature which would allow spectrophotometer cell contents to equilibrate to 63.0 °C (see Fig. A1.8 in Appendix 1). The thiol availability assay solution was prepared by mixing 2.55 mL freshly degassed pH 6.7 phosphate buffer and 300 μL freshly prepared 7.77 mM DTNB solution in a 4 mL spectrophotometer cell that had been previously rinsed 10 times with water. The reference sample was prepared by mixing 2.70 mL of pH 6.7 phosphate buffer and 300 μL freshly prepared 7.77 mM DTNB solution in a matching 4 mL spectrophotometer cell that had been previously rinsed in the same way. Both cells were then placed in the cell holder of the spectrophotometer and allowed to equilibrate for 15 min to 63.0 °C. After this time the temperature controller of the bath was adjusted to 65.5 °C so that cell contents would cool to 60.0 °C (see Fig. A1.8 in Appendix 1) and exactly 1 min later the chart recorder was started. The waterbath temperature was adjusted in this manner to minimise the extent of the temperature decrease which occured when BLG solution at room temperatures was added to the pre-warmed assay solution. The selection of conditions which minimise the extent of this decrease are discussed in Section A2.1.5 of Appendix 2.

Exactly 3 min after the temperature of the waterbath had been re-adjusted, 150 μL of a 29.5 mg/mL solution of BLG at pH 6.7 was injected into the sample cell, quickly mixed using a glass rod, and the increase in A_{412} was monitored. Thiol availability assays at pH 6.7 were thus made at a BLG concentration of 1.47 mg/mL in the presence of approximately a 10-fold molar excess of DTNB. Assays for each variant were made in duplicate.

For BLG samples at pH 7.4, thiol availability assays were made in a manner similar to that described above, except that the waterbath was initially set at 57.0 °C, which allows cell contents to equilibrate to 53.0 °C (see Fig. A1.8 in Appendix 1). Then, 3 min before the addition of 33.2 mg/mL BLG solution to the assay mixture, the waterbath was re-set to 54.0 °C so that cell contents would cool to 50.0 °C (see Fig. A1.8 in Appendix 1). The stock BLG solutions were thus diluted to 1.66 mg/mL for assays at pH 7.4. Measurements were also made at 60.0 °C using a solution of BLG A at pH 7.4.

Reduced Glutathione Standard Solutions.

Estimates of the A_{412} values which corresponded to 100 % thiol group exposure were obtained from values for A_{412} for the reaction of DTNB with reduced glutathione solutions which had molar thiol concentrations equivalent to those in the 29.5 mg/mL and 33.2 mg/mL BLG solutions used in pH 6.7 and pH 7.4 thiol availability assays respectively. For pH 6.7 measurements stock reduced glutathione solution, prepared at a concentration of 2.01 mM, was diluted to 1.76 mM with freshly degassed pH 6.7 phosphate buffer. In the case of pH 7.4 measurements stock reduced glutathione

solutions were used undiluted. Determinations were then made using a modified version of the procedure described above; only the final value for A_{412} was measured because reduced glutathione reacts very rapidly with DTNB. On each day that thiol availability assays were made at a given pH the value for A_{412} for the appropriate reduced glutathione standard was determined. Equation 6.2.2.1 was used to calculate the percentage of solvent-exposed thiol groups in BLG solutions at time *t*, (% exposure) after temperature increase:-

$$\text{Percentage of Total Thiols Exposed at Time } t = \frac{A_{412}(\text{Protein Solution at Time } t)}{A_{412}(\text{Reduced Glutathione Standard})} \times 100 \qquad (6.2.2.1)$$

Development of the Protocol for Following the Reaction of the Thiol Group of β-Lactoglobulin with DTNB at Elevated Temperatures.

The development of the protocol for following the reaction of the thiol group of BLG with DTNB at elevated temperatures is discussed in Appendix 2.

6.2.2.2. Additional Assays: The Reaction of the Thiol Group of β-Lactoglobulin with DTNB at 20 °C.

Data were acquired at a cell contents temperature of 20 °C using a procedure similar to that described in Section 6.2.2.1. Estimates of the A_{412} value which corresponded to 100 % thiol group exposure in BLG solutions were obtained using solutions of reduced glutathione as described in Section 6.2.2.1.

6.2.2.3. Additional Assays: Effect of Heat-treating β-Lactoglobulin A Solutions at 60 °C at pH 6.7 for Various Times on Thiol Exposure.

The assays described below were made to determine whether irreversible structural change in BLG molecules which occurs as a consequence of heat treatment at 60 °C could be detected by measuring rates and extents of thiol exposure. Solutions of BLG A at pH 6.7 at 1.47 mg/mL were heat-treated at this temperature in spectrophotometer cells in the temperature-controlled cell holder of the Shimadzu spectrophotometer for 1 min, 3 min, 5 min or 10 min so that thiol availability assays could be started immediately after heat treatment, and so that gelation did not occur during heat treatment. However, because these heat-treated BLG solutions had been diluted prior to the commencement of thiol availability assays, measurements were made using the procedure of Section 6.2.2.1 with the following modifications:

The reference and sample cells, containing 2.70 mL and 2.55 mL pH 6.7 phosphate buffer respectively, were equilibrated to 63 °C in the spectrophotometer cell holder. During thermal equilibration the "baseline" absorbance value was recorded. After 15 min the temperature of the waterbath was re-adjusted so that cell contents would cool to 60 °C. Exactly 3 min later a 150 μL volume of 29.5 mg/mL BLG A solution was added and then quickly mixed into the sample cell as described in Section 6.2.2.1. After protein addition, 300 μL DTNB solution (7.77 mM) was added and mixed into the contents of the reference cell and the chart recorder was started. Exactly 1 min after the BLG A solution had been mixed into the pre-warmed buffer, a 300 μL volume of DTNB solution (7.77 mM) was injected and quickly mixed into the sample cell as described in Section 6.2.2.1. A total of four such assays were made in this manner and in the second, third and fourth assays, the 300 μL volume of DTNB solution was added to the sample cell 3 min, 5 min and 10 min respectively after the BLG A solution had been added to this cell (i.e. BLG A heat treatment periods of 3 min, 5 min and 10 min respectively).

6.2.2.4. Additional Assays: Effect of Heat Treatment at 85 °C.

A thiol availability assay was also made using a solution of BLG A at pH 7.4 previously heat-treated at 85 °C for 12.5 min. Although this solution was heat-treated immediately before assay measurements were made, it was cooled to a temperature below 50 °C before being added to the thiol availability assay solution. This ensured that the results obtained in the assay made using this solution could be compared to those obtained from measurements made as described in Section 6.2.2.1.

6.2.2.5. Reaction Between Solvent-exposed β-Lactoglobulin Thiol Groups and ODNB.

Thiol availabilities in solutions of BLGs A, B and C at pH 6.7 and pH 7.4 were also assayed using the thiol reagent ODNB in a similar manner to that described in Section 6.2.2.1. Solutions of ODNB were prepared daily in a manner similar to that used to prepare DTNB solutions.

6.2.3. TIME-DEPENDENT CHANGES IN $\Delta\varepsilon_{293}$ AT ELEVATED TEMPERATURES.

The stock solutions of BLGs A, B and C at pH 6.7 and pH 7.4 prepared as described in Section 6.2.1 were diluted to 32.4 mg/mL with the appropriate phosphate buffer prior to use. Time-course CD measurements were made at 80 °C and the data for each variant at each pH value were collected in turn.

In preparation for data collection, the waterbath was set at 82.0 °C, the temperature which allows water-jacketed CD cell contents to equilibrate to 81.0 °C (see Fig. A1.4 of Appendix 1). The cell was then rinsed using the procedure described in Section 3.2.4.2, filled with 717 μL of pH 6.7 or pH 7.4 phosphate buffer, and then placed in the cell holder of the spectropolarimeter. After a thermal equilibration period of 10 min, the waterbath was adjusted to 81.0 °C so that the cell contents would cool to 80.0 °C (see Fig. A1.4 of Appendix 1). Exactly 1 min later, 58 μL of a particular BLG variant solution was quickly mixed into the contents of the water-jacketed CD cell, and was thus diluted to 2.42 mg/mL. During the 6 s mixing period the cell was not removed from the cell holder of the spectropolarimeter. Values for CD at 293 nm were then recorded every 10 s for at least 40 min, using a spectral band width of 1.0 nm and a 2 s time constant.

The data for each assay were stored electronically as plots of CD at 293 nm versus time. Measurements on each variant at each pH value were made in duplicate. Before these CD data were analysed, units of optical rotation were converted from millidegrees to molar circular dichroism ($\Delta\varepsilon$), as described in Section 3.2.4.2.

6.2.4. TIME-DEPENDENT INCREASES IN TRYPTOPHAN EMISSION INTENSITY AT ELEVATED TEMPERATURES.

Time-dependent changes in tryptophan emission intensity were measured at 80 °C using the stock solutions of BLGs A, B and C at pH 6.7 or pH 7.4 prepared as described in Section 6.2.1. However, prior to use, samples from each stock solution were diluted to 20.0 mg/mL with pH 6.7 or pH 7.4 phosphate buffer. The data for BLGs A, B and C at a particular pH value were collected in parallel.

Prior to data collection the Neslab waterbath was set at 85.8 °C, the temperature which allows fluorimeter cell contents to equilibrate to 80.0 °C (see Fig. A1.6 in Appendix 1). Fluorimeter cells, previously rinsed using the procedure described in Section 3.2.5.2 and then filled with 2.85 mL of either pH 6.7 or pH 7.4 phosphate buffer, were then placed in positions 2, 3 and 4 of the fluorimeter cell holder and left to equilibrate to 80.0 °C for 15 min. Initially, the excitation shutter of the fluorimeter was left closed and the cell holder was oriented so that the cell in position 4 would be in the light beam when the excitation shutter was opened.

Exactly 14.5 min after the cells had been placed in the fluorimeter cell holder the chart recorder was started. Precisely 30 s later, a 150 μL volume of 20.0 mg/mL BLG A solution at pH 6.7 or pH 7.4 was mixed into the contents of the cell in position 4 of the fluorimeter cell holder as described in Section 6.2.2.1, yielding a final BLG concentration of 1.00 mg/mL. During addition and mixing of BLG solution, which took no longer than 6 s, cells were left in the fluorimeter cell holder. The solution in position 4 was then excited at 295 nm by opening the excitation shutter and the time-dependent change in emission intensity at 336 nm was followed. Measurements were made at sensitivity 5 using excitation and emission band widths of 8 nm. Changes in tryptophan emission intensity were followed for exactly 4.75 min. After this time, the cell in position 3 of the fluorimeter cell block, was shifted into the light beam. The excitation shutter was then closed.

Exactly 5 min after injection of BLG A solution, a 150 μL aliquot of BLG B solution at the same pH as the BLG A solution was quickly mixed into the cell in position 3 and data were collected as described above. Precisely 10 min after the commencement of BLG A measurements, a 150 μL volume of BLG C solution at the same pH as the BLG A and B solutions was quickly mixed into the contents of the cell in position 2 of the fluorimeter cell holder, and tryptophan emission intensity measurements were made as described for BLGs A and B (see above).

Exactly 15 min after the commencement of BLG A measurements, emission intensities from the A, B and C variant solutions were monitored, in turn, for 30 s each. After each 30 s period the fluorimeter cell holder was rotated, bringing the next BLG solution into the light path. Although the excitation shutter was left open during the entire 90 s period, it was closed after measurements on the C variant solution had been made. This procedure was then repeated at 5 min intervals, but 30 s before measurements were made on the solution of BLG A the chart recorder was started so that the baseline emission intensity value could be determined with the excitation shutter closed. Measurements on BLGs A, B and C at each pH value were made in duplicate.

6.3. RESULTS AND DISCUSSION: TIME-DEPENDENT CHANGES IN THE STRUCTURES OF β-LACTOGLOBULINS AT ELEVATED TEMPERATURES.

6.3.1. EXPOSURE OF THE THIOL GROUP OF β-LACTOGLOBULIN TO SOLVENT AT ELEVATED TEMPERATURES.

In the crystal structures of Brownlow *et al.* (1997), Bewley *et al.* (1998) and Qin *et al.* (1998), the thiol group of BLG, that of Cys^{121}, is solvent-inaccessible. However, the results in Section 5.3.5 indicate that a thiol group becomes solvent-exposed as a consequence of heat treatment at temperatures greater than approximately 65 °C at pH 6.7 and 55 °C at pH 7.4 and is available for reaction with DTNB. This is indicative of structural change in BLG molecules. Rates of thiol exposure in BLGs A, B and C, which occurred as a consequence of rapid temperature increase, were measured by following time-dependent increases in A_{412}.

6.3.1.1. Reaction of Reduced Glutathione with DTNB.

Before thiol availability data were collected from solutions of BLG the rate of reaction between solvent-exposed thiol groups and DTNB was measured. In the thiol availability assay procedure described in Section 6.2.2.1, it is assumed that rates of A_{412} increase reflect rates of BLG thiol group exposure and that exposed thiol groups react very rapidly with DTNB (Section 5.3.5.6). The measurements described below were therefore made to confirm that the rate of reaction of DTNB with thiol groups is very rapid compared to the rate of heat-induced BLG thiol group exposure.

Reduced glutathione was used to examine rates of reaction of DTNB with thiol groups because its thiol group is permanently solvent-exposed. The plot in Fig. 6.3.1.1 indicates that the reaction between reduced glutathione and DTNB is essentially complete within the time of mixing (approximately 6 s). This indicates that the reaction between thiol groups and DTNB is very rapid, suggesting that rates of BLG thiol group exposure at elevated temperatures can be measured using the assay procedure described in Section 6.2.2.1.

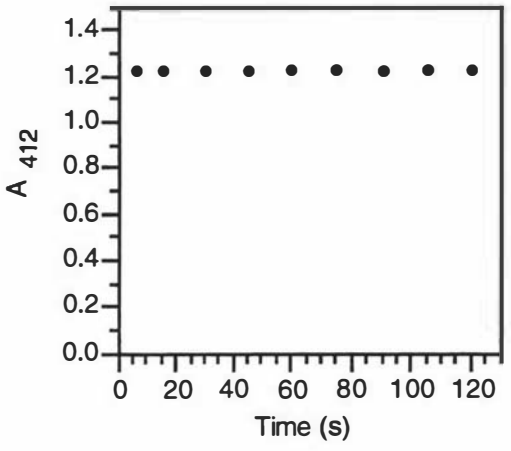


Fig. 6.3.1.1. Reaction of reduced glutathione (88 μM) with DTNB (777 μM) at 60 °C at pH 6.7. A 150 μL volume of stock reduced glutathione solution was mixed into a pre-warmed mixture of pH 6.7 phosphate buffer (2.55 mL) and stock DTNB solution (300 μL) and the increase in A_{412} was followed using the Shimadzu spectrophotometer. Values for A_{412} were measured from chart recordings. The assay procedure is described in full in Section 6.2.2.1.

6.3.1.2. Reaction of the Thiol Group of β-Lactoglobulins with DTNB at 20 °C.

Plots of the time-dependent increase in A_{412} which occurred after solutions of BLGs A, B and C at pH 6.7 and pH 7.4 had been mixed into DTNB solution at 20 °C are shown in Fig. 6.3.1.2.

Because the concentration of thiol groups in the BLG solutions used in these measurements is similar to that in the reduced glutathione solution which gave an A_{412} value of 1.2 (Section 6.3.1.1), the results in Fig. 6.3.1.2 indicate that at 20 °C at pH 6.7 the extent of reaction between the thiol group of BLGs A, B and C and DTNB is low. Therefore, the majority of molecules of BLGs A, B and C do not possess a solvent-exposed thiol group at 20 °C at pH 6.7.

At 20 °C the reaction of DTNB with the thiol group of BLGs A, B and C is slightly faster at pH 7.4 than at pH 6.7 (Fig. 6.3.1.2), suggesting that at this temperature the proportions of BLG molecules which possess a solvent-exposed thiol group (subsequently referred to as reversibly unfolded BLG molecules) are slightly greater at pH 7.4 than at pH 6.7. Therefore, the structures of these variants may be more open at the higher pH. However, the rate of spontaneous BLG thiol group exposure is unlikely to be more rapid at pH 7.4 than at pH 6.7, because an initial rapid increase in A_{412} (a "burst") was not observed in the assay results for BLGs A, B and C at pH 7.4. The BLG solutions used in thiol availability assays were dialysed against pH 7.4 phosphate buffer several days before measurements were made. An initial A_{412} burst should therefore have been observed if the rate of spontaneous thiol exposure was more rapid at pH 7.4 than at pH 6.7.

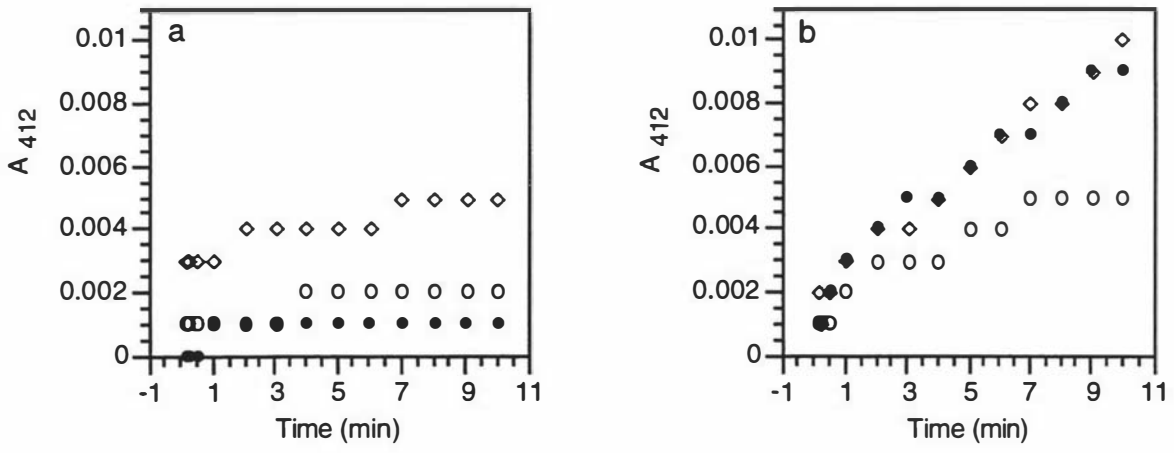


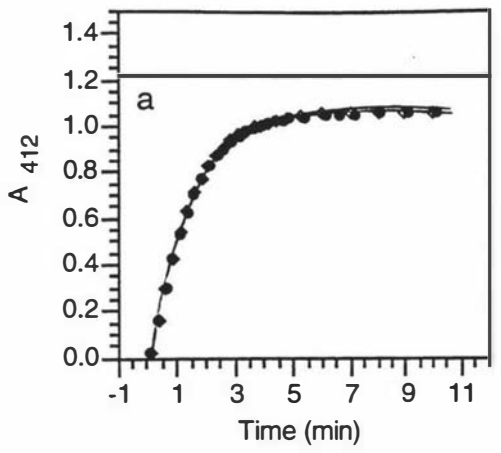
Fig. 6.3.1.2. Reaction of DTNB with the thiol group of BLGs A, B and C at pH 6.7 and pH 7.4 at 20 °C. Reactions were started by mixing 150 μL stock BLG solution into a mixture of pH 6.7 or pH 7.4 phosphate buffer (2.55 mL) and stock DTNB solution (300 μL) at 20 °C and the increase in A_{412} was then followed using the Shimadzu spectrophotometer. The plots are as follows: BLGs A (•), B (◇) and C (o) at pH 6.7, panel a; and at pH 7.4, panel b. Measurements were made using a procedure similar to that described in Section 6.2.2.1.

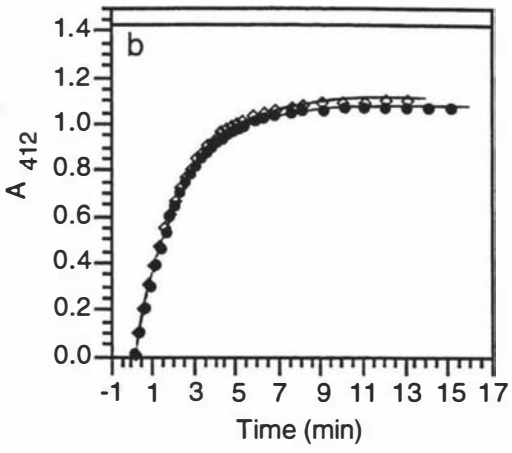
6.3.1.3. β-Lactoglobulin Thiol Exposure at 60 °C at pH 6.7 and at 50 °C at pH 7.4.

Plots of the time-dependent increase in A_{412} which occurred after solutions of BLGs A, B and C at 20 °C at pH 6.7 and pH 7.4 had been mixed into DTNB solution at 60 °C and 50 °C respectively are shown in Fig. 6.3.1.3. The A_{412} value which corresponds to 100 % thiol group exposure for each BLG solution (shown as a horizontal line in each plot) was determined as described in Section 6.2.2.1. The results obtained in a series of preliminary experiments (see Section A2.1.4 in Appendix 2) indicated that 60 °C and 50 °C are appropriate temperatures for measuring rates of BLG thiol group exposure at pH 6.7 and pH 7.4 respectively, in agreement with the results of Iametti *et al.* (1996).

The coincidence of the experimental A_{412} data with the fitted curves for first order kinetics (Fig. 6.3.1.3) suggests that the appropriate reaction kinetics model had been selected. Therefore the fitted values for the parameters k^*, A_{412}(initial) and A_{412}(final) shown in Table 6.3.1.1 can be used as measures of rates and extents of heat-induced thiol group exposure in BLG solutions in the presence of DTNB. In Table 6.3.1.1, errors in the values for k^*, A_{412}(initial) and A_{412}(final) are given. These are standard errors calculated by "Enzfitter" and are likely to be underestimates of the true error in the values for these parameters.

Fig. 6.3.1.3. Reaction of DTNB with the thiol group of BLGs A, B and C at pH 6.7 at 60 °C and at pH 7.4 at 50 °C. Reactions were started by mixing 150 μL stock BLG solution into a mixture of either pH 6.7 phosphate buffer (2.55 mL) and stock DTNB solution (300 μL) at 60 °C, or a mixture of pH 7.4 phosphate buffer (2.55 mL) and stock DTNB solution (300 μL) at 50 °C. The increase in A_{412} was then followed using the Shimadzu spectrophotometer. The plots of duplicate experimental A_{412} data measured from chart recordings, denoted by (•) and (◇), are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The assay procedure is described in full in Section 6.2.2.1. The curves were obtained by fitting experimental A_{412} data to a model for first order reaction kinetics using the computer program "Enzfitter" (Leatherbarrow, 1987). The horizontal line in each plot denotes the A_{412} value which represents 100 % thiol group exposure. These were determined by measuring the extent of A_{412} increase which occurred when a 10-fold molar excess of DTNB was added to solutions of reduced glutathione with thiol concentrations equivalent to those of the BLG solutions used in thiol availability assays (Section 6.2.2.1).


a
A 412
Time (min)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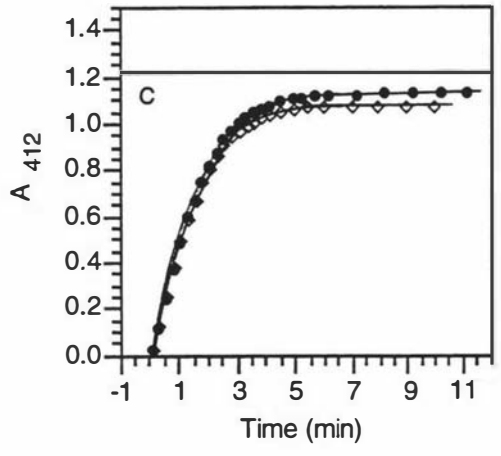
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A 412
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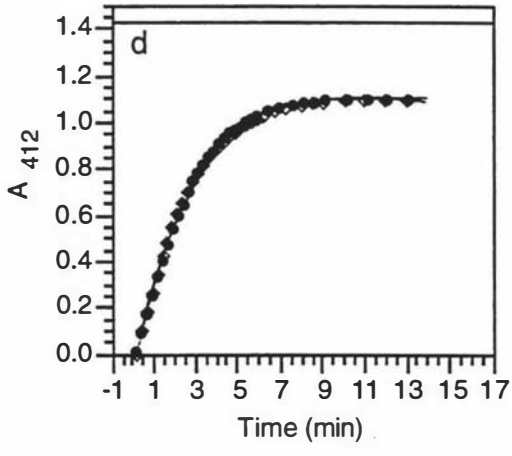
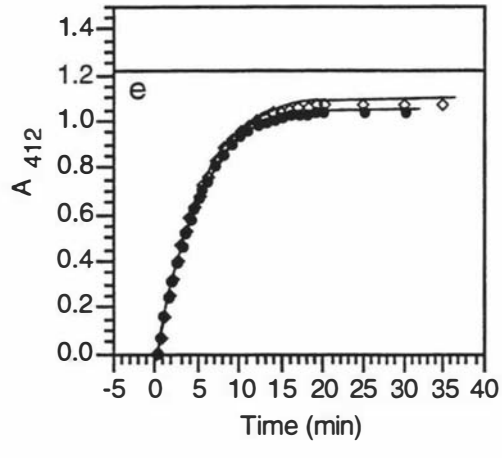

d
A 412
Time (min)


e
A 412
Time (min)

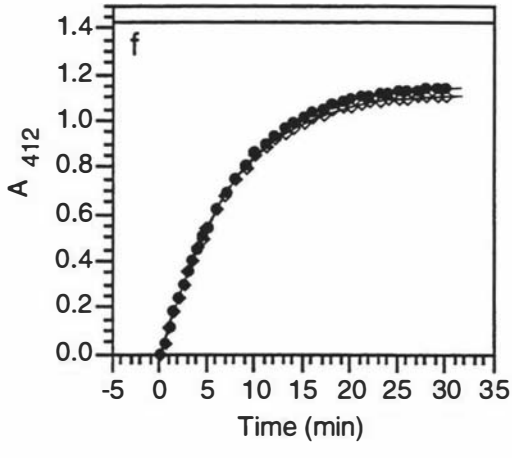

f
A 412
Time (min)

Table 6.3.1.1. Kinetic Parameters for the Exposure of Thiol Groups in Solutions of β-Lactoglobulins A, B and C at 60 °C at pH 6.7 and at 50 °C at pH 7.4.

Sample	k^* (min^{-1})	A_{412}(initial)	A_{412}(final)	k^*(pH 7.4)/k^*(pH 6.7)	% exposure
pH 6.7, BLG A	0.733 ± 0.010	-0.084 ± 0.007	1.065 ± 0.007		86.4
	0.732 ± 0.010	-0.077 ± 0.008	1.079 ± 0.007		86.7
pH 6.7, BLG B	0.655 ± 0.013	-0.085 ± 0.011	1.149 ± 0.011		95.4
	0.668 ± 0.015	-0.089 ± 0.012	1.109 ± 0.015		92.1
pH 6.7, BLG C	0.203 ± 0.002	-0.044 ± 0.003	1.064 ± 0.003		88.4
	0.203 ± 0.001	-0.050 ± 0.003	1.100 ± 0.003		91.4
pH 7.4, BLG A	0.474 ± 0.003	-0.055 ± 0.003	1.080 ± 0.003	0.64	77.6
	0.480 ± 0.003	-0.056 ± 0.003	1.113 ± 0.003	0.65	79.9
pH 7.4, BLG B	0.403 ± 0.006	-0.076 ± 0.007	1.126 ± 0.007	0.61	80.9
	0.405 ± 0.004	-0.063 ± 0.005	1.124 ± 0.005	0.61	80.7
pH 7.4, BLG C	0.132 ± 0.001	-0.034 ± 0.001	1.173 ± 0.002	0.65	84.7
	0.135 ± 0.001	-0.034 ± 0.002	1.143 ± 0.002	0.67	82.1

Where:

k^* is the first order rate constant for the increase in A_{412} and is assumed to be the rate constant for heat-induced BLG thiol group exposure in the presence of DTNB.

A_{412}(initial) and A_{412}(final) are the computer calculated values for A_{412} at zero time and at the conclusion of the assay respectively. The computer program "Enzfitter" (Leatherbarrow, 1987) was used for all fitting.

% exposure is the maximum percentage of BLG molecules in assay solutions whose thiol group reacts with DTNB during assays. Values for % exposure were calculated as described in Section 6.2.2.1. Solutions of each variant at each pH value were assayed in duplicate. The errors in k^*, A_{412}(initial) and A_{412}(final) values are standard errors calculated by "Enzfitter".

A comparison of the plots in Figs 6.3.1.2 and 6.3.1.3 indicates that in the presence of DTNB the extent of exposure of the thiol group of BLGs A, B and C at 20 °C at pH 6.7 and pH 7.4 after 10 min is approximately 1 % of the maximum extent of thiol group exposure that occurs during heat treatment at 60 °C at pH 6.7 and at 50 °C at pH 7.4 (i.e. the contribution of the rate of thiol group exposure at 20 °C is negligible).

The small negative values for A_{412}(initial) in Table 6.3.1.1 suggest that the proportion of BLG molecules which possessed a solvent-exposed thiol group in solutions of all three variants at both pH 6.7 and pH 7.4 was initially very low. If thiol groups in these solutions had been exposed prior to temperature increase, they would have reacted very rapidly with the DTNB, causing an initial A_{412} "burst".

At 60 °C at pH 6.7 in the presence of DTNB, the values for k^* for thiol group exposure for BLGs A and B are approximately 3 - 4 × greater than those for BLG C (Table 6.3.1.1). This is also true at pH 7.4 at 50 °C. Similar differences in the rates of thiol group exposure for BLGs A and B and BLG C were reported by Hill *et al.* (1996). β-Lactoglobulin C therefore appears to be significantly less susceptible to heat-induced structural change in the vicinity of Cys^{121} than BLGs A and B. The significance of this will be discussed in Section 8.5. Although rates of BLG thiol group exposure at pH 6.7 and pH 7.4 were measured at different temperatures the fact that the values for k^* for all three variants at pH 7.4 are approximately 65 % of those at pH 6.7 (Table 6.3.1.3) suggests that the susceptibilities of BLGs A, B and C to heat-induced structural change show a similar dependence on pH between 6.7 and 7.4.

At the conclusion of the assays at pH 6.7, the thiol group in approximately 90 % of molecules of BLGs A, B and C had reacted with DTNB (Table 6.3.1.1). These values are consistent with the results of Iametti *et al.* (1996), who reported that one thiol group per BLG monomer reacts with DTNB during heat treatment at approximately 60 °C. At pH 7.4 the thiol group in approximately 80 % of molecules of BLGs A, B and C had reacted with DTNB by the conclusion of thiol availability assays (Table 6.3.1.1). The lower proportion of BLG thiol groups which reacted with DTNB at pH 7.4 is likely to reflect the instability of TNB at pH 7.4 (Section 5.3.5.4).

Although the thiol group in fewer than 100 % of BLG molecules had reacted with DTNB by the conclusion of thiol availability assays at both pH 6.7 and pH 7.4 (Table 6.3.1.1), it is unlikely that thiol oxidation had occurred before measurements were made. This is because the results in Section 5.3.5.4 indicate that at pH 6.7 in the presence of 5.0 M urea, the thiol group in close to 100 % of BLG B molecules is available for reaction with DTNB. Therefore, the 10 % - 20 % of BLG thiol groups which did not react with DTNB may have been oxidised during thiol availability assays, rather than before.

6.3.1.4. β-Lactoglobulin A Thiol Exposure at pH 7.4 at 60 °C.

Because thiol availabilities in BLG solutions at pH 6.7 and pH 7.4 were assayed at different temperatures, the effect of pH on rates of thiol exposure could not be investigated quantitatively by comparing these assay results. However, a brief quantitative examination of the effect of pH on these rates was made by following time-dependent increases in thiol group exposure in a solution of BLG A at pH 7.4 at 60 °C, the temperature at which pH 6.7 measurements were normally made. At 60 °C the reaction of the thiol group of BLG A with DTNB occurs approximately 5 × more rapidly at pH 7.4 than at pH 6.7 (Fig. 6.3.1.4, Table 6.3.1.2). This suggests that BLG A is more susceptible to heat-induced structural change at pH 7.4 than at pH 6.7. These results are consistent with those of Kella and Kinsella (1988a). Similarly, the results of Chapter 5 indicate that BLGs A, B and C are more susceptible to heat-induced irreversible structural change at pH 7.4 than at pH 6.7.

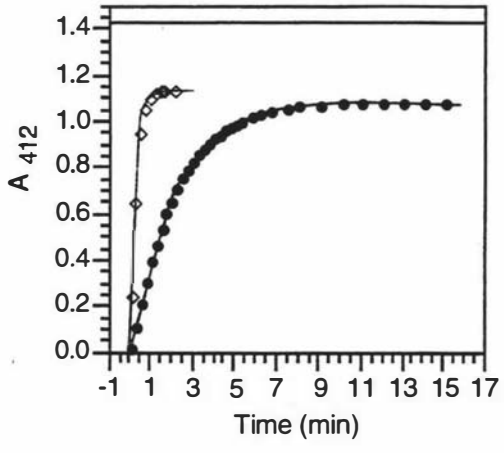


Fig. 6.3.1.4. Reaction of DTNB with the thiol group of BLG A at pH 7.4 at 50 °C and at pH 7.4 at 60 °C. Reactions were started by mixing 150 µL stock BLG solution at pH 7.4 into a mixture of pH 7.4 phosphate buffer (2.55 mL) and stock DTNB solution (300 µL) at either 50 °C (•) or 60 °C (◇). The increase in A_{412} was then followed using the Shimadzu spectrophotometer. The assay procedure is described in full in Section 6.2.2.1. The symbols denote experimental A_{412} data measured from chart recordings. The curves were obtained by fitting experimental A_{412} data to a model for first order kinetics using the computer program "Enzfitter" (Leatherbarrow, 1987). See Fig. 6.3.1.3 for the significance of the horizontal line in the plots.

Table 6.3.1.2. Kinetic Parameters for the Exposure of Thiol Groups in Solutions of β-Lactoglobulin A at pH 6.7 at 60 °C, at pH 7.4 at 60 °C and at pH 7.4 at 50 °C.

Sample	k^* (min^{-1})	A_{412}(initial)	A_{412}(final)	% exposure
pH 6.7, 60 °C #	0.733 ± 0.010	-0.084 ± 0.007	1.065 ± 0.007	86.4
pH 6.7, 60 °C #	0.732 ± 0.010	-0.077 ± 0.008	1.079 ± 0.007	87.6
pH 7.4, 60 °C	3.760 ± 0.088	-0.017 ± 0.015	1.135 ± 0.014	81.1
pH 7.4, 50 °C #	0.474 ± 0.003	-0.055 ± 0.003	1.080 ± 0.003	77.6
pH 7.4, 50 °C #	0.480 ± 0.003	-0.056 ± 0.003	1.113 ± 0.003	79.9

The kinetic parameters for these duplicate runs are also shown in Table 6.3.1.1.

Definitions for k^*, A_{412}(initial), A_{412}(final) and % exposure are given in the caption of Table 6.3.1.1. The errors in k^*, A_{412}(initial) and A_{412}(final) values are standard errors calculated by "Enzfitter" (Leatherbarrow, 1987).

For BLG A at pH 7.4 the rate constant for thiol group exposure at 60 °C is approximately 8 × greater than that at 50 °C (Table 6.3.1.2). This confirms the report of Iametti *et al.* (1996) that rates of thiol exposure for BLG A/B increase with increasing temperature between 40 °C and 70 °C.

By the conclusion of the assays at both 50 °C and 60 °C, the thiol group in close to 80 % of the BLG A molecules at pH 7.4 had reacted with DTNB (Table 6.3.1.2). This suggests that temperature increase from 50 °C to 60 °C has no appreciable effect on the extent of thiol oxidation which occurs during thiol availability assays.

The thiol availability assay results in this chapter differ appreciably to those in Section 5.3.5. **During** heat treatment in the presence of DTNB for 12.5 min at 60 °C at pH 6.7 or at 50 °C at pH 7.4, the thiol group in 80 % - 90 % of molecules of BLGs A, B and C becomes solvent-exposed and reacts with DTNB. In contrast, **after** heat treatment at 60 °C at pH 6.7 or at 50 °C at pH 7.4, the proportion of BLG A, B and C molecules in heat-treated solutions which possess a permanently solvent-exposed thiol group is negligible (Fig. 5.3.5.2). These differences can be explained in terms of the reaction of solvent-exposed BLG thiol groups with DTNB during heat treatment. Under the conditions in which thiol availability assays (this section) were made, equilibrium between reversibly unfolded and folded BLG species was not established because the reversibly unfolded species was removed from the system as a consequence

of the reaction with DTNB (Section 5.3.5.6). Therefore, after sufficiently long heat treatment times, for example approximately 12.5 min at 60 °C at pH 6.7, the thiol group in the majority of BLG molecules had reacted with DTNB (Fig. 6.3.1.3); extents of thiol group exposure are increased as a consequence of heat treatment in the presence of DTNB. For the same reasons thiol oxidation, which may have occurred during thiol availability assays, would also prevent equilibrium between reversibly unfolded and folded BLG species from being established.

The results in Section 5.3.5, on the other hand, suggest that BLG thiol group exposure at 60 °C at pH 6.7 and at 50 °C at pH 7.4 is reversible in the absence of DTNB. This is because the proportions of BLG molecules which possess a permanently solvent-exposed thiol group after heat treatment under such conditions are negligible. Alternatively, the results in Section 5.3.5 may indicate that BLG thiol groups do become solvent-exposed during heat treatment in the absence of DTNB, but are oxidised. Therefore, it may be impossible to detect irreversible structural change which occurs as a consequence of heat treatment under the above conditions by reacting BLG molecules with DTNB. The possibility that irreversible structural change in molecules of BLG A occurs as a consequence of heat treatment at 60 °C at pH 6.7 is examined in Section 6.3.1.5.

6.3.1.5. Effect of Heat-treating β-Lactoglobulin A Solutions at 60 °C at pH 6.7 for Various Times on Thiol Exposure.

Thiol availability assays were made using solutions of BLG A previously heat-treated at 60 °C at pH 6.7 for various times to determine whether irreversible structural change occurs as a consequence of heat treatment under these conditions. If, as suggested in Section 6.3.1.4, thiol group exposure at 60 °C is reversible, then initial A_{412} bursts should not be observed during assays made using BLG solutions previously heat-treated at this temperature. Alternatively, if thiol groups are oxidised during heat treatment at 60 °C (Section 6.3.1.4), then the thiol group in fewer than approximately 90 % of BLG molecules in solutions previously heat-treated at this temperature should have reacted with DTNB by the conclusion of thiol availability assays.

Plots of the time-dependent increase in A_{412} for the reaction of DTNB with the thiol group of heat-treated BLG A are shown in Fig. 6.3.1.5. Because these BLG solutions were heat-treated at 1.47 mg/mL (the pH 6.7 thiol availability assay concentration) in the temperature-controlled cell holder of the Shimadzu spectrophotometer, assays could not be started by adding a small volume of BLG solution to pre-warmed DTNB solution. However, by diluting BLG solutions to this concentration, gelation did not occur during heat treatment and assays could be started

immediately after the desired heating period. The coincidence of the experimental A_{412} data in Fig. 6.3.1.5 with the fitted curves for first order kinetics suggests that, after heat treatment at 60 °C, thiol group exposure in BLG A at this temperature still follows first order kinetics. The fitted values for the parameters k^*, A_{412}(initial) and A_{412}(final) for these data are shown in Table 6.3.1.3.

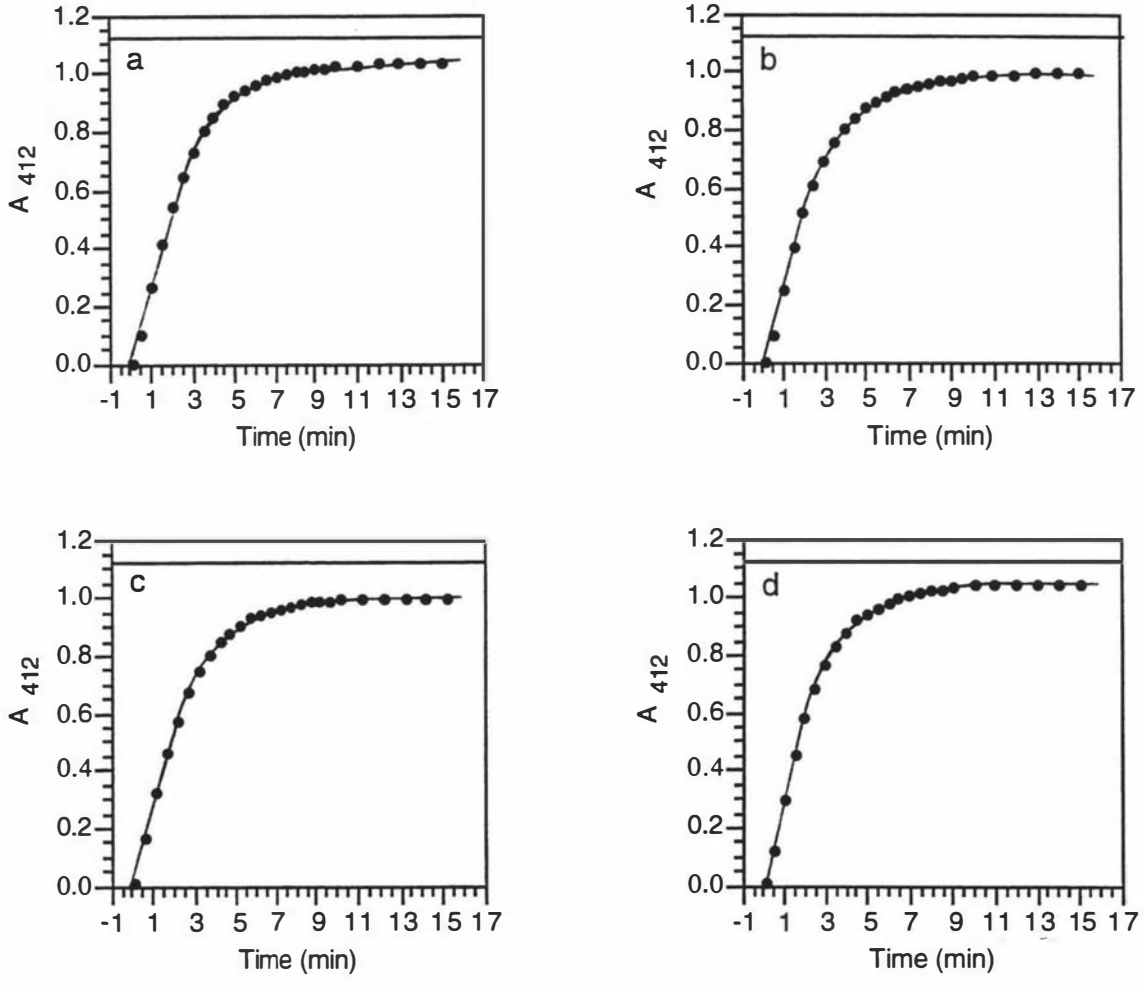


Fig. 6.3.1.5. Reaction of DTNB with the thiol group of BLG A at pH 6.7 at 60 °C after heat treatment at this temperature for various times. Reactions were started by mixing 300 µL stock DTNB solution into 1.47 mg/mL solutions of BLG A (2.70 mL) at 60 °C previously heat-treated at this concentration at this temperature for 1 min, panel a; 3 min, panel b; 5 min, panel c; and 10 min, panel d. The increase in A_{412} was then followed using the Shimadzu spectrophotometer. The assay procedure is described in full in Section 6.2.2.3. The symbols denote experimental A_{412} data measured from chart recordings. The curves were obtained by fitting experimental A_{412} data to a model for first order kinetics using the computer program "Enzfitter" (Leatherbarrow, 1987). See Fig. 6.3.1.3 for the significance of the horizontal line in each plot.

Table 6.3.1.3. Kinetic Parameters for the Exposure of Thiol Groups in Solutions of β-Lactoglobulin A at pH 6.7 Previously Heat-treated for Various Times at 60 °C.

Heating Time (min)	k^* (min^{-1})	A_{412}(initial)	A_{412}(final)	% exposure
1	0.427± 0.008	-0.096 ± 0.010	1.041 ± 0.010	92.9
3	0.419 ± 0.006	-0.077 ± 0.007	0.999 ± 0.007	89.2
5	0.447 ± 0.008	-0.094 ± 0.009	1.006 ± 0.009	89.8
10	0.459 ± 0.006	-0.072 ± 0.007	1.051 ± 0.007	93.8

Definitions for k^*, A_{412}(initial), A_{412}(final) and % exposure are given in the caption of Table 6.3.1.1. The errors in k^*, A_{412}(initial) and A_{412}(final) values are standard errors calculated by "Enzfitter" (Leatherbarrow, 1987).

Initial A_{412} bursts are not observed in any of the plots in Fig. 6.3.1.5 and all of the values for A_{412}(initial) in Table 6.3.1.3 are small. This suggests that the proportions of BLG molecules which possess a permanently solvent-exposed thiol group in solutions previously heat-treated at 60 °C for up to 10 min are negligible prior to DTNB addition. Furthermore, the rate constants in Table 6.3.1.3 are similar, suggesting that heat treatment at 60 °C for periods of up to 10 min does not have an appreciable effect on rates of thiol group exposure during subsequent periods of heat treatment at this temperature.

In the solutions of BLG A at pH 6.7 previously heat treated at 60 °C for 1 min, 3 min, 5 min and 10 min, the thiol group in approximately 90 % of protein molecules had reacted with DTNB by the conclusion of assays (Table 6.3.1.3). These percentages are similar to those for unheated BLG A at pH 6.7 (Table 6.3.1.1), suggesting that heat-treatment at 60 °C at pH 6.7 for periods of up to approximately 10 min does not lead to a decrease in the proportion of BLG A molecules which possess a thiol group available for reaction with DTNB.

The results in Fig. 6.3.1.5 and Table 6.3.1.3 suggest that irreversible structural change (as indicated by an initial A_{412} burst, a change in the rate constant for thiol group exposure, or a decrease in the proportion of thiol groups which become available for reaction with DTNB during assays) does not occur as a consequence of heat treatment at 60 °C at pH 6.7. Therefore, it is concluded that any thiol group exposure which occurs during heat treatment at this temperature at this pH is reversible.

6.3.1.6. Effect of Heat-treating β-Lactoglobulin A Solutions at 85 °C at pH 7.4 for 12.5 Minutes on Thiol Exposure.

The effect of heat treatment at 85 °C at pH 7.4 for 12.5 min on rates and extents of thiol group exposure in a solution of BLG A was examined briefly because the results presented in Section 5.3.5 indicate that extents of irreversible thiol group exposure are appreciable after heat treatment at this temperature and pH. In thiol availability assays, this phenomenon was expected to be reflected in an initial A_{412} burst and an A_{412}(final) value that would indicate that fewer than 80 % - 90 % of BLG molecules in the heat-treated solution possessed a thiol group available for reaction with DTNB.

A plot of the time-dependent increase in A_{412} for the reaction of DTNB with the thiol groups in the heat-treated BLG solution is shown in Fig. 6.3.1.6. Measurements were made as outlined in the caption of this figure because the BLG solution was diluted to the concentration at which assays were made (1.66 mg/mL) so that gelation did not occur during heat treatment at 85 °C. Within the 4.5 s mixing period, the value for A_{412} had increased to 0.64, in agreement with the A_{412}(initial) value (0.65, Table 6.3.1.4) obtained from a fit of experimental A_{412} data to a model for first order kinetics. This A_{412}(initial) value suggests that prior to the addition of DTNB a large proportion of the molecules of BLG A in the heat-treated solution possessed a solvent-exposed thiol group, which had probably become exposed as a consequence of heat-treatment. The results in Fig. 6.3.1.6 and Table 6.3.1.4 are therefore consistent with those in Section 5.3.5, which indicate that at pH 7.4, the thiol group of BLG A becomes irreversibly solvent-exposed as a consequence of heat treatment at temperatures greater than 51 °C (Fig. 5.3.5.2).

The thiol group in 66 % of the BLG A molecules in the solution heat-treated at 85 °C had reacted with DTNB by the conclusion of the assay (Table 6.3.1.4). This value is lower than that for unheated BLG A at pH 7.4 (78.7 %, Table 6.3.1.4). Thus, the number of BLG A molecules which possess a thiol group appears to decrease as a consequence of heat treatment at 85 °C at pH 7.4 for 12.5 min. This suggests that thiol oxidation occurs during heat treatment under these heating conditions. Similar conclusions were drawn in Section 5.3.5.4 for BLG solutions previously heat-treated at approximately 85 °C.

For the solution of BLG A previously heat-treated at pH 7.4 at 85 °C for 12.5 min, the rate constant for thiol group exposure is greater than that for the corresponding unheated solution. This may indicate that the thiol groups in BLG A solutions previously heat-treated in this manner are exposed more rapidly during subsequent heat treatment periods than in those in the corresponding unheated solutions. Therefore, heat treatment at pH 7.4 at 85 °C for 12.5 min leads to irreversible structural change in BLG molecules.

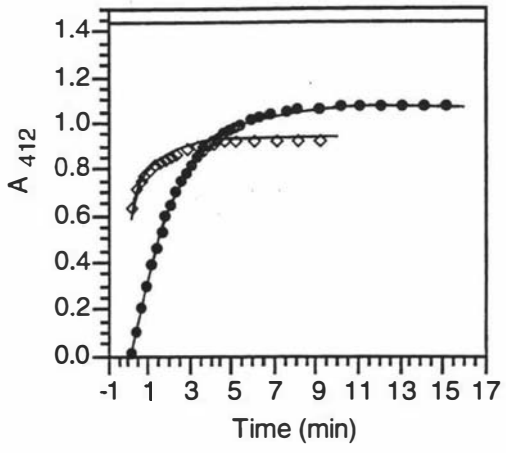


Fig. 6.3.1.6. Reaction of DTNB with the thiol group of unheated BLG A at pH 7.4 and BLG A previously heat-treated at this pH at 85 °C for 12.5 min. For the heat-treated solution (◇) the reaction was started by mixing 300 μL stock DTNB solution into 1.66 mg/mL BLG A solution (2.70 mL) at 50 °C (see Section 6.2.2.4 for additional detail). For the unheated solution (•) the reaction was started as described in the caption of Fig. 6.3.1.3. Increases in A_{412} were then followed using the Shimadzu spectrophotometer. The symbols denote experimental A_{412} data measured from chart recordings. The curves were obtained by fitting experimental A_{412} data to a model for first order kinetics using the computer program "Enzfitter" (Leatherbarrow, 1987). See Fig. 6.3.1.3 for the significance of the horizontal line in the plot.

Table 6.3.1.4. Kinetic Parameters for the Exposure of Thiol Groups at pH 7.4 and 50 °C in Solutions of Unheated and Heat-treated β-Lactoglobulin A.

Sample	k^* (min^{-1})	A_{412}(initial)	A_{412}(final)	% exposure
Heat-treated BLG A	0.678 ± 0.022	0.650 ± 0.004	0.929 ± 0.004	66.4
Unheated BLG A	0.474 ± 0.003	-0.055 ± 0.003	1.080 ± 0.003	78.7*

* Average of the duplicate values shown in Table 6.3.1.1.

Definitions for k^*, A_{412}(initial), A_{412}(final) and % exposure are given in the caption of Table 6.3.1.1. The errors in k^*, A_{412}(initial) and A_{412}(final) values are standard errors calculated by "Enzfitter" (Leatherbarrow, 1987).

6.3.1.7. Reaction of the β-Lactoglobulin Thiol Group with ODNB.

The thiol group in a maximum of approximately 90 % and 80 % of BLG molecules in solutions at pH 6.7 and pH 7.4 respectively becomes exposed during heat treatment and reacts with DTNB (Fig. 6.3.1.3 and Table 6.3.1.1). To confirm that DTNB had reacted with all of the thiol groups present in these solutions another series of assays was made using the thiol reagent ODNB.

Faulstich *et al.* (1993) reported that ODNB, in which one thionitrobenzoate group is substituted by an *n*-octyl chain, can react with partially buried thiol groups which are inaccessible to DTNB. Furthermore, the reaction of protein thiol groups with ODNB is usually more rapid than with DTNB. The differences in the reactivities of these two thiol reagents may reflect the greater hydrophobicity and lower negative charge of ODNB (Faulstich *et al.* 1993), which allows molecules of ODNB to enter partially folded protein structures. Therefore, the thiol group in the 10 % - 20 % of BLG molecules that was unavailable for reaction with DTNB may be available for reaction with ODNB.

Kinetic data obtained from ODNB assays were not interpreted quantitatively because they could not be fitted to a model for first order reaction kinetics or any of the other reaction kinetics models offered by "Enzfitter" (see Fig. 6.3.1.7 for an example). The assay data acquired using ODNB are difficult to fit because the kinetics of the reaction between asymmetric disulphides and thiol groups are extremely complex (Brocklehurst, 1979).

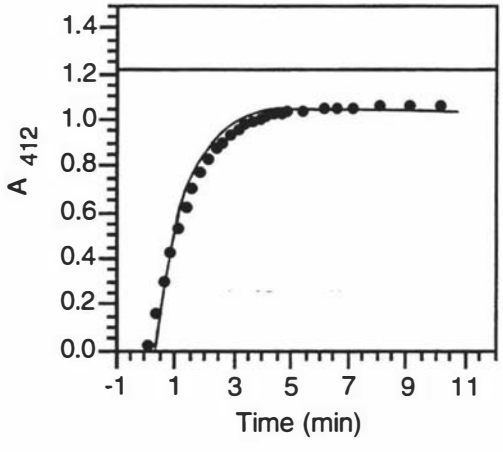


Fig. 6.3.1.7. Reaction of ODNB with the thiol group of BLG A at pH 6.7 at 60 °C. The reaction was started by mixing 150 μL stock BLG solution into a mixture of pH 6.7 phosphate buffer (2.55 mL) and stock ODNB solution (300 μL) at 60 °C. The increase in A_{412} was then followed using the Shimadzu spectrophotometer. The assay procedure is described in full in Sections 6.2.2.1 and 6.2.2.5. The symbols denote experimental A_{412} data measured from chart recordings. The curve was obtained by fitting experimental A_{412} data to a model for first order kinetics using the computer program "Enzfitter" (Leatherbarrow, 1987).

6.3.1.7.1. Reaction of Reduced Glutathione with ODNB.

The reaction of ODNB with reduced glutathione was followed to determine whether the rate of the reaction of thiol reagent with thiol group is very rapid compared to that for the exposure of the thiol group of BLG to the solvent.

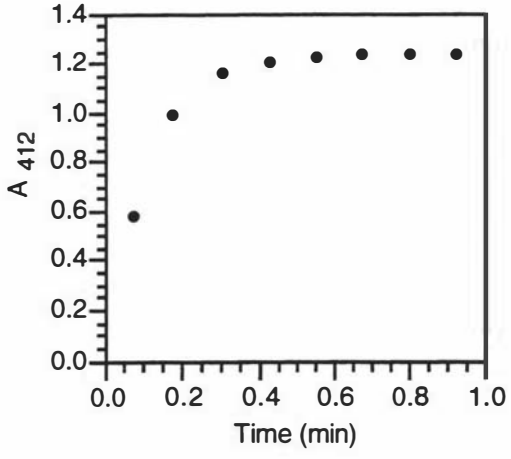


Fig. 6.3.1.8. Reaction of ODNB (777 μM) with reduced glutathione (88 μM) at 60 °C at pH 6.7. A 150 μL volume of stock reduced glutathione solution was mixed into a pre-warmed mixture of pH 6.7 phosphate buffer (2.55 mL) and stock ODNB solution (300 μL) and the increase in A_{412} was followed using the Shimadzu spectrophotometer. The assay procedure is described in full in Sections 6.2.2.1 and 6.2.2.5.

About 45 s was required for the reaction between reduced glutathione and ODNB to go essentially to completion (Fig. 6.3.1.8). Therefore the time required for the reaction of solvent-exposed thiol groups with ODNB to go to completion is longer than that for the reaction of solvent-exposed thiol groups with DTNB. This complicates the kinetic analysis of ODNB assay data further because ODNB reacts with solvent-exposed thiol groups (Fig. 6.3.1.8) and the thiol group of BLG (Fig. 6.3.1.7) at similar rates.

6.3.1.7.2. Reaction of the β-Lactoglobulin Thiol Group with ODNB at Elevated Temperatures.

Plots of A_{412} versus time for ODNB assays made using solutions of BLGs A, B and C at pH 6.7 and pH 7.4 are shown in Fig. 6.3.1.9. The A_{412} values increase from approximately zero, suggesting that in all twelve unheated solutions the proportion of BLG molecules which possessed a solvent-exposed thiol group was negligible, in agreement with the results obtained using DTNB.

At both pH 6.7 and pH 7.4, the thiol group in approximately 80 % of BLG molecules had reacted with ODNB by the conclusion of assays (Fig 6.3.1.9). This proportion is approximately 10 % lower and equivalent to that determined using DTNB

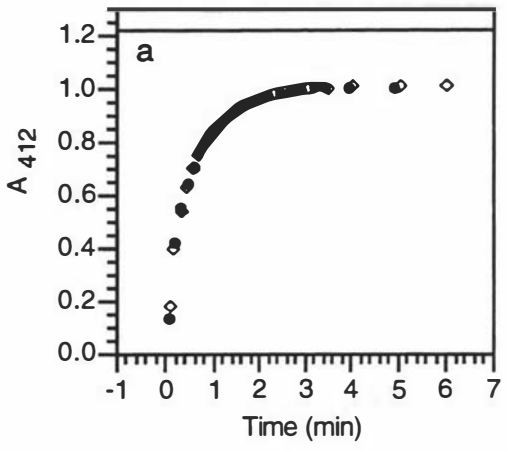
thiol availability assay data at pH 6.7 and pH 7.4 respectively. The proportions of thiol groups in solutions of BLGs A, B and C available for reaction with ODNB are therefore no greater than those available for reaction with DTNB. This suggests that during heat treatment at 60 °C at pH 6.7 and at 50 °C at pH 7.4 the proportions of molecules of BLGs A, B and C whose thiol group was unavailable for reaction with DTNB were very low, and therefore that the 10 % - 20 % of thiol groups in BLG solutions unavailable for reaction with both DTNB and ODNB were oxidised during assays at 60 °C and 50 °C.

6.3.1.8. Thiol Availability Discussion.

The results in Section 6.3.1.3 suggest that the thiol group of BLG becomes solvent-exposed during heat treatment at 60 °C at pH 6.7 and at 50 °C at pH 7.4. Therefore, because Qi *et al.* (1997) have reported that the amount of α-helix in molecules of BLG does not start to decrease until temperatures are raised above 60 °C at pH 6.75, the thiol availability assay results suggest that thiol exposure occurs as a consequence of a peeling of the α-helix from the exterior of the BLG calyx (Section 5.3.5.3). Thus it is likely that thiol availability assay results describe heat-induced structural change primarily in the vicinity of Cys^{121}.

The maximum extent of thiol group exposure in solutions of BLGs A, B and C which occurs **during** heat treatment at 60 °C at pH 6.7 in the presence of DTNB (Section 6.3.1.3) is significantly greater than the extent of irreversible thiol group exposure which occurs **as a consequence** of heat treatment under these conditions in the absence of DTNB (Section 5.3.5.4). These differences arise because reversibly exposed thiol groups react with DTNB and are oxidised to disulphide groups during thiol availability assays.

Fig. 6.3.1.9. Reaction of ODNB with the thiol group of BLGs A, B and C at pH 6.7 at 60 °C and at pH 7.4 at 50 °C. Reactions were started by mixing 150 μL stock BLG solution into either a mixture of pH 6.7 phosphate buffer (2.55 mL) and stock ODNB solution (300 μL) at 60 °C, or a mixture of pH 7.4 phosphate buffer (2.55 mL) and stock ODNB solution (300 μL) at 50 °C. The increase in A_{412} was then followed using the Shimadzu spectrophotometer. The plots of duplicate experimental A_{412} data measured from chart recordings, denoted by (•) and (◇), are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The assay procedure is described in full in Sections 6.2.2.1 and 6.2.2.5. See Fig. 6.3.1.3 for the significance of the horizontal line in each plot.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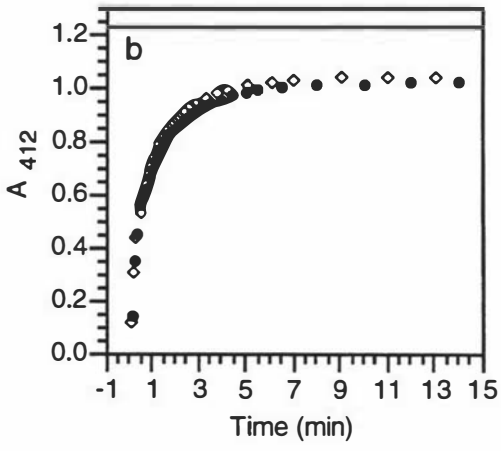
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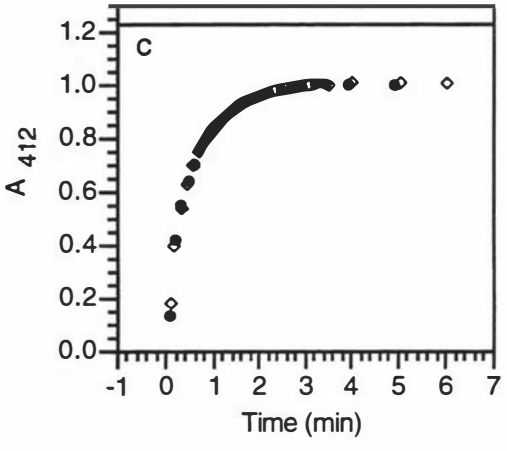
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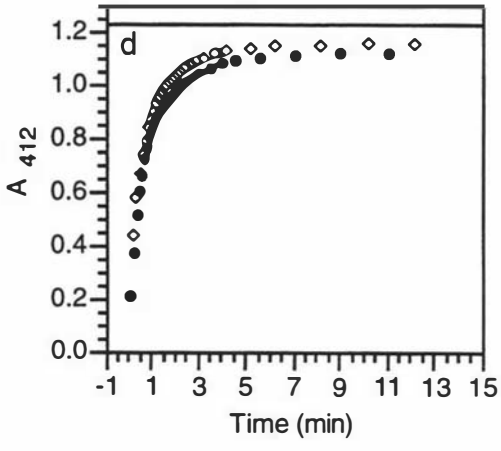
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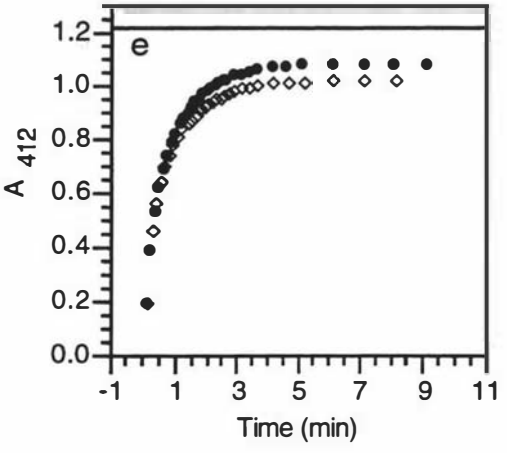
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A 412
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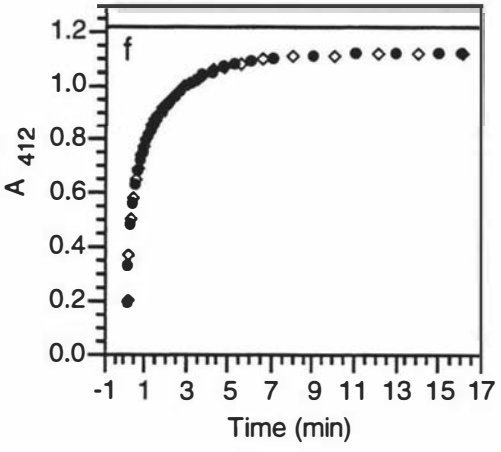

f
A 412
Time (min)

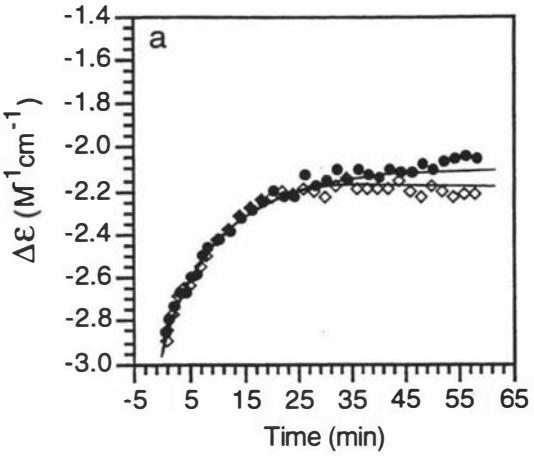
6.3.2. TIME-DEPENDENT CHANGES IN $\Delta\varepsilon_{293}$ AT ELEVATED TEMPERATURES.

Time-dependent structural change in molecules of BLGs A, B and C at pH 6.7 and pH 7.4 which occurs as a consequence of rapid temperature increase was also studied by following changes in the intensity of $\Delta\varepsilon_{293}$. This technique was used to obtain estimates of the rate constant for heat-induced structural change in BLG molecules in the vicinity of Trp^{19}, which is a considerable distance from Cys^{121} (Section 5.3.1.7).

The results obtained in a series of preliminary experiments indicated that rates of decrease in the intensity of $\Delta\varepsilon_{293}$ are negligible at temperatures less than approximately 80 °C. Therefore, time-dependent changes in $\Delta\varepsilon_{293}$ which occurred after solutions of BLG at both pH 6.7 and pH 7.4 at 20 °C had been mixed into phosphate buffer at 80 °C were followed. The decrease in the intensity of $\Delta\varepsilon_{293}$ is approximately 90 % complete within 12.5 min of temperature increase to 80 °C, and after approximately 30 min the decrease in the intensity of $\Delta\varepsilon_{293}$ is, in most instances, very gradual (Fig. 6.3.2.1). For the reasons discussed in Section 5.3.1.3, this decrease suggests that the environment of the side chain of Trp^{19} becomes increasingly less chiral during heat treatment. This in turn suggests that the extent of structural change in the vicinity of this residue increases with increasing heat treatment time. Similarly, the results obtained from near UV CD measurements made at room temperature using BLG solutions previously heat-treated indicate that a loosening of side chain packing in the vicinity of Trp^{19} occurs as a consequence of heat treatment (Section 5.3.1.3). In contrast to the results in Fig. 6.3.2.1, Iametti *et al.* (1996) reported that heat-induced changes in the near UV CD spectrum of BLG are complete within seconds of temperature increase to values between 30 °C and 80 °C. However, they used pre-warmed BLG solutions in some of their measurements but do not specifically state whether their results were for native BLG or pre-warmed BLG. Therefore, it cannot be concluded that the results shown in Fig. 6.3.2.1 are inconsistent with those of Iametti *et al.* (1996).

The coincidence of the experimental $\Delta\varepsilon_{293}$ data with the fitted curves for first order kinetics (Fig. 6.3.2.1) suggests that the appropriate reaction kinetics model had been selected. Therefore, it is assumed that the fitted values for the parameters k^*, $\Delta\varepsilon_{293}$(initial) and $\Delta\varepsilon_{293}$(final) in Table 6.3.2.1 represent measures of the rates and extents of heat-induced structural change in the vicinity of Trp^{19} in molecules of BLGs A, B and C.

Fig. 6.3.2.1. Time-dependent decreases in the intensity of $\Delta\varepsilon_{293}$ in 2.42 mg/mL solutions of BLGs A, B and C at pH 6.7 and pH 7.4 after rapid temperature increase to 80 °C. Reactions were started by mixing 58 μL stock BLG solution into pH 6.7 or pH 7.4 phosphate buffer (717 μL) at 80 °C. Duplicate runs are shown by different symbols, as follows: BLG A at pH 6.7, panel a; BLG B at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. Measurements were made using a spectral band width of 1.0 nm and a 2 s time constant with the Jasco J-720 spectropolarimeter set up in "time-scan" mode (Section 3.1.2.6). The experimental procedure is described in full in Section 6.2.3. The curves were obtained by fitting experimental $\Delta\varepsilon_{293}$ data to a model for first order kinetics using the computer program "Enzfitter" (Leatherbarrow, 1987).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-2.6
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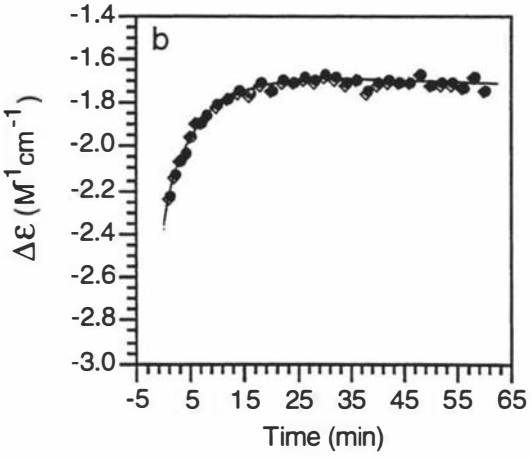
Time (min)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-1.6
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-2.4
-2.6
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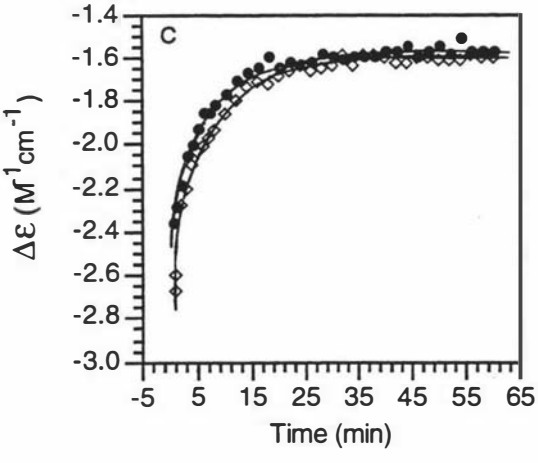
-5 5 15 25 35 45 55 65
Time (min)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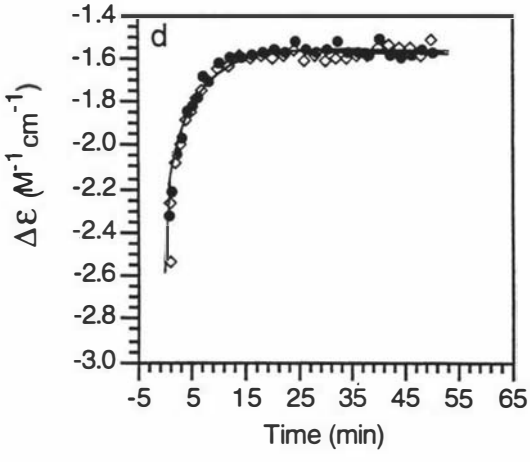
Time (min)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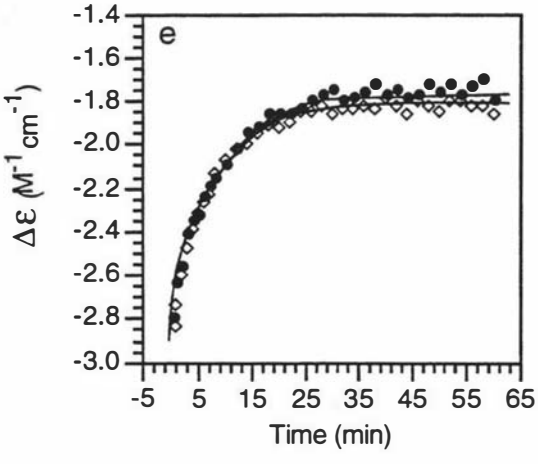
Time (min)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-3.0
-5 5 15 25 35 45 55 65
Time (min)

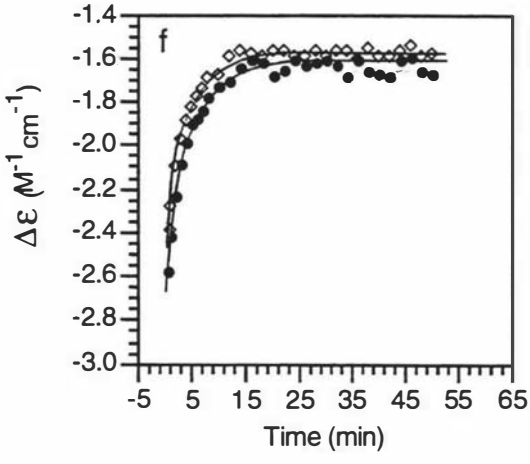

f
Δε (M⁻¹cm⁻¹)
-1.4
-1.6
-1.8
-2.0
-2.2
-2.4
-2.6
-2.8
-3.0
-5 5 15 25 35 45 55 65
Time (min)

Table 6.3.2.1. Kinetic Parameters for Time-dependent Changes in $\Delta\varepsilon_{293}$ at 80 °C in Solutions of β-Lactoglobulins A, B and C.

Sample	k^* (min^{-1})	$\Delta\varepsilon_{293}$(Initial)	$\Delta\varepsilon_{293}$(Final)	$\Delta\varepsilon_{293}$(Final)-$\Delta\varepsilon_{293}$(Initial)
pH 6.7, BLG A	0.084 ± 0.002	-2.867 ± 0.009	-2.077 ± 0.009	0.790
	0.115 ± 0.003	-2.945 ± 0.010	-2.161 ± 0.011	0.784
pH 6.7, BLG B	0.158 ± 0.004	-2.396 ± 0.012	-1.591 ± 0.011	0.805
	0.145 ± 0.004	-2.550 ± 0.012	-1.616 ± 0.012	0.934
pH 6.7, BLG C	0.121 ± 0.003	-2.773 ± 0.011	-1.760 ± 0.011	1.013
	0.137 ± 0.003	-2.843 ± 0.013	-1.826 ± 0.012	1.017
pH 7.4, BLG A	0.173 ± 0.006	-2.312 ± 0.013	-1.708 ± 0.012	0.486
	0.107 ± 0.005	-2.451 ± 0.010	-1.809 ± 0.009	0.642
pH 7.4, BLG B	0.244 ± 0.009	-2.389 ± 0.019	-1.569 ± 0.018	0.793
	0.293 ± 0.010	-2.577 ± 0.019	-1.581 ± 0.018	0.996
pH 7.4, BLG C	0.245 ± 0.008	-2.657 ± 0.021	-1.637 ± 0.020	1.020
	0.266 ± 0.009	-2.479 ± 0.019	-1.571 ± 0.018	0.908

Where:

k^* is the first order rate constant for the decrease in the intensity of $\Delta\varepsilon_{293}$ and is assumed to be the rate constant for structural change in the vicinity of Trp^{19}.

$\Delta\varepsilon_{293}$(initial) and $\Delta\varepsilon_{293}$(final) are the computer calculated values for $\Delta\varepsilon_{293}$ at zero time and at the conclusion of measurements respectively. The computer program "Enzfitter" (Leatherbarrow, 1987) was used for all fitting.

$\Delta\varepsilon_{293}$(final)-$\Delta\varepsilon_{293}$(initial) is the difference between $\Delta\varepsilon_{293}$(final) and $\Delta\varepsilon_{293}$(initial) values. Solutions of each variant at each pH value were assayed in duplicate. The errors in k^*, $\Delta\varepsilon_{293}$(initial) and $\Delta\varepsilon_{293}$(final) values are standard errors calculated by "Enzfitter".

At both pH 6.7 and pH 7.4, the values for the rate constant, k^*, for the heat-induced decrease in the intensity of $\Delta\varepsilon_{293}$ for BLGs B and C are equivalent, but are greater than those for BLG A (Table 6.3.2.1). This suggests that at both pH 6.7 and pH 7.4 at 80 °C, the susceptibilities of BLGs B and C to heat-induced structural change in the vicinity of Trp^{19} are equivalent, but are greater than that of BLG A.

The values for the rate constant for the heat-induced decrease in the intensity of $\Delta\varepsilon_{293}$ for BLGs B and C are greater at pH 7.4 than at pH 6.7 (Table 6.3.2.1). This suggests that these variants are more susceptible to heat-induced structural change at pH 7.4 than at pH 6.7. For BLG A, the poor agreement between duplicate k^* values does not allow a conclusion to be made about the relative susceptibilities to heat-induced structural change at pH 6.7 and pH 7.4.

For BLGs B and C the magnitudes of the differences in the values for $\Delta\varepsilon_{293}$(initial) and $\Delta\varepsilon_{293}$(final) at pH 6.7 are similar to those at pH 7.4 (Table 6.3.2.1). However, for BLG A the difference between $\Delta\varepsilon_{293}$(initial) and $\Delta\varepsilon_{293}$(final) values at pH 7.4 is less than that at pH 6.7. This may indicate that BLG A responds to both heat treatment and pH increase in a different manner to BLGs B and C, and is consistent with the effects on the rate constant discussed above. The $\Delta\varepsilon_{293}$ and PAGE results in Sections 5.3.1 and 5.3.9 respectively also suggest that BLG A responds to heat treatment and pH change in a different manner to BLGs B and C.

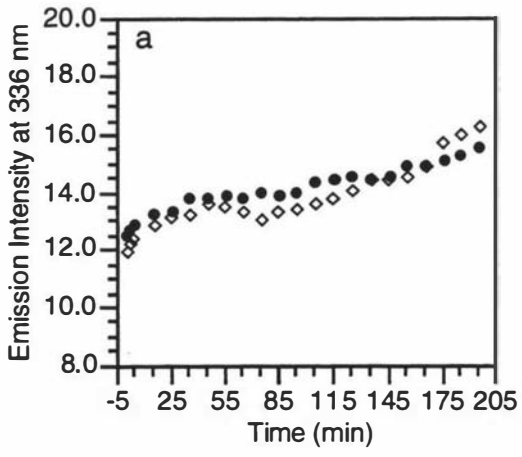
6.3.3. TIME-DEPENDENT INCREASES IN TRYPTOPHAN EMISSION INTENSITY AT ELEVATED TEMPERATURES.

Heat-induced structural change in molecules of BLGs A, B and C was also studied by following time-dependent changes in tryptophan fluorescence emission intensity (I_{Trp}).

Plots of the time-dependent change in I_{Trp} which occurred after solutions of BLGs A, B and C at pH 6.7 and pH 7.4 at 20 °C were rapidly warmed to 80 °C are shown in Fig. 6.3.3.1. Measurements were made at 80 °C because the results from a series of preliminary experiments indicated that rates of the time-dependent change in I_{Trp} are negligible below this temperature.

The heat-induced increases in I_{Trp} appear to progress in two distinct phases, occurring more rapidly between 0 min and approximately 10 min after temperature increase to 80 °C than during the remainder of the 3.5 hr of data collection (Fig. 6.3.3.1). Because the first phase of the time-dependent increase in I_{Trp} occurs on a similar time scale to the heat-induced decrease in the intensity of $\Delta\varepsilon_{293}$ (Section 6.3.2), the former may occur as a consequence of structural change in the vicinity of Trp^{19} (Section 5.3.1.3).

Fig. 6.3.3.1. Time-dependent increases in I_{Trp} in 1.0 mg/mL solutions of BLGs A, B and C at pH 6.7 and pH 7.4 after rapid temperature increase to 80 °C. Reactions were started by mixing 150 μL stock BLG solution into pH 6.7 or pH 7.4 phosphate buffer (2.85 mL) at 80 °C, after which the excitation shutter of the fluorimeter was opened. Duplicate runs are shown by different symbols, as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The solutions of BLG were excited at 295 nm and emission intensity at 336 nm was recorded. Measurements were made using excitation and emission band widths of 8 nm. The experimental procedure is described in full in Section 6.2.4.


a
Emission Intensity at 336 nm
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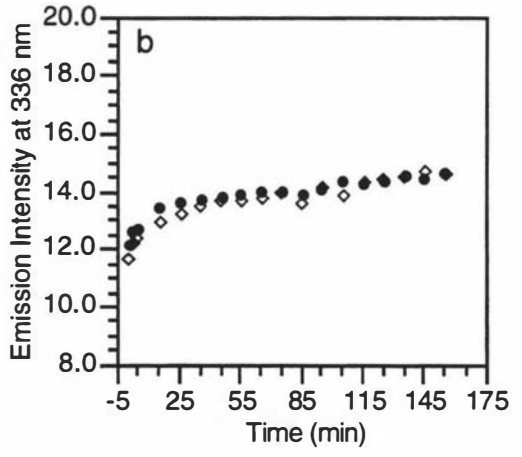
18.0
16.0
14.0
12.0
10.0
8.0
-5 25 55 85 115 145 175 205
Time (min)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Emission Intensity at 336 nm
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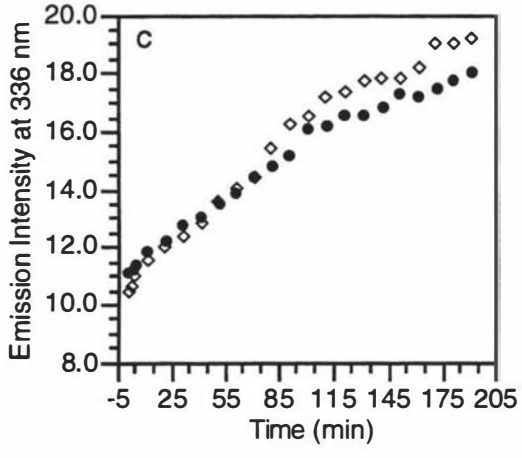
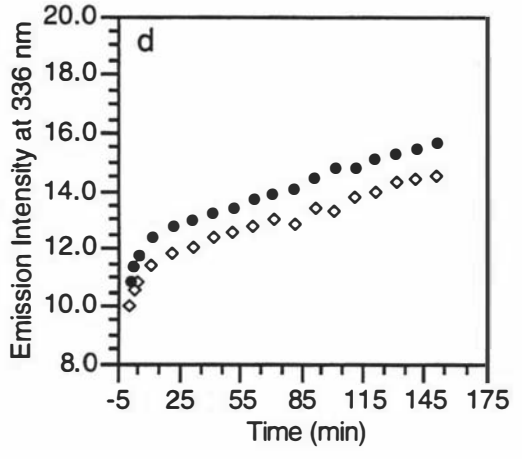
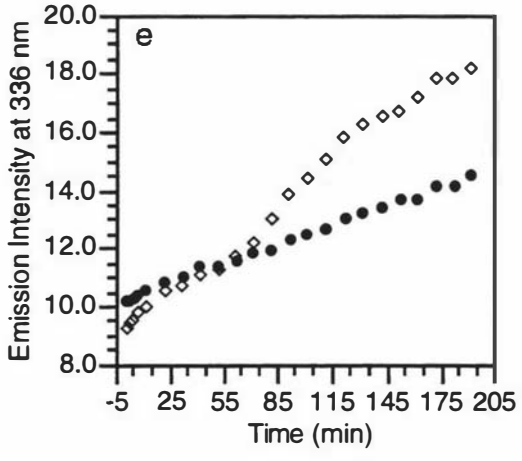
16.0
14.0
12.0
10.0
8.0
-5 25 55 85 115 145 175
Time (min)


c
Emission Intensity at 336 nm
20.0
18.0
16.0
14.0
12.0
10.0
8.0
-5 25 55 85 115 145 175 205
Time (min)


d
Emission Intensity at 336 nm
20.0
18.0
16.0
14.0
12.0
10.0
8.0
-5 25 55 85 115 145 175
Time (min)


e
Emission Intensity at 336 nm
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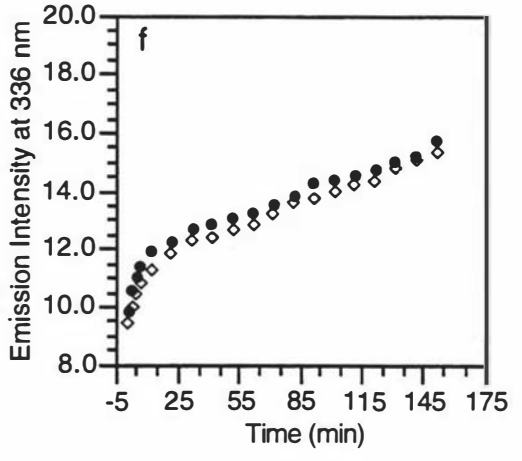
18.0
16.0
14.0
12.0
10.0
8.0
-5 25 55 85 115 145 175 205
Time (min)


f
Emission Intensity at 336 nm
20.0
18.0
16.0
14.0
12.0
10.0
8.0
-5 25 55 85 115 145 175
Time (min)

The second phase of the time-dependent increase in I_{Trp} (Fig. 6.3.3.1) occurs over a much longer period than that for the decrease in the intensity of $\Delta\varepsilon_{293}$, and may therefore reflect a process other than a change in the tertiary and secondary structure of BLG molecules. This process is not instrumental drift or indole ring photolysis because after an initial 5 min thermal equilibration period, I_{Trp} from a solution of N-acetyl-tryptophanamide (i.e. aqueous tryptophan) remained constant (data not shown). Therefore, the second phase of the time-dependent increase in I_{Trp} for BLG may reflect aggregate formation which, as shown by the PAGE results in Section 5.3.9, also occurs over a period of several hours. Because non-native disulphide bonds are formed during aggregate formation, the second phase of the time-dependent increase in I_{Trp} may reflect a decrease in the extent of Trp^{61} fluorescence quenching produced by the loss of the disulphide bond Cys^{66}-Cys^{160} (Section 5.3.3.8).

The trends in time-course I_{Trp} results (Fig. 6.3.3.1) are consistent with the PAGE (Section 5.3.9) and $\Delta\varepsilon_{270}$ (Section 5.3.1.6) results, which also pertain to BLG aggregate formation. For BLGs B and C in particular, the increase in I_{Trp} with increasing heat treatment time is less marked at pH 7.4 than at pH 6.7 (Fig. 6.3.3.1). For the reasons given above this is consistent with the suggestion that large (i.e. M_r greater than approximately 200 000) disulphide-linked aggregates of BLG are formed less readily during heat treatment at pH 7.4 than during heat treatment at pH 6.7 (Section 5.3.9). Furthermore, at both pH 6.7 and pH 7.4 the increase in I_{Trp} with increasing heat treatment time is less marked for BLG A than for BLGs B and C and this is consistent with the suggestion that large disulphide-linked aggregates are formed more slowly from BLG A than from BLGs B and C (Section 5.3.9.2).

6.3.4. SUMMARY: MEASUREMENT OF TIME-DEPENDENT CHANGES IN β-LACTOGLOBULIN STRUCTURE AT ELEVATED TEMPERATURES.

6.3.4.1. Extents of Heat-induced Structural Change.

Heat-induced changes in $\Delta\varepsilon_{293}$ and thiol group exposure are likely to occur as a consequence of changes in tertiary and secondary structure in the vicinities of Trp^{19} and Cys^{121} respectively. In contrast, heat-induced changes in I_{Trp} may occur as a consequence of both changes in tertiary and secondary structure (in the vicinities of Trp^{19} and Trp^{61}) and aggregate formation. The thiol group of 80 % - 90 % of BLG molecules becomes solvent-exposed and reacts with DTNB within approximately 12.5 min of temperature increase to 60 °C at pH 6.7 and 50 °C at pH 7.4 (Section 6.3.1.3).

Time-dependent decreases in the intensity of $\Delta\varepsilon_{293}$ are complete within approximately 12.5 min of temperature increase to 80 °C (Section 6.3.2). At this temperature, time-dependent increases in I_{Trp} are observed over much longer periods (Section 6.3.3). Thus, because extents of heat-induced thiol group exposure are increased due to heat treatment in the presence of DTNB (Section 6.3.1.4), and because heat-induced changes in I_{Trp} reflect aggregate formation, extents of heat-induced structural change in BLG molecules are probably best described by changes in $\Delta\varepsilon_{293}$. Therefore, in the case of the BLG solutions used in the measurements discussed in Chapter 5, time-course $\Delta\varepsilon_{293}$ results indicate that approximately 90 % of the protein molecules in these solutions would have been converted to a non-native conformation during heat treatment for 12.5 min or 13.5 min.

6.3.4.2. Rates of Heat-induced Structural Change.

Heat-induced thiol group exposure (Section 6.3.1) occurs at lower temperatures than heat-induced structural change in the vicinity of Trp^{19} ($\Delta\varepsilon_{293}$ results, Section 6.3.2). This suggests that BLG molecules are more susceptible to structural change in the vicinity of Cys^{121} than in the vicinity of Trp^{19}. Therefore, initial thiol group exposure may precede structural change in other parts of the BLG molecule during heat treatment.

Chapter 7.

EXTENTS OF STRUCTURAL CHANGE IN β-LACTOGLOBULIN MOLECULES AT ELEVATED TEMPERATURES.

7.1. INTRODUCTION.

Near UV CD and tryptophan fluorescence were used to study the structures of BLGs A, B and C at elevated temperatures to complement the studies in Chapter 5 in which heat-induced irreversible structural change and aggregate formation was examined.

7.2. METHODS.

7.2.1. PREPARATION OF PROTEIN.

Solutions of BLGs A, B and C at pH 6.7 and pH 7.4 of known concentration were prepared as described in Section 5.2.1.1 and then diluted with either pH 6.7 or pH 7.4 phosphate buffer to 1.00 mg/mL for near UV CD measurements and to 1.08 mg/mL for tryptophan fluorescence measurements.

7.2.2. NEAR UV CD SPECTROSCOPY.

Prior to data collection the water-jacketed CD cell was rinsed using the procedure described in Section 3.2.4.2. The cell was then allowed to attain thermal equilibrium at a waterbath temperature setting of 24 °C, filled with a particular BLG variant solution, stoppered, placed in the cell holder of the spectropolarimeter and the BLG solution given 10 min to attain thermal equilibrium. During all equilibration periods cell contents were shielded from the light source. After 10 min the BLG solution was irradiated and the near UV CD spectrum was recorded. Measurements were made using the instrument settings described in Section 3.2.4.2. Data were collected between 310 nm and 260 nm and the final spectrum represented the average of 2 scans. The temperature of the waterbath was then increased stepwise by 2 °C increments to 92 °C and at each new temperature measurements were made as described immediately above.

Because data were collected at elevated temperatures and because the data collection protocol was fully automated the actual temperatures of the BLG solutions could not be determined during measurements. β-Lactoglobulin solution temperatures were therefore estimated by measuring the temperature of a volume of pH 6.7 phosphate buffer heated in the water-jacketed CD cell to the same waterbath temperatures as those used for BLG measurements.

Treatment of Data.

Values for CD at 293 nm in millidegrees were determined and converted to $\Delta\varepsilon_{293}$ manually using equation 3.2.4.1 in Section 3.2.4.2 because this manipulation could not be made using the Jasco J-720 software (Section 3.1.2.6). These data were then fitted to the modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) described in Section 5.2.8.2.

7.2.3. TRYPTOPHAN FLUORESCENCE.

Volumes of BLG solution (3.0 mL) at a particular pH were delivered into separate fluorimeter cells which had been previously rinsed as described in Section 3.2.5.2. The cells containing BLG A, B and C solution were then stoppered, placed in positions 4, 3 and 2 respectively in the temperature-controlled cell holder of the fluorimeter, and given 10 min to attain thermal equilibrium at a waterbath temperature setting of 40 °C. Initially, the excitation shutter of the fluorimeter was left closed and the cell holder was oriented so that the cell in position 4 would be in the light beam when the excitation shutter was opened.

After 10 min, the BLG solutions were excited, sequentially, at 295 nm and emission spectra were recorded. Data were acquired using the instrument settings given in Section 5.2.1.5, after which the excitation shutter was closed. Data for the three variants at a particular pH were thus collected in parallel.

The temperature of the waterbath was then increased stepwise by 2 °C increments to 94 °C for solutions at pH 6.7, and to 92 °C for solutions at pH 7.4. After each temperature increase emission spectra for the three variants were recorded as described above. The temperatures attained by BLG solutions during data collection were estimated as described in Section 7.2.2.

Treatment of Data.

Values for tryptophan emission intensity at λ_{max} were measured from chart recordings. Emission intensity data were then fitted to the modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) described in Section 5.2.8.2.

7.3. RESULTS AND DISCUSSION: EXTENTS OF STRUCTURAL CHANGE IN β-LACTOGLOBULIN MOLECULES AT ELEVATED TEMPERATURES.

7.3.1. NEAR UV CD SPECTROSCOPY.

7.3.1.1. Qualitative Study of Temperature-dependent Changes in the Near UV CD Spectrum of β-Lactoglobulin B.

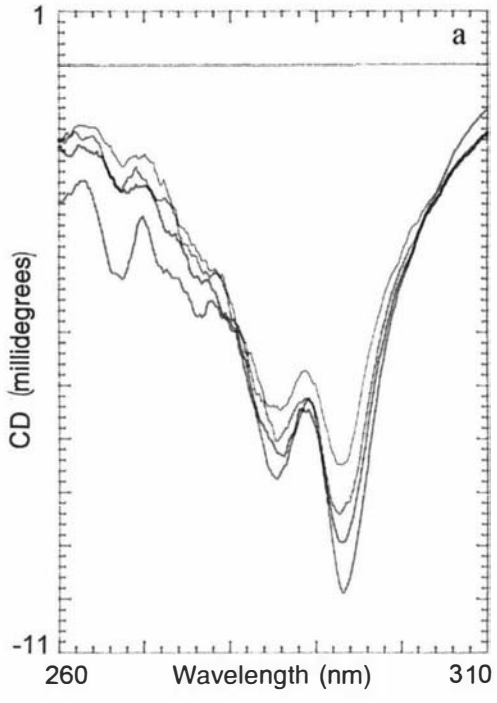
The near UV CD spectra of BLG B at pH 6.7 at temperatures between 24 °C and 90 °C are shown in Fig. 7.3.1.1. In these spectra the intensities of bands, particularly those of tryptophan at 285 nm and 293 nm, decrease with increasing temperature up to 80 °C. Thus, for the reasons discussed in Section 5.3.1.3, these spectra suggest that the environment of Trp^{19} is less chiral at elevated temperatures than at room temperature. This is consistent with the results in Section 5.3.1, which indicate that Trp^{19} is permanently shifted into a less chiral environment as a consequence of heat treatment.

The intensity of a broad trough centred at approximately 277 nm increases with increasing temperature above approximately 76 °C in the spectra in Fig. 7.3.1.1. Thus, for the reasons discussed in Section 5.3.1.3, this suggests that the formation of non-native disulphide bonds occurs during heat treatment. This is consistent with the results in Section 5.3.1.3, which suggest that non-native disulphide bonds remain upon cooling.

The spectra of BLG B at pH 7.4 at elevated temperatures (Fig. 7.3.1.2) show similarities to those described above, except that at 24 °C the two tryptophan bands are less intense at pH 7.4 than at pH 6.7. This confirms the results in Section 5.3.1.3, which suggest that at 20 °C the structure of BLG in the vicinity of Trp^{19} is less compact at pH 7.4 than at pH 6.7.

The trends in the spectra in Figs 7.3.1.1 and 7.3.1.2 are consistent with those reported in other near UV CD studies made at elevated temperatures at neutral pH using BLG solutions at concentrations of approximately 1 mg/mL (Arakawa, 1989; Griffin *et al.*, 1993; Iametti *et al.*, 1996). In agreement with the present results these authors reported that the intensities of the two tryptophan bands in the near UV CD spectra of solutions of BLG A/B, BLG A and BLG A/B respectively decrease with increasing temperature. However, none of these authors reported that the depth of a broad trough centred at approximately 277 nm increases with increasing temperature. Matsuura and Manning (1994) reported that at a BLG concentration of 70 mg/mL the intensities of the tryptophan CD bands decrease with increasing temperature, while the intensity of a broad trough centred at approximately 277 nm increases with increasing temperature.

Fig. 7.3.1.1. The effect of temperature increase on the near UV CD spectrum of BLG B at pH 6.7. In panel a spectra were recorded at 24 °C, blue; 54 °C, red; 58 °C, green; and 64 °C, purple. In panel b spectra were recorded at 66 °C, blue; 70 °C, red; 76 °C, green; and 80 °C, purple. In panel c spectra were recorded at 82 °C, blue; 84 °C, red; and 90 °C, green. All spectra were acquired from a 1.00 mg/mL solution of BLG B in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) in a 10 mm path length water-jacketed cell using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 50 nm/min, using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. Each final spectrum represents the average of two scans. The solution preparation protocol is described in Section 7.2.1 and the heating and data collection protocol (which was completely automated) is described in full in Section 7.2.2.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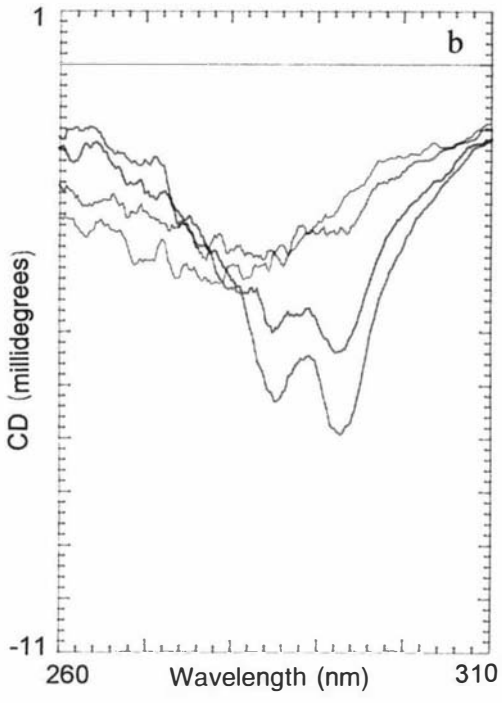
Wavelength (nm)
310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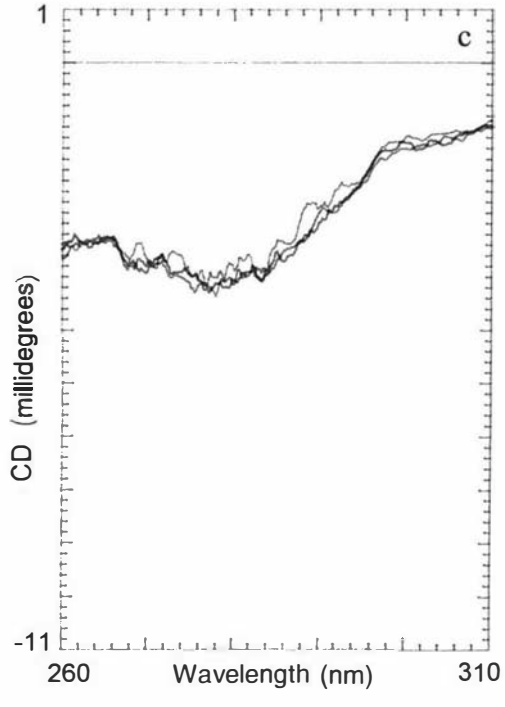
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1
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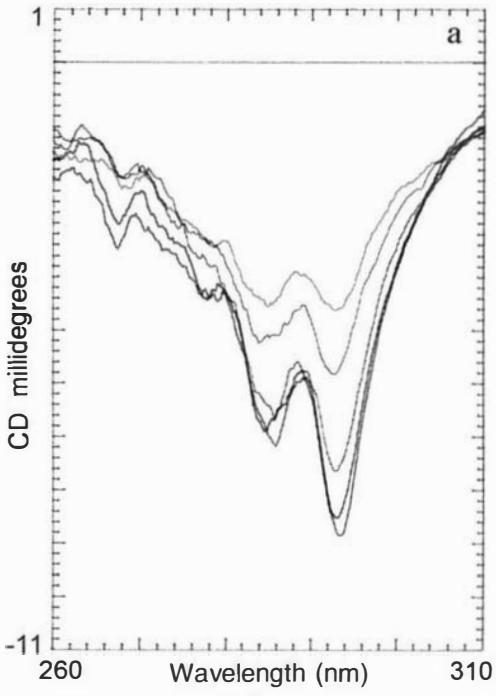
CD (millidegrees)
260
Wavelength (nm)
310

Fig. 7.3.1.2. The effect of temperature increase on the near UV CD spectrum of BLG B at pH 7.4. In panel a spectra were recorded at 24 °C, blue; 40 °C, red; 50 °C, green; 60 °C, purple; and 64 °C, orange. In panel b spectra were recorded at 66 °C, blue; 70 °C, red; 78 °C, green; and 80 °C, purple. In panel c spectra were recorded at 82 °C, blue; 86 °C, red; and 90 °C, green. All spectra were acquired from a 1.00 mg/mL solution of BLG B in pH 7.4 phosphate buffer (26 mM, with 68 mM NaCl) as described in Fig. 7.3.1.1.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Wavelength (nm)
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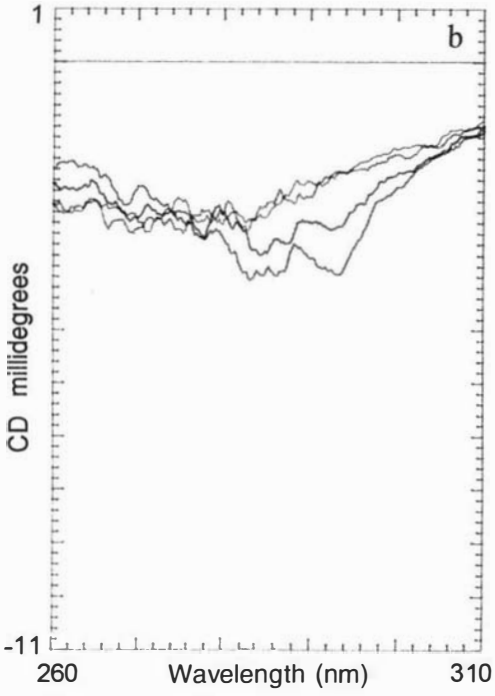
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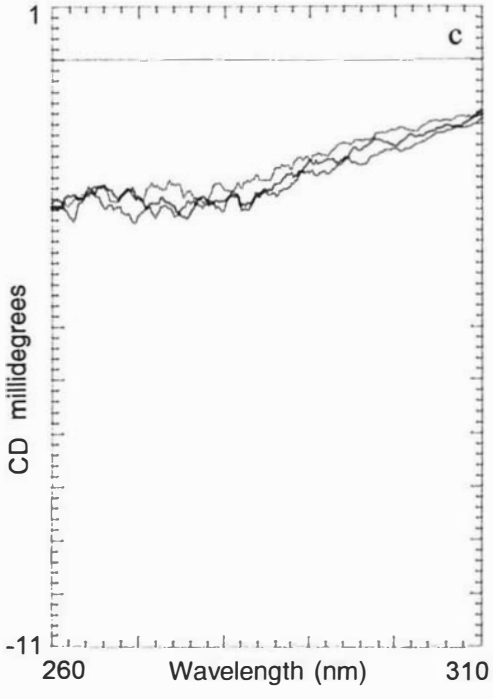
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CD millidegrees
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Wavelength (nm)
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7.3.1.2. Quantitative Study of the Effect of Temperature Increase on the Structures of β-Lactoglobulins A, B and C.

A quantitative study of the susceptibilities of BLGs A, B and C at pH 6.7 and pH 7.4 to heat-induced structural change, in which $\Delta\varepsilon_{293}$ data were fitted to the modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) described in Section 5.2.8.2, was made. Plots of experimental $\Delta\varepsilon_{293}$ data versus temperature and the curves obtained by fitting these data to equations 5.2.8.2 and 5.2.8.3 using the computer program "Enzfitter" are shown in Fig. 7.3.1.3.

The coincidence of the fitted curves with experimental $\Delta\varepsilon_{293}$ data is poorer in the case of the data collected at elevated temperatures (this section) than in the case of the data collected at 20 °C using previously heat-treated BLG solutions (Section 5.3.1). Nevertheless, a comparison of T_{midET} values for BLGs A, B and C at pH 6.7 and pH 7.4 (Table 7.3.1.1) was made. For a particular variant at a particular pH, the value for T_{midET} was assumed to represent the midpoint temperature of the transition for heat-induced structural change in the vicinity of Trp^{19}. In Table 7.3.1.1 the errors in T_{midET} values are standard errors calculated by "Enzfitter", and they are likely to be underestimates of the true errors in the values for this parameter.

The T_{midET} values for BLG A in Table 7.3.1.1 are slightly lower than those estimated from the plots of $\Delta\varepsilon_{293}$ versus temperature shown in Fig. 7B of Griffin *et al.* (1993), which are 74 °C at pH 6.4, 70 °C at pH 7.0 and 63 °C at pH 7.5[1]. Similarly, the T_{midET} values in Table 7.3.1.1 are slightly lower than those determined by Kella and Kinsella (1988a), which are 71.9 °C at pH 6.5, 69.2 °C at pH 7.0 and 64.8 °C at pH 7.4. Kella and Kinsella (1988a) used UV difference spectroscopy to measure extents of structural change in molecules of BLG A/B at elevated temperatures.

For a particular variant at a particular pH, the T_{midET} value in Table 7.3.1.1 is lower than the T_{mid} value in Table 5.3.1.1. This suggests that reversible structural change in the vicinity of Trp^{19} occurs at elevated temperatures which are lower than those heat treatment temperatures required to induce irreversible structural change in the vicinity of this residue. A comparison of the plots in Figs 5.3.1.5 and 7.3.1.3 is consistent with this suggestion. Decreases in the intensity of $\Delta\varepsilon_{293}$ are observed at lower elevated temperatures (Fig. 7.3.1.3) than those heat treatment temperatures which lead to an irreversible decrease in the intensity of $\Delta\varepsilon_{293}$ (Fig. 5.3.1.5).

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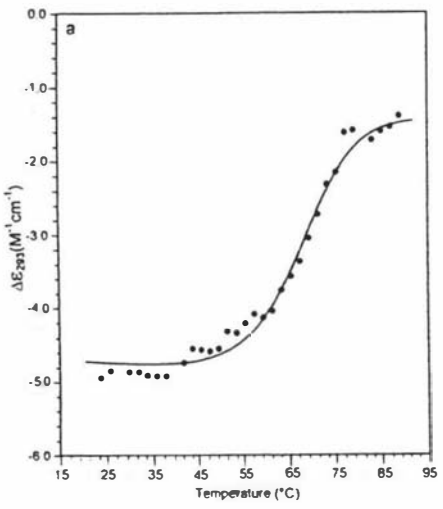
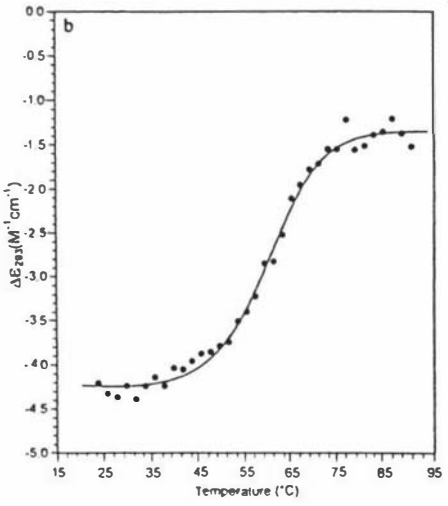
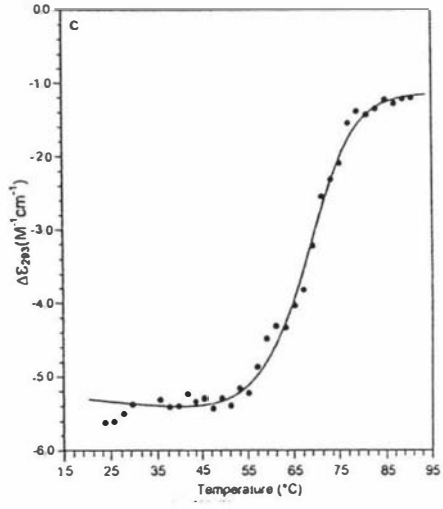
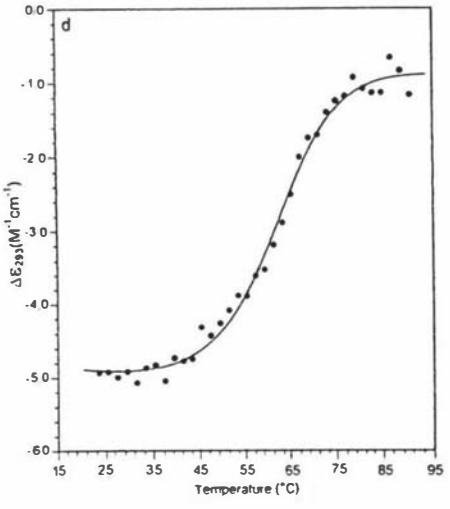
[1] In Fig. 7B of Griffin *et al.* (1993), the plots of pH 6.5 and pH 7.5 data appear to have been labelled the wrong way around.

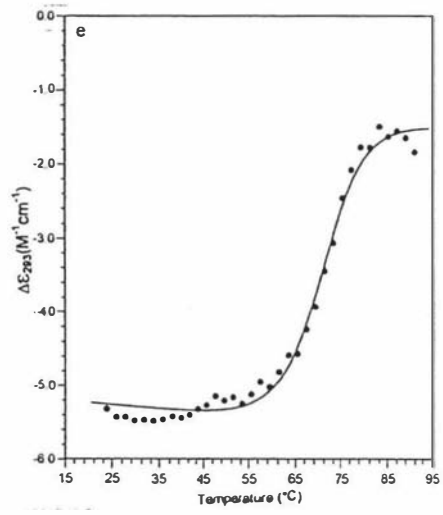
Fig. 7.3.1.3. The effect of temperature on $\Delta\varepsilon_{293}$ in the spectra of BLGs A, B and C at pH 6.7 and pH 7.4. The plots are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The symbols denote experimental $\Delta\varepsilon_{293}$ data which were obtained from spectra recorded as described in Fig. 7.3.1.1. The curves were obtained by fitting experimental $\Delta\varepsilon_{293}$ data to equations 5.2.8.2 and 5.2.8.3 as described in Section 5.2.8.2 using the computer program "Enzfitter" (Leatherbarrow, 1987). The values for the fitting parameters are given in Appendix 3.


a
$\Delta\varepsilon_{293}(M^{-1}cm^{-1})$
Temperature (°C)


b
$\Delta\varepsilon_{293}(M^{-1}cm^{-1})$
Temperature (°C)


c
$\Delta\varepsilon_{293}(M^{-1}cm^{-1})$
Temperature (°C)


d
$\Delta\varepsilon_{293}(M^{-1}cm^{-1})$
Temperature (°C)


e
$\Delta\varepsilon_{293}(M^{-1}cm^{-1})$
Temperature (°C)

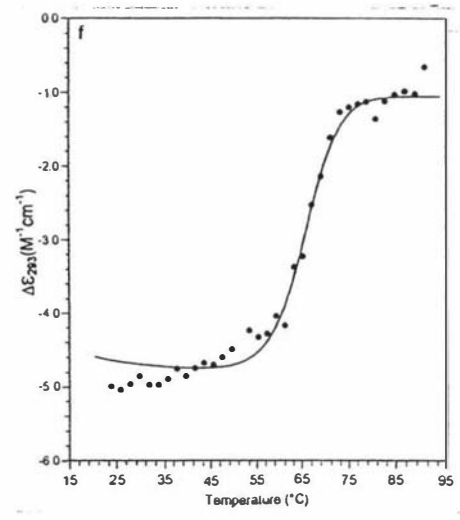

f
$\Delta\varepsilon_{293}(M^{-1}cm^{-1})$
Temperature (°C)

Table 7.3.1.1. Values for the Midpoint Temperature (T_{midET}) for Heat induced Structural Change in the Vicinity of Tryptophan ^{19} in β-Lactoglobulins A, B and C at pH 6.7 and pH 7.4.

Sample	T_{midET} (°C)
pH 6.7, BLG A	67.0 ± 0.3
pH 6.7, BLG B	67.4 ± 0.2
pH 6.7, BLG C	70.3 ± 0.8
pH 7.4, BLG A	59.6 ± 0.2
pH 7.4, BLG B	60.3 ± 1.2
pH 7.4, BLG C	65.0 ± 0.3

Where:

T_{midET} is the midpoint temperature of the transition for heat-induced structural change in the vicinity of Trp^{19} (i.e. the temperature at which $\Delta\varepsilon_{293}$ is half way between the average values for $\Delta\varepsilon_{293}$ on the low and high temperature sides of the transition for structural change in the plots shown in Fig. 7.3.1.3). T_{midET} is one of the parameters used to fit experimental $\Delta\varepsilon_{293}$ data to equations 5.2.8.2 and 5.2.8.3 (Section 5.2.8.2) using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in T_{midET} are standard errors calculated by "Enzfitter".

At pH 6.7 the T_{midET} value for BLG A is 0.4 °C lower than that for BLG B, which is 2.9 °C lower than that for BLG C (Table 7.3.1.1). However, the difference for BLGs A and B is within the error shown in this table. At pH 7.4 the T_{midET} value for BLG A is 0.7 °C lower than that for BLG B, which is 4.7 °C lower than that for BLG C, but the difference for BLGs A and B is within the error shown in Table 7.3.1.1. Thus, at both pH 6.7 and pH 7.4 the susceptibilities of BLGs A and B to heat-induced structural change may be similar, but are both less less than that of BLG C.

The order of variant "thermostabilities" shown by the values for T_{midET} in Table 7.3.1.1 is different from that shown by the T_{mid} values summarised in Table 5.3.6.1. However, in the studies discussed in Chapter 5 the "thermostabilities" of BLGs A, B and C were assessed by measuring, at 20 °C, extents of heat-induced irreversible structural change using BLG solutions that had been previously heat-treated. The significance of the differences between the results in Tables 5.3.6.1 and 7.3.1.1 will be discussed further in Section 8.5.

In the case of the T_{midET} values in Table 7.3.1.1, those for BLGs A, B and C at pH 6.7 are 7.4 °C, 7.1 °C and 5.3 °C higher, respectively, than those at pH 7.4. This indicates that BLGs A, B and C are more susceptible to heat-induced structural change at pH 7.4 than at pH 6.7, in agreement with the results presented in Chapters 5 and 6 (time-dependent changes in BLG structure). The results in Table 7.3.1.1 are also consistent with those of Mills (1976), Kella and Kinsella (1988a) and Griffin *et al.* (1993), which indicate that the thermostabilities of BLG B, BLG A/B and BLG A respectively decrease with increasing pH between approximately 6.5 and 7.5 (Section 2.6.4).

7.3.1.3. Thermodynamic Analysis of $\Delta\varepsilon_{293}$ Data Collected at Elevated Temperatures.

A thermodynamic analysis of fitted $\Delta\varepsilon_{293}$ data collected at elevated temperatures was made. However, because these data were acquired under conditions where the unfolding of BLG is not reversible, only estimates of values for the apparent change in free energy of unfolding, ΔG_{app}, could be calculated (Section 5.3.1.5). For fitted $\Delta\varepsilon_{293}$ data collected at elevated temperatures, approximate values for ΔG_{app} were calculated at 67 °C at pH 6.7 and at 59 °C at pH 7.4 (Table 7.3.1.2). These temperatures were selected because they are close to the T_{midET} values for all three variants at the appropriate pH, where differences in thermostabilities are greatest.

Table 7.3.1.2. Values for ΔG_{app} at 67 °C at pH 6.7 and at 59 °C at pH 7.4 for Structural Change in the Vicinity of Tryptophan[19] in β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6.7, BLG A	0.261 ± 0.002	-0.33*
pH 6.7, BLG B	0.589 ± 0.002	0 (by definition)
pH 6.7, BLG C	2.354 ± 0.030	1.77#
pH 7.4, BLG A	0.300 ± 0.001	-1.23*
pH 7.4, BLG B	1.528 ± 0.030	0 (by definition)
pH 7.4, BLG C	3.999 ± 0.020	2.47#

* ΔG_{app}(BLG A) - ΔG_{app}(BLG B)

\# ΔG_{app} (BLG C) - ΔG_{app} (BLG B).

ΔG_{app} is assumed to represent the change in free energy at 67 °C at pH 6.7 and at 59 °C at pH 7.4 for structural change in the vicinity of Trp^{19}. Values for ΔG_{app} were calculated as described in Section 5.2.8.3 using fitted values for $\Delta\varepsilon_{293}$ at these temperatures and pH values. The fitted values for $\Delta\varepsilon_{293}$ were obtained from the fitted curves shown in Fig. 7.3.1.3 using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in ΔG_{app} values were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 7.3.1.1).

The values for ΔG_{app} for BLG A are less than those for BLG B, which are less than those for BLG C (Table 7.3.1.2). However, because the T_{mid} values for BLGs A and B are equivalent within the limits of experimental error, this must also be true for the corresponding ΔG_{app} values. Therefore, it is concluded that the susceptibilities of BLGs A and B to heat-induced structural change are equivalent, but are both less than that of BLG C. The significance of the differences in ΔG_{app} values, shown as $\Delta\Delta G_{app}$ values in Table 7.3.1.2, will be discussed in Section 8.5.

7.3.2. TRYPTOPHAN FLUORESCENCE.

7.3.2.1. The Tryptophan Fluorescence Emission Spectrum of β-Lactoglobulin B at Elevated Temperatures.

The λ_{max} values in the tryptophan fluorescence emission spectra of BLG B at pH 6.7 increase with increasing temperature (Fig. 7.3.2.1). Similarly, in spectra recorded at 20 °C using previously heat-treated solutions of BLG, tryptophan emission λ_{max} values increase with increasing heat treatment temperature between approximately 65 °C and 80 °C (Fig. 5.3.3.3). Thus, for the reasons discussed in Section 5.3.3.2, the spectra in Fig. 7.3.2.1 suggest that the solvent-accessibility of the side chain of at least one tryptophan residue increases with increasing temperature.

Tryptophan emission intensity (I_{Trp}) for BLG B is also affected by temperature increase (Fig. 7.3.2.1), decreasing with increasing temperature between 40 °C and 60 °C and then increasing with increasing temperature between 60 °C and 80 °C. This indicates that the dependence of I_{Trp} on temperature for BLG B is affected by more than one factor. A quantitative study of the dependence of I_{Trp} on temperature is presented in Section 7.3.2.2.

7.3.2.2. Quantitative Study of the Effect of Temperature Increase on Tryptophan Fluorescence Emission Intensity for β-Lactoglobulins A, B and C.

Plots of I_{Trp} versus temperature for BLGs A, B and C at pH 6.7 and pH 7.4 are shown in Fig. 7.3.2.2. For all three variants at pH 6.7, I_{Trp} decreases with increasing temperature below approximately 64 °C and above approximately 82 °C, but increases with increasing temperature between these temperatures. For BLGs B and C at pH 7.4, I_{Trp} changes with increasing temperature in a similar manner to that observed at pH 6.7 (Fig. 7.3.2.2). For BLG A at pH 7.4, however, I_{Trp} decreases with increasing temperature except between 56 °C and 64 °C (Fig. 7.3.2.2). Measurements on solutions of BLG A at pH 7.4 were made in triplicate (data not shown) to confirm that the dependence of I_{Trp} on temperature for this variant at this pH differs appreciably from that for BLGs B and C at pH 6.7 and pH 7.4 and for BLG A at pH 6.7. For all three variants at both pH values, the decrease in I_{Trp} with increasing temperature is more marked below 50 °C than above 84 °C (Fig. 7.3.2.2).

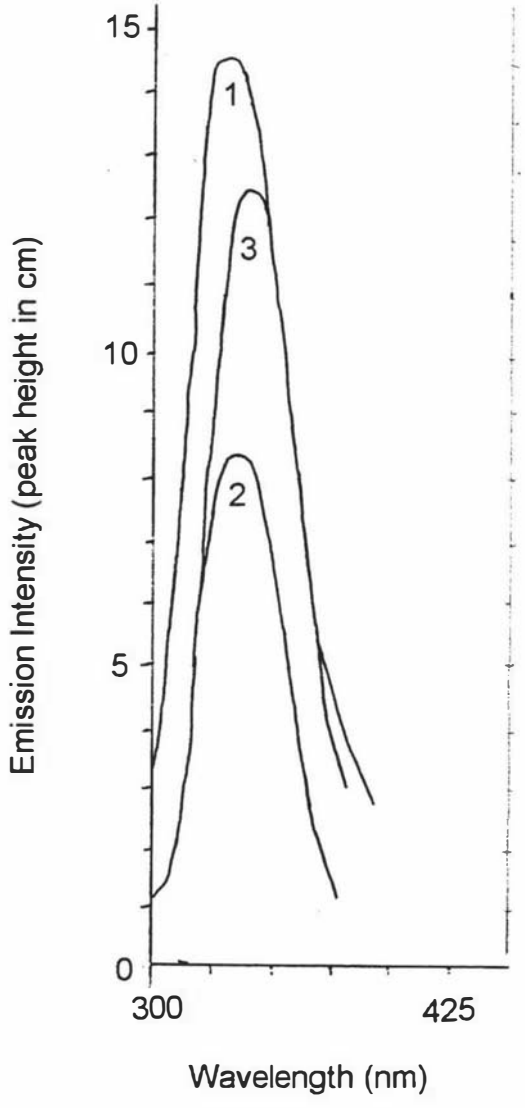
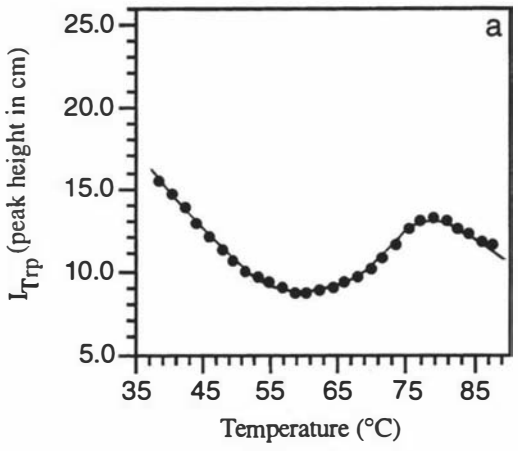
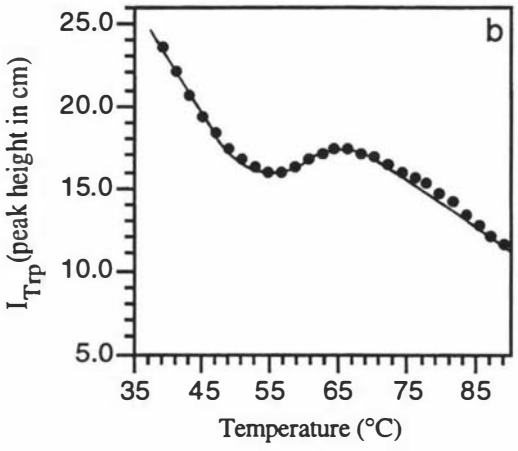
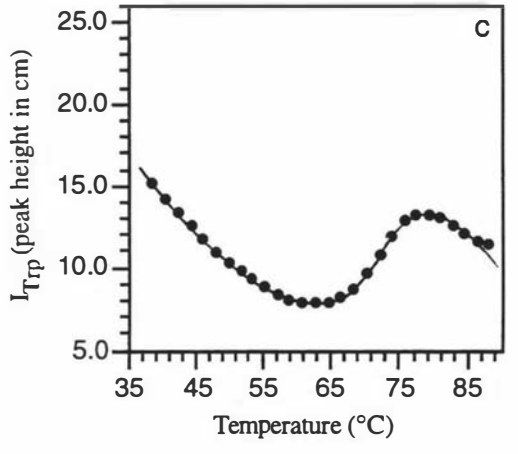


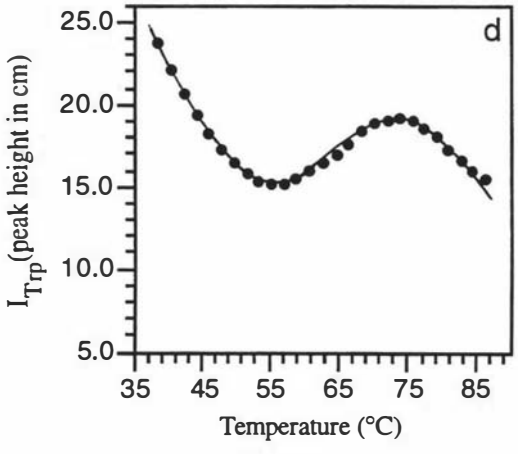
Fig. 7.3.2.1. The effect of temperature increase on the tryptophan fluorescence emission spectrum of BLG B at pH 6.7. Spectra were acquired at 40 °C, spectrum 1; 60 °C, spectrum 2; and 80 °C, spectrum 3; from 1.0 mg/mL solutions in 10 mm path length cells excited at 295 nm using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. Measurements were made using excitation and emission slit widths of 8 nm at a scan speed of 1 cm/min. The solution preparation and data collection protocols are described in Sections 7.2.1 and 7.2.3 respectively.

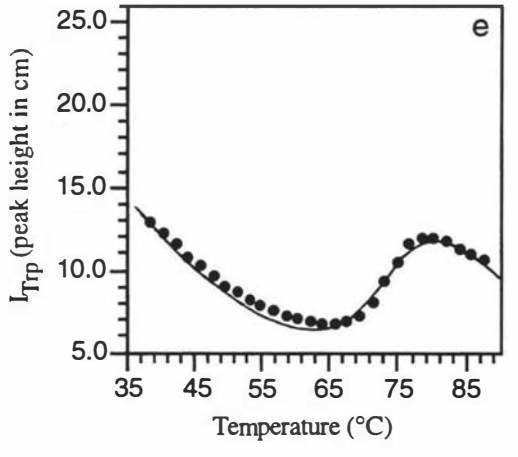
Fig. 7.3.2.2. The effect of temperature on I_{Trp} for BLGs A, B and C at pH 6.7 and pH 7.4. The panels are as follows: BLG A at pH 6.7, panel a; BLG A at pH 7.4, panel b; BLG B at pH 6.7, panel c; BLG B at pH 7.4, panel d; BLG C at pH 6.7, panel e; and BLG C at pH 7.4, panel f. The symbols denote experimental I_{Trp} data which were obtained from spectra recorded as described in Fig. 7.3.2.1. The curves were obtained by fitting experimental I_{Trp} data to equations 5.2.8.2 and 5.2.8.3 as described in Section 5.2.8.2 using the computer program "Enzfitter" (Leatherbarrow, 1987). The values for the fitting parameters are given in Appendix 3.

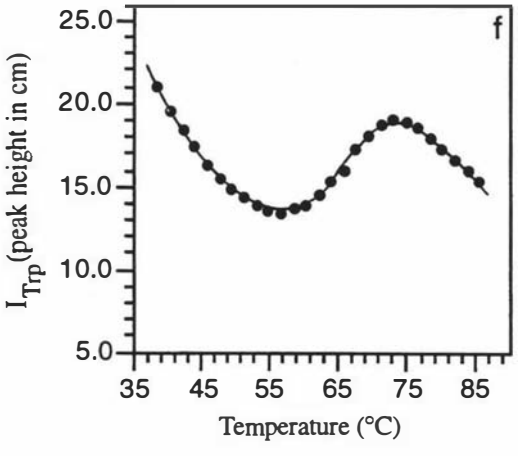

a
25.0
20.0
15.0
10.0
5.0
35 45 55 65 75 85
Temperature (°C)
I_{Trp} (peak height in cm)


b
25.0
20.0
15.0
10.0
5.0
35 45 55 65 75 85
Temperature (°C)
I_{Trp} (peak height in cm)


c
25.0
20.0
15.0
10.0
5.0
35 45 55 65 75 85
Temperature (°C)
I_{Trp} (peak height in cm)


d
25.0
20.0
15.0
10.0
5.0
35 45 55 65 75 85
Temperature (°C)
I_{Trp} (peak height in cm)


e
25.0
20.0
15.0
10.0
5.0
35 45 55 65 75 85
Temperature (°C)
I_{Trp} (peak height in cm)


f
25.0
20.0
15.0
10.0
5.0
35 45 55 65 75 85
Temperature (°C)
I_{Trp} (peak height in cm)

With the exception of BLG A at pH 7.4, the temperature ranges in which I_{Trp} increases with increasing temperature (Fig. 7.3.2.2) are similar to, although wider than, those over which I_{Trp} increases irreversibly with increasing heat treatment temperature (Section 5.3.3.4). Therefore, the process which causes I_{Trp} to increase with increasing temperature may resemble that responsible for the irreversible increases in I_{Trp} which occur as a consequence of heat treatment. Increases in I_{Trp} with increasing temperature may therefore reflect an increase in the distance between the side chain of Trp^{61} and the disulphide bond Cys^{66}-Cys^{160} or the loss of this disulphide bond (Section 5.3.3.2).

The regions of negative slope in the plots in Fig 7.3.2.2 probably reflect a decrease in tryptophan fluorescence quantum yield with increasing temperature (Lakowicz, 1983), and indicate that the proportion of fluorophores in the excited state which return to the ground state by emitting a photon decreases with increasing temperature. Although I_{Trp} appears to increase with increasing temperature between approximately 64 °C and 82 °C at pH 6.7 and between approximately 56 °C and 76 °C at pH 7.4 in the plots shown in Fig. 7.3.2.2, quantum yield decreases continuously with increasing temperature (Lakowicz, 1983). This is illustrated in the plot of N-acetyltryptophanamide (NATA) fluorescence emission intensity versus temperature shown in Fig. 7.3.2.3. Because the indole ring of NATA is not part of the structure of a protein, the extent of its solvent exposure and its proximity to quenching groups do not change during temperature increase. Therefore, changes in NATA fluorescence emission intensity with increasing temperature can only be due to changes in fluorescence quantum yield. The NATA fluorescence results therefore suggest that for BLG, increases in I_{Trp} with increasing temperature, which probably reflect structural change, occur concomitantly with a continuous decrease in tryptophan fluorescence quantum yield (emission intensity decrease) with increasing temperature. This complicates the quantitative interpretation of I_{Trp} data BLGs A, B and C collected at elevated temperatures.

Experimental I_{Trp} data were fitted to the modified version of the 2-state thermal unfolding model of Luo *et al.* (1995) described in Section 5.2.8.2. In this modified model two fitting parameters, s_i and s_f, are used to allow for the regions of negative slope on the low and high temperature sides of the transition for structural change. In most instances the plots of experimental I_{Trp} data (Fig. 7.3.2.2) coincide with the curves obtained by fitting these data to equations 5.2.8.2 and 5.2.8.3 using the computer program "Enzfitter" (Leatherbarrow, 1987). Therefore, the fitted value for the parameter T_{midET} for a particular variant at a particular pH (Table 7.3.2.1) was assumed to represent the midpoint temperature of the transition for heat-induced structural change in the vicinities of Trp^{19} and Trp^{61}, and a comparison of the T_{midET} values for BLGs A, B and C at pH 6.7 and pH 7.4 was made.

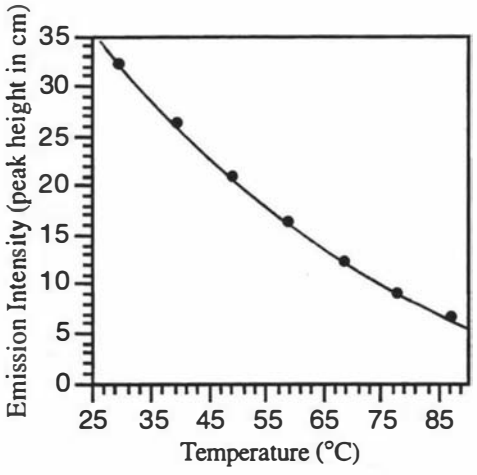


Fig. 7.3.2.3. Effect of temperature on the fluorescence emission intensity from a 100 μM solution of NATA in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl). The symbols denote experimental I_{Trp} data which were obtained from spectra recorded in a manner similar to that described in the caption of Fig. 7.3.2.1. The curve was obtained by fitting experimental I_{Trp} data to a quadratic equation using the computer program "Cricket Graph" (Rafferty and Norling, 1986).

A number of alternative data fitting protocols, in which experimental I_{Trp} data were corrected for temperature-dependent changes in fluorescence quantum yield before fitting, were investigated. A plot of experimental I_{Trp} data corrected using the functions for the linear decreases in I_{Trp} with increasing temperature below 50 °C and above 84 °C (i.e. a data correction protocol similar to that used by Hawkes *et al.*, 1984) is shown in Fig. 7.3.2.4. The data shown in this plot have therefore been treated in a similar manner to data fitted using equations 5.2.8.2 and 5.2.8.3 (which allow for non-zero slope in regions other than that for the transition for structural change). None of the alternative fitting protocols investigated gave better fits than those shown in Fig. 7.3.2.2 and were therefore not used.

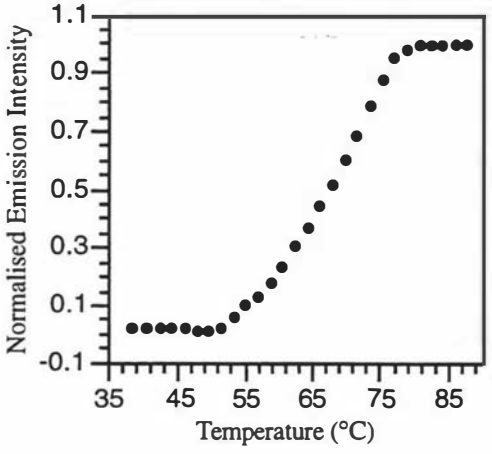


Fig. 7.3.2.4. Plot of I_{Trp} versus temperature prepared using I_{Trp} data corrected for the linear decreases in I_{Trp} with increasing temperature below 50 °C and above 84 °C. The correction was made as described in the text in this section.

Table 7.3.2.1. Values for the Midpoint Temperature (T_{midET}) for Heat-induced Structural Change in the Vicinities of Tryptophan^{19} and Tryptophan^{61} in β-Lactoglobulins A, B and C at pH 6.7 and pH 7.4

Sample	T_{midET} (°C)
pH 6.7, BLG A	67.9 ± 0.3
pH 6.7, BLG B	69.4 ± 0.1
pH 6.7, BLG C	70.5 ± 0.3
pH 7.4, BLG A	53.4 ± 2.2
pH 7.4, BLG B	61.2 ± 0.8
pH 7.4, BLG C	62.8 ± 0.2

Where:

T_{midET} is the midpoint temperature of the transition for heat-induced structural change in the vicinities of Trp^{19} and Trp^{61}. T_{midET} is one of the parameters used to fit experimental I_{Trp} data to equations 5.2.8.2 and 5.2.8.3 (Section 5.2.8.2) using the computer program "Enzfitter" (Leatherbarrow, 1987). The errors in T_{midET} are standard errors calculated using "Enzfitter".

At pH 6.7 the T_{midET} for BLG A is 1.5 °C lower than that for BLG B, which is 1.1 °C lower than that for BLG C (Table 7.3.2.1), indicating that at this pH at elevated temperatures, BLGs A, B and C exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced structural change. In contrast, the T_{midET} values determined using $\Delta\varepsilon_{293}$ data suggest that at elevated temperatures the susceptibilities of BLGs A and B to heat-induced structural change are equivalent, but are both greater than that of BLG C (Table 7.3.1.1).

At pH 7.4 the T_{midET} value for BLG A is 7.8 °C lower than that for BLG B, which is 1.6 °C lower than that for BLG C (Table 7.3.2.1). The unusually large difference between the T_{midET} values for BLGs A and B probably reflects the different shape of the plot for BLG A rather than a large difference in the susceptibilities of these variants to heat-induced structural change. Therefore, although the difference in T_{midET} values for BLGs A and B at pH 7.4 still needs to be quantified, it appears that BLG A is more susceptible to heat-induced structural change than BLG B. Furthermore, BLG B is more susceptible to heat-induced structural change than BLG C (Table 7.3.2.1). This order of variant thermostabilities is the same as that shown by the T_{midET} values determined using I_{Trp} data at pH 6.7 (Table 7.3.2.1), but is different to that shown by the T_{midET} values determined using $\Delta\varepsilon_{293}$ data (Table 7.3.1.1).

The order of variant "thermostabilities" shown by the values for T_{midET} in Table 7.3.2.1 is also different from that shown by all of the T_{mid} values summarised in Table 5.3.6.1, except for those determined using I_{Trp} data collected at pH 7.4. The significance of this difference will be discussed further in Section 8.5.

The T_{midET} values determined using I_{Trp} data collected at elevated temperatures (Table 7.3.2.1) for BLGs A, B and C at pH 6.7 are, respectively, 14.5 °C, 8.2 °C and 7.7 °C higher than those at pH 7.4. This indicates that BLGs A, B and C are more susceptible to heat-induced structural change at pH 7.4 than at pH 6.7, in agreement with the T_{midET} values determined using $\Delta\varepsilon_{293}$ data collected at elevated temperatures (Table 7.3.1.1). The results in Table 7.3.2.1 are therefore consistent with those of Mills (1976), Kella and Kinsella (1988a) and Griffin *et al.* (1993) discussed in Section 7.3.1.2.

7.3.2.3. Thermodynamic Analysis of Tryptophan Emission Intensity Data Collected at Elevated Temperatures.

A thermodynamic analysis of fitted I_{Trp} data collected at elevated temperatures was made. These data were, however, acquired under conditions in which the unfolding of BLG is not reversible and therefore, only estimates of values for the apparent change in free energy of unfolding, ΔG_{app}, could be calculated (Section 5.3.1.5). For fitted I_{Trp} data collected at elevated temperatures, approximate values for ΔG_{app} were calculated at 67 °C at pH 6.7 and at 59 °C pH 7.4. These temperatures were selected because they are close to the T_{midET} values for all three variants at the appropriate pH and because they are those at which ΔG_{app} values were calculated using $\Delta\varepsilon_{293}$ data collected at elevated temperatures.

The values for ΔG_{app} for BLG A are less than those for BLG B, which are less than those for BLG C (Table 7.3.2.2). This suggests that the A, B and C variants exhibit the greatest, intermediate and lowest susceptibilities respectively to heat-induced structural change, and this is consistent with the T_{midET} values shown in Table 7.3.2.1. The differences in ΔG_{app} values for BLGs A, B and C are shown as $\Delta\Delta G_{app}$ values in Table 7.3.2.2 and the significance of these values will be discussed in Section 8.5.

Table 7.3.2.2. Values for ΔG_{app} at 67 °C at pH 6.7 and at 59 °C at pH 7.4 for Structural Change in the Vicinities of Tryptophan[19] and Tryptophan[61] in β-Lactoglobulins A, B and C.

Sample	ΔG_{app} (kJ/mol)	$\Delta\Delta G_{app}$ (kJ/mol)
pH 6,7, BLG A	3.482 ± 0.020	-0.68*
pH 6.7, BLG B	4.164 ± 0.006	0 (by definition)
pH 6.7, BLG C	5.131 ± 0.020	0.97 #
pH 7.4, BLG A	0.430 ± 0.020	-3.58*
pH 7.4, BLG B	4.005 ± 0.050	0 (by definition)
pH 7.4, BLG C	4.163 ± 0.010	0.16#

* ΔG_{app}(BLG A) - ΔG_{app}(BLG B)

ΔG_{app} (BLG C) - ΔG_{app} (BLG B).

ΔG_{app} is assumed to represent the change in free energy at 67 °C at pH 6.7 and at 59 °C at pH 7.4 for structural change in the vicinities of Trp^{19} and Trp^{61}. ΔG_{app} values were calculated using fitted I_{Trp} data (Section 7.3.2.2) as follows: The fitted I_{Trp} data were normalised so that the maximum and minimum values in each set were 0 and 1 respectively. Values for ΔG_{app} were then calculated using the normalised fitted I_{Trp} data at the temperatures given above as described in Section 5.2.8.3. The errors in ΔG_{app} values were calculated from percentage errors in T_{mid} values, which in turn were calculated using the values for T_{mid} and the corresponding standard errors determined by "Enzfitter" (Table 7.3.2.1).

Chapter 8.

DISCUSSION.

8.1. BOVINE β-LACTOGLOBULIN AGGREGATION MODELS POST 1994.

8.1.1. CAIROLI *et al.* (1994), IAMETTI *et al.* (1995) and IAMETTI *et al.* (1996).

Prior to 1994, several models for the formation of macroscopic aggregates of bovine BLG had been proposed (McKenzie, 1971; Mulvihill and Donovan, 1987; Griffin *et al.*, 1993; McSwiney *et al.*, 1994b, Section 2.7). However, the nature of the heat-induced structural modifications in BLG molecules, which are thought to occur before aggregation can commence, is not described in detail in these models. Heat-induced structural change in BLG A/B has since been studied by Iametti and co-workers (Cairoli *et al.*, 1994; Iametti *et al.*, 1995; Iametti *et al.*, 1996) and the findings from these studies are summarised and compared in Iametti *et al.* (1996). Heat-induced structural change in BLG A/B at pH 6.8 proceeds through different steps which each exhibit their own dependence on temperature (Iametti *et al.*, 1996).

During temperature increase from room temperature to approximately 70 °C, dimers dissociate to monomers, the thiol group and the side chains of tryptophan residues become solvent-accessible, the intensity of fluorescence from bound ANS increases, and near UV CD spectra suggest that the tertiary structure of BLG changes (Iametti *et al.*, 1996). They suggested that the observed increase in fluorescence emission intensity from bound ANS is indicative of an increase in the solvent-accessibility of the hydrophobic side chains of BLG and that this may occur as a consequence of a loosening, or "swelling", of the BLG structure (Section 5.3.4.7). Furthermore, all of the above changes, except possibly thiol group exposure, are reversible upon cooling from temperatures less than approximately 70 °C to room temperature (Iametti *et al.*, 1996). They also suggest a correlation between rates of thiol group exposure and BLG "swelling".

The structural changes observed at lower temperatures become irreversible above approximately 70 °C (Iametti *et al.*, 1996). Furthermore, at temperatures in this range, emission intensity from bound ANS first increases and then decreases with increasing heating time (Cairoli *et al.*, 1994; Iametti *et al.*, 1995). The decrease in ANS fluorescence emission intensity was described as "hydrophobic collapse" by Iametti and co-workers and may reflect a gradual decrease in the amount of ANS bound to the

surface or interior of "heat-swollen" BLG molecules which occurs as a consequence of aggregate formation (Section 5.3.4.7). Iametti *et al.* (1996) also reported, from SDS-PAGE and gel permeation chromatography results, that both disulphide-linked and non-covalently-linked aggregates of BLG A/B are formed at temperatures greater than approximately 70 °C. Additionally, a correlation between rates of "hydrophobic collapse" and aggregate formation was suggested by Iametti *et al.* (1996). They also theorised that during heat treatment, BLG monomers initially associate to form non-covalently-linked aggregates, and that thiol-disulphide interchange reactions lead to the stabilisation of these aggregates.

Iametti *et al.* (1996) also studied BLG aggregation in the presence of the thiol blocking agent iodoacetamide and found that only non-covalently-linked aggregates were formed. They also found that dimers could not re-form after BLG A/B had been heat-treated in the presence of iodoacetamide, and that extents of irreversible structural change, measured using near UV CD, were less than those which occurred after heat treatment in the absence of iodoacetamide. From these results, and from the observed correlation between rates of thiol group exposure and surface hydrophobicity increase ("swelling"), Iametti *et al.* (1996) suggested that both the thiol group and a large number of hydrophobic side chains are located close to the dimer interface, as previously suggested by Lontie and Preaux (1966). However, these suggestions are incompatible with the crystal structures (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998), which show that the two Cys^{121} residues of the BLG dimer cannot be close to the dimer interface because they are 25.6 Å apart.

Cairoli *et al.* (1994) and Iametti *et al.* (1996) suggested that the structure of BLG at elevated temperatures resembles that of a molten globule. Fink (1995) defined a molten globule as a species which exhibits the five following characteristics and structural features: a) it possesses nearly as much secondary structure as the corresponding native species, b) it possesses appreciably less tertiary structure than the corresponding native species, c) it is only slightly less compact than the corresponding native species, d) it exhibits a tendency to aggregate and e) it binds ANS in such a manner that the intensity of emission from bound probe is greater than for the corresponding native species. Thus, many of the results of Iametti and co-workers suggest that BLG forms a molten globule-like species at elevated temperatures. Their near UV CD, intrinsic protein fluorescence and thiol exposure results suggest that heat treatment leads to changes in tertiary structure. Furthermore, a heat-induced increase in emission intensity from bound ANS and the formation of aggregates was reported. Although Iametti and co-workers do not show results which indicate the presence of a core of secondary structure at elevated temperatures, the far UV CD results of Griffin *et al.* (1993) suggest that molecules of BLG A possess similar amounts of β-sheet structure at both elevated

temperatures and at room temperature. This is therefore consistent with the suggestion that BLG exists in a molten globule-like conformation at elevated temperatures. Finally, because Iametti and co-workers do not discuss the apparent size of BLG molecules at elevated temperatures, the formation of a true molten globule by BLG has not yet been proved.

Iametti *et al.* (1996) also published a model for the heat-induced aggregation of BLG (Fig. 8.1.1). This model is similar to that of McSwiney *et al.* (1994b), which was developed to explain aggregation phenomena observed when BLG solutions at concentrations 50 mg/mL and greater are heat-treated. In the model of McSwiney *et al.* (1994b), BLG dimers dissociate, monomers then undergo structural change, re-associate in a non-covalent manner, and the resulting non-covalently-linked aggregates are then stabilised by intermolecular disulphide bonds (Section 2.7.2). Similarly, Iametti *et al.* (1996) suggested that aggregate formation cannot commence until the structures of monomers are modified (Fig. 8.1.1).

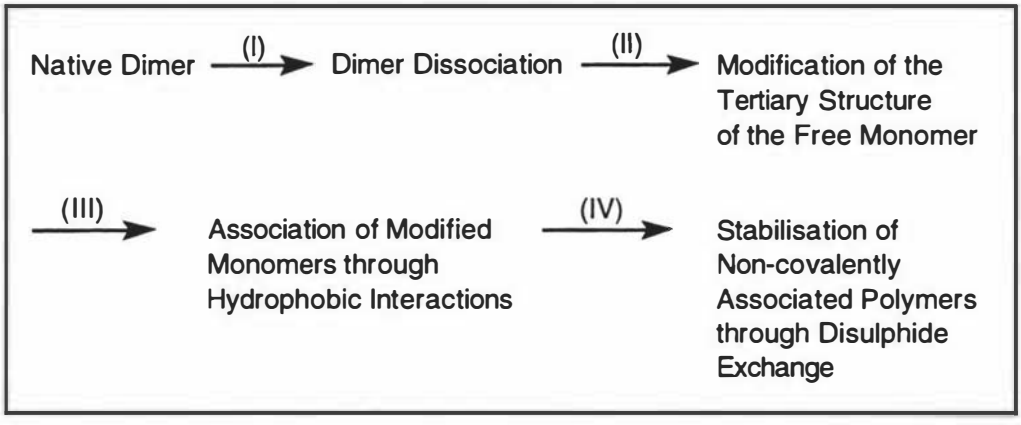


Fig. 8.1.1. The model for the heat-induced aggregation of BLG of Iametti *et al.* (1996).

8.1.2. QI *et al.* (1995) and QI *et al.* (1997).

The work of Qi *et al.* (1995, 1997) has contributed to an understanding of the heat-induced structural changes in BLG A molecules which precede aggregate formation. From results obtained in their 1995 differential scanning calorimetry (DSC) study, they suggested that the concentration at which BLG A solutions are heat-treated influences whether unfolding occurs from the dimeric or the monomeric state. Qi *et al.* (1995) found that the endotherm peaks in the thermograms of BLG A solutions at concentrations less than 25 mg/mL were broad and exhibited fine structure. This suggests that the majority of BLG A molecules unfold from the monomeric state at

concentrations less than 25 mg/mL (Qi *et al.*, 1995), in agreement with the aggregation models discussed in Section 2.7 and that of Iametti *et al.* (1996) discussed in Section 8.1.1. However, in the thermograms of BLG A solutions at concentrations greater than 25 mg/mL, the endotherm peak was sharp and did not exhibit fine structure (Qi *et al.*, 1995). This suggests that at these higher concentrations, where the proportion of dimeric protein is higher than at lower concentrations, the majority of BLG A molecules unfold from the dimeric state (Qi *et al.*, 1995).

Qi *et al.* (1997) found that the proportion of β-sheet in BLG decreases only slightly during temperature increase to 60 °C and have suggested that BLG forms a molten globule-like species at elevated temperatures. They also suggested that the heat-induced aggregation of BLG may be explained in terms of the formation of a molten globule-like species, which are more susceptible to aggregation than their native counterparts (Section 8.1.1). Furthermore, Qi *et al.* (1997) cite others who have reported that thiol-disulphide interchange reactions can occur when other proteins which possess thiol groups and disulphide bonds are converted to a molten globule state, and suggest that this may explain why thiol-disulphide interchange occurs at elevated temperatures for BLG.

Qi *et al.* (1997) have published a model for the aggregation of BLG (Fig. 8.1.2) which is similar to that of Griffin *et al.* (1993). However, Qi *et al.* (1997) suggested that after dimer dissociation (reaction I), up to three conformational changes may occur before BLG monomers form aggregates. Griffin *et al.* (1993) suggested that one conformational change occurs between dimer dissociation and aggregation (Section 2.7.1.2). In the model of Qi *et al.* (1997), reaction IV is an interpretation of the results of de Wit and Klarenbeek (1981), who reported from DSC results that BLG unfolds at approximately 130 °C in solutions at concentrations of approximately 100 mg/mL. Qi *et al.* (1997) also suggested that the BLG aggregation mechanism is affected by protein concentration (see above).

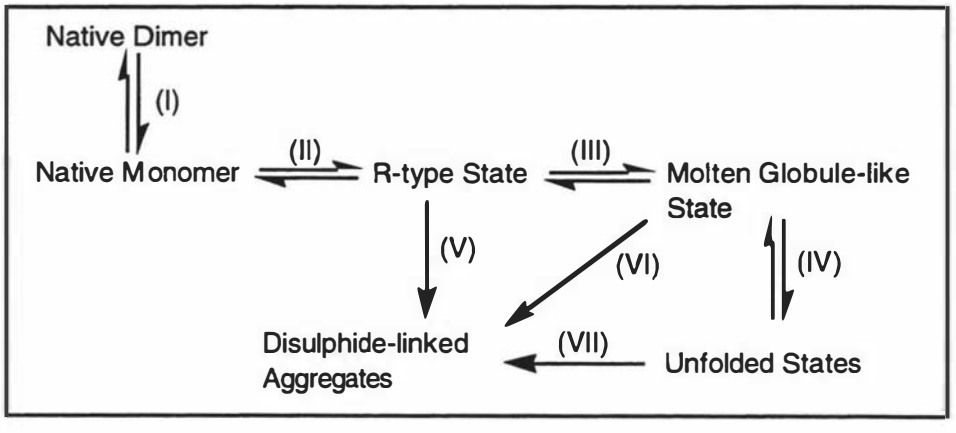


Fig. 8.1.2. The model for the heat-induced aggregation of BLG of Qi *et al.* (1997).

8.1.3. ELOFSSON *et al.* (1996a) and ELOFSSON *et al.* (1996b).

Elofsson *et al.* (1996a, b) used *in situ* dynamic light scattering (DLS) to follow the increase in the size of BLG aggregates during heat treatment of solutions at pH values between 6.50 and 6.94. Elofsson *et al.* (1996b) used microcalorimetry to identify the T_{max} values of the different events which occur when solutions of BLG are heat-treated. A comparison of the aggregation behaviour of BLGs A and B was also made by Elofsson *et al.* (1996b).

Elofsson *et al.* (1996b) found that the length of a lag phase which precedes aggregate formation increases with decreasing temperature, and suggested that this phenomenon can be explained in terms of the amount of unfolded BLG available for aggregate formation. The amount of unfolded BLG decreases as solution temperatures are decreased, which in turn means that there is less unfolded BLG available for aggregate formation. From the results obtained in their microcalorimetric study, Elofsson *et al.* (1996b) suggest that BLG aggregation proceeds via two transitions: the T_{max} of the first (62 °C and 68 °C for BLGs A and B respectively) coincides with the onset temperature of the second (which has a T_{max} of 74 °C for both BLGs A and B). Furthermore, they suggested that, for both BLGs A and B, the first and the second transitions correspond to BLG unfolding and "thiol activation" respectively. Elofsson *et al.* (1996b) also suggested that because the T_{max} for BLG unfolding is lower than that for "thiol activation", their results are consistent with those of McSwiney *et al.* (1994b), which indicate that rates of loss of monomeric BLG during heat treatment are greater than those for the formation of disulphide-linked aggregates.

The T_{max} of the transition which Elofsson *et al.* (1996b) believe corresponds to BLG unfolding is similar to that of the transition which Qi *et al.* (1997) have suggested corresponds to the formation of a molten globule-like species (Section 8.1.2). Therefore, transitions observed at approximately 65 °C may correspond to structural change in BLG molecules.

8.1.4. ROEFS and de KRUIF (1994).

The BLG aggregation model of Roefs and de Kruif (1994) differs appreciably from those discussed in Sections 2.7 and 8.1.1 - 8.1.3. Roefs and de Kruif (1994) have suggested that the BLG aggregation mechanism is analogous to that for the free radical polymerisation of ethylene, with thiol exposure and thiol-disulphide interchange corresponding to initiation and propagation respectively. They suggested that termination occurs when two exposed thiol groups meet and are oxidised to a disulphide (Fig. 8.1.3).

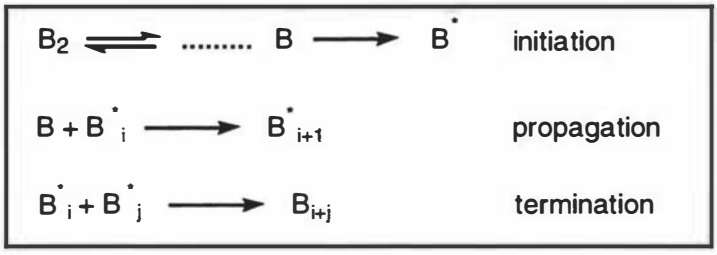


Fig. 8.1.3. The model for the heat-induced aggregation of BLG of Roefs and de Kruif (1994).

The Roefs and de Kruif (1994) model assumes that initiation occurs via a number of reversible reactions which eventually result in the formation of the species which possesses a solvent-exposed thiol group. Although the basic premise of this model is that the thiol group and one disulphide bond react to form linear aggregates, Roefs and de Kruif (1994) state that the other disulphide bond may participate in thiol-disulphide interchange reactions, thus introducing branching into BLG aggregate structures.

Roefs and de Kruif (1994) found, using high performance gel permeation chromatography to measure the concentration in heat-treated solutions of BLG soluble at pH 4.7, that their model underestimates rates of decrease in the concentration of monomeric BLG during heat treatment, particularly at concentrations 50 mg/mL and greater. They explained this inconsistency in terms of the Trommsdorf effect[1]. Similarly, the model of Roefs and de Kruif (1994) does not appear to fit particularly well the experimental data of Hoffmann and van Mil (1997), who used similar experimental techniques to Roefs and de Kruif (1994). The model of Roefs and de Kruif (1994) may underestimate rates of decrease in the concentration of monomeric BLG during heating because this decrease occurs as a consequence of the formation of both disulphide-linked and non-covalently-linked aggregates (McSwiney *et al.*, 1994a, b), and this model only considers aggregate formation via thiol-disulphide interchange.

[1] As the length of a polymer chain increases, the viscosity of the solution increases. This in turn, leads to a decrease in the rate of diffusion of polymer molecules in solution. Thus, as polymerisation proceeds, the probability that two free radicals will meet, resulting in termination, decreases. This in turn, leads to an apparent autocatalysis of the polymerisation reaction and therefore, to an increase in the rate for aggregate formation.

8.1.5. GEZIMATI *et al.* (1997).

Gezimati *et al.* (1997) used the results and aggregation models discussed in Sections 2.7 and 8.1.1 - 8.1.4 to describe in detail how disulphide-linked aggregates of BLG, the end product of BLG aggregation reactions which occur in the absence of reducing agents, are formed from both non-covalently-linked and disulphide-linked precursors (Fig. 8.1.4).

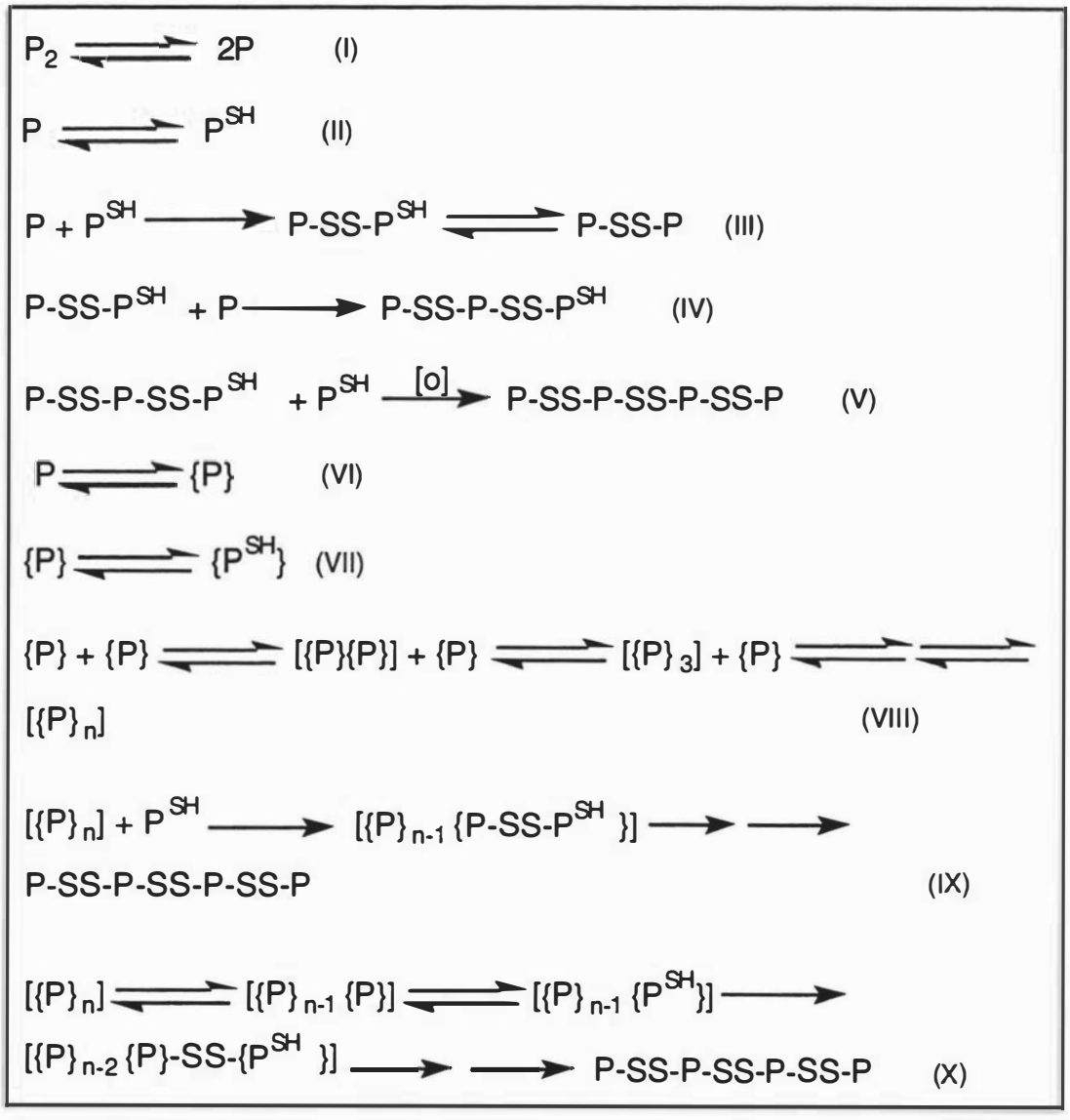


Fig. 8.1.4. The model for the heat-induced aggregation of BLG of Gezimati *et al.* (1997). For a description of the reactions and species, see the text.

Reactions I - V depict aggregate formation via thiol-disulphide interchange. Initially, heat treatment leads to dimer dissociation (reaction I), and then to thiol exposure in some BLG monomers (reaction II). Via reaction (III), exposed thiol groups can then catalyse thiol-disulphide interchange with the monomers formed in reaction (I). If the thiol group of the resulting disulphide-linked dimer remains exposed, then this species can catalyse further thiol-disulphide interchange reactions (reaction IV). The exposed thiol groups in different polymeric clusters may also react with each other, resulting in oxidation to an intermolecular disulphide bond (reaction V). This portion of the model of Gezimati *et al.* (1997) is therefore similar to that of Roefs and de Kruif (1994). Gezimati *et al.* (1997) also suggested that after disulphide-linked dimers have formed via thiol-disulphide interchange, the new solvent-exposed thiol group may become re-buried. This will prevent the newly formed disulphide-linked dimer from taking part in further thiol-disulphide interchange reactions.

Gezimati *et al.* (1997) suggest that the relatively solvent-exposed disulphide bond Cys^{66}-Cys^{160} (Fig. 2.4.9) participates in more intermolecular thiol-disulphide interchange reactions than the disulphide bond Cys^{106}-Cys^{119}, which is located in the interior of the BLG structure (Fig. 2.4.8). This suggestion is consistent with the results of Morgan *et al.* (1997), who discovered a disulphide bonded peptide comprising the residues 102 - 124 and 149 - 162 in BLG solutions previously heat-treated at 60 °C at pH 7.0 for periods up to 1 hr. The disulphide bond in this peptide is not found in native BLG and may thus have formed between Cys^{121} and Cys^{160} as a consequence of heat-induced thiol-disulphide interchange. Gezimati *et al.* (1997) also suggest that extensive intramolecular thiol-disulphide interchange may not be possible until BLG molecules have unfolded.

In their BLG aggregation model, Gezimati *et al.* (1997) also postulate the conversion of BLG monomers to a molten globule-like species (reaction VI, Fig. 8.1.4) and the subsequent formation of non-covalently-linked aggregates (reaction VIII). These non-covalently-linked aggregates may then be converted to disulphide-linked aggregates via two pathways (Gezimati *et al.*, 1997). In the first pathway, they suggest that the thiol group of a monomeric BLG molecule becomes solvent-exposed (reaction VII, Fig. 8.1.4) and catalyses thiol-disulphide interchange reactions in non-covalently-linked aggregates (reaction IX). In the second pathway suggested by Gezimati *et al.* (1997), thiol exposure may occur from within non-covalently-linked aggregates, initiating the conversion of these species to disulphide-linked aggregates (reaction X). The second portion of the BLG aggregation model of Gezimati *et al.* (1996) is therefore similar to the models of McSwiney *et al.* (1994b) and Iametti *et al.* (1996), in which the formation of non-covalently-linked aggregates precedes thiol-disulphide interchange.

8.1.6. CONCLUSION.

In all but one of the models discussed in Sections 2.7 and 8.1.1 - 8.1.5, dimer dissociation is assumed to be the first step on the aggregation pathway of BLG. Qi *et al.* (1995), however, have suggested that dimer dissociation may occur after unfolding events in BLG solutions at concentrations greater than 25 mg/mL.

All of the aggregation models discussed in Sections 2.7 and 8.1.1 - 8.1.5 include steps after dimer dissociation which correspond to structural change in BLG monomers. Studies in which heat-induced structural change and aggregation were examined suggest that BLG is converted to a molten globule-like species at elevated temperatures (McSwiney *et al.*, 1994b; Iametti *et al.*, 1996; Qi *et al.*, 1997; Gezimati *et al.*, 1997). This species appears to possess no α-helix and slightly less β-sheet than native BLG (Qi *et al.*, 1997), and may be more hydrophobic than the corresponding native species (Iametti *et al.*, 1996). Heat treatment also leads to thiol exposure and changes in the environments of aromatic side chains, suggesting a loss of tertiary structure (Iametti *et al.*, 1996).

An examination of the BLG aggregation models published to date indicates that there is still some doubt as to how aggregates form. Some models assume that the solvent-exposed thiol group of partially unfolded BLG monomers catalyses thiol-disulphide interchange reactions, leading to the formation of disulphide-linked aggregates (Roefs and de Kruif, 1994; Gezimati *et al.*, 1997). In other models, it is suggested that, initially, non-covalently-linked aggregates are formed from molten globule-like species, and that thiol-disulphide interchange reactions lead to the stabilisation of these aggregate species (McSwiney *et al.*, 1994b; Iametti *et al.*, 1996, Qi *et al.*, 1997). The formation of disulphide-linked aggregates of BLG may also occur via both thiol-disulphide interchange reactions and the association of molten globule-like species, as suggested by Gezimati *et al.* (1997).

8.2. SUMMARY OF EXPERIMENTAL RESULTS.

The near UV CD, tryptophan fluorescence and thiol availability assay results in Chapters 5, 6 and 7 suggest that the following structural changes occur during heat treatment: the side chain of Trp^{19} is shifted into a less chiral and more solvent-exposed environment, the side chain of Trp^{61} is shifted into a more solvent-exposed environment and fluoresces more intensely, and the thiol group becomes solvent-exposed. These structural changes are irreversible after heat treatment for 12.5 min or 13.5 min at temperatures greater than approximately 65 °C at pH 6.7 and 55 °C at pH 7.4. These heating conditions will be subsequently referred to as "high temperatures". The ANS fluorescence results also indicate that the structure of BLG is altered as a consequence of heat treatment at high temperatures. The far UV CD results suggest that the proportion of β-sheet in molecules of BLG increases slightly, while the proportion of random structure increases appreciably as a consequence of heat treatment at high temperatures. The alkaline native-PAGE and SDS-PAGE results indicate that heat-treated solutions of BLG contain both non-covalently-linked and disulphide-linked aggregates, and that non-covalently-linked aggregates as well as "unfolded" monomeric and disulphide-linked dimeric species are important intermediary species on the BLG aggregation pathway.

8.3. HEAT-INDUCED STRUCTURAL CHANGE IN MOLECULES OF BOVINE β-LACTOGLOBULIN AND THE HEAT-INDUCED AGGREGATION OF BOVINE β-LACTOGLOBULIN.

The mechanism by which BLG forms aggregates during heat treatment has been studied extensively (Sections 2.7 and 8.1). However, the results in Chapters 5, 6 and 7 (summarised in Section 8.2) contribute to an increased understanding of this mechanism in several ways: confirming, extending, and in some cases, contradicting the results of earlier studies. In the discussion presented below, the results in Chapters 5, 6 and 7 are compared with those obtained in earlier studies, with the aim of re-evaluating how heat treatment affects the structure of BLG and leads to the formation of aggregates. At the end of this section, a new model for the heat-induced aggregation of bovine BLG, which takes into account both the results in Chapters 5, 6 and 7 and those of others, is presented.

8.3.1. NATURE OF THE HEAT-INDUCED STRUCTURAL CHANGES IN β-LACTOGLOBULIN MOLECULES.

8.3.1.1. Tertiary Structure Modification.

The results in Chapters 6 and 7 indicate that a number of structural changes occur in BLG molecules during heat treatment. Firstly, near UV CD results suggest that the side chain of Trp^{19} is in a less chiral environment at temperatures of 54 °C and higher than at room temperature (Section 7.3.1.2). Secondly, tryptophan fluorescence emission λ_{max} results suggest that the side chain of at least one tryptophan residue is more solvent-exposed at elevated temperatures than at room temperature (Section 7.3.2.1). Thirdly, I_{Trp} results suggest that the extent of Trp^{61} fluorescence quenching at elevated temperatures is less than that at room temperature (Sections 7.3.2.1 and 7.3.2.2). The structural changes which may be responsible for this phenomenon are described in Section 5.3.3.2. Fourthly, thiol availability assay results indicate that a thiol group becomes solvent-exposed at elevated temperatures (Section 6.3.1.3).

The spectroscopic results in Chapter 5 were obtained at 20 °C using BLG solutions previously heat-treated. Near UV CD (Section 5.3.1), tryptophan fluorescence emission λ_{max} (Section 5.3.3) and thiol availability (Sections 5.3.9 and 6.3.1.6) results indicate that the structural changes discussed above become irreversible after heat treatment at high temperatures. Furthermore, the far UV CD results in Section 5.3.2 suggest that heat treatment at higher temperatures leads to an irreversible increase in the proportion of random structure in BLG molecules.

All of the results discussed above, except possibly those obtained in thiol availability measurements, appear consistent with a loosening of side chain packing in molecules of BLG. They may therefore indicate that some of the native tertiary structure of BLG is lost during heat treatment, this loss being irreversible after heat treatment at higher temperatures. From near UV CD results obtained from measurements made at elevated temperatures, Griffin *et al.* (1993) and Iametti *et al.* (1996) have suggested that BLG tertiary structure is modified during heat treatment. Furthermore, Iametti *et al.* (1996) suggested that at temperatures greater than 70 °C these modifications become irreversible.

8.3.1.2. Thiol Group Exposure.

The nature of the heat-induced structural change which leads to the solvent-exposure of the thiol group of Cys^{121} may be dependent on heat treatment temperature. The thiol group of Cys^{121} in native BLG is sandwiched between the α-helix and the exterior of the calyx (Fig. 2.4.8). Therefore, heat-induced thiol group exposure may occur as a consequence of a peeling of the α-helix from the exterior of the calyx (Section 5.3.5.3), and thus a modification of tertiary structure. However, because the proportion of α-helix in BLG molecules decreases with increasing temperature above 60 °C (Qi *et al.*, 1997), thiol group exposure can also occur as a consequence of a change in secondary structure. Thus, thiol group exposure at temperatures less than approximately 60 °C may occur as a consequence of a loss of tertiary structure, while at temperatures greater than approximately 60 °C thiol group exposure may occur as a consequence of a loss in both tertiary and secondary structure.

8.3.1.3. ANS Fluorescence.

ANS fluorescence emission results for previously heat-treated BLG solutions (Section 5.3.4) also indicate that the structures of BLG molecules are irreversibly altered as a consequence of heat treatment. Emission λ_{max} results suggest that heat treatment at temperatures greater than approximately 55 °C leads to an irreversible increase in the hydrophobicity of the ANS binding site of BLG, which is therefore indicative of irreversible structural change (Section 5.3.4.3). The intensity of fluorescence emission from ANS bound to BLG increases as a consequence of protein heat treatment at high temperatures (Section 5.3.4.5). These results are more difficult to interpret than ANS emission λ_{max} results, but may indicate that the ANS binding site of heat-treated BLG is more hydrophobic and binds ANS molecules in a more planar conformation than the ANS binding site of the corresponding unheated species. Therefore, ANS fluorescence emission intensity results could only be interpreted as indicating that structural change in molecules of BLG occurs as a consequence of heat treatment.

8.3.1.4. Secondary Structure Modification.

The far UV CD results also suggest that the proportion of β-sheet in molecules of BLG increases slightly as a consequence of heat treatment at approximately 3 mg/mL (Section 5.3.2.3). This is inconsistent with the results obtained in other far UV CD studies in which spectra were recorded at room temperature using BLG solutions previously heat-treated (Sawyer *et al.*, 1971; Matsuura and Manning, 1994). They reported that the proportion of β-sheet in BLG molecules increases appreciably as a consequence of heat treatment. However, because Matsuura and Manning (1994) heat-treated BLG solutions at a concentration of 70 mg/mL, wherein gelation occurs, a quantitative comparison of the results in Section 5.3.2 with those of Matsuura and Manning (1994) may be inappropriate. Sawyer *et al.* (1971) heat-treated BLG solutions at a concentration of 5 mg/mL (Section 5.3.2.3). This concentration is similar to that at which the BLG solutions used in the present study were heat-treated (Section 5.3.2). The large heat-induced increase in the proportion of β-sheet in BLG molecules reported by Sawyer *et al.* (1971) may therefore require re-evaluation.

The results in Section 5.3.2 are consistent with those obtained in far UV CD studies made at elevated temperatures (Griffin *et al.*, 1993; Qi *et al.*, 1997), which show that the intensity of $[\theta]_{216}$ does not change appreciably during temperature increase. This is indicative of only a small change (Griffin *et al.*, 1993) and a small decrease (Qi *et al.*, 1997) in the proportion of β-sheet in BLG molecules. The conclusion drawn in Section 5.3.2.3 is thus similar to those drawn by Griffin *et al.* (1993) and Qi *et al.* (1997). In contrast, Lapanje and Poklar (1989) reported that $[\theta]_{216}$ decreases appreciably with increasing temperature.

8.3.1.5. Irreversible Structural Change in Molecules of β-Lactoglobulin in Relation to Molten Globule-like Conformations.

The results in Chapter 5 suggest that BLG molecules previously heat-treated at high temperatures (see Section 8.2 for definition) exhibit four of the five characteristics of a molten globule (Section 8.1.1). Firstly, after heat treatment under these conditions, BLG appears to possess less native tertiary structure than the corresponding unheated species (Section 8.3.1.1). Secondly, BLG appears to possess slightly more secondary structure after heat treatment at high temperatures than the corresponding unheated species (Section 8.3.1.4). Thirdly, the intensity of fluorescence emission from ANS bound to BLG previously heat-treated at high temperatures is greater than that from ANS bound to the corresponding unheated species (Section 8.3.1.3). Fourthly, the PAGE results in Section 5.3.9 suggest that BLG exhibits a tendency to form aggregates during heat treatment at high temperatures. Although these PAGE results suggest that aggregates of BLG are eventually stabilised by intermolecular disulphide bonds, they also suggest that initially some are stabilised in only a non-covalent manner, the standard mode of stabilisation of aggregates formed by proteins in a molten globule conformation (Fink, 1995).

A protein in a molten globule conformation is also slightly less compact than it is in the native state (Fink, 1995). In the study presented in this thesis, the molecular size of BLG was not measured at elevated temperatures, or at room temperature after heat treatment. Nevertheless, the alkaline native-PAGE results in Figs 5.3.9.4 and 5.3.9.5 show that a BLG species with an electrophoretic mobility intermediate between those of "native-like" monomers[1] and dimers is formed as a consequence of heat treatment. This species is probably a stable unfolded form of monomeric BLG (Sections 5.3.9.4 and 5.3.9.5). Its presence in heat-treated solutions therefore indicates that at least some BLG molecules in heat-treated solutions are unfolded to a considerable extent and are therefore much less compact than the corresponding native species. For this reason, the alkaline native-PAGE results indicate that at least some BLG molecules in heat-treated samples do not exhibit all five characteristics of a molten globule, and therefore do not exist in a molten globule-like conformation.

Although some BLG molecules in previously heat-treated solutions do not exist in a molten globule-like conformation, it is not possible to ascertain from alkaline native-PAGE results alone whether other BLG molecules in these solutions are found in a molten globule-like conformation. This is because BLG molecules retained in a molten globule-like conformation after heat treatment would have an electrophoretic mobility on alkaline native gels similar to that of "native-like" monomeric BLG. The correlations of temperature-dependent changes in monomeric BLG band intensity with temperature-dependent changes in spectroscopic signal intensity (Section 5.3.10.1) may, however, indicate that BLG is not retained in a molten globule-like conformation after heat treatment. The proportion of BLG molecules in heat-treated solutions which runs as a monomer on alkaline native gels decreases linearly with the proportion of BLG molecules in heat-treated solutions which exhibit the spectral characteristics (i.e. $\Delta\varepsilon_{293}$ $[\theta]_{205}$, I_{Trp} and I_{ANS}) and the thiol availability of the unheated species. This suggests that the monomeric BLG band on alkaline native gels contains only "native-like" and not molten globule-like BLG molecules. Therefore, the proportion of BLG molecules in heat-treated solutions which exist in a molten globule-like conformation may be negligible.

Although the results discussed above suggest that the monomeric BLG band on alkaline native gels contains only "native-like" BLG, they may instead indicate that concentrations of this species and also of a molten globule-like species both decrease linearly with increasing heat treatment temperature in a similar manner. However, this behaviour is unlikely. Time-course alkaline native-PAGE results (Figs 5.3.9.11 and

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[1] The BLG molecules in the monomeric band on native gels are assumed to be only "native-like" because these gels are run at pH 8.8, where denaturation can occur at room temperature (Groves *et al.*, 1951; Casal *et al.*, 1988).

5.3.9.12) indicate that the concentrations of non "native-like" BLG species with M_r values less than approximately 200 000 in BLG solutions decrease with increasing heat treatment time after approximately 10 min. Therefore, it is likely that the concentration of a molten globule-like species in heat-treated solutions, which would also be non-"native-like", would exhibit a similar dependence on heat treatment time (i.e. the concentration of the molten globule-like species would not decrease linearly with increasing heat treatment time). Therefore, because the correlations discussed in Section 5.3.10.1 are linear, it is unlikely that BLG molecules in a molten globule-like conformation run with "native-like" monomers on alkaline native gels. If the opposite were true, then the correlations discussed here and in Section 5.3.10.1 would probably not have been linear. Therefore, it is likely that the monomeric BLG band on alkaline native gels contains only "native-like" BLG. This in turn increases the likelihood that alkaline native-PAGE results (Section 5.3.9) indicate that heat-treated BLG solutions do not contain a partially folded species which is only slightly less compact than the corresponding native species. Thus, it appears that the proportion of BLG molecules retained in a molten globule-like conformation as a consequence of heat treatment is negligible.

8.3.1.6. Formation of a Molten Globule-like Conformation at Elevated Temperatures.

The results discussed in Section 8.3.1.5 suggest that the BLG molecules in previously heat-treated solutions do not exist in a molten globule-like conformation but are partially unfolded. However, BLG may exist in a molten globule-like conformation at some stage during heat treatment under conditions which lead to irreversible structural change. The results in Chapters 6 and 7 suggest that BLG possesses less native tertiary structure at elevated temperatures than at room temperature (Section 8.3.1.1). Iametti *et al.* (1996) have also suggested that BLG assumes a molten globule-like conformation at elevated temperatures. They found that temperature increase leads to a modification of tertiary structure and for short periods of time, an increase in the intensity of fluorescence emission from bound ANS (Section 8.1.1). Similarly, Qi *et al.* (1997) have suggested that BLG exists in a molten globule-like conformation at temperatures greater than approximately 70 °C. Their far UV CD results suggest that at these temperatures, BLG contains no α-helix and slightly less β-sheet than at room temperature.

8.3.1.7. Further Evidence Which Suggests that Molecules of β-Lactoglobulin in Solutions Previously Heat-treated are only Partially Unfolded.

A comparison of the results in Chapter 5 with those obtained in the urea unfolding study of Creamer (1995) is consistent with the suggestion that BLG molecules in previously heat-treated solutions are partially folded. Bands are still visible in the near UV CD spectrum of BLG previously heat-treated at temperatures greater than approximately 84 °C. In contrast, no bands are observed when the near UV CD spectrum of BLG is recorded in the presence of 7.24 M urea (Creamer, 1995). A comparison suggests that the side chain of Trp^{19} is located in a semi-chiral environment after heat treatment, which in turn may be indicative of only a partial loss of native structure.

Intrinsic protein fluorescence results obtained using an excitation wavelength of 275 nm (Section 5.3.5.7) indicate that the efficiency of RET from tyrosine to tryptophan is still high after heat treatment at temperatures greater than approximately 84 °C. Therefore, at least some tyrosine and tryptophan side chains are located within 14 Å of each other in BLG molecules in previously heat-treated solutions. This indicates that the BLG molecules in heat-treated solutions are only partially unfolded. In contrast, the efficiency of RET from tyrosine to tryptophan is diminished appreciably in the presence of 9.48 M urea (Creamer, 1995). A comparison therefore suggests that the extent of BLG unfolding in the presence of 9.48 M urea is greater than that which occurs as a consequence of heat treatment.

The ANS fluorescence emission intensity is enhanced in the presence of heat-treated BLG (Section 5.3.4). This indicates that the BLG molecules in previously heat-treated solutions are capable of binding ANS, suggesting only partial protein unfolding. The ANS binding site of heat-treated BLG, however, is probably different from that of native BLG (Section 5.3.4.3). Conversely, in the presence of 8.1 M urea BLG does not enhance ANS emission intensity, suggesting a loss of the integrity of the ANS binding site of BLG (Creamer, 1995). A comparison therefore suggests that the extent of BLG unfolding in the presence of 8.1 M urea is greater than that which occurs as a consequence of heat treatment.

The far UV CD results in Section 5.3.2 suggest that the proportion of β-sheet in molecules of BLG increases slightly as a consequence of heat treatment. However, the proportion of β-sheet in BLG is negligible in the presence of 7.24 M urea (Creamer, 1995). These results also suggest that heat and urea treatment lead to partial and near-complete unfolding respectively. To summarise, the comparison of the results in Chapter 5 with those obtained in the urea unfolding study of Creamer (1995) is consistent with the suggestion that BLG molecules in heat-treated solutions exist in a partially folded conformation.

8.3.2. TIME-DEPENDENT CHANGES IN β-LACTOGLOBULIN STRUCTURE DURING HEAT TREATMENT.

The results in Chapter 6 show that thiol group exposure occurs at lower temperatures than heat-induced structural change in the vicinity of Trp^{19} ($\Delta\varepsilon_{293}$ results). Therefore, BLG is likely to be more susceptible to structural change in the vicinity of Cys^{121} than in the vicinity of Trp^{19} during heat treatment. This in turn suggests that initial thiol group exposure may precede structural change in other parts of the BLG molecule during heat treatment.

Time-dependent increases in I_{Trp} appear to occur as a consequence of two phenomena. Approximately 10 min after rapid temperature increase to 80 °C, the rate of increase in I_{Trp} decreased (Section 6.3.3). The first phase of this increase was assumed to reflect changes in tertiary and secondary structure in BLG molecules because at 80 °C time-dependent changes in $\Delta\varepsilon_{293}$ also occur over periods of approximately 10 min (Section 6.3.2). The second phase of the time-dependent increase in I_{Trp}, which continued for at least 3.5 hr, may occur as a consequence of aggregate formation. This is because time-course PAGE results indicated that aggregate formation occurs on a similar time-scale to the second phase of the time-dependent increase in I_{Trp} (Section 6.3.3).

The rates of change in I_{Trp} determined in the study in Chapter 6 are very much slower than those determined by Iametti and co-workers, who reported that changes in I_{Trp} were complete within seconds of temperature increase to 80 °C - 85 °C. However, Iametti and co-workers followed changes in I_{Trp} for only 2 min and therefore would not have observed the second phase of the time-dependent increase in I_{Trp} identified in Chapter 6.

8.3.3. EVIDENCE FOR NON-NATIVE DISULPHIDE BOND FORMATION FROM SPECTROSCOPIC RESULTS.

Many of the results obtained from spectroscopic measurements suggest that non-native disulphide bonds, both intramolecular and intermolecular, are formed in BLG molecules during and as a consequence of heat treatment. Firstly, near UV CD results indicate that the intensity of $\Delta\varepsilon_{270}$ in the spectra of BLG solutions previously heat-treated at temperatures greater than approximately 75 °C is greater than that in the spectra of unheated solutions. This suggests that non-native disulphide bonds are formed as a consequence of heat treatment. Secondly, increases in I_{Trp}, observed

during heat treatment (Chapters 6 and 7) and at 20 °C **after heat treatment** (Chapter 5) may reflect the loss of the disulphide bond Cys^{66}-Cys^{160}. This disulphide bond is thought to be the main quencher of tryptophan fluorescence in native BLG, and its loss may occur when non-native intramolecular and intermolecular disulphide bonds are formed (Sections 5.3.3.2 and 5.3.3.8).

Creamer (1995) reported that I_{Trp} increases as the urea concentration in BLG solutions is increased. However, the extent of aggregate formation which occurs in the presence of urea is significantly less than that which occurs during heat treatment (Creamer, personal communication). Furthermore, the comparisons discussed in Section 8.3.1.7 suggest that BLG molecules in solutions of concentrated urea are more unfolded than those in solutions previously heat-treated under conditions which lead to irreversible structural change. Therefore, it is likely that the increases in I_{Trp} observed by Creamer (1995) reflect an increase in the distance between the side chain of Trp^{61} and the disulphide bond Cys^{66}-Cys^{160} rather than the loss of this disulphide bond. In contrast, the opposite may be true in the case of increases in I_{Trp} which occur as a consequence of heat treatment (Section 5.3.3.8).

Both the thiol availability assay results in Section 6.3.1.6 and the results obtained at 20 °C for BLG solutions previously heat-treated (Section 5.3.5) suggest that the proportions of BLG molecules which possess a thiol group decrease as a consequence of heat treatment. This suggests that non-native disulphide bonds in BLG molecules are formed via thiol oxidation reactions during heat treatment. However, there is no actual proof that this interpretation is correct. For example, thiol groups exposed at elevated temperatures may have become re-buried during cooling. Nevertheless, although the results obtained from thiol availability measurements made using BLG solutions previously heat-treated may reflect, in part, a re-burying of thiol groups, they probably also indicate that non-native disulphide bonds are formed during heat treatment.

8.3.4. AGGREGATION.

The PAGE results in Section 5.3.9 appear most consistent with the BLG aggregation models of McSwiney *et al.* (1994b) and Gezimati *et al.* (1997) because they suggest that BLG forms both disulphide-linked and non-covalently-linked aggregates during heat treatment (Section 5.3.9.7). However, many of the results in Section 5.3.9 pertinent to aggregate formation are also consistent with the results obtained in studies other than those made by McSwiney *et al.* (1994a, b) and Gezimati *et al.* (1997). A comparison of the results obtained in the PAGE study in Section 5.3.9 with those obtained in earlier BLG aggregation studies will now be made.

8.3.4.1. Comparison of Present Results with those of Others and the Aggregation Models of Others.

8.3.4.1.1. Formation of Disulphide-linked Aggregates During Heat Treatment.

In agreement with the results obtained in all of the earlier BLG aggregation studies and reviews discussed in Sections 2.7 and 8.1, a comparison of the SDS-PAGE results obtained in the presence and absence of 2-mercaptoethanol indicates that disulphide-linked aggregates of BLG are formed when solutions of BLG are heat-treated in the absence of reducing agents and thiol blocking reagents (Section 5.3.9.2) . Furthermore, disulphide-intact SDS-PAGE results indicate that the concentrations of these aggregates increase with both increasing heat treatment temperature (Sections 5.3.9.2.2 and 5.3.9.3) and heat treatment time (Section 5.3.9.8), i.e. as the severity of heat treatment conditions is increased, in agreement with the results of Iametti *et al.* (1995).

8.3.4.1.2. Formation of Non-covalently-linked Aggregates During Heat Treatment.

The comparison of the alkaline native-PAGE and disulphide-intact SDS-PAGE results discussed in Section 4.3.9.7 shows that non-covalently-linked aggregates of BLG are formed during heat treatment, in agreement with the PAGE results of McSwiney *et al.* (1994a, b) and Gezimati *et al.* (1997). Similarly, Sawyer (1968) and Elofsson *et al.* (1996b) reported that BLG forms non-covalently-linked aggregates in the presence of the thiol blocking agent N-ethylmaleimide, although the extent of BLG aggregation was less in its presence than in its absence. From a comparison of SDS-PAGE and gel permeation chromatography results, Iametti *et al.* (1996) also reported that not all BLG aggregates are stabilised in a covalent manner. However, from gel permeation chromatography results, Iametti *et al.* (1996) would not have been able to distinguish between native dimers, non-covalently-linked non-native dimers and disulphide-linked dimers. Nevertheless, it is likely that their conclusion, that not all aggregates of BLG are stabilised in a covalent manner, is valid.

8.3.4.1.3. Dimer Dissociation and the Stepwise Incorporation of Monomers into Aggregate Structures.

The alkaline native-PAGE and disulphide-intact SDS-PAGE results in Section 5.3.9 may be consistent with the suggestion of Qi *et al.* (1995) that, when BLG solutions are heat-treated at concentrations less than approximately 25 mg/mL, dimer dissociation precedes aggregation. Although native dimers of BLG are dissociated to monomers on both alkaline native (Section 5.3.9.4) and disulphide-intact SDS gels,

these gels indicate that heat-treated BLG solutions contain a stable trimeric species, which cannot form unless dimers dissociate to monomers during heat treatment. Cairoli *et al.* (1994) also reported the presence of trimeric BLG species in heat-treated solutions of BLG.

The alkaline native-PAGE results (Section 5.3.9) may also indicate that BLG monomers are incorporated into growing aggregate structures in a step-wise manner during heat treatment, as suggested by Cairoli *et al.* (1994) and Iametti *et al.* (1996). The present research results indicate that dimers, trimers, tetramers etc. are present in heat-treated BLG solutions, and are therefore consistent with this manner of aggregate growth.

8.3.4.1.4. Increase in Aggregate Molecular Weight During Heat Treatment.

All of the PAGE results in Section 5.3.9 indicate that the extent of conversion of low M_r aggregates to high M_r aggregates increases with both increasing heat treatment temperature and heat treatment time. This is consistent with the results obtained in the photon correlation spectroscopic study of Griffin *et al.* (1993), and the *in situ* DLS studies of Elofsson *et al.* (1996 a, b) and Hoffmann *et al.* (1996), which indicate that the size of BLG aggregates increases with increasing heat treatment time.

Elofsson *et al.* (1996a, b) reported a lag phase prior to the onset of aggregation when 8 mg/mL solutions of BLG were heat-treated at temperatures between 59 °C and 63 °C. This may be consistent with the PAGE results in Section 5.3.9, which indicate that the concentration of aggregates in BLG solutions previously heat-treated at a similar concentration at temperatures less than approximately 65 °C for 12.5 min are negligible. However, the results of Elofsson *et al.* (1996 a, b) suggest that if the BLG solutions used in the PAGE study had been heat-treated in this temperature range for longer periods of time, then aggregation may have occurred.

8.3.4.2. Novel Results.

Some of the results obtained in the BLG aggregation study in Section 5.3.9 do not appear to correspond to steps in any of the earlier BLG aggregation models discussed in Sections 2.7 and 8.1. These will now be identified.

8.3.4.2.1. Stability of Non-covalently-linked Aggregates.

The quantitative comparison of time-course alkaline native-PAGE and disulphide-intact SDS-PAGE results (Section 5.3.9.8) indicates that non-covalently-linked aggregates of BLG exist as reaction intermediates during heat treatment. Concentrations of these species start to decrease with increasing heat treatment time after approximately 10 min. These results are therefore consistent with the BLG aggregation models of McSwiney *et al.* (1994b) and Gezimati *et al.* (1997).

8.3.4.2.2. "Unfolded" Monomers and Disulphide-linked Dimers.

The alkaline native-PAGE and the 2D-PAGE results in Section 5.3.9 suggest the presence in heat-treated BLG solutions of stable "unfolded" monomeric BLG species which have mobilities intermediate between those of monomers and dimers on alkaline native gels, but usually run as a monomer on SDS gels. Because the 2D-PAGE results show that these species do not always run as monomers in an SDS-PAGE system, some "unfolded" monomers are probably retained in partially unfolded conformations by non-native intramolecular disulphide bonds (Section 5.3.9.6). These "unfolded" monomeric BLG species also appear to exist as intermediate species during heat treatment because their concentrations vary with heat treatment temperature and heat treatment time in a manner similar to that observed for non-covalently-linked BLG aggregates (Section 8.3.4.2.1). The multiple bands observed in the regions of alkaline native and SDS gels where monomers, dimers, trimers etc. run may also be consistent with the suggestion that "unfolded" monomers of BLG which possess non-native intramolecular disulphide bonds are formed during heat treatment (Section 5.3.9.5).

The 2D-PAGE results in Section 5.3.9.6 suggest that disulphide-linked dimers are another important intermediate species on the BLG aggregation pathway. Using disulphide-intact SDS-PAGE, this species was resolved from all BLG aggregate bands previously separated by alkaline native-PAGE.

8.3.4.2.3. Comparison of PAGE and Spectroscopic Results.

The extent of the loss due to aggregate formation of the BLG species that run as monomers on alkaline native gels correlates linearly with the extent of the heat-induced loss of the BLG species which exhibit the spectral characteristics of native BLG (Section 5.3.10.1). This suggests that only the species which run as "native-like" monomers on alkaline native gels exhibit the spectral characteristics of native BLG.

Normalised disulphide-intact SDS-PAGE monomer band intensity data do not correlate as well with normalised spectroscopic data as do normalised alkaline native-PAGE monomer band intensity data (Section 5.3.10.1). Therefore, the correlations in this section indicate that alkaline native-PAGE is a better technique than disulphide-intact SDS-PAGE for measuring losses of native BLG caused by heat treatment.

The threshold temperatures for irreversible structural change and aggregate formation are similar. The PAGE results in Section 5.3.9 indicate that concentrations of aggregate species are negligible in BLG solutions previously heat-treated for 12.5 min at temperatures less than approximately 65 °C at pH 6.7 and 55 °C at pH 7.4. Furthermore, most of the spectroscopic results in Chapter 5 indicate that extents of irreversible structural change in BLG molecules previously heat-treated under similar conditions are negligible.

8.3.4.3. Contradictions to Other β-Lactoglobulin Aggregation Models.

The PAGE results in Section 5.3.9 are not consistent with the BLG aggregation model of Roefs and de Kruif (1994) which is discussed in Section 8.1.4. The results in Section 5.3.9 clearly demonstrate that heat-treated BLG solutions contain non-covalently-linked aggregates. This is difficult to explain if it is assumed that aggregates of BLG can only form via intermolecular thiol/disulphide interchange, as suggested by Roefs and de Kruif (1994). The results in Section 5.3.9 also suggest that stable "unfolded" monomeric BLG species, which probably contain non-native intramolecular disulphide bonds, are formed during heat treatment. Although Roefs and de Kruif (1994) suggest that thiol group activation is dependent on protein unfolding, the retention of moderate concentrations of stable "unfolded" monomeric species in BLG solutions after heat treatment seems inconsistent with the suggestion that an "unfolded" monomeric BLG species initiates aggregation in a manner analogous to the free radical polymerisation mechanism of ethylene (Section 8.1.4). The results in Section 5.3.9 also indicate that disulphide-linked dimers are associated non-covalently in larger BLG aggregates. Once again, this is difficult to explain in terms of the aggregation model of Roefs and de Kruif (1994), which assumes that BLG monomers are added to linear aggregates in a step-wise manner via thiol-disulphide interchange.

8.3.5. PROPOSED MODEL FOR THE HEAT-INDUCED AGGREGATION OF BOVINE β-LACTOGLOBULIN.

Some of the results discussed in Section 8.3.4 appear inconsistent with published bovine BLG aggregation models and may therefore indicate that the bovine BLG aggregation mechanism is more complex than was previously thought. For this reason, it must be assumed that published aggregation models (Sections 2.7 and 8.1) do not accurately described the mechanism by which bovine BLG forms aggregates during heat treatment. A new model for the heat-induced aggregation of bovine BLG, based on that of Gezimati *et al.* (1997), but which takes into account the results presented and discussed in this thesis, will now be described (Fig. 8.3.5.1).

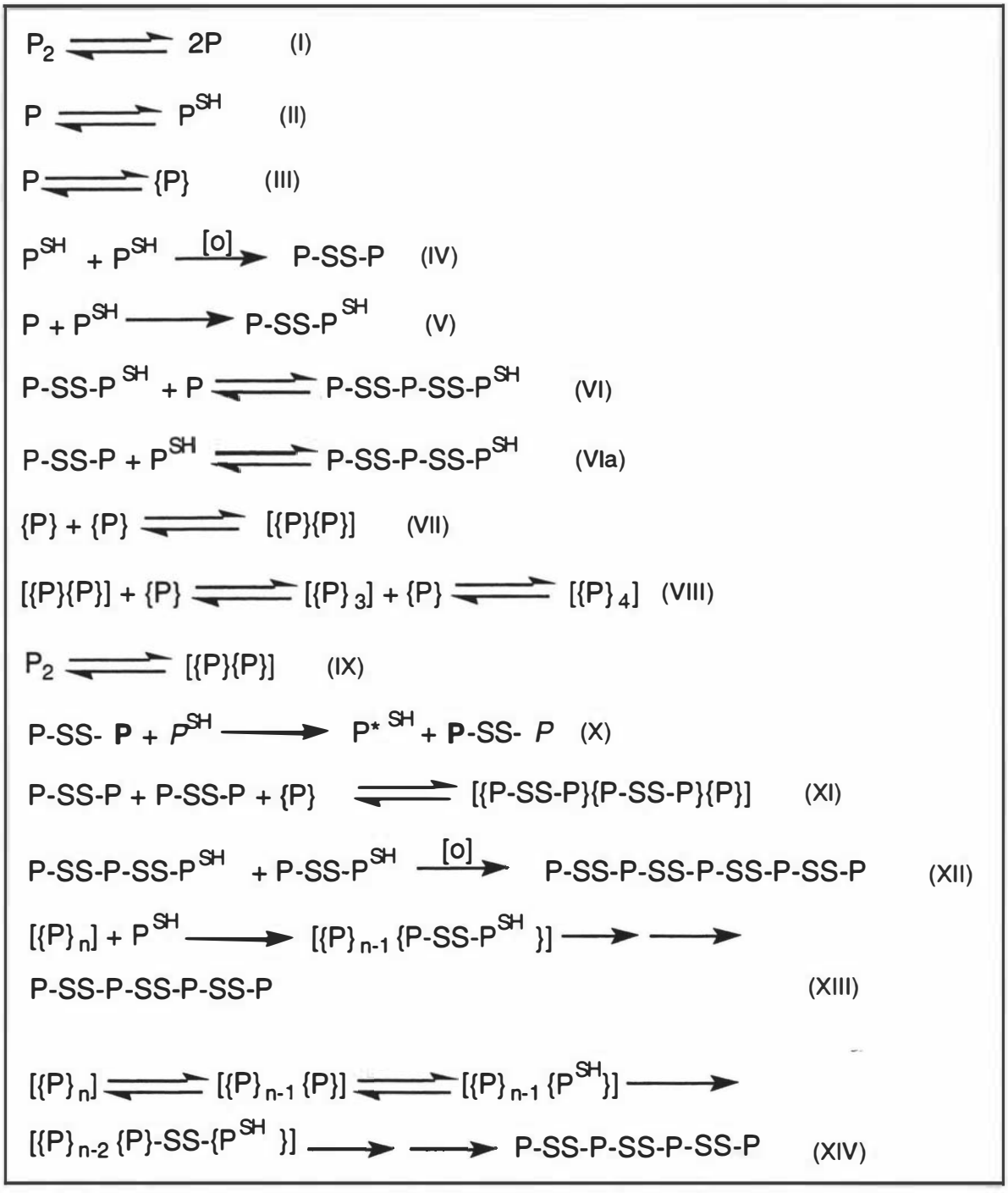

Fig. 8.3.5.1. Proposed model for the heat-induced aggregation of bovine BLG. For a description of the reactions and species, see the text.

1) Dimer Dissociation.

Reaction (I) depicts the reversible dissociation of BLG dimers to monomers.

2) Loss of Native Structure in Molecules of β-Lactoglobulin and the Initiation of Aggregation.

Aggregation may be initiated by reversible structural change in BLG monomers. These structural changes lead to a slight peeling of the α-helix from the calyx (formation of species P^{SH} via reaction II) and therefore an increase in the exposure of the thiol of Cys^{121}. Structural change is also likely to lead to the transient exposure of a few hydrophobic side chains (formation of species {P} via reaction III) which may serve as nucleation sites for both further unfolding and aggregation. From the results in both the literature and this thesis, it is not possible to ascertain whether or not P^{SH} and {P} are the same species. However, data suggest that thiol exposure may precede other structural changes during heat treatment (Section 8.3.2).

3) Alternative Pathways for the Loss of Native Structure in β-Lactoglobulin Molecules.

Native BLG molecules may also be converted to non-native conformations as a consequence of the formation of non-native intramolecular disulphide bonds via thiol-disulphide interchange. This will be discussed in 5) below.

4) The Initial Stages of Aggregation.

β-Lactoglobulin monomers in the form P^{SH} can form aggregates via thiol oxidation (reaction IV) or thiol-disulphide interchange (reactions V and VI). Alone, disulphide-linked dimers formed via thiol oxidation (P-SS-P) cannot aggregate further. This may be the reason why this species is present in larger BLG aggregates associated in mainly a non-covalent manner. However, in the presence of BLG species which possess solvent-exposed thiol groups, disulphide linked dimers may aggregate further via thiol-disulphide interchange (reaction VIa). As suggested by Gezimati *et al.* (1997), it is most likely that the solvent-exposed thiol group of a BLG monomer in the form P^{SH} reacts with the disulphide bond Cys^{66}-Cys^{160} of another BLG molecule because this disulphide bond is more solvent-exposed than that which forms between Cys^{106} and Cys^{119}.

Because BLG monomers in the form {P} possess more solvent-exposed hydrophobic side chains than native BLG, the former may associate via hydrophobic interactions (reactions VII and VIII).

Qi *et al.* (1995) have suggested that at concentrations greater than 25 mg/mL, changes in tertiary and secondary structure can occur before dimer dissociation. Therefore, non-covalently-linked dimers may also form via reaction (IX). These species may then be converted to disulphide-linked dimers via thiol oxidation (similar to reaction IV) or via thiol-disulphide interchange (similar to reaction V).

5) Growth of Aggregates.

Aggregates larger than dimers may form via two major pathways: thiol-disulphide interchange (continuation of reaction VI, i.e. a similar manner to that suggested by Roefs and de Kruif, 1994), and non-covalent association via hydrophobic interactions (continuation of reaction VIII).

In addition, intramolecular thiol-disulphide interchange may occur after disulphide-linked dimers have formed. Molecules of BLG will be retained in a non-native conformations as a consequence of such reactions.

Monomers of BLG retained in a "non-native" conformation by non-native disulphide bonds may also be liberated from disulphide-linked aggregates via thiol-disulphide interchange, as shown by reaction (X), (each BLG molecule is shown differently and the "non-native" monomer is denoted by P*). This is likely to be the reaction which leads to the formation of stable "unfolded" monomers. These species can then be re-incorporated into aggregates via non-covalent association (similar to reaction VIII) or thiol-disulphide interchange (similar to reaction VI).

5a) Combination of Thiol-disulphide Interchange and Non-covalent Association via Hydrophobic Interactions.

In addition to the two pathways described above, BLG aggregate M_r may increase via a combination of thiol-disulphide interchange and non-covalent association as shown by reaction (XI). The product of this reaction will be large aggregates which contain non-native monomers and small disulphide-linked aggregates associated in primarily a non-covalent manner.

6) Advanced Stages of Aggregation.

In the advanced stages of aggregation, it is likely that thiol-disulphide interchange reactions will continue in a manner similar to that shown by reaction (VI). However, as suggested by Roefs and de Kruif (1994), the probability that an oxidation reaction will terminate aggregate growth (reaction XII) will decrease with increasing aggregate M_r (i.e. the Trommsdorf effect, Section 8.1.4). This will lead to an increase in the rate of aggregate growth.

Non-covalently-linked aggregates may be converted to disulphide-linked aggregates via thiol-disulphide interchange in the advanced stages of aggregation. This could occur in two ways. Firstly, as suggested by Gezimati *et al.* (1997), the solvent-exposed thiol group of another aggregate or monomer may react with a disulphide bond in a non-covalently-linked aggregate, thus initiating thiol-disulphide interchange in the non-covalently-linked aggregate (reaction XIII). Secondly, as suggested by Gezimati *et al.* (1997), non-covalently-linked aggregates may be converted to disulphide-linked aggregates via intramolecular thiol-disulphide interchange reactions which occur within the non-covalently-linked aggregate (reaction XIV).

The results in Section 5.3.9 suggest that when BLG solutions are heat-treated at a concentration of approximately 2 mg/mL at pH 6.7 and pH 7.4, both non-covalently-linked and disulphide-linked aggregates are formed. However, in the early stages of aggregation, the relative importance of hydrophobic interactions and thiol-disulphide interchange may be influenced by the initial BLG concentration (Creamer, personal communication). At concentrations greater than approximately 50 mg/mL, the concentrations used in the studies of McSwiney *et al.* (1994a, b), hydrophobic interactions may be more important than thiol-disulphide interchange because significant quantities of non-covalently-linked aggregates are formed during heat treatment under these conditions. However, at BLG concentrations less than approximately 50 mg/mL, as in the case of the study in Section 5.3.9, the opposite may be true. Nevertheless, irrespective of the initial BLG concentration, it is likely that after prolonged heat treatment, the majority of native BLG molecules are incorporated into disulphide-linked aggregates.

8.4. THE EFFECT OF pH ON THE STRUCTURE, THERMOSTABILITY AND AGGREGATION BEHAVIOUR OF β-LACTOGLOBULINS.

The results in Chapters 5, 6 and 7 suggest that the structures, thermostabilities and aggregation behaviour of BLGs differ at pH 6.7 and pH 7.4. In the discussion below a comparison of the results obtained at pH 6.7 and pH 7.4 is made.

8.4.1. THERMOSTABILITY AT pH 6.7 AND pH 7.4.

Many of the results in Chapters 5, 6 and 7 indicate that the thermostability of BLG at pH 6.7 differs from that at pH 7.4. Three sets of evidence support this conclusion.

8.4.1.1. T_{midET} Values from Measurements at Elevated Temperatures.

The majority of the values for T_{midET} for BLGs A, B and C at pH 7.4 determined using $\Delta\varepsilon_{293}$ and I_{Trp} data acquired at elevated temperatures (Chapter 7) are between 5 °C and 8 °C lower than the corresponding values at pH 6.7 (Table 8.4.1.1). This indicates that BLG molecules are less thermostable[1] at pH 7.4 than at pH 6.7. It should be stressed that the values for T_{midET} determined using the data in Chapter 7 do not represent midpoint temperatures for the unfolding of BLG. Instead, the T_{midET} values are defined as the midpoint temperatures of the transition for structural change (Section 7.3.1.2). Although the T_{midET} values in Table 8.4.1.1 do not represent the midpoint temperatures for unfolding, they should give measures of the relative thermostabilities of BLGs at pH 6.7 and pH 7.4.

8.4.1.2. T_{mid} Values from Measurements Made using Pre-heated β-Lactoglobulin.

The majority of the T_{mid} values determined using $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and thiol availability data collected at 20 °C from solutions of BLGs A, B and C previously heat-treated at pH 7.4 are between 6 °C and 7 °C lower than those determined using the corresponding sets of pH 6.7 data (Table 8.4.1.2). This indicates that BLG is more susceptible to heat-induced irreversible structural change[2] at pH 7.4 than at pH 6.7. It should be stressed that, for similar reasons to those discussed above, the values for T_{mid} determined using the data in Chapter 5 do not represent midpoint temperatures for the unfolding of BLG. Instead, they are assumed to represent the midpoint temperatures of the transition for heat-induced irreversible structural change.

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[1] The term thermostability will be used when discussing how readily the structure of a particular variant is altered during heat treatment.

[2] The term susceptibility to heat-induced irreversible structural change will be used when discussing the collective effects of thermostability and how readily non-native BLG molecules are incorporated and retained in aggregate structures.

Table. 8.4.1.1. Comparison of Midpoint Temperatures (T_{midET}) Determined using $\Delta\varepsilon_{293}$ and I_{Trp} Data Collected at Elevated Temperatures.

Sample	T_{midET} (°C)	
	$\Delta\varepsilon_{293}$	I_{Trp}
pH 6.7, BLG A	67.0 ± 0.3	67.9 ± 0.3
pH 6.7, BLG B	67.5 ± 0.2	69.4 ± 0.1
pH 6.7, BLG C	70.3 ± 0.9	70.5 ± 0.3
pH 7.4, BLG A	59.7 ± 0.2	53.4 ± 2.2
pH 7.4, BLG B	60.3 ± 1.2	61.2 ± 0.8
pH 7.4, BLG C	65.0 ± 0.3	62.9 ± 0.2

Where: T_{midET} is the midpoint temperature of the transition for heat-induced structural change (Tables 7.3.1.1 and 7.3.2.1). The T_{mid} values were determined as described in the captions of the tables listed immediately above.

Table 8.4.1.2. Comparison of Midpoint Temperatures (T_{mid}) Determined using $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and Thiol Availability Data Collected from Pre-heated β-Lactoglobulin Solutions.

Sample	T_{mid} (°C)				
	$\Delta\varepsilon_{293}$	$[\theta]_{205}$	I_{Trp}	I_{ANS}	Thiol Availability
pH 6.7, BLG A	74.7 ± 0.3	75.7 ± 0.7	77.4 ± 0.7	74.7 ± 0.2	74.3 ± 0.8
pH 6.7, BLG B	72.8 ± 0.1	74.8 ± 1.6	75.7 ± 0.8	72.5 ± 0.1	72.8 ± 0.2
pH 6.7, BLG C	76.0 ± 0.1	77.0 ± 0.1	77.7 ± 0.7	76.3 ± 0.1	75.6 ± 0.1
pH 7.4, BLG A	67.0 ± 0.2	69.4 ± 0.9	67.4 ± 0.4	67.7 ± 0.1	65.4 ± 0.4
pH 7.4, BLG B	66.2 ± 0.1	64.6 ± 0.8	69.0 ± 0.2	65.3 ± 0.4	63.5 ± 0.3
pH 7.4, BLG C	69.5 ± 0.1	70.0 ± 0.4	72.5 ± 0.5	69.9 ± 0.2	66.7 ± 0.2
pH 8.1, BLG A	62.1 ± 0.9	65.4 ± 0.7	60.7 ± 0.2	67.3 ± 1.2	-

Where: T_{mid} is the midpoint temperature of the transition for heat-induced irreversible structural change (Tables 5.3.1.1, 5.3.2.1, 5.3.3.1, 5.3.4.1 and 5.3.5.2). In the cases of I_{Trp}, I_{ANS} and A_{412} data, the average of the two T_{mid} values for a particular variant at a particular pH value and the corresponding average standard error are shown above. The T_{mid} values were determined as described in the captions of the tables listed immediately above.

The results in Tables 8.4.1.1 and 8.4.1.2 are consistent with those obtained in a number of earlier studies. Mills (1976) reported that the point of inflexion in plots of I_{Trp} versus temperature for BLG B decreased with increasing pH between 5.6 and 7.4 (Section 2.6.4). This indicates that the thermostability of BLG B decreases with increasing pH in this range. Kella and Kinsella (1988a) found, using UV difference spectroscopy, that the thermostability of BLG A/B decreases with increasing pH between 6.5 and 7.5 (Section 2.6.4). The near UV CD results of Griffin *et al.* (1993) show that the thermostability of BLG A decreases with increasing pH between pH 6.5 and pH 7.5.

The results obtained in a number of DSC studies also indicate that the thermostability of BLG decreases with increasing pH. de Wit and Klarenbeek (1981) found that the T_{max} for BLG A/B decreases with increasing pH between 6.0 and 8.0 (Section 2.6.4). The results obtained for BLG A and BLG A/B in the DSC studies of Qi *et al.* (1995) and Lapanje and Poklar (1989) respectively are consistent with those obtained by de Wit and Klarenbeek (1981). Park and Lund (1984) reported that BLG A/B is more thermostable at pH values between 5.0 and 8.0 than at pH values outside this range. Therefore, in the case of pH values greater than the isoelectric point of BLG (approximately 5.2), their results appear consistent with those obtained in the other studies.

8.4.1.3. Rate Constants for Heat-induced Structural Change.

A comparison of the values of the rate constants (k^*) for heat-induced structural change in molecules BLGs A, B and C (Chapter 6) also suggests that the thermostability of this protein exhibits a dependence on pH. The values of k^* determined using $\Delta\varepsilon_{293}$ data (Table 6.3.2.1) indicate that heat-induced structural change in the vicinity of Trp^{19} in molecules of these variants occurs more rapidly at pH 7.4 than at pH 6.7. This suggests that BLG is less thermostable at pH 7.4 than at pH 6.7. Furthermore, the values of k^* determined using thiol availability assay data for BLG A indicate that after rapid temperature increase to 60 °C, the thiol group reacts more rapidly with DTNB at pH 7.4 than at pH 6.7 (Section 6.3.1.4). This suggests that for BLG A, the structural change which leads to thiol group exposure during heat treatment (Section 8.3.1.2) occurs more rapidly at pH 7.4 than at pH 6.7 and therefore, that this variant is less thermostable at pH 7.4 than at pH 6.7.

8.4.2. THE STRUCTURES OF β-LACTOGLOBULIN MOLECULES AT ROOM TEMPERATURE AT pH 6.7 AND pH 7.4.

The near UV CD results and the thiol availability assay results in Chapters 5 and 6 respectively suggest that the structures of BLG molecules in unheated solutions at pH 6.7 differ slightly to those of BLG molecules in unheated solutions at pH 7.4.

8.4.2.1. Near UV CD Results.

The $\Delta\varepsilon_{293}$ results in Chapter 5 suggest that the side chain of Trp^{19} is in a slightly less chiral environment at pH 7.4 than at pH 6.7. Therefore, the structure of BLG in the vicinity of Trp^{19} is slightly more expanded at pH 7.4 than at pH 6.7 (Section 5.3.1.2). This is consistent with the crystallographic results of Qin *et al.* (1998), which show that the dimensions of the unit cell of crystals of BLG A (lattice Z) at pH 6.2 are smaller than those at pH 7.1, which are smaller than those at pH 8.2, and therefore indicate that the entire BLG A structure expands with increasing pH in this range.

8.4.2.2. Thiol Availability Results.

The thiol availability assay results in Chapter 6 indicate that rates of thiol group exposure at 20 °C in molecules of BLGs A, B and C are slightly faster at pH 7.4 than at pH 6.7 (Section 6.3.1.2). This may indicate that at room temperature, the structure of BLG in the vicinity of Cys^{121} is more expanded at pH 7.4 than at pH 6.7, thus allowing the reaction of DTNB with thiol groups to proceed more rapidly at the higher pH (Section 6.3.1.2). However, because initial A_{412} bursts were not observed during measurements made at pH 7.4, it is unlikely that rates of spontaneous thiol group exposure at 20 °C are significantly greater at pH 7.4 than at pH 6.7. Thus, although thiol availability assay results suggest that the structure of BLG in the vicinity of Cys^{121} differs at pH 6.7 and pH 7.4, they also suggest that the magnitude of this difference is not great. This is therefore consistent with the comparison of $\Delta\varepsilon_{293}$ results discussed above. An explanation for how a pH shift can alter the structure of BLG is given in Section 8.4.3.

The comparisons discussed above may be consistent with the results of Groves *et al.* (1951) and Casal *et al.* (1988). These authors reported that BLG denatures at room temperature at pH values 8.0 and higher (Section 5.3.1.2). Therefore, although the results in Chapters 5 and 6 suggest that the differences in the structure of BLG at pH 6.7 and pH 7.4 are not large, these structural differences may be of a similar type to those which lead to denaturation at higher pH values.

8.4.3. MAXIMUM EXTENTS OF HEAT-INDUCED IRREVERSIBLE STRUCTURAL CHANGE IN β-LACTOGLOBULIN MOLECULES AT pH 6.7 and pH 7.4.

The maximum extents of heat-induced irreversible change in $\Delta\varepsilon_{293}$, and therefore the maximum extents of heat-induced irreversible structural change in the vicinity of Trp^{19} in BLG molecules, appear to be less at pH 7.4 than at pH 6.7. The values for $\Delta\varepsilon_{293}$ suggest that the structures of unheated BLGs A, B and C in the vicinity of Trp^{19} are more expanded at pH 7.4 than at pH 6.7 (Section 8.4.2). However, the values for $\Delta\varepsilon_{293}$ for BLGs A, B and C heat-treated at pH 6.7 and pH 7.4 are similar, suggesting that the structures of BLG molecules in solutions heat-treated at these pH values are similar. These comparisons therefore suggest that the maximum extent of irreversible structural change in BLG molecules that was caused by heat treatment is less at pH 7.4 than at pH 6.7.

Dissimilarities in the structure and thermostability of BLG at different pH values can be explained in terms of the overall negative charge on BLG molecules. This will be greater at pH 7.4 than at pH 6.7 because the lower pH value is closer to the isoelectric point of this protein (approximately 5.2). Thus, the strength of electrostatic repulsions between the negatively charged side chains of BLG, and therefore the strength of the interactions which oppose folding, will be greater at pH 7.4 than at pH 6.7. In contrast, the strength of the interactions which favour folding (hydrophobic interactions and to a lesser extent, hydrogen bonds) are not affected by pH increase. The overall effect of raising pH will therefore be an increase in the strength of the interactions which oppose folding, and therefore, a decrease in BLG thermostability.

8.4.4. AGGREGATES OF β-LACTOGLOBULIN FORMED AS A CONSEQUENCE OF HEAT TREATMENT AT pH 6.7 AND pH 7.4.

The average M_r of aggregates of BLG and also the temperatures and rates at which these aggregates are formed exhibit a dependence on pH. Both the disulphide-intact SDS-PAGE and the alkaline native-PAGE results in Section 5.3.9 indicate that aggregates of BLGs A, B and C are formed at lower temperatures at pH 7.4 than at pH 6.7. This may reflect the lower thermostabilities of BLGs A, B and C at pH 7.4 than at pH 6.7. Furthermore, because both structural change and aggregate formation occur at lower temperatures at pH 7.4 than at pH 6.7, a comparison of PAGE and spectroscopic results may be consistent with the suggestion that aggregate formation is dependent on changes in tertiary and secondary structure in BLG molecules.

The PAGE results in Section 5.3.9 also indicate that the average M_r of BLG aggregates formed as a consequence of heat treatment is less at pH 7.4 than at pH 6.7. This is consistent with the results obtained in the *in situ* DLS study of Hoffmann *et al.* (1996) discussed in Section 5.3.9.2.2. Furthermore, time-course PAGE results indicate that after an initial rapid increase, concentrations of aggregates of BLGs A, B and C with M_r values less than approximately 200 000 (i.e. those in bands which migrate further than the stacking/resolving gel interface, subsequently referred to as small aggregates) decrease more slowly during heat treatment at pH 7.4 than at pH 6.7 (Section 5.3.9.8). This suggests that small aggregates are more stable at pH 7.4 than at pH 6.7. Therefore, although aggregates of BLG are formed at lower temperatures at pH 7.4 than at pH 6.7, those aggregates with M_r values greater than approximately 200 000 (subsequently referred to as larger aggregates) are formed less readily at the higher pH.

The comparison of the alkaline native-PAGE and disulphide-intact SDS-PAGE results discussed in Section 5.3.9.7 suggests that at pH 6.7, concentrations of non-covalently-linked aggregates are higher in BLG solutions previously heat-treated at T5 (i.e. approximately 80 °C, see Section 5.3.9.1 for definition) than in those previously heat-treated at T6 (i.e. approximately 90 °C). In contrast, concentrations of non-covalently-linked aggregates are similar in BLG solutions previously heat-treated at pH 7.4 at T5 and T6 (i.e. approximately 75 °C and 80 °C respectively). This suggests that non-covalently-linked aggregates of BLG are more stable at pH 7.4 than at pH 6.7 during heat treatment at higher temperatures. Therefore, disulphide-linked aggregates of BLG appear to be formed less readily at pH 7.4 than at pH 6.7 during heat treatment at higher temperatures.

Differences in the aggregation behaviour of BLG at pH 6.7 and pH 7.4 are also reflected in the $\Delta\varepsilon_{270}$ results in Chapter 5 and the I_{Trp} results in Chapter 6. For BLGs A, B and C, the maximum extent of the change in $\Delta\varepsilon_{270}$ which occurs as a consequence of heat treatment is less at pH 7.4 than at pH 6.7. Additionally, the time-dependent increase in I_{Trp} caused by temperature increase is more gradual at pH 7.4 than at pH 6.7. For the reasons discussed in Sections 5.3.10.2 ($\Delta\varepsilon_{270}$ results) and 6.3.3 (I_{Trp} results), this behaviour suggests that disulphide-linked aggregates of BLG are formed less readily at pH 7.4 than at pH 6.7.

To summarise, the PAGE results discussed above indicate that aggregates of BLGs A, B and C are formed at lower temperatures at pH 7.4 than at pH 6.7. Furthermore, both disulphide-linked aggregates and large aggregates are formed less readily at pH 7.4 than at pH 6.7.

The dissimilarities in the aggregation behaviour of BLG molecules at different pH values are affected by two factors: thermostability and net charge.

i) Thermostability

Aggregates of BLG are formed at lower temperatures at pH 7.4 than at pH 6.7 probably because BLG molecules are less thermostable at the higher pH, as indicated by spectroscopic results (Chapters 5, 6 and 7).

ii) Net Charge

The average M_r of BLG aggregates formed as a consequence of heat treatment is less at pH 7.4 than at pH 6.7 and this may reflect the greater net charge on BLG molecules at the higher pH (Section 8.4.3). Therefore, the strength of electrostatic repulsions between BLG molecules will be greater at pH 7.4 than at pH 6.7, thus making it less likely that molecules of this protein will associate at the higher pH.

The PAGE results in Section 5.3.9 also suggest that the contributions of the two factors discussed above to the overall aggregation behaviour of BLG differ at different heat treatment temperatures. Although BLG molecules carry a greater net charge at pH 7.4 than at pH 6.7, which in theory should make aggregate formation more difficult at the higher pH, aggregates are formed at lower temperatures at the higher pH. This suggests that the aggregation behaviour of BLG is more dependent on thermostability than on net charge during heat treatment at lower temperatures. However, because aggregates in BLG solutions heat-treated at pH 7.4 never attain the size of those in BLG solutions heat-treated at pH 6.7, the aggregation behaviour of BLG is probably more dependent on net charge than on thermostability during heat treatment at higher temperatures.

8.5. THE EFFECT OF VARIANT TYPE ON THE THERMOSTABILITY AND AGGREGATION BEHAVIOUR OF β-LACTOGLOBULINS.

One of the major research aims (Section 2.9) was to identify differences in the thermostabilities and aggregation behaviour of BLGs A, B and C. The studies in Chapters 5, 6 and 7 were made using these variants. β-Lactoglobulins A, B and C were selected because they are the three genetic variants that have been identified in the milk of New Zealand dairy herds. The A variant has an aspartate at position 64 and a valine at position 118, while in the B variant, glycine and alanine are found at positions 64 and 118 respectively. β-Lactoglobulin C has a histidine at position 59, while BLGs A and B have a glutamine at this position. In the following discussion, the results from Chapters 5, 6 and 7 which indicate that the thermostabilities and aggregation behaviour of BLGs A, B and C differ are examined and compared with the results obtained in published studies. Differences in thermostabilities and aggregation behaviour of BLGs A and B have been studied extensively (Gough and Jenness, 1962; Sawyer, 1968; Sawyer *et al.*, 1971; Hillier and Lyster, 1979; Imafidon *et al.*, 1991; McSwiney *et al.*, 1994b; Huang *et al.*, 1995a; Dong *et al.*, 1996; Nielsen *et al.*, 1996; Elofsson *et al.*, 1996b). However, the thermostability and aggregation behaviour of BLG C does not appear to have been studied in as much detail. The studies listed above that were published before 1994 are discussed in Section 2.8. Furthermore, in the discussion presented below, the high resolution crystal structures of BLGs A, B and C are used to suggest why the thermostabilities and aggregation behaviour of these variants differ.

8.5.1. AGGREGATES OF β-LACTOGLOBULINS A, B AND C.

8.5.1.1. Comparison of the Aggregation Behaviour of β-Lactoglobulin A with that of β-Lactoglobulins B and C.

The following experimental evidence suggests that BLG A forms aggregates, particularly large disulphide-linked aggregates, more slowly than BLGs B and C. Firstly, for BLG solutions heat-treated for 12.5 min at pH 6.7 in particular, the decrease in the intensity of the monomer band with increasing heat treatment temperature on both alkaline native and the disulphide-intact portions of SDS gels is less marked for BLG A than for BLGs B and C (Section 5.3.9.7). This suggests that at pH 6.7 in particular, "native-like" monomers and "unfolded" monomers of BLG A are incorporated into aggregate structures more slowly than those of BLGs B and C. Secondly, the maximum extent of change in $\Delta\varepsilon_{270}$ which occurs as a consequence of heat treatment is less

for BLG A than for BLGs B and C, and the time-dependent increase in I_{Trp} caused by temperature increase is more gradual for BLG A than for BLGs B and C. For the reasons given in Sections 5.3.10.2 ($\Delta\varepsilon_{270}$ results) and 6.3.3 (I_{Trp} results), these results suggest that disulphide-linked aggregates are formed more slowly from BLG A than from BLGs B and C. Thirdly, concentrations of "unfolded" monomers (see Section 5.3.9.4 for definition), dimers and non-covalently-linked aggregates are higher in heat-treated solutions of BLG A than those of BLGs B and C (Sections 5.3.9.7 and 5.3.9.8). This suggests that these species are retained for longer periods of time during heat treatment in solutions of BLG A than in those of BLGs B and C, and therefore, that "unfolded" monomers, dimers and non-covalently-linked aggregates formed from BLG A are more stable than those formed from BLGs B and C. Finally, both the disulphide-intact SDS-PAGE and alkaline native-PAGE results in Section 5.3.9 suggest that at pH 6.7 in particular, concentrations of small aggregates are higher in heat-treated solutions of BLG A than in those of BLGs B and C. This suggests that small aggregates formed from BLG A are more stable than those formed from BLGs B and C. The above results, which collectively suggest that BLG A forms aggregates, particularly large disulphide-linked aggregates, more slowly than BLGs B and C, may be consistent with the results obtained in the PAGE study of McSwiney *et al.* (1994b). Their results indicate that after heat treatment for periods longer than approximately 13.3 min, concentrations of monomeric BLG A are greater than those of monomeric BLG B.

The differences between the aggregation behaviour of BLG A and that of BLGs B and C may be due to the amino acid substitution at position 64, in which the glycine of BLGs B and C is replaced by an aspartate in BLG A. At both pH 6.7 and pH 7.4, the pH values at which the BLG solutions used in the PAGE studies were heat-treated, the side chain of Asp^{64} will be charged. Therefore, molecules of BLG A will carry a greater net negative charge than those of BLGs B and C and the strength of electrostatic repulsions between molecules of the A variant will be greater than those between molecules of the B and C variants. This may explain why large aggregates form more slowly from BLG A than from BLGs B and C.

The negative charge on the side chain of Asp^{64} of BLG A at both pH 6.7 and pH 7.4 may also be the reason why non-covalently-linked aggregates of this variant appear more stable than those of BLGs B and C. In the crystal structure of BLG A at pH 6.2 (Qin *et al.*, 1998), the end of the side chain of Glu^{65} is 9.0 Å from the disulphide bond Cys^{66}-Cys^{160} and 8.0 Å from the end of the side chain of Asp^{64} (which is 11.5 Å from this disulphide bond, Fig. 2.4.9). In solution, the side chain of Glu^{65} is therefore probably repelled towards the disulphide bond Cys^{66}-Cys^{160} by the negative charge on Asp^{64}. Therefore, the magnitude of negative charge in the vicinity of this disulphide bond will be greater in molecules of the A variant than in those of the B

and C variants. This in turn may increase the strength of electrostatic repulsions between BLG molecules in the vicinity of this disulphide bond. Therefore, the formation of intermolecular disulphide bonds which involve either Cys^{66} or Cys^{160} and a solvent-exposed thiol group (as a thiolate anion) in an adjacent BLG molecule may be less likely in the case of BLG A than in the case of BLGs B and C. Consequently, this may lessen the overall susceptibility of BLG A to intermolecular disulphide bond formation during heat treatment, thus explaining why non-covalently-linked aggregates of this variant appear to be more stable than those of BLGs B and C.

Some of the results discussed above appear consistent with those obtained in the *in situ* DLS study of Elofsson *et al.* (1996b), while others do not. Elofsson *et al.* (1996b) found that the lag phase which precedes aggregation is longer for BLG A than for BLG B. This is consistent with the PAGE results discussed above which suggest that aggregates are formed more slowly from BLG A than from BLGs B and C. However, Elofsson *et al.* (1996b) also found that once aggregation had started the rate of particle size increase was faster for BLG A than for BLG B. This appears inconsistent with the results discussed above which suggest that large aggregates are formed more slowly from BLG A than from BLGs B and C.

Elofsson *et al.* (1996b) offered two hypotheses to explain why rates of increase in aggregate particle size during heat treatment differ for BLGs A and B. In their first hypothesis, they concluded from DSC results that BLG A is less thermostable than BLG B and suggested that this is the reason why rates of aggregate particle size increase are faster in solutions of BLG A than in those of BLG B.

In their second hypothesis, Elofsson *et al.* (1996b) explained why rates of increase in aggregate particle size during heat treatment differ for BLGs A and B in terms of differences in extents of dimer dissociation. Elofsson *et al.* (1996b) made their *in situ* DLS measurements using 8 mg/mL solutions of BLG. They calculated, using the results of Qi *et al.* (1995), that an appreciable proportion of molecules of BLG A will unfold from the monomeric state at this concentration. However, from similar calculations made for BLG B, Elofsson *et al.* (1996b) suggest that dimer dissociation, unfolding, and aggregation occur simultaneously at 8 mg/mL. Therefore, because the majority of molecules of BLG A unfold from the monomeric state during heat treatment at this concentration, their thiol and disulphide groups will be more available to take part in thiol-disulphide interchange reactions than those of BLG B (Elofsson *et al.* 1996b). This, they suggest, is the reason why rates of increase in aggregate particle size during heat treatment are greater for BLG A than for BLG B. Thus, the results of Elofsson *et al.* (1996b) may reflect differences in the extents of dissociation of dimers of BLGs A and B as well as differences in the aggregation behaviour of these variants. Therefore, it is not concluded that the results in Section 5.3.9 are inconsistent with those of Elofsson *et al.* (1996b).

8.5.1.2. Comparison of the Aggregation Behaviour of β-Lactoglobulins B and C.

The PAGE results in Section 5.3.9 suggest that concentrations of large aggregates are slightly higher in heat-treated solutions of BLG C than in those of BLG B. This phenomenon can be explained in terms of the amino acid substitution at position 59. Because His^{59} forms a salt bridge with Glu^{44} in BLG C, it is likely that the majority of His^{59} side chains are protonated at both pH 6.7 and pH 7.4. Therefore, because the isoelectric point of BLG is approximately 5.2, molecules of the C variant will carry a lower net negative charge at both pH 6.7 and pH 7.4 than molecules of the B variant. Aggregates should therefore be more readily formed from BLG C than from BLG B in this pH range, in agreement with the results in Section 5.3.9.

8.5.2. THE THERMOSTABILITIES OF β-LACTOGLOBULINS A, B AND C.

The results obtained from the spectroscopic measurements discussed in Chapters 5, 6 and 7 indicate that the thermostabilities of BLGs A, B and C differ. These differences were identified from values for T_{midET} (Table 8.4.1.1), midpoint temperatures of the transition for structural change (Section 8.4.1.1), T_{mid} (Table 8.4.1.2), midpoint temperatures of the transition for **irreversible** structural change (Section 8.4.1.2) and k^*, the apparent rate constants for structural change (Table 8.5.2.1). Although the values for T_{mid} and T_{midET} do not represent the midpoint temperatures for the unfolding of BLGs A, B and C it is likely that they can be used to obtain measures of the relative thermostabilities of these variants.

For similar reasons to those discussed in Sections 8.4.1.1 and 8.4.1.2 it should be stressed that values for k^* (determined using the data in Chapter 6) are unlikely to represent rate constants for the heat-induced unfolding of BLGs A, B and C. The values for k^* are instead defined as apparent rate constants for structural change and it is assumed that they can be used to compare relative rates of heat-induced structural change in molecules of BLGs A, B and C.

The differences in the values for ΔG_{app} in Chapters 5 and 7 are also assumed to represent measures of the relative thermostabilities of BLGs A, B and C. The magnitude of the increased thermostabilities of BLGs A and C relative to that of BLG B are shown as values for $\Delta\Delta G_{app}$ values in Tables 8.5.2.2 (measurements made at elevated temperatures, Chapter 7) and 8.5.2.3 (measurements made at 20 °C using BLG solutions previously heat-treated, Chapter 5). However, it must be stressed that these $\Delta\Delta G_{app}$ values may not reflect differences in the change in free energy for the unfolding

of BLGs A, B and C because the data used to calculate these values were collected under heating conditions where the unfolding of BLG is neither complete nor reversible (Section 5.3.1.5). Nevertheless, the results in Tables 8.5.2.2 and 8.5.2.3 are likely to represent reasonable measures of the relative thermostabilities of BLGs A, B and C.

Table 8.5.2.1. Rate Constants for Heat-induced Structural Change in Molecules of β-Lactoglobulins A, B and C.

Sample	Rate Constant for Structural Change (k^*, min^{-1})	
	Thiol Availability	$\Delta\varepsilon_{293}$
pH 6.7, BLG A	0.733 ± 0.010	0.099 ± 0.003
pH 6.7, BLG B	0.662 ± 0.015	0.152 ± 0.004
pH 6.7, BLG C	0.203 ± 0.002	0.129 ± 0.003
pH 7.4, BLG A	0.477 ± 0.003	0.140 ± 0.006
pH 7.4, BLG B	0.404 ± 0.006	0.268 ± 0.010
pH 7.4, BLG C	0.134 ± 0.001	0.255 ± 0.009

Where:

k^* is the rate constant for heat-induced structural change (Tables 6.3.1.1 and 6.3.2.1). The values for k^* were determined as described in the captions of the tables listed immediately above.

Table 8.5.2.2. Differences Between ΔG_{app} Values for β-Lactoglobulins A, B and C at Temperatures Close to T_{midET}.

Comparison	$\Delta\Delta G_{app}$ (kJ/mol)	
	$\Delta\varepsilon_{293}$	I_{Trp}
pH 6.7, ΔG_{app}(BLG A)-ΔG_{app}(BLG B)	-0.33	-0.68
pH 6.7, ΔG_{app}(BLG C)-ΔG_{app}(BLG B)	1.77	0.97
pH 7.4, ΔG_{app}(BLG A)-ΔG_{app}(BLG B)	-1.23	-3.58
pH 7.4, ΔG_{app}(BLG C)-ΔG_{app}(BLG B)	2.47	0.16

Where:

ΔG_{app} is assumed to represent the change in free energy at 67 °C at pH 6.7 and at 59 °C at pH 7.4 for structural change (Tables 7.3.1.2 and 7.3.2.2). The values for ΔG_{app} were calculated as described in the captions of the tables listed immediately above.

Table 8.5.2.3. Differences Between ΔG_{app} Values for β-Lactoglobulins A, B and C at Temperatures Close to T_{mid}.

Comparison	$\Delta\Delta G_{app}$ (kJ/mol)				
	$\Delta\varepsilon_{293}$	$[\theta]_{205}$	I_{Trp}	I_{ANS}	Thiol Availability
pH 6.7, ΔG_{app}(BLG A)- ΔG_{app}(BLG B)	1.40	1.54	3.93	2.41	1.16
pH 6.7, ΔG_{app}(BLG C)- ΔG_{app}(BLG B)	4.60	3.90	5.61	4.93	3.40
pH 7.4, ΔG_{app}(BLG A)- ΔG_{app}(BLG B)	0.53	1.95	-1.61	2.44	1.43
pH 7.4, ΔG_{app}(BLG C)- ΔG_{app}(BLG B)	3.10	3.75	4.40	5.01	3.21

Where:

With the exception of I_{Trp} results, ΔG_{app} is assumed to represent the change in free energy at 72 °C at pH 6.7 and at 66 °C at pH 7.4 for irreversible structural change (Tables 5.3.1.2, 5.3.2.2, 5.3.4.2 and 5.3.5.3). For I_{Trp} results, ΔG_{app} is assumed to represent the change in free energy at 75 °C at pH 6.7 and at 67 °C at pH 7.4 for irreversible structural change (Table 5.3.3.2). The values for ΔG_{app} were calculated as described in the captions of the tables listed immediately above.

8.5.2.1. The Thermostability of β-Lactoglobulin C Compared to those of β-Lactoglobulins A and B.

The majority of the T_{midET} and T_{mid} values for BLG C in Tables 8.4.1.1 and 8.4.1.2 respectively are between 0.3 °C and 5.1 °C higher than those for BLGs A and B. Furthermore, the values for k^* for BLG C in Table 8.5.2.1 determined using thiol availability assay data are between 28 % and 33 % of those for BLGs A and B. In contrast, the values for k^* for BLG C determined using $\Delta\varepsilon_{293}$ data are intermediate between those for BLGs A and B. The majority of values for T_{midET} determined using data acquired at elevated temperatures (Table 8.4.1.1) therefore suggest that BLG C is less susceptible to heat-induced structural change than BLGs A and B. Similarly, most of the values for T_{mid} determined from data acquired at 20 °C using BLG solutions previously heat-treated (Table 8.4.1.2) suggest that BLG C is less susceptible to heat-induced **irreversible** structural change than BLGs A and B. In addition, the values for k^* in Table 8.5.2.1 determined using thiol availability assay data suggest that heat-induced structural change in the vicinity of Cys^{121} occurs more slowly in molecules of BLG C than in those of BLGs A and B. Finally, the values for $\Delta\Delta G_{app}$ in Tables 8.5.2.2 and 8.5.2.3 show that the ΔG_{app} values for BLG C are between 0.67 kJ/mol and 5.61 kJ/mol greater than those for BLGs A and B. The majority of the

spectroscopic results in Chapters 5, 6 and 7 therefore suggest that BLG C is more thermostable than BLGs A and B. Furthermore, the results obtained in the confirmatory experiments (Sections 5.3.7 and 5.3.8) are consistent with this conclusion.

The greater thermostability of BLG C compared to BLGs A and B must result from the replacement of Gln^{59} with His^{59}. However, when the crystal structures of BLGs A, B and C (Bewley *et al.*, 1998) are examined it is difficult to ascertain how this amino acid substitution could produce this effect. This is because the side chain of residue 59 and neighbouring side chains are on the surface of the BLG structure and their positions are not well defined (Bewley *et al.*, 1998). Therefore, the replacement of the hydrogen bond between residue 59 and Glu^{44} with a salt bridge, which occurs when Gln^{59} becomes His^{59}, may not affect the thermostability of BLG appreciably because residue 59 is likely to be in a flexible region of the BLG structure. Furthermore, because the sizes and hydrophobicities of the side chains of histidine and glutamine are similar, it is unlikely that the substitution of one for the other would have an appreciable affect on the entropy of the unfolded state of BLG, and therefore thermostability.

The greater thermostability of BLG C compared to BLGs A and B may be explained in terms of the overall charge on molecules of these variants and dimer dissociation. For the reasons given in Section 8.5.1.2, molecules of BLG C will carry a lower negative charge than molecules of BLGs A and B at pH 6.7 and pH 7.4. Dimers of BLG C should therefore dissociate less readily than those of BLGs A and B during heat treatment. For this reason, and because dimer dissociation precedes changes in tertiary and secondary structure when BLG molecules are heat-treated in solutions at concentrations less than 25 mg/mL (Section 8.1), the C variant will appear more thermostable than the A and B variants. That is, BLG C is likely to be more thermostable than BLGs A and B because dimers of the C variant dissociate less readily than those of the A and B variants during heat treatment.

The above conclusion, that BLG C is more thermostable than BLGs A and B, appears to contradict the conclusion of Sawyer (1968) that BLG C is less thermostable than BLGs A and B because solutions of the C variant are more turbid than those of the A and B variant after heat treatment . However, measuring the opacity of a suspension of aggregates of a protein formed as a consequence of heat treatment is probably not a precise way to determine protein thermostability.

8.5.2.2. Comparison of the Thermostabilities of β-Lactoglobulins A and B.

This section emphasises that the apparent relative "thermostabilities" of BLGs A and B, as determined by comparing T_{midET}, T_{mid} and k^* values (Tables 8.4.1.1, 8.4.1.2 and 8.5.2.1 respectively), are dependent on the experimental technique used to measure structural change. This is also true for the results of earlier studies. These show that the apparent relative "thermostabilities" of BLGs A and B depend on the concentration at which their solutions were heat-treated and whether measurements were made at elevated temperatures or at room temperature using BLG solutions previously heat-treated. In the discussion below, the results in Chapters 5, 6 and 7 are compared with each other and with those obtained in earlier studies, and similarities and dissimilarities between different sets of results are identified. The PAGE results in Section 5.3.9 and the crystal structures of Bewley *et al.* (1998) are then used to suggest which experimental results are likely to best describe BLG variant "thermostabilities". Possible reasons why some experimental techniques provide better measures of BLG variant "thermostabilities" than others are then discussed, thus permitting a conclusion to be made regarding whether BLG A is more "thermostable" than BLG B (or *vice versa*). The results obtained in earlier studies where the structural stabilities of native BLGs A and B were compared are then discussed and compared with those obtained in heating studies. The discussion below is structured as follows:

- Comparison of results obtained at room temperature for BLG solutions previously heat-treated at low concentrations.
- Comparison of results obtained at room temperature for BLG solutions previously heat-treated at both low and high concentrations.
- Effect of heat treatment concentration on the relative susceptibilities of BLGs A and B to structural change.
- Comparison of the results in Chapters 6 and 7 (measurements at elevated temperatures).
- Comparison of the results in Chapters 6 and 7 with those of others.
- Comparison of the results obtained at elevated temperatures with the crystal structures of Bewley *et al.* (1998).
- Selection of appropriate experimental techniques for comparing the relative thermostabilities of BLGs A and B.
- Interpretation of the results obtained at room temperature using previously heat-treated BLG solutions.
- Importance of heat treatment concentration.
- The structural stabilities of native BLGs A and B in relation to their "intrinsic themostabilities".

8.5.2.2.1. Comparison of Results Obtained at Room Temperature for β-Lactoglobulin Solutions Previously Heat-treated at Low Concentrations.

The majority of the T_{mid} values determined using $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{Trp}, I_{ANS} and thiol availability data collected at 20 °C from BLG solutions previously heat-treated (Table 8.4.1.2) for BLG A are between 0.8 °C and 4.8 °C higher than those for BLG B. This suggests that BLG A is less susceptible to heat-induced irreversible structural change than BLG B, in agreement with the results obtained in the confirmatory experiments (Sections 5.3.7 and 5.3.8). Furthermore, the values for $\Delta\Delta G_{app}$ in Table 8.5.2.3 show that the ΔG_{app} values for BLG A are between 0.53 kJ/mol and 3.93 kJ/mol greater than those for BLG B, in agreement with the above suggestion.

The results summarised in Tables 8.4.1.2 and 8.5.2.3 are consistent with those obtained by Gough and Jenness (1962), Sawyer (1968), Sawyer *et al.* (1971) and those obtained by Hillier and Lyster (1979) at temperatures less than 95 °C. In these studies, variant thermostabilities were assessed by measuring, at room temperature, the concentrations of native BLG in solutions previously heat-treated at concentrations 10 mg/mL and less (Section 2.8.1). Griffin *et al.* (1993) reported that in solutions of BLG A heat-treated for a fixed period of time at pH 7.0, aggregate particle size increased with increasing heat treatment temperature between 73 °C and 86 °C and decreased with increasing heat treatment temperature above 86 °C. To date, similar behaviour has not been reported for BLG B. Therefore, with respect to the study of Sawyer (1968), the results of Griffin *et al.* (1993) suggest that comparing the opacities of BLG solutions previously heat-treated at 97.5 °C may not be the most appropriate way to determine the relative thermostabilities of the A and B variants.

8.5.2.2.2. Comparison of Results Obtained at Room Temperature for β-Lactoglobulin Solutions Previously Heat-treated at both Low and High Concentrations.

McSwiney *et al.* (1994b) suggested that BLG A is less stable to heat treatment than BLG B. They found, using alkaline native-PAGE and disulphide-intact SDS-PAGE to measure concentrations of monomeric BLG in solutions previously heat-treated at a concentration of 100 mg/mL, that the decrease in the concentration of monomeric BLG with increasing heat treatment time was more marked for the A variant than for the B. The DSC results obtained by Imafidon *et al.* (1991) may also indicate that BLG A is less thermostable than BLG B at a concentration of 100 mg/mL in a range of buffers. The results of these two groups therefore appear inconsistent with those discussed in Section 8.5.2.2.1.

8.5.2.2.3. Effect of Heat Treatment Concentration on the Relative Susceptibilities of β-Lactoglobulins A and B to Structural Change.

The differences between the results of McSwiney *et al.* (1994b) and Imafidon *et al.* (1991) and those discussed in Section 8.5.2.2.1 can be explained in terms of the concentration at which BLG solutions were heat-treated. Nielsen *et al.* (1996) reported that at concentrations less than approximately 50 mg/mL at pH 7.0, BLG A is less sensitive to heat treatment than BLG B. Using HPLC to measure, at room temperature, concentrations of native BLG in solutions previously heat-treated for different times at 75 °C, they found that the decrease in the concentration of native protein with increasing heat treatment time was less marked for BLG A than for BLG B. Nielsen *et al.* (1996) also reported that BLG A is more sensitive to heat treatment than BLG B at concentrations greater than approximately 50 mg/mL. Finally, at a concentration of approximately 50 mg/mL, they found that the sensitivities of BLGs A and B to heat treatment are equivalent. Therefore, a comparison of the results summarised in Tables 8.4.1.2 and 8.5.2.3 with those of McSwiney *et al.* (1994b) and Imafidon *et al.* (1991) may be inappropriate because the present study was made using BLG solutions previously heat-treated at a concentration lower than approximately 50 mg/mL.

8.5.2.2.4. Comparison of the Results in Chapters 6 and 7 (Measurements at Elevated Temperatures).

The order of "thermostabilities" for BLGs A and B shown by the results summarised in Tables 8.4.1.1 and 8.5.2.2 (obtained from measurements on solutions of BLGs A and B at elevated temperatures, Chapter 7) differs from that shown by the results summarised in Tables 8.4.1.2 and Table 8.5.2.3 (obtained from measurements made at 20 °C using solutions of these variants previously heat-treated, Chapter 5). The T_{midET} values for BLGs A and B determined using $\Delta\varepsilon_{293}$ data (Table 8.4.1.1) appear equivalent within the limits of the standard error calculated by "Enzfitter". However, the T_{midET} values determined using I_{Trp} data (Table 8.4.1.1) for BLG A are 1.0 °C and 7.8 °C lower than those for BLG B at pH 6.7 and pH 7.4 respectively. The T_{midET} value for BLG A at pH 7.4 determined using I_{Trp} data is considered unusually low because increases in I_{Trp} with increasing temperature were observed over a lower and a narrower temperature range than for BLGs B and C at both pH 6.7 and pH 7.4 and for BLG A at pH 6.7 (Section 7.3.2.2). The values for $\Delta\Delta G_{app}$ in Table 8.5.2.2 show that the ΔG_{app} values for BLG A are between 0.33 kJ/mol and 3.58 kJ/mol lower than those for BLG B. However, the differences between the ΔG_{app}

values calculated using $\Delta\varepsilon_{293}$ data are equivalent within the limits of the standard error calculated by "Enzfitter". Thus, the T_{midET} and $\Delta\Delta G_{app}$ values determined using $\Delta\varepsilon_{293}$ data suggest that the "thermostabilities" of BLGs A and B are equivalent, while those determined using I_{Trp} data suggest that the "thermostability" of BLG A is slightly less than that of BLG B.

The order of "thermostabilities" for BLGs A and B shown by the values for k^* (apparent rate constants for heat-induced structural change in BLG molecules summarised in Table 8.5.2.1) is the same as that shown by the $\Delta\varepsilon_{293}$ results in Tables 8.4.1.1 and 8.5.2.2. The values for k^* indicate that heat-induced thiol group exposure occurs at similar rates in molecules of BLGs A and B. This is also true for heat-induced structural change in the vicinity of Trp^{19} (as shown by changes in $\Delta\varepsilon_{293}$).

To summarise, the majority of the results obtained from measurements made at elevated temperatures suggest that either BLG A is slightly less "thermostable" than BLG B, or that the "thermostabilities" of these variants are equivalent.

8.5.2.2.5. Comparison of the Results in Chapters 6 and 7 with those of Others.

The results in Chapters 6 and 7 (summarised in Tables 8.4.1.1, 8.5.2.1 and 8.5.2.2) were obtained from measurements made using BLG solutions at a concentration of approximately 2 mg/mL. Therefore, the orders of "thermostabilities" shown by these results differs from that shown by the results of Gough and Jenness (1962), Sawyer (1968), Sawyer *et al.* (1971), those obtained by Hillier and Lyster (1979) at temperatures less than 95 °C, and those obtained by Nielsen *et al.* (1996) for BLG solutions at concentrations less than approximately 50 mg/mL. The results obtained in these earlier studies were interpreted as indicating the BLG A is more stable towards heat treatment than BLG B. However the order of "thermostabilities" for BLGs A and B shown by the results summarised in Tables 8.4.1.1, 8.5.2.1 and 8.5.2.2 appears consistent with that shown by the results of Huang *et al.* (1995a) and Elofsson *et al.* (1996b). From their DSC results, Huang *et al.* (1995a) found that at pH 8.0 at 20 mg/mL, the T_{max} (defined in Section 2.6.4) for BLG A is 5 °C lower than that for BLG B, indicating that BLG A is less thermostable than BLG B. Elofsson *et al.* (1996b) have suggested from DSC results that the T_{max} of the unfolding transition for BLG A is 6 °C lower than that for BLG B. The present results suggest that BLG A is, at best, slightly less "thermostable" than BLG B.

8.5.2.2.6. Comparison of the Results Obtained at Elevated Temperatures with the Crystal Structures (Bewley *et al.*, 1998).

The results of Huang *et al.* (1995a), Elofsson *et al.* (1996b) and those summarised in Tables 8.4.1.1, 8.5.2.1 and 8.5.2.2 appear consistent with the crystal structures of BLGs A and B of Bewley *et al.* (1998). These structures indicate that when Ala^{118} is replaced with Val^{118} several nearby side chains shift slightly to accommodate the two extra methyl groups (Fig. 2.4.11). Therefore, the conformational changes which are likely to occur before the larger side chain of valine can be accommodated in the hydrophobic core of the BLG structure could lead to a small decrease in the thermostability of this protein in aqueous solution.

Decreases in protein thermostability which occur when the size of a side chain in the hydrophobic core of a protein is altered have been reported before. For example, Eriksson *et al.* (1993) found that when Leu^{99} of bacteriophage T4 lysosyme was replaced with Phe^{99}, the T_{mid} decreased by 1.0 °C at pH 3.0 and 0.7 °C at pH 5.7. Therefore, it is not unreasonable to assume that when Ala^{118} of BLG is replaced with Val^{118}, thermostability decreases slightly.

The difference in the hydrophobicities of the side chains of alanine and valine is not, however, likely to be the reason why BLG A appears less thermostable than BLG B. In theory, an increase in the hydrophobicity of the side chain at position 118 caused by the replacement of alanine with valine could lead to an increase in the ΔS_{app} for the transfer of this side chain to an aqueous environment and thus an increase in thermostability. Pakula and Sauer (1990) have shown that this is possible for λCro. However, in the case of position 118 in BLG, it appears that when the smaller less hydrophobic side chain of alanine is replaced with the larger more hydrophobic side chain of valine thermostability decreases slightly. Therefore, a difference in the ΔS_{app} for the transfer of the side chain of residue 118 of BLG to an aqueous environment is unlikely to be the reason why the A variant is less thermostable than the B variant.

β-Lactoglobulins A and B possess an aspartate and a glycine respectively at position 64. However, it is unlikely that this amino acid substitution affects the thermostability of BLG appreciably because residue 64 is located in a flexible surface loop (loop CD, Fig. 2.4.10). Furthermore, when residue 64 is aspartate, the side chain points away from the other side chains in loop CD (Bewley *et al.*, 1988; Qin *et al.*, 1998). Any hydrogen bonds or salt bridges formed between Asp^{64} and other side chains are therefore unlikely to increase the thermostability of BLG appreciably.

8.5.2.2.7. Selection of Appropriate Experimental Techniques for Comparing the Relative Thermostabilities of β-Lactoglobulins A and B.

The suggestions discussed in Section 8.5.2.2.6 do not appear consistent with the results obtained by Gough and Jenness (1962), Sawyer (1968), Sawyer *et al.* (1971), those obtained by Hillier and Lyster (1979) at temperatures less than 95 °C and those obtained by Nielsen *et al.* (1996) for BLG solutions at concentrations less than approximately 50 mg/mL. These results were interpreted as indicating that at concentrations less than approximately 50 mg/mL, BLG A is more stable towards heat treatment than BLG B. The results in Chapter 5 (summarised in Tables 8.4.1.2 and 8.5.2.3) also suggest that at concentrations less than approximately 50 mg/mL, BLG A is more stable towards heat treatment than BLG B. However, in all of the earlier studies, the thermostabilities of these variants were assessed by measuring the extents of the loss of native protein to aggregates formed as a consequence of heat treatment (Section 2.8.1). The spectroscopic results in Chapter 5 can also be considered as measures of the concentration of native BLG in solutions previously heat-treated because the correlation of PAGE and spectroscopic results suggest that only the BLG species which run as monomers on alkaline native gels (i.e. "native-like" BLG species) exhibit the spectral characteristics of native BLG. Thus, the results obtained in these studies may provide better measures of extents of aggregate formation in heat-treated BLG solutions than BLG variant thermostabilities.

The results of Huang *et al.* (1995a), Elofsson *et al.* (1996b) and possibly those in Chapters 6 and 7 (summarised in Tables 8.4.1.1, 8.5.2.1 and 8.5.2.2) may, however, provide more accurate measures of the relative thermostabilities of BLGs A and B. DSC results describe the amount of thermal energy needed to induce structural change in protein molecules. The results in Chapter 7 describe the proportion of BLG molecules in a solution at a particular temperature which contain non native tertiary and secondary structure. For these reasons, it is concluded that the "intrinsic thermostability" of BLG A is slightly less than that of BLG B.

8.5.2.2.8. Interpretation of the Results Obtained at Room Temperature using Previously Heat-treated β-Lactoglobulin Solutions.

The results summarised in Tables 8.4.1.2 and 8.5.2.3, those of Gough and Jenness (1962), Sawyer (1968), Sawyer *et al.* (1971), those obtained by Hillier and Lyster (1979) at temperatures less than 95 °C, and those obtained by Nielsen *et al.* (1996) for BLG solutions at less than approximately 50 mg/mL may reflect the slower rate at which BLG A forms aggregates compared to that for BLG B (Section 8.5.1.1). This behaviour was explained in terms of the greater net charge on BLG A compared

to that on BLG B at pH 6.7 and pH 7.4. Therefore, in the case of the results obtained from studies in which BLG variant "thermostabilities" were assessed by measuring extents of loss of native protein which occurred as a consequence of heat treatment, it is possible that BLG A appears more "thermostable" than BLG B, only because the A variant forms aggregates more slowly than the B variant. For this reason, only the interpretation of results of the studies listed above may be inconsistent with those obtained in the DSC studies of Huang *et al.* (1995a) and Elofsson *et al.* (1996b) and those summarised in Tables 8.4.2.1, 8.5.2.1 and 8.5.2.2 (i.e. results of obtained from measurements made at elevated temperatures). Therefore, it is also concluded that BLG A is less susceptible to "irreversible structural change" than BLG B.

8.5.2.2.9. Importance of Heat Treatment Concentration.

The results of McSwiney *et al.* (1994b) and Imafidon *et al.* (1991), those obtained by Hillier and Lyster (1979) at temperatures greater than 95 °C and those of Nielsen *et al.* (1996) for BLGs A and B at concentrations greater than approximately 50 mg/mL (all obtained from measurements made at room temperature on BLG solutions previously heat-treated) appear consistent with the suggestion made above that the "intrinsic thermostability" BLG A is slightly less than that of BLG B. Nielsen *et al.* (1996) suggested that at concentrations greater than approximately 50 mg/mL, BLG A is more sensitive to heat treatment than BLG B (Section 8.5.2.2.3). Therefore, during heat treatment at BLG concentrations greater than approximately 50 mg/mL or at temperatures higher than 95 °C, it appears that the rate of decrease in the concentration of native protein is more dependent on the "intrinsic thermostability" of the variant in question rather than on its susceptibility to aggregate formation. In contrast, during heat treatment at concentrations less than approximately 50 mg/mL or at temperatures less than 95 °C, the opposite may be true.

8.5.2.2.10. The Structural Stabilities of Native β-Lactoglobulins A and B in Relation to their "Intrinsic Thermostabilities".

The hypothesis that the "intrinsic thermostability" of BLG A is slightly less than that of BLG B is also consistent with the results obtained in the tryptic hydrolysis study of Huang *et al.* (1995a) and the fourier transform infrared (FTIR) study of Dong *et al.* (1996). Huang *et al.* (1995a) found that, at pH 8.0 at 5 mg/mL, BLG A is hydrolysed more rapidly by immobilised trypsin than BLG B. From these results, and also their DSC results (Section 8.5.2.2.3), Huang *et al.* (1995a) suggested that the structural stability of BLG A is less than that of BLG B. Dong *et al.* (1996) found that the rate of H-D exchange was greater for BLG A than for BLG B, and suggested that the structural

mobility of the A variant is greater than that of the B. This also suggests that the structural stability of BLG A is less than that of BLG B. The lower stability of the structure of BLG A compared to that of BLG B may therefore explain why the "intrinsic thermostability" of the A variant is less than that of the B.

8.5.2.3. Summary: The Effect of Variant Type on the Thermostability and Aggregation Behaviour of β-Lactoglobulins.

A comparison of published results and those in Chapters 5, 6 and 7 suggests that the "intrinsic thermostability" of BLG A is slightly less than that of BLG B, which is less than that of BLG C. In addition, BLG A appears to form aggregates more slowly than BLGs B and C. Finally, concentrations of large aggregates are slightly higher in heat-treated solutions of BLG C than in those of BLG B.

8.5.2.4. The Effect of Differences in the Aggregation Behaviour of β-Lactoglobulins A, B and C on the Determination of the Order of "Intrinsic Thermostabilities".

The conclusions regarding the "intrinsic thermostabilities" of BLGs A, B and C (Section 8.5.2.3) appear valid. Almost all of the results in Chapters 5, 6 and 7 suggest that the "intrinsic thermostability" of BLG C is greater than those of BLGs A and B, and the results of Huang *et al.* (1995a) and Dong *et al.* (1996) suggest that the structural stability of BLG A is less than that of BLG B. However, further studies are required to establish whether or not the DSC results of Huang *et al.* (1995a) and Elofsson *et al.* (1996b), and those summarised in Tables 8.4.2.1, 8.5.2.1 and 8.5.2.2 are affected by aggregate formation. This is because the results obtained in the PAGE study in Section 5.3.9 suggest that structural change in BLG molecules can occur as a consequence of intramolecular thiol-disulphide interchange (Section 8.3.5). Therefore, DSC results for example may reflect both the heat-induced "unfolding" of BLG molecules and "unfolding" which occurs as a consequence of intramolecular thiol-disulphide interchange. This in turn suggests that the standard experimental techniques used to determine protein thermostability may not be appropriate for proteins that undergo thiol-disulphide interchange reactions at elevated temperatures.

8.5.2.5. Slopes at T_{mid} in Plots of $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{ANS} and A_{412} Versus Heat Treatment Temperature.

In many instances, the slopes at T_{mid} in plots of $\Delta\varepsilon_{293}$, $[\theta]_{205}$, I_{ANS} and A_{412} versus heat treatment temperature for BLG A differ significantly to those for BLGs B and C, suggesting that the A variant responds to heat treatment in a different manner to the B and C variants. These observations appear consistent with the results discussed in Section 8.5.1.1, which suggest that monomeric species of BLG A are incorporated in aggregate structures more slowly than those of BLGs B and C during heat treatment, but are difficult to explain in terms of "intrinsic thermostability".

8.6. THE FOLDING PATHWAY OF β-LACTOGLOBULIN.

In recent years, significant advances have been made towards deciphering the folding pathway of BLG. The structures of partially folded BLG species formed during folding (subsequently referred to as folding intermediates[1]) appear similar to those of stable partially unfolded forms of BLG (subsequently referred to as equilibrium unfolding intermediates[2]). Determining the structures of the latter has assisted in the identification of the most stable part of the BLG structure and, in a broader context, may have relevance to the folding pathways of all-β proteins.

8.6.1. Folding Intermediates of Bovine β-Lactoglobulin.

The results obtained in the stopped-flow CD study of Kuwajima *et al.* (1996) suggest that in the early stages of refolding (i.e. within approximately 18 ms), BLG forms a molten globule-like intermediate which contains approximately 30 % α-helix, approximately 20 % more than native BLG. Kuwajima *et al.* (1996) have offered two explanations for why an intermediate rich in α-helix forms on the folding pathway of BLG and the results in this thesis may help identify which of these explanations is the most likely, as discussed below.

8.6.2. Equilibrium Unfolding Intermediates of Bovine β-Lactoglobulin.

The results obtained in a number of other studies suggest that equilibrium unfolding intermediates also contain more α-helix than the corresponding native species. Hamada and Goto (1997) reported that in Gdn-HCl, BLG forms an equilibrium unfolding intermediate which contains an appreciable amount of β-structure, but more α-helix than the corresponding native species. They found that the midpoint Gdn-HCl concentration in plots of $[\theta]_{222}$ (i.e. α-helix) versus [Gdn-HCl] is greater than that in plots of $[\theta]_{295}$ (i.e. tertiary structure) versus [Gdn-HCl]. Similarly, Kuwajima *et al.* (1996) reported that BLG forms an equilibrium unfolding intermediate which possesses significantly more secondary structure than tertiary structure. They found that at pH 3.2 at 4.5 °C, the midpoint Gdn-HCl concentration in plots of $[\theta]_{219}$ (i.e. β-sheet) versus [Gdn-HCl] was 2.95 M, while that in plots of $\Delta\varepsilon_{293}$ and A_{293} (i.e. tertiary structure) versus [Gdn-HCl] was 2.64 M.

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[1] Folding intermediates exist transiently on the folding or re-folding pathways of proteins. They are most commonly identified in stopped-flow studies in which a solution of protein in concentrated Gdn-HCl or urea (i.e. solution conditions which disfavour folding) is rapidly diluted into an aqueous solution (i.e. solution conditions which favour folding).

[2] Equilibrium unfolding intermediates are stable partially unfolded conformations of a protein which form in moderate concentrations of denaturant (e.g. 2 M Gdn-HCl), or at low pH (e.g. pH 1.5) or at moderately high temperatures (e.g. 65 °C), (Fink, 1995). If the unfolding of the protein in question is reversible, then the equilibrium unfolding intermediate will be stable providing the conditions which led to its formation are retained.

8.6.3. Equilibrium Unfolding Intermediates of Other Lipocalycins.

Under non-physiological conditions, equine BLG and other lipocalycins also assume partially unfolded conformations which contain significant amounts of β-structure and also more α-helix than the corresponding native species. Ikeguchi *et al.* (1996) reported that equine BLG, which is monomeric and does not possess a thiol group, assumes a molten globule-like conformation with these structural features at pH 1.5. Another lipocalin, plasma retinol-binding protein, assumes a molten globule-like conformation with more α-helix than the corresponding native species at elevated temperatures and at low pH (Bychkova *et al.*, 1992; Muccio *et al.*, 1992). Finally, cellular retinoic acid-binding protein contains more α-helix at low pH than at approximately neutral pH (Liu *et al.*, 1994). This protein also exhibits a strong propensity to form α-helices in trifluoroethanol (Liu *et al.*, 1994).

8.6.4. Formation of α-Helices by β-Lactoglobulin.

The amino acid sequence of bovine BLG is predicted to form α-helices (Creamer *et al.*, 1983; Sawyer and Holt, 1993) and this may explain why BLG forms folding intermediates and equilibrium unfolding intermediates with more α-helix than the corresponding native species. The strong "tendency" of BLG to form α-helices is consistent with the results of Shiraki *et al.* (1995), Hamada *et al.* (1995) and Dufour *et al.* (1993, 1994). Shiraki *et al.* (1995) found that the structure of BLG contains more α-helix in the presence of trifluoroethanol than in its absence. Hamada *et al.* (1995) found that three synthesised peptides from BLG, residues 11 - 28, 61 - 71 and 127 - 142, exhibit marked α-helical propensities. Dufour *et al.* (1993, 1994) found that β-sheet in molecules of BLG is converted to α-helix in the presence of alcohol.

8.6.5. The Structure of Bovine β-Lactoglobulin at Elevated Temperatures and at Room Temperature After Heat Treatment.

In contrast to the folding intermediates and equilibrium unfolding intermediates discussed above, the species present in BLG solutions at elevated temperatures or in BLG solutions at room temperature previously heat-treated do not possess more α-helix than the corresponding native species. Qi *et al.* (1997) found, from far UV CD results, that the structure of BLG contains a negligible amount of α-helix and less β-sheet at temperatures greater than approximately 70 °C than at room temperature. Furthermore, the far UV CD results in Section 5.3.2 suggest that the amount of α-helix in the structure of BLG does not increase appreciably as a consequence of heat treatment.

8.6.6. The Structure of Bovine β-Lactoglobulin in Concentrated Solutions of Chaotropes and at Low pH.

In agreement with the results obtained in the thermal unfolding studies discussed above, the results obtained in a number of chaotrope-induced BLG unfolding studies suggest that the β-structure of this protein is more stable than both the α-helical and tertiary structure. Civera *et al.* (1996) found that BLG possesses some β-structure at pH 2.1 in 8 M urea. Similarly, Kuwajima *et al.* (1996) found that BLG possesses a small amount of β-structure and no α-helix at pH 3.2 in 4 M Gdn-HCl. Finally, Molinari *et al.* (1996) found that at pH 2.1 in the absence of chaotropes, BLG has less tertiary structure than at approximately neutral pH, but possesses a core of β-sheet.

8.6.7. The Role of α-Helix on the Folding Pathway of Bovine β-Lactoglobulin.

As mentioned in Section 8.6.1, Kuwajima *et al.* (1996) have offered two explanations for why an intermediate species which contains approximately 30 % α-helix is formed during the BLG folding process. Firstly, this species may be an intermediate on the BLG folding pathway. If this is the case, then the formation of α-helix may dominate the early stages of BLG folding, suggesting that folding follows a non-sequential mechanism. However, this does not necessarily mean that BLG should form partially unfolded species rich in α-helix during heat-induced unfolding. The results of Qi *et al.* (1997) indicate that at temperatures greater than approximately 70 °C BLG possesses a negligible amount of α-helix. Secondly, Kuwajima *et al.* (1996) have suggested that the folding of BLG is guided by the stable β-structure which these molecules possess at pH 3.2 in 4 M Gdn-HCl, and that non-native α-helix forms transiently in regions of the BLG structure with a strong propensity to form the latter type of secondary structure. Kuwajima *et al.* (1996) favour their second explanation for why a highly α-helical intermediate forms on the folding pathway of BLG because only β-structure is found at pH 3.2 in 4 M Gdn-HCl. This suggests that BLG folds via a sequential folding mechanism. The results obtained in heating studies (Section 5.3.2; Qi *et al.*, 1997) are therefore consistent with this supposition because they indicate that heat treatment does not lead to an appreciable decrease in the amount of β-sheet in molecules of BLG.

8.6.8. The Folding Pathways of Other All-β Proteins.

The formation of an intermediate in the early stages of the re-folding of bovine BLG which contains a significant amount of β-structure has important implications for the folding pathways of all-β proteins. Barber *et al.* (1995) have suggested that these proteins can fold in manners consistent with both the molten globule model, in which

global interactions are more important than local interactions, and the framework model, in which the opposite is true. In the molten globule model, the hydrophobic side chains of a protein are buried in the core of a molten globule in the earliest stages of folding. The majority of the secondary structure of the protein then forms, thus minimising the effect of transferring polar amide and carbonyl groups to a hydrophobic environment. In the final stages of folding, specific tertiary interactions develop. If folding occurs in this manner, then β-sheets may not form until the constituent β-strands have aligned properly. Therefore, the protein may have to assume a relatively compact conformation before β-sheets can form, which is consistent with the hypothesis that during the early stages of the folding of all-β proteins, global interactions are more important than local interactions.

In the framework model, small pieces of secondary structure, for example β-turns, form in the earliest stages of folding. These small pieces of secondary structure may then serve as folding nucleation sites which permit the remaining secondary structure, for example β-sheets, to form. In the final stages of folding, specific tertiary interactions develop. Thus, if the β-turns of all-β proteins are formed first, then the protein may not have to assume a relatively compact conformation before the majority of the secondary structure has formed. This is therefore consistent with the hypothesis that in the framework model, local interactions are more important than global interactions in the early stages of the folding of all β-proteins.

The results obtained by Kuwajima *et al.* (1996) for the folding of BLG appear most consistent with the molten globule model for protein folding because the results obtained in the equilibrium unfolding studies discussed in Sections 8.6.2 and 8.6.3 and those of the heating study in Section 5.3.2 suggest that BLG molecules possess some β-structure under non-physiological conditions. This suggests that at least some of the β-structure of BLG is very stable, which may in turn be consistent with the rapid formation of this type of secondary structure during folding. Such behaviour would require that non-local interactions are more important than local interactions in the early stages of folding.

The results obtained by Kuwajima *et al.* (1996) for the re-folding of BLG appear consistent with those obtained in a number of other studies in which the re-folding of all-β proteins was studied. Staphylococcal nuclease (Jacobs and Fox, 1994), interleukin-1-β (Varley *et al.*, 1993), apoplastacyanin (Koide *et al.*, 1993), and *E. coli* dihydrofolate reductase (Jones and Matthews, 1995) also form β-sheet structure in the early stages of re-folding. The results obtained in these studies and also that by Kuwajima *et al.* (1996) therefore suggest that several all-β proteins, including BLG, commonly fold in a manner consistent with the molten globule model.

Chapter 9.

CONCLUSIONS.

In the study presented and discussed in this thesis, differences in the susceptibilities of bovine BLGs A, B and C to heat-induced structural change were examined using the spectroscopic techniques of near and far UV CD, intrinsic protein and ANS fluorescence and by measuring the availability of the thiol group of these variants for reaction with the thiol reagent DTNB. Measurements were made on BLG solutions at both elevated temperatures and 20 °C using BLG solutions that had been previously heat-treated. Differences in the aggregation behaviour of BLGs A, B and C were examined using alkaline native-PAGE and SDS-PAGE (both disulphide-reduced and disulphide-intact) and 2-dimensional (alkaline native-, then disulphide-intact SDS-PAGE). The results obtained in these examinations were interpreted as follows:-

9.1. Aggregation Mechanism.

When BLGs A, B and C were heated in solution at pH 6.7 or pH 7.4 both disulphide-linked and non-covalently-linked aggregates were formed. The proportions of native protein, non-native monomers, disulphide-linked dimers and larger aggregates (both non-covalently-linked and disulphide-linked) in heat-treated solutions depends on the variant type, solution pH and heat treatment temperature and time. Concurrently, many of the spectral properties of these variants changed, indicating that their tertiary structure in particular had been altered. Although most of these structural changes could be fitted to 2-state models, the overall pathway for the formation of aggregates of BLG has multiple steps. These appear to occur in the following order:

1) Dimers dissociate to monomers.

2) The thiol group is reversibly exposed and this may precede structural changes in other parts of the BLG structure.

3) The solvent-exposed thiol group of non-native BLG molecules then participates in oxidation and thiol-disulphide interchange reactions with other BLG molecules. Intramolecular thiol-disulphide interchange reactions also occur. As a consequence of these reactions, BLG molecules are retained in non-native conformations which exhibit the following structural characteristics:

a) a solvent-exposed thiol group

b) Trp^{19} and Trp^{61} side chains in non-native environments

c) more random structure than native BLG

d) an ANS binding site with a structure different to that of native BLG.

Thiol oxidation and thiol-disulphide interchange reactions lead to a decrease in the concentration of monomeric BLG and the concomitant formation of disulphide-linked dimers, trimers, tetramers etc. and stable "unfolded" monomers.

4) Small disulphide-linked aggregates and also "unfolded" monomers and native BLG continue to aggregate via thiol-disulphide interchange reactions, as shown by PAGE results, time-dependent increases in tryptophan emission intensity and an increase in the intensity of a trough near 277 nm in near UV CD spectra.

A parallel pathway for the formation of BLG aggregates exists. Non-covalently-linked aggregates are also formed during heat treatment. These aggregates exist as reaction intermediates and are converted to disulphide-linked aggregates via thiol-disulphide interchange reactions after prolonged heat treatment, or after heat treatment at high temperatures.

I tentatively conclude that previously heat-treated solutions of BLG do not contain a molten globule-like species.

9.2. Effect of pH on the Susceptibility to Heat-induced Structural Change and the Aggregation Behaviour of β-Lactoglobulin.

The greater net negative charge on BLG molecules, and therefore stronger electrostatic repulsions between monomeric units at pH 7.4 than at pH 6.7 decreases the T_{mid} values for all structural changes examined but also shifts the distribution of aggregates to lower M_r despite the greater thiol reactivity at pH 7.4.

9.3. Effect of Variant Type on the Aggregation Behaviour of β-Lactoglobulin.

The following experimental evidence suggests that BLG A forms aggregates, particularly large ones stabilised by disulphide bonds, more slowly than BLGs B and C:

a) The decrease in the concentration of monomers and "unfolded" monomers with increasing heat treatment temperature is less marked for BLG A than for BLGs B and C, suggesting that monomeric BLG A species are incorporated into aggregates less readily.

b) The maximum extent of the heat-induced change in $\Delta\varepsilon_{270}$ is less for BLG A.

c) Tryptophan emission intensity increases more slowly for BLG A after temperature increase.

d) Concentrations of non-covalently-linked aggregates are higher in heat-treated solutions of BLG A.

e) BLG A forms smaller aggregates.

These differences probably reflect the greater net negative charge at pH 6.7 and pH 7.4 on molecules of BLG A than on those of BLGs B and C. In addition, the proximity of residue 64, which is an aspartate in BLG A and a glycine in BLGs B and C, to the side chain of Glu^{65} and the disulphide bond Cys^{66}-Cys^{160} probably affects the aggregation behaviour of BLG. At pH 6.7 and pH 7.4 both Asp^{64} and Glu^{65} will carry a negative charge and the side chain of Glu^{65} will be repelled towards the disulphide bond Cys^{66}-Cys^{160}. The negative charge close to the disulphide bond Cys^{66}-Cys^{160} will therefore be greater for BLG A than for BLGs B and C, making it less likely that molecules of BLG A will participate in intermolecular thiol-disulphide interchange reactions with thiolate anions.

β-Lactoglobulin C forms large aggregates slightly more readily than BLG B. Concentrations of aggregate species with M_r values greater than approximately 200 000 are slightly higher in heat-treated solutions of BLG C than in those of BLG B. This difference probably reflects the lower net negative charge at pH 6.7 and pH 7.4 on molecules of BLG C.

9.4. Effect of Variant Type on the Susceptibility of β-Lactoglobulin to Heat-induced Structural Change.

The "intrinsic thermostability" of BLG C is appreciably greater than that of BLG B, which is slightly greater than that of BLG A (as shown by near and far UV CD, tryptophan and ANS fluorescence and thiol availability results). However, further studies are required to confirm that the results from which the above order of variant thermostabilities was determined do not also reflect differences in the aggregation behaviour of these variants. This is because thiol-disulphide interchange reactions, which occur during heat-induced aggregate formation, may also lead to irreversible structural change in BLG molecules. The differences in the "intrinsic thermostabilities" of BLGs A, B and C are not readily explained in terms of the published crystal structures of these variants.

Spectroscopic and thiol availability results also suggest that the extent of "irreversible structural change" in molecules of BLG C which occurs as a consequence of heat treatment is less than that for BLG A, which is less than that for BLG B. Thus, the order of the susceptibilities of BLGs A and B to "heat-induced irreversible structural change" is different to the order of their "intrinsic thermostabilities".

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APPENDIX 1. TEMPERATURE CONTROL.

A1.0. INTRODUCTION.

Near UV CD, intrinsic protein fluorescence and thiol availability measurements were made on BLG solutions at elevated temperatures using a water-jacketed cell (near UV CD) or standard 10 mm path length cells in a temperature-controlled cell holder (fluorimetry and thiol availability). In fluorescence and thiol availability measurements, cell contents were maintained at the required temperature by a flow of water, heated in the Neslab waterbath, through the cell holder.

At temperatures greater than ambient, heat was dissipated from waterbath tubing, cell holders etc. to the surroundings. Thus, the temperatures of the solutions under study were always lower than that of the waterbath. Therefore, the relationship between waterbath temperature settings and actual cell contents temperatures was examined at a range of temperatures. In addition, protocols were developed to minimise temperature shifts caused by the mixing of cold and hot solutions within CD, fluorimeter and spectrophotometer and cells. These protocols will be discussed in Appendix 2.

A thermocouple was used to routinely measure the temperatures of cell contents. However, prior to initial use, this instrument was calibrated by setting the waterbath to a series of known temperatures and comparing these with thermocouple readings. The waterbath, in turn, was calibrated using an RT-200 resistance thermometer (see below).

A1.1. INSTRUMENT CALIBRATION.

A1.1.1. Neslab Waterbath.

A resistance thermometer (RT-200 Resistance Thermometer, Industrial Research Ltd, Gracefield Research Centre, Gracefield Rd, Lower Hutt, New Zealand) was used to check the calibration of the Neslab waterbath. This thermometer is the NZDRI secondary thermometric standard.

The waterbath was set to 20.0 °C, the probe of the resistance thermometer was placed in the water, and the temperature recorded. The temperature setting of the waterbath was then adjusted to 25.0 °C, and the new water temperature was recorded. For the remainder of the calibration, the waterbath temperature setting was raised, in 5 °C increments, and water temperatures were recorded. The calibration curve is shown in Fig. A1.1.

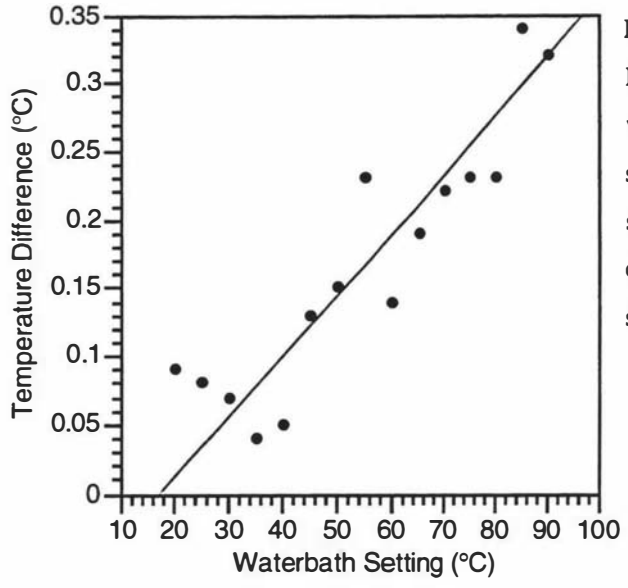

Fig. A1.1. Calibration curve for the Neslab RTE-200 waterbath. Temperature difference values represent actual water temperatures subtracted from the waterbath temperature setting values. Refer to the text above for calibration protocol and information on the standard.

A1.1.2. Thermocouple.

The thermocouple was initially calibrated by setting the waterbath to a series of temperatures and comparing the thermocouple reading with that of the RT-200 resistance thermometer. Subsequently, the thermocouple calibration was checked against the waterbath using the calibration curve shown in Fig. A1.1. Prior to commencing each phase of work which required temperature measurement, the calibration of the thermocouple was re-checked. A typical calibration curve is shown in Fig. A1.2.

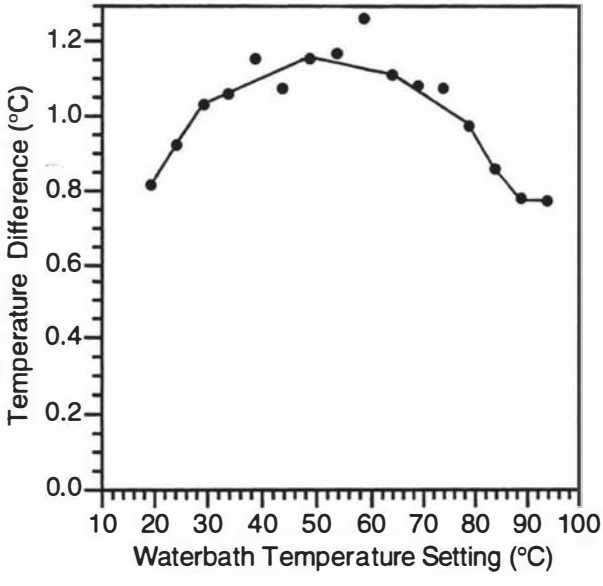

Fig. A1.2. Calibration curve for the thermocouple. Values for the temperature difference represent water temperatures, as measured using the thermocouple, subtracted from water temperatures measured using the resistance thermometer. Refer to the text for calibration protocols.

A1.2. TEMPERATURES OF CELL CONTENTS DURING DATA COLLECTION.

A1.2.1. Water-jacketed CD Cell.

When solutions were heated in 10 mm path length CD cells using a temperature controlled cell holder, the cell contents took up to 30 min to attain thermal equilibrium (data not shown). For this reason, all elevated temperature CD measurements were made using a water-jacketed CD cell despite the low signal:noise ratio due to the smaller cell window diameter.. Periods from temperature re-adjustment to when the rate of temperature change first became less than 0.01 °C per 3 min (defined as attainment of thermal equilibrium) after both temperature increase and decrease are shown in Fig. A1.3.

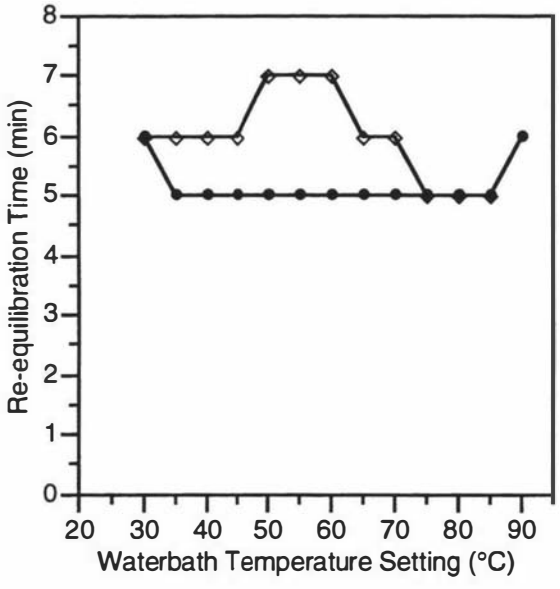

Fig. A1.3. Times required for the contents (pH 6.7 phosphate buffer) of the water-jacketed CD cell to re-attain thermal equilibrium (see text for definition) after temperature increase and decrease. The symbols (●) and (◇) denote temperature increase and decrease respectively. The CD cell was connected to the waterbath and the temperature of the latter was first increased, in 5 °C increments, from 20 °C to 90 °C, and then decreased, in 5 °C increments, back to 20 °C.

A plot of the difference between the cell contents temperatures at thermal equilibrium and the waterbath temperature setting values versus waterbath temperature setting values is shown in Fig. A1.4, and was used to estimate cell contents temperatures that had not been measured directly.

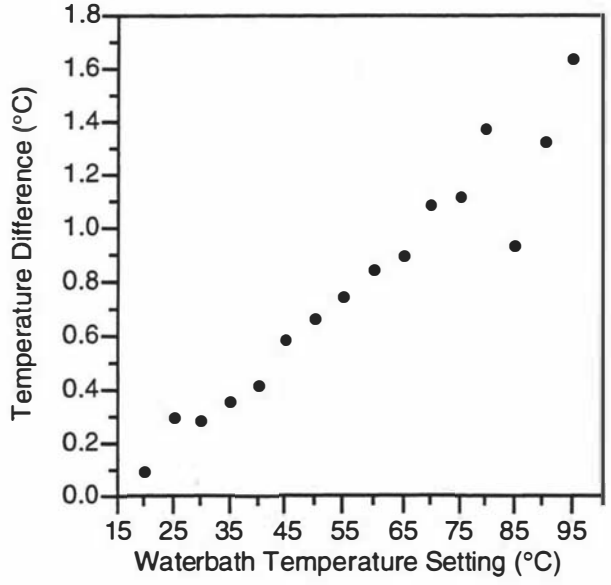

Fig. A1.4. Plot of the difference between water-jacketed CD cell contents temperatures at thermal equilibrium and the waterbath temperature setting values versus the waterbath temperature setting values. Cell contents temperatures, measured using the thermocouple, were calculated using a calibration curve similar to that shown in Fig. A1.2.

A1.2.2. Fluorimeter Cells.

A1.2.2.1. Thermal Re-equilibration Times.

The times required for the contents of fluorimeter cells to re-attain thermal equilibrium after temperature increase (Fig. A1.5) were determined using a procedure similar to that described in Fig. A1.3. The contents of fluorimeter cells were warmed in positions 2, 3 and 4 of the fluorimeter temperature controlled cell holder. Data were acquired from cells placed in each of these positions on different days.

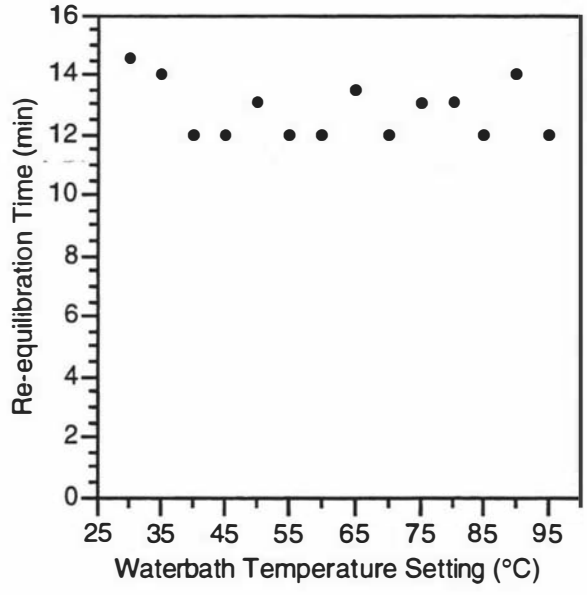

Fig. A1.5. Times required for the contents (pH 6.7 phosphate buffer) of a fluorimeter cell placed in position 4 of the fluorimeter temperature-controlled cell holder to re-attain thermal equilibrium after temperature increase (see Section A1.2.1 for definition). The cell holder was connected to the waterbath and the temperature of the latter was increased, from 30 °C to 95 °C, in 5 °C increments.

A1.2.2.2. Temperature Differential Within Fluorimeter Cells.

The measurements made as described in Section A1.2.2.1 indicated that the contents of fluorimeter cells are significantly cooler than the waterbath. Therefore, the temperatures of fluorimeter cell contents were also likely to be non-uniform.

The results in Table A1.1 indicate that at higher temperatures, the top portion of fluorimeter cell contents was appreciably warmer than the bottom portion.

Table A1.1. Temperature Differential Within Fluorimeter Cells.

Waterbath Temperature Settings (°C)	Cell Contents Temperature: Top Portion (°C)	Cell contents Temperature: Bottom Portion (°C)	Temperature Differential (°C)
20	20.5	20.5	0
40	39.8	39.8	0
60	59.0	58.7	0.3
80	77.6	76.9	0.7

Data were collected from 3 mL of pH 6.7 phosphate buffer, contained in a fluorimeter cell, in position 4 of the fluorimeter temperature controlled cell holder. The cell contents temperature, at a position approximately 5 mm from each side and 5 mm from the bottom or top of the cell contents, was measured 15 min after the waterbath temperature has been set.

The light beam of the fluorimeter traverses the bottom portion of the cell, and for this reason temperatures measured at the bottom of fluorimeter cells were used to prepare the calibration curves for fluorimeter cell contents (see Section A1.2.2.3).

A1.2.2.3. Cell Contents Temperatures.

The temperatures of the lower portion of fluorimeter cell contents, warmed in positions 2, 3 and 4 of the fluorimeter temperature-controlled cell holder, were determined using a procedure similar to that described in Fig. A1.3.

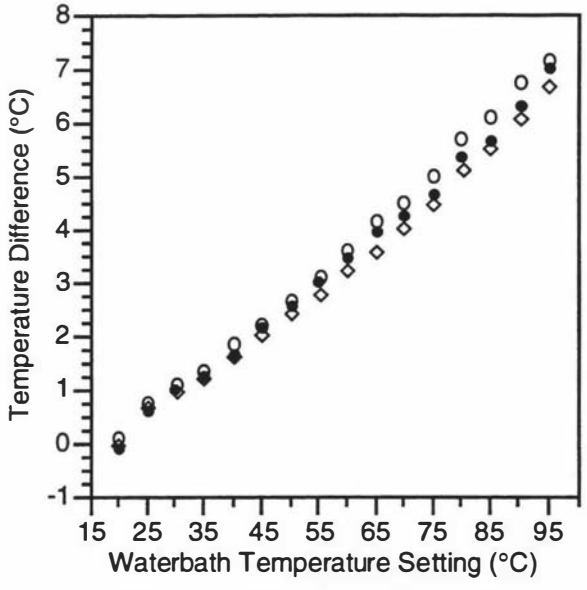

Fig. A1.6. Plots of the difference between fluorimeter cell contents temperatures at thermal equilibrium and the waterbath temperature setting values versus the waterbath temperature setting values. The symbols (•), (◇) and (o) denote data acquired from cells warmed in positions 4, 3 and 2 respectively of the fluorimeter temperature controlled cell holder. Cell contents temperatures, measured using the thermocouple, were calculated using a calibration curve similar to that shown in Fig. A1.2.

The temperature differences shown in Fig. A1.6 are larger than those shown in Fig. A1.4. For example, when the waterbath was set at 90 °C, fluorimeter cell contents warmed in positions 2, 3 and 4 of the cell holder were 6.8 °C, 6.1 °C and 6.3 °C cooler respectively than this temperature. In contrast, at a waterbath temperature setting of 90 °C, CD cell contents attained a temperature 1.3 °C lower than this value.

A1.2.3. Spectrophotometer Cells.

The times required for the contents of spectrophotometer cells to re-attain thermal equilibrium (see Section A1.2.1 for definition) after temperature increase are shown in Fig. A1.7. A plot of the difference between the cell contents temperatures at thermal equilibrium and the waterbath temperature setting versus the latter is shown in Fig. A1.8. These data were collected from a cell placed in the sample position closest to where water flowed into the temperature controlled cell holder of the Shimadzu UV 260-spectrophotometer. All spectrophotometric data were acquired from cells placed in this same cell holder position. The temperature differences shown in Fig. A1.8 exceed those for fluorimeter cells (Fig. A1.6).

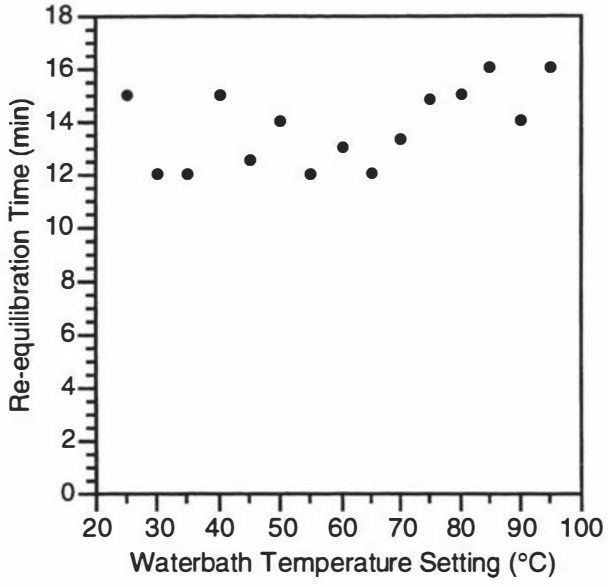

Fig. A1.7. Times required for the contents of a spectrophotometer cell (pH 6.7 phosphate buffer) to re-attain thermal equilibrium (see Section A1.2.1 for definition) after temperature increase. The cell holder was connected to the waterbath and the temperature of the latter was increased, in 5°C increments, from 25 °C to 95 °C.

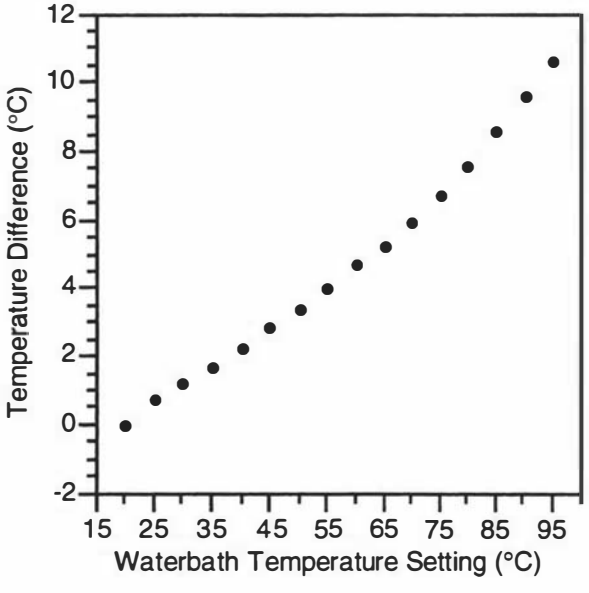

Fig. A1.8. Plot of the difference between spectrophotometer cell contents temperatures at thermal equilibrium and the waterbath temperature setting values versus the waterbath temperature setting values. Cell contents temperatures, measured using the thermocouple, were calculated using a calibration curve similar to that shown in Fig. A1.2.

A1.2.4. Conclusions.

The plots in Figs A1.4, A1.6 and A1.8 indicate that at a particular waterbath temperature setting the temperatures attained by the contents of the water-jacketed CD cell, fluorimeter and spectrophotometer cells differ from each other and are all less than the waterbath temperature setting value. Nevertheless, by using the calibration curves shown in these figures, it was possible to determine the actual temperatures of cell contents. Using the plots in Figs A1.4, A1.6 and A1.8, it was also possible to calculate those waterbath temperature settings which would allow the contents of spectrophotometer, fluorimeter and water-jacketed CD cells to attain equivalent temperatures.

APPENDIX 2. THIOL AVAILABILITY.

A2.1. DEVELOPMENT OF THE PROTOCOL USED TO MEASURE TIME-DEPENDENT CHANGES IN β-LACTOGLOBULIN THIOL AVAILABILITY AT ELEVATED TEMPERATURES.

Before the thiol availabiliy assay results discussed in Section 6.3.1 could be obtained, a number of parameters, including heating temperatures and protein and reagent concentrations at which heat-induced structural change could be readily followed, were determined.

A2.1.1. Justification of Reagent Concentrations and Selected Assay Conditions.

Assuming complete availability of one cysteine per molecule of monomeric BLG, the BLG concentration that would give an A_{412} of 1.0 in a 10 mm path length cell was calculated using a molar extinction coefficient of 14 150 $M^{-1}cm^{-1}$ (Riddles, 1979) to be 1.30 mg/mL.

The most appropriate method of starting the reaction seemed to be to inject the protein solution directly into 10 × 10 × 45 mm spectrophotometer cells containing hot buffered DTNB solution. However, if BLG solutions of concentration 50 mg/mL or higher were used, there was a danger that gelation would occur when the protein came into contact with the hot buffer (McSwiney *et al.*, 1994a). For this reason, BLG solutions of concentration 29.6 mg/mL and 33.3 mg/mL at pH 6.7 and pH 7.4 respectively were used in all thiol availability assays. Mixing 150 μL of stock BLG solutions with 2.85 mL of hot buffered DTNB in the cells gave final assay concentrations of 1.47 mg/mL and 1.66 mg/mL at pH 6.7 and pH 7.4 respectively. A 10-fold molar excess of DTNB (0.78 mM) was used to simplify reaction kinetics.

When solutions of BLG are heated in the presence of oxygen, the concentration of free thiol groups decreases (Watanabe and Klostermeyer, 1976), probably due to oxidation to disulphides (Patrick and Swaisgood, 1976). Therefore, all buffers and DTNB solutions were de-aerated at the beginning of each day on which thiol availability measurements were made.

A2.1.2. Stability of Aqueous DTNB at pH 7.0.

Values for A_{412} in 0.78 mM solutions of DTNB at pH 7.0 and 20 °C increased spontaneously with time (Fig. A2.1) at a rate of 0.0060 hr^{-1}. The below results confirm the report of Riddles *et al.*, 1979. Consequently, reagent blank solutions, prepared immediately before each assay, were used for all measurements.

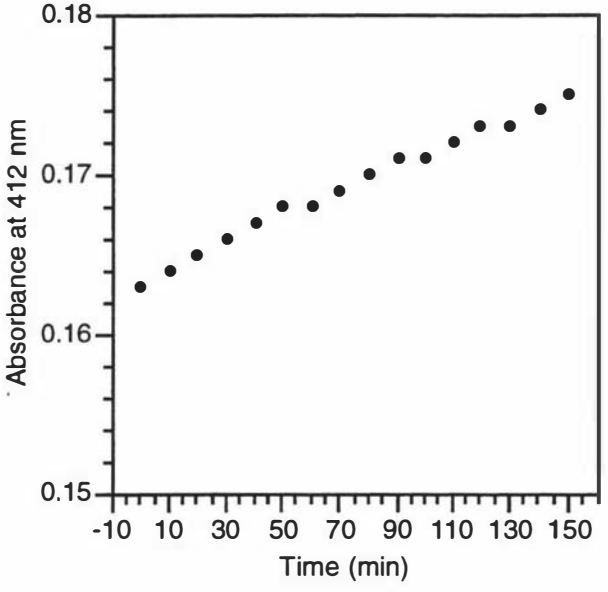

Fig. A2.1. Time-dependent increase in A_{412} in a 0.78 mM solution of DTNB in pH 7.0 phosphate buffer at 20 °C. The A_{412} of the DTNB solution was compared with an air blank. The cell compartment was flushed with dry oxygen-free nitrogen. Measurements were made using a 10 mm path length cell and a double-beam Shimadzu UV-260 spectrophotometer.

A2.1.3. Effect of Heat on Stability of Aqueous DTNB.

A2.1.3.1. Methods.

Aliquots (2 mL) of freshly degassed 7.77 mM DTNB solution in nitrogen-flushed and sealed 8 mL screw-cap glass vials were heat-treated in the waterbath at 40 °C, 60 °C or 80 °C for 10 min. At the conclusion of the heating period, vials were cooled rapidly in crushed ice.

The cooled solutions were then diluted 1:20 DTNB:pH 7.0 phosphate buffer, and the A_{412} of each solution was measured exactly 1 hr after heat treatment so that time-dependent changes in A_{412} were standardised. Values for A_{412} were read against a buffer blank approximately 1 min after cells had been placed in the cell holder of the double-beam Shimadzu UV-260 spectrophotometer.

A2.1.3.2. Results.

The absorbance at 412 nm of 0.39 mM DTNB solutions was not affected by temperature (Table A2.1), suggesting that DTNB had similar stabilities at 20 °C, 40 °C, 60 °C and 80 °C. Thus, the stability of DTNB at elevated temperatures was equivalent to that at 20 °C.

Table A2.1. Absorbance at 412 nm of Heated-treated DTNB Solutions.

Heating Temperature (°C)	A_{412}
Control (held at 20 °C)	0.053
40	0.055
60	0.056
80	0.056

Refer to text for data collection protocol.

A2.1.4. Selection of Appropriate Temperatures for Measuring Rates of Thiol Exposure in Solutions of β-Lactoglobulin.

A2.1.4.1. Methods.

β-Lactoglobulin A/B solutions (Sigma A/B, 3 × crystallised, L 0130, lot 51H7210) were prepared by dissolving 175 mg lyophilised protein in either pH 6.7 or pH 7.4 phosphate buffer and then diluting to 5 mL. The protein concentrations, 32.2 mg/mL, were determined from measured A_{280} values as described in Section 4.2.1.7.

Rates of Thiol Exposure in Solutions of β-Lactoglobulin.

Cells were placed in the reference and sample positions of the heated cell holder and 2.70 mL (sample cell) or 2.85 mL (reference cell) of phosphate buffer at either pH 6.7 or pH 7.4 added. DTNB solution (7.77 mM, 150 μL) was then mixed in. Cells were given 14 min to attain thermal equilibrium, the chart recorder was started, 150 μL of 32.2 mg/mL BLG solution at 20 °C was injected into the sample cell, quickly mixed using a glass rod, and the increase of A_{412} was followed until the end point was reached.

A2.1.4.2. Results.

Plots of A_{412} versus time for measurements made at pH 6.7 are shown in Fig. A2.2a. At 60 °C, reaction between exposed BLG thiol groups and DTNB proceeded at a convenient rate. Consequently, this temperature was selected for the comparative studies at pH 6.7 (Section 6.3.1.3). From comparable experiments at pH 7.4 (Fig. A2.2b), the temperature 50 °C was selected for the comparative studies at pH 7.4 (Section 6.3.1.3). At both pH 6.7 and pH 7.4, final A_{412} values decreased with decreasing assay temperature (Fig. A2.2a, b), indicating that the proportion of thiol groups able to react with DTNB decreased.

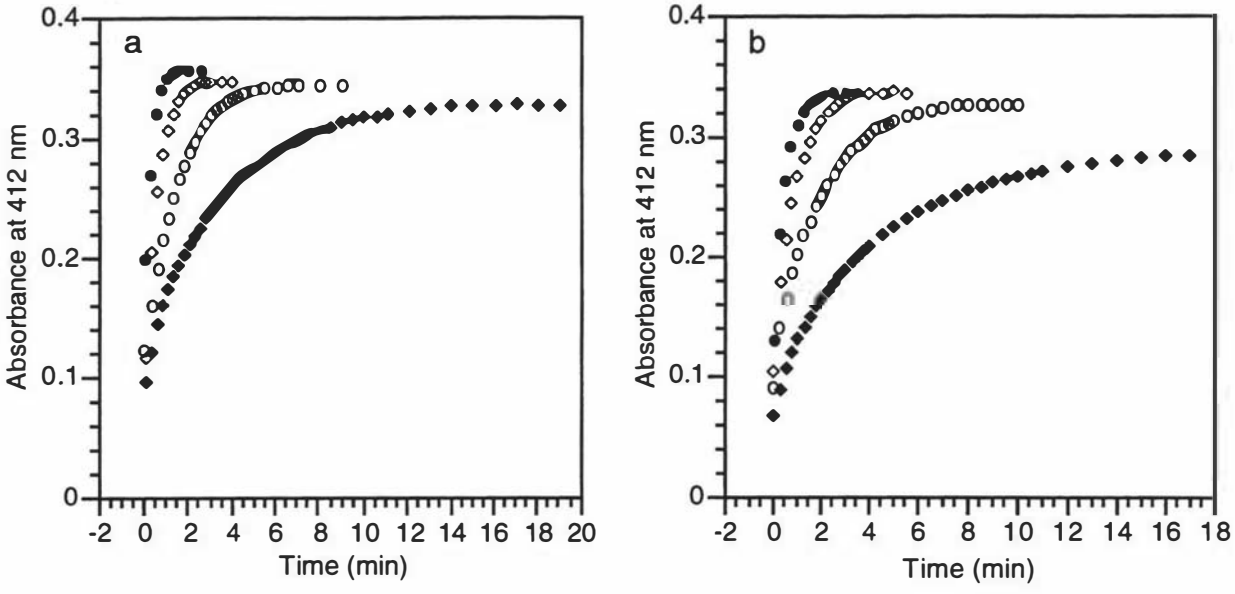

Fig. A2.2. The reaction of DTNB with the thiol group of BLG A/B at pH 6.7 and pH 7.4 at various temperatures. For the results in panel a, reactions were started by mixing 150 µL of 32.2 mg/mL BLG A/B solution at pH 6.7 into 2.85 mg/mL of 388 µM DTNB solution at the same pH at 68 °C, (•), 64 °C, (◇), 60 °C, (o) or 56 °C, (♦). For the results in panel b, reactions were started by mixing 150 µL of 32.2 mg/mL BLG A/B solution at pH 7.4 into 2.85 mg/mL of 388 µM DTNB solution at the same pH at 58 °C, (•), 54 °C, (◇), 50 °C, (o), or 46 °C, (♦). Measurements were made using 10 mm path length solutions and a double-beam Shimadzu UV-260 spectrophotometer.

A2.1.5. Temperature Changes After Reagent Mixing.

The changes in the temperature of the cell contents after the addition of 150 µL BLG solution at 20 °C to 2.85 mL phosphate buffer at 60 °C were followed (Fig. A2.3). Clearly, the temperature decrease of approximately 3 °C, and the subsequent increase to 60 °C over approximately 3 min will introduce a systematic error into experimental data.

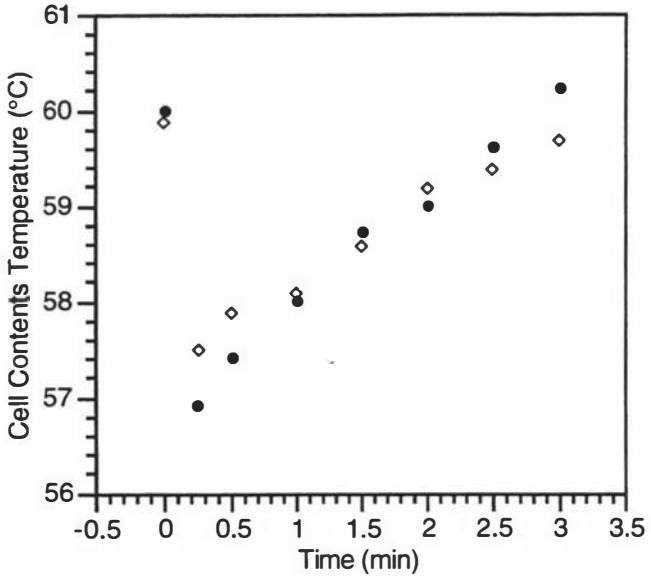

Fig. A2.3. Temperature fluctuation in spectrophotometer cells. The effect on the temperature of spectrophotometer cell contents of adding 150 µL of BLG solution at 20 °C to 2.85 mL phosphate buffer previously equilibrated to 60 °C. Buffer was heated in the temperature controlled cell holder of the Shimadzu UV-260 spectrophotometer. The symbols (•) and (◇) denote duplicate measurements. The point at zero time in each plot represents the temperature of cell contents prior to addition of BLG solution. β-Lactoglobulin solution was then added and, as soon as possible after mixing, the temperature of cell contents was re-determined (point at 0.25 min). Temperatures were measured using the thermocouple (Section 1.1.2 of Appendix 1).

Minimisation of the Temperature Change Following the Mixing of Hot and Cold Solutions in the Heated Cell.

If the reaction times at 60 °C at pH 6.7 were approximately 5 min (Fig. A2.2a), then the major part of the reaction would take place at temperatures below 60 °C (Fig. A2.3). In an attempt to have the reaction take place at a known temperature, the mixing protocol was modified. The cell holder was heated to 63.4 °C, the waterbath temperature was then lowered, and after a short time, protein solution was mixed into the reaction mixture. The effect of varying the time between waterbath temperature setting re-adjustment and BLG solution addition on the subsequent changes in the temperature of the cell contents is shown in Fig. A2.4. Clearly, this approach diminished, but did not eliminate, the temperature fluctuation.

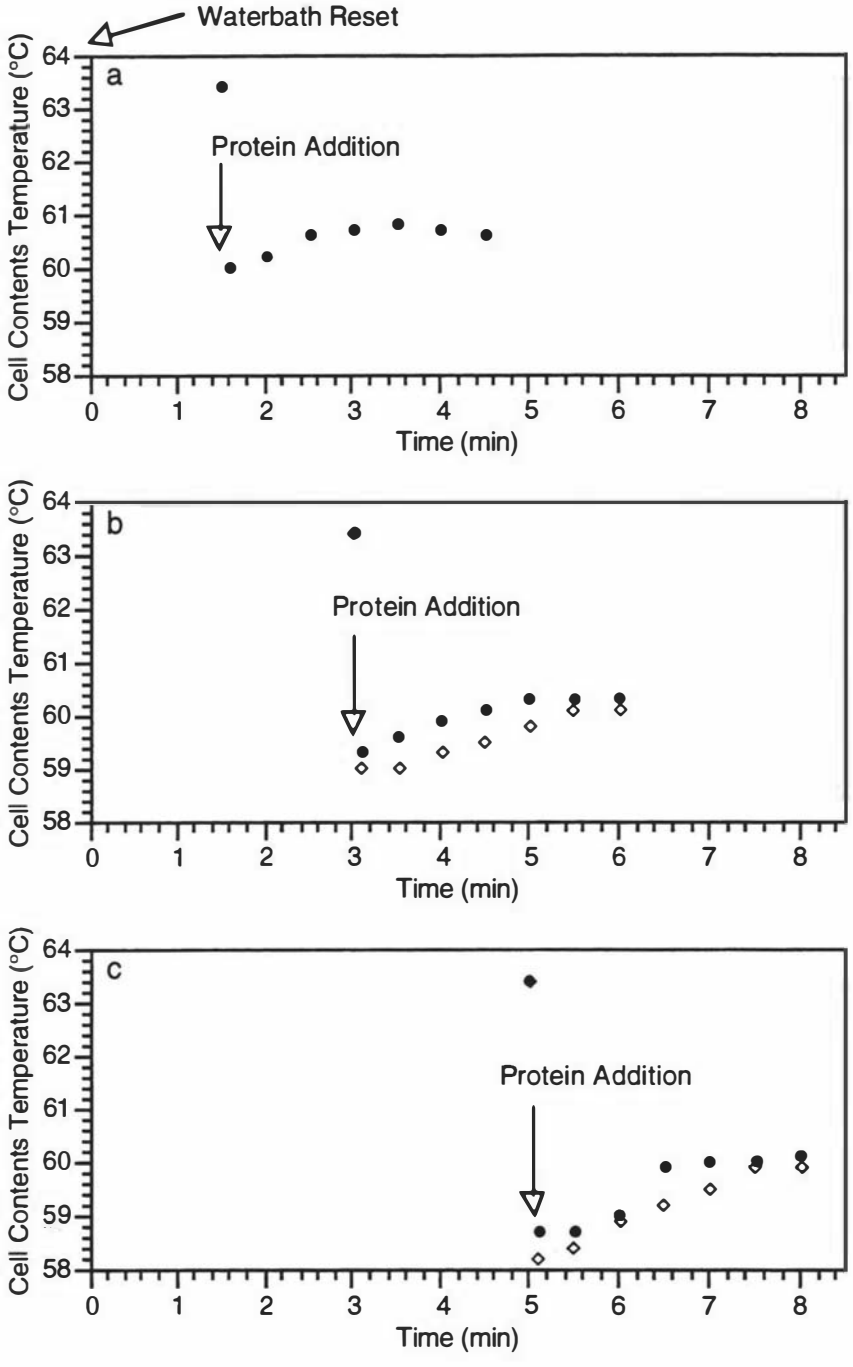

Fig. A2.4. Minimisation of temperature fluctuations. The effect of varying the time intervals between waterbath temperature setting re-adjustment and BLG solution addition on the subsequent changes in the temperature of the cell contents caused by the addition of a 150 µL volume of BLG solution at 20 °C to 2.85 mL pre-warmed buffer initially equilibrated to 63.4 °C was examined. The waterbath was re-set 1.5 min (panel a), 3 min (panel b), or 5 min (panel c) before the BLG solution was added so that cell contents would cool to 60.0 °C. The symbols (•) and (◇) denote duplicate measurements made as described in Fig. A2.3.

A 3 min delay was found to cause minimal temperature fluctuation (Fig. A2.4b). Therefore, in the thiol availability assays made as described in Section 6.2.2.1, the temperature of the waterbath was decreased by 3.4 °C exactly 3 min before the BLG solution was mixed in. By keeping to this protocol, reproducible results at temperatures close to those expected were obtained.

A2.1.6. Glutathione Standard Solutions.

Before thiol availability measurements were made using BLG solutions, the assay procedure was checked to ensure that A_{412} values obtained experimentally at reaction end points corresponded to those predicted to represent exposure of the thiol group in 100 % of BLG molecules. These checks were made using solutions of reduced glutathione with thiol concentrations equivalent to those of the BLG solutions used in assay measurments. Reduced glutathione was selected because it reacts quickly and quantitatively with DTNB to form TNB, the species which absorbs at 412 nm. The molar extinction coefficient for TNB of 14 150 $M^{-1}cm^{-1}$ (Riddles *et al.*, 1979) was taken as the primary standard. Measurements were made using a 5-fold molar excess of DTNB to ensure that all thiol groups reacted to liberate TNB. Therefore, the purity of the DTNB preparation used was not important.

The thiol availabilities of solutions of reduced glutathione prepared as described above were determined daily as a means of following variation in instrument perfomance which may have occurred during the month-long period over which assays were made. Furthermore, standard determinations were made using reduced glutathione solutions at these concentrations so that daily estimates could be obtained of the A_{412} value which corresponded to 100 % exposure of BLG thiol groups.

A2.1.6.1. Purity of the Preparation of Reduced Glutathione.

In a 3 mL cell, 2.70 mL of pH 6.7 phosphate buffer was mixed with 150 µL freshly prepared 7.77 mM DTNB solution and 150 µL freshly prepared reduced glutathione solution (0.618 mg solid per mL). The A_{412} of the mixture was read against a reference sample consisting of 2.85 mL pH 6.7 phosphate buffer and 150 µL DTNB solution, and the concentration of reduced glutathione was calculated, from the measured A_{412} value, to be 0.565mg/mL. The preparation therefore contained 91 % reduced glutathione by weight.

A2.1.6.2. Standard A_{412} Measurements.

Stock 2.01 mM reduced glutathione solution was diluted to 1.76 mM with pH 6.7 phosphate buffer in order to obtain thiol concentrations equivalent to those in the BLG solutions used in pH 6.7 assays. For pH 7.4 measurments the stock reduced glutathione solution was used undiluted. Determinations were then made using a modified version of the thiol availability assay procedure described in Section 6.2.2.1 in which only the final value for A_{412} was measured.

In all instances, experimentally determined A_{412} values which corresponded to 100 % BLG thiol group exposure were equivalent to those predicted. Thus, accurate estimates of extents of BLG thiol group exposure could be obtained from data acquired using the assay procedure of Section 6.2.2.1.

A2.1.7. Experimental Results Used in the Development of the Final Thiol Availability Assay Protocol.

a) Thiol availability assays at pH 6.7 and pH 7.4 were made using BLG solutions at concentrations of 1.47 mg/mL and 1.66 mg/mL respectively.

b) A procedure was developed to help minimise the temperature decrease which occurred when cold BLG solution was mixed into hot DTNB solution. Buffers were initially equilibrated to either 63.4 °C or 53.4 °C. Then, 3 min before BLG addition, the waterbath was re-set to the temperature which would allow cell contents to return to the appropriate assay temperature.

c) Rates of reaction between DTNB and thiol groups of BLG at pH 6.7 and pH 7.4 were measured at 60 °C and 50 °C respectively.

d) To simplify reaction kinetics, BLG thiol groups were reacted with a 10-fold molar excess of DTNB.

e) DTNB solutions were prepared daily using degassed buffers.

A2.2. DEVELOPMENT OF THE PROTOCOL USED TO MEASURE EXTENTS OF THIOL EXPOSURE IN SOLUTIONS OF PREVIOUSLY HEAT-TREATED β-LACTOGLOBULIN.

Before the the results of Section 5.3.5 could be obtained, measurements additional to those of Section A2.1 of this Appendix were required, including times required for complete reaction between exposed thiol groups and DTNB at room temperature, time-dependent change in TNB concentration, and the effect of delayed sampling.

A2.2.1. Justification of Reagent Concentrations and Selected Measurement Conditions.

1) To compensate for spontaneous A_{412} increase (see Section A2.1.2), a reagent blank was prepared at the same time as each heat-treated BLG solution was mixed with DTNB.

2) To further minimise thiol group oxidation, BLG solutions were heat-treated and reacted with DTNB under dry oxygen-free nitrogen in sealed glass vials.

A2.2.2. Reaction of DTNB with the Thiol Group of Native β-Lactoglobulin at pH 6.7.

A frozen solution of BLG A previously dialysed against 4 changes of pH 6.7 phosphate buffer was thawed at 20 °C, and then filtered (see Section 3.1.2.9). Immediately after filtration, the solution was degassed in a Büchner flask using a water pump. The BLG concentration was then calculated from the measured value for A_{280} (see Section 4.2.1.7), after which the solution was diluted to 1.43 mg/mL with degassed pH 6.7 phosphate buffer.

A 2.70 mL aliquot of diluted protein solution was mixed with 300 µL of 7.77 mM DTNB solution in a 3 mL cell. The absorbance at 412 nm was then measured at 20 °C against a reagent blank which consisted of 2.70 mL pH 6.7 phosphate buffer and 300 µL DTNB solution. Values for A_{412} were re-determined 20 min and 40 min later. The cell compartment of the spectrophotometer was flushed with dry oxygen-free nitrogen during this period.

The A_{412} values obtained immediately, 20 min and 40 min after addition of DTNB to the BLG solution were 0.010, 0.012 and 0.014 respectively. These results show that TNB was not formed in appreciable quantities in the presence of unheated BLG, and therefore that the thiol group of unheated BLG is unavailable for reaction with DTNB and probably solvent-inaccessible. This in turn suggests that the unheated BLG was native because in the crystal structures (Brownlow *et al.*, 1997; Bewley *et al.*, 1998; Qin *et al.*, 1998) the thiol group of Cys^{121} is sandwhiched between the α-helix and the exterior of the calyx and is therefore solvent-inaccessible (see Section 2.4.2).

A2.2.3. Time Required for Complete Reaction between DTNB and the Thiol Group of β-Lactoglobulin Heat-treated at pH 6.7.

A2.2.3.1. Methods.

A 15 mL volume of diluted protein solution, prepared as described in Section A2.2.2, was delivered into a 50 mL screw-top bottle. Prior to sealing, air in the headspace of the bottle was displaced using a stream of dry oxygen-free nitrogen. The bottle was then heated for 12.5 min at 95 °C in a waterbath, and then cooled rapidly by placing in crushed ice.

Immediately after the BLG solution had cooled, a 2.70 mL aliquot was removed, the headspace of the bottle was re-flushed with dry oxygen-free nitrogen, and then re-sealed. Thiol availability measurments were then made as described in Section A2.2.2.

A2.2.3.2. Results.

The time-dependent increase in A_{412} after heat-treated BLG A at pH 6.7 had been mixed into DTNB solution (Fig. A2.5) shows that TNB was formed in appreciable quantities in the presence of heat-treated BLG. Thus, BLG thiol groups became exposed as a consequence of heat treatment.

The values for A_{412} were both stable and maximal between 30 min and 3 hr after DTNB had been added to heat-treated BLG solutions (Fig. A2.5). For this reason, A_{412} values from solutions of BLG that had been heat-treated at pH 6.7 were measured between 30 min and 2.5 hours after addition of DTNB.

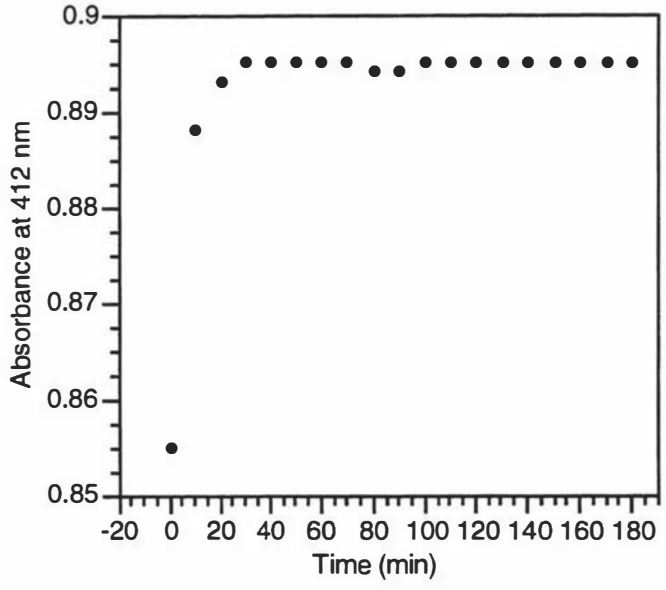

Fig. A2.5. The reaction at 20 °C at pH 6.7 of DTNB with the thiol group of previously heat-treated BLG A. The reaction was started by mixing 300 μL of 7.77 mM DTNB solution into a 2.70 mL aliquot of 1.43 mg/mL BLG A solution previously heat-treated at 95 °C at pH 6.7 for 12.5 min.

A2.2.4. Effect of Delayed Sampling.

When thiol groups in BLG become solvent-exposed, they are oxidised to disulphides and are thus unable to react with DTNB (Patrick and Swaisgood, 1967). Furthermore, the rate of this conversion is most rapid during the first 24 hours after heat treatment (Patrick and Swaisgood, 1976). In the study discussed in this section, thiol availability measurements were made 3 hrs and 12 hrs after a solution of BLG had been heat-treated to determine how rapidly exposed thiol groups were lost during holding periods. The heat-treated BLG solution used in these measurments was the one used in the study discussed in Section A2.2.3.

A2.2.4.1. Methods.

A second 2.70 mL aliquot was taken from the solution used in the study discussed in Section A2.2.3.1 3 hr after heat treatment. After removal of this second aliquot, the lid of the bottle was not replaced. Thiol availability measurements were then made using the procedure described in Section A2.2.2.

The remaining portion of the heat-treated solution was then held at 4 °C for approximately 12 hr. After this time, a further thiol availability measurment was made.

A2.2.4.2. Results.

When DTNB was added to the 3 hr old heat-treated BLG A solution, the maximal A_{412} attained was 0.845, which is slightly lower than that attained when DTNB was added immediately after heat treatment (0.895). Therefore, 5 % of the thiol groups exposed as a consequence of heat treatment were lost over the 3 hr holding period.

The maximal A_{412} value attained when measurements were made on the 12 hr old heat-treated BLG A solution was 0.529. Therefore, 41 % of the thiol groups which became exposed as a consequence of heat treatment were lost over the 12 hr period holding period. As a consequence of these findings, DTNB was added to BLG solutions as soon as possible after heat treatment.

A2.2.5. Time Required for Complete Reaction between DTNB and the Thiol Group of β-Lactoglobulin Heat-treated at pH 7.4.

After DTNB had been added to a solution of BLG A heat-treated at pH 7.4, the value for A_{412} increased over time, reaching a maximum after approximately 30 min. However, between 40 min and 50 min after DTNB addition, A_{412} values started to decrease (Fig. A2.6). This suggested that TNB was unstable at pH 7.4, and that the maximum concentration of TNB, formed as a consequence of the reaction between DTNB and thiol groups, was lower than that of solvent-exposed thiol groups in the BLG solution heat-treated at pH 7.4. For thiol availability determinations at pH 7.4, A_{412} values were read between 30 min and 40 min after DTNB addition.

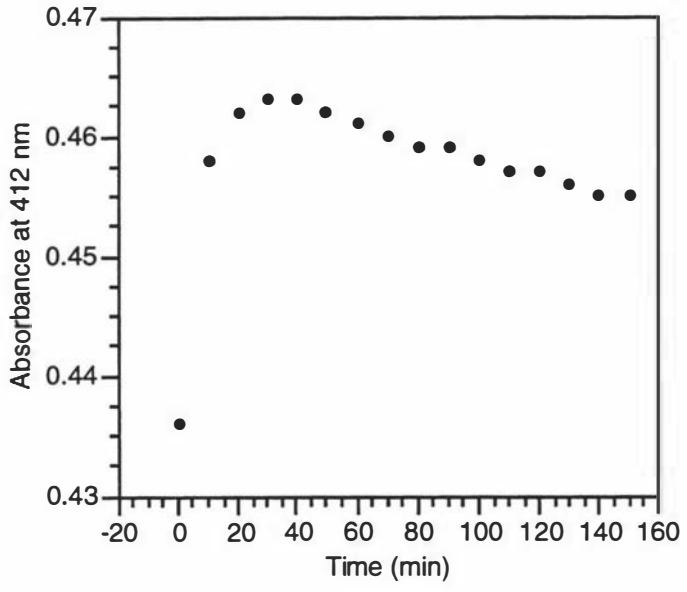

Fig. A2.6. The reaction at 20 °C at pH 7.4 of DTNB with the thiol group of previously heat-treated BLG A. The reaction was started by mixing 300 µL of 7.77 mM DTNB solution into a 2.70 mL aliquot of 1.43 mg/mL BLG A solution previously heat-treated at 95 °C at pH 7.4 for 12.5 min.

A2.2.6. Experimental Results Used in the Development of the Final Thiol Availability Measurement Protocol.

a) DTNB was mixed into BLG solutions as soon as possible after heat treatment to minimise the length of time during which exposed thiol groups could be oxidised to disulphides (Patrick and Swaisgood, 1976).

b) Values for A_{412} from BLG solutions at pH 6.7 and pH 7.4 were measured between 30 min and 3 hr, and between 30 min and 40 min respectively after DTNB addition.

c) Samples of thermally denatured BLG were prepared in an anaerobic environment to minimise the loss of free thiol groups which occurs during aerobic heat treatment (Watanabe and Klostermeyer, 1976). Thiol availability determinations were also made in an anaerobic environment.

APPENDIX 3. COMPUTER FITTING OF SPECTROSCOPIC DATA.

The spectroscopic data in Chapters 5 and 7 were fitted to the modified versions of the 2-state protein unfolding model of Luo *et al.* (1995) discussed in Sections 5.2.8.1 and 5.2.8.2. The fitted values for the parameters S_{low}, S_{high} (the spectroscopic signal intensities for, respectively, native BLG and that for the sample in which the proportion of BLG molecules whose structures had been altered due to heat treatment was greatest), T_{mid} (or T_{midET}), (the midpoint temperature of the transition for structural change), ΔS°_{mid} (the change in entropy at T_{mid}), ΔC_p (the heat capacity difference between the native species and the heat-treated species which gave the signal S_{high}), s_i and s_f (non-zero slopes on the low and high temperature sides of the transition for heat-induced structural change respectively) are tabulated below. All of the errors given below are standard errors calculated by "Enzfitter".

1. $\Delta\varepsilon_{293}$ Data in Chapter 5.

Sample	T_{mid} (°C)	ΔS°_{mid} ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low} ($M^{-1}cm^{-1}$)	S_{high} ($M^{-1}cm^{-1}$)
pH 6.7, BLG A	74.7 ± 0.3	864 ± 70	-361 ± 8475	-4.950 ± 0.035	-1.413 ± 0.134
pH 6.7, BLG B	72.8 ± 0.1	1 148 ± 17	17 087 ± 1 296	-4.964 ± 0.027	-1.310 ± 0.020
pH 6.7, BLG C	76.0 ± 0.1	1 289 ± 41	-1 389 ± 5 430	-4.857 ± 0.013	-1.183 ± 0.046
pH 7.4, BLG A	67.0 ± 0.2	734 ± 26	9 314 ± 1 810	-4.571 ± 0.039	-1.623 ± 0.025
pH 7.4, BLG B	66.2 ± 0.1	845 ± 16	950 ± 2 282	-4.532 ± 0.017	-1.178 ± 0.018
pH 7.4, BLG C	69.5 ± 0.1	990 ± 37	11 330 ± 6 069	-4.566 ± 0.058	-1.272 ± 0.019
pH 8.1, BLG A	62.1 ± 0.9	423 ± 30	-1 668 ± 2 685	-4.552 ± 0.074	-1.483 ± 0.018

2. I_{Trp} Data in Chapter 5.

Sample	T_{mid} (°C)	$\Delta S°_{mid}$ ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low}*	S_{high}*
pH 6.7, BLG A	77.2 ± 0.3	1 744 ± 228	35 086 ± 5 195	19.01 ± 7.73	9.01 ± 7.73
	77.7 ± 1.0	1 522 ± 631	-83 114 ± 19 605	21.33 ± 8.35	11.33 ± 8.35
pH 6.7, BLG B	75.4 ± 0.2	1 059 ± 36	-41 205 ± 3 191	26.83 ± 0.08	16.83 ± 0.08
	76.0 ± 1.4	919 ± 336	-36 150 ± 5 263	27.05 ± 4.51	17.05 ± 4.51
pH 6.7, BLG C	77.7 ± 0.1	1 643 ± 126	-51 430 ± 4 774	21.83 ± 12.23	11.83 ± 12.23
	77.8 ± 1.3	1 638 ± 112	-55 494 ± 3 939	21.01 ± 3.55	11.01 ± 3.55
pH 7.4, BLG A	67.4 ± 0.4	1.483 ± 301	-40 715 ± 12 040	21.32 ± 26.74	11.32 ± 26.74
	67.4 ± 0.3	1 504 ± 284	18 647 ± 41 594	22.53 ± 260.27	11.53 ± 260.27
pH 7.4, BLG B	69.2 ± 0.2	932 ± 62	-12 594 ± 5 741	32.93 ± 0.16	22.93 ± 0.16
	68.8 ± 0.2	973 ± 62	-7 823 ± 9 116	32.74 ± 3.20	22.74 ± 3.20
pH 7.4, BLG C	72.5 ± 0.5	933 ± 136	-31 343 ± 3 707	26.31 ± 6.71	16.31 ± 6.71
	72.6 ± 0.4	1 011 ± 123	-36 246 ± 3 831	20.96 ± 14.94	10.96 ± 14.94
pH 8.1, BLG A	60.7 ± 0.2	731 ± 36	-8 507 ± 1 973	26.93 ± 0.11	16.93 ± 0.11
	60.7 ± 0.2	710 ± 35	-7 885 ± 2 054	27.77 ± 0.11	17.77 ± 0.11

Sample	$^{s}_{i}$ (I_{Trp}/°C)	$^{s}_{f}$ (I_{Trp}/°C)
pH 6.7, BLG A	0.038 ± 0.026	-0.003 ± 0.013
	0.059 ± 0.024	-0.011 ± 0.013
pH 6.7, BLG B	0.150 ± 0.001	-0.059 ± 0.001
	0.151 ± 0.015	-0.058 ± 0.008
pH 6.7, BLG C	0.053 ± 0.017	-0.005 ± 0.008
	0.043 ± 0.011	-0.002 ± 0.005
pH 7.4, BLG A	0.073 ± 0.059	-0.020 ± 0.028
	0.083 ± 0.083	-0.025 ± 0.039
pH 7.4, BLG B	0.247 ± 0.001	-0.109 ± 0.001
	0.245 ± 0.013	-0.109 ± 0.006
pH 7.4, BLG C	0.098 ± 0.028	-0.024 ± 0.013
	0.037 ± 0.039	0.006 ± 0.018
pH 8.1, BLG A	0.175 ± 0.001	-0.075 ± 0.001
	0.165 ± 0.001	-0.070 ± 0.001

* Fluorescence emission intenisty extrapolated to 0 °K.

3. I_{ANS} Data in Chapter 5.

Sample	T_{mid} (°C)	$\Delta S°_{mid}$ ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low}*	S_{high}*
pH 6.7, BLG A	74.6 ± 0.2	82 ± 85	-32 975 ± 2 081	-35.26 ± 2.15	9.74 ± 2.15
	74.7 ± 0.2	82.2 ± 72.8	-27 690 ± 1 679	-39.24 ± 1.80	5.52 ± 1.80
pH 6.7, BLG B	72.5 ± 0.1	1 287 ± 35	20 801 ± 4 806	-15.02 ± 250	29.88 ± 250
	72.4 ± 0.1	1 298 ± 58	16 047 ± 8 247	-18.94 ± 303.72	25.06 ± 303.72
pH 6.7, BLG C	76.3 ± 0.1	1 362 ± 102	21 290 ± 7 186	-18.39 ± 50.87	26.61 ± 50.87
	76.3 ±0.1	1 382 ± 77	21 696 ± 3 644	-18.41 ± 5.33	26.60 ± 5.33
pH 7.4, BLG A	67.9 ± 0.1	518 ± 139	-16 938 ± 1 705	-17.70 ± 1.3	28.30 ± 9.13
	67.4 ± 0.1	80.69 ± 127	-16 515 ± 897	-14.81 ± 2.67	30.19 ± 2.67
pH 7.4, BLG B	65.2 ± 0.4	670 ± 78	-21 765 ± 1 875	-3.83 ± 2.22	42.17 ± 2.22
	65.4 ± 0.1	468.40 ± 66	-17 261 ± 1 474	0.18 ± 2.24	46.19 ± 2.24
pH 7.4, BLG C	69.9 ± 0.2	969 ± 101	-32 074 ± 3 120	-23.47 ± 10.0	22.93 ± 10.0
	69.8 ± 0.1	978 ± 136	-29 725 ± 3 659	-16.18 ± 11.73	29.82 ± 11.73
pH 8.1, BLG A	68.7 ± 0.3	238 ± 106	-7 819 ± 995	2.70 ± 7.00	48.70 ± 7.00
	65.8 ± 1.2	361 ± 73	-8 376 ± 899	5.68 ± 6.47	51.98 ± 6.47

Sample	$^{s}_{i}$ (I_{ANS}/°C)	$^{s}_{f}$ (I_{ANS}/°C)
pH 6.7, BLG A	-0.454 ± 0.008	0.351 ± 0.005
	-0.505 ± 0.007	0.377 ± 0.004
pH 6.7, BLG B	-0.112 ± 0.002	0.137 ± 0.001
	-0.168 ± 0.091	0.165 ± 0.043
pH 6.7, BLG C	-0.020 ± 0.050	0.035 ± 0.022
	-0.020 ± 0.020	0.036 ± 0.010
pH 7.4, BLG A	-0.126 ± 0.037	0.114 ± 0.018
	-0.139 ± 0.018	0.142 ± 0.006
pH 7.4, BLG B	0.031 ± 0.010	0.048 ± 0.061
	0.061 ± 0.010	0.043 ± 0.007
pH 7.4, BLG C	-0.120 ± 0.001	0.098 ± 0.001
	-0.036 ± 0.050	0.057 ± 0.024
pH 8.1, BLG A	0.108 ± 0.032	-0.011 ± 0.016
	0.159 ± 0.031	-0.044 ± 0.014

* Fluorescence emission intensity extrapolated to 0 °K.

4. $[\theta]_{205}$ Data in Chapter 5.

Sample	T_{mid} (°C)	$\Delta S°_{mid}$ ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low} *	S_{high} *
pH 6.7, BLG A	75.7 ± 0.7	766 ± 109	-14 600 ± 5 874	-3 950 ± 107	-17 310 ± 1 126
pH 6.7, BLG B	74.8 ± 1.6	539 ± 137	-11 126 ± 4 450	-2 260 ± 104	-8 067 ± 2 760
pH 6.7, BLG C	77.0 ± 0.1	519 ± 8	-5 756 ± 404	-2 950 ± 66	-10 377 ± 897
pH 7.4, BLG A	69.4 ± 0.9	407 ± 45	-4 851 ± 2 555	-4 538 ± 239	-14 330 ± 559
pH 7.4, BLG B	64.6 ± 0.8	562 ± 76	-5 950 ± 4 087	-4 526 ± 216	-10 887 ± 365
pH 7.4, BLG C	70.0 ± 0.4	1 050 ± 99	-10 380 ± 15 200	-3 539 ± 226	-12 111 ± 1 086
pH 8.1, BLG A	65.4 ± 0.7	531 ± 61	-5 445 ± 2 856	-6 908 ± 190	-13 660 ± 969

* degree.cm^2 $dmol^{-1}$

5. Thiol Availability Data in Chapter 5.

Sample	T_{mid} (°C)	$\Delta S°_{mid}$ ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low}*	S_{high}*
pH 6.7, BLG A	74.2 ± 0.8	809 ± 141	11 400 ± 11 390	0.014 ± 0.077	0.738 ± 0.014
	74.3 ± 0.3	947 ± 77	11 770 ± 8 212	0.037 ± 0.032	0.714 ± 0.007
pH 6.7, BLG B	72.6 ± 0.1	1 142 ± 46	8 887 ± 8 050	0.022 ± 0.007	0.794 ± 0.007
	73.0 ± 0.2	1 123 ± 65	19 960 ± 3 880	0.007 ± 0.018	0.800 ± 0.007
pH 6.7, BLG C	75.4 ± 0.1	1 192 ± 62	8 203 ± 10 420	0.014 ± 0.007	0.794 ± 0.090
	75.8 ± 0.1	1 355 ± 50	23 170 ± 3 243	0.014 ± 0.008	0.800 ± 0.006
pH 7.4, BLG A	65.4 ± 0.4	817 ± 79	-3 040 ± 10 650	0.073 ± 0.014	0.800 ± 0.006
	65.3 ± 0.2	829 ± 41	3 296 ± 5 504	0.067 ± 0.009	0.794 ± 0.090
pH 7.4, BLG B	63.6 ± 0.3	1 048 ± 85	19 840 ± 4 822	0.059 ± 0.018	0.687 ± 0.008
	63.4 ± 0.2	1 044 ± 60	20 600 ± 2 934	0.059 ± 0.012	0.669 ± 0.005
pH 7.4, BLG C	66.6 ± 0.2	1 120 ± 72	17 480 ± 9 238	0.049 ± 0.019	0.674 ± 0.005
	66.8 ± 0.2	995 ± 50	3.939 ± 7.529	0.056 ± 0.007	0.685 ± 0.007

* "Absorbance Units"

6. $\Delta\varepsilon_{293}$ Data in Chapter 6.

Sample	T_{mid} (°C)	$\Delta S°_{mid}$ ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low} ($M^{-1}cm^{-1}$)	S_{high} ($M^{-1}cm^{-1}$)
pH 6.7, BLG A	67.0 ± 0.3	438 ± 16	6 170 ± 616	-4.758 ± 0.038	-1.200 ± 0.038
pH 6.7, BLG B	67.4 ± 0.2	550 ± 18	4 222 ± 905	-5.324 ± 0.050	-1.012 ± 0.050
pH 6.7, BLG C	70.3 ± 0.8	648 ± 10	5 062 ± 548	-5.252 ± 0.015	-0.604 ± 0.015
pH 7.4, BLG A	59.6 ± 0.2	466 ± 14	3 301 ± 662	-4.233 ± 0.020	-1.091 ± 0.020
pH 7.4, BLG B	60.3 ± 1.2	368 ± 42	3 116 ± 1 116	-4.917 ± 0.308	-1.787 ± 0.308
pH 7.4, BLG C	65.0 ± 0.3	628 ± 30	5 557 ± 1 579	-4.743 ± 0.046	-1.617 ± 0.046

Sample	s_i ($\Delta\varepsilon_{293}$/°C)	s_f ($\Delta\varepsilon_{293}$/°C)
pH 6.7, BLG A	0.0051 ± 0.0004	0.0034 ± 0.0003
pH 6.7, BLG B	0.0053 ± 0.0001	0.0040 ± 0.0009
pH 6.7, BLG C	0.0042 ± 0.0001	0.0035 ± 0.009
pH 7.4, BLG A	0.0036 ± 0.0001	0.0033 ± 0.0001
pH 7.4, BLG B	0.0057 ± 0.0012	0.0038 ± 0.0006
pH 7.4, BLG C	0.0053 ± 0.0001	0.0034 ± 0.0001

7. I_{Trp} Data in Chapter 6.

Sample	T_{mid} (°C)	$\Delta S°_{mid}$ ($JK^{-1}mol^{-1}$)	ΔC_p ($JK^{-1}mol^{-1}$)	S_{low} *	S_{high} *
pH 6.7, BLG A	67.9 ± 0.3	486 ± 26	7 573 ± 368	67.98 ± 2.75	67.98 ± 2.75
pH 6.7, BLG B	69.4 ± 0.1	772 ± 4	13 520 ± 100	56.48 ± 0.03	56.45 ± 0.03
pH 6.7, BLG C	70.5 ± 0.3	599 ± 28	6 686 ± 1 381	64.80 ± 0.19	64.79 ± 0.19
pH 7.4, BLG A	53.4 ± 2.2	288 ± 144	18 701 ± 1 435	63.81 ± 2.11	63.81 ± 2.11
pH 7.4, BLG B	61.2 ± 0.8	-364 ± 25	8 824 ± 63	92.34 ± 40.06	92.34 ± 40.06
pH 7.4, BLG C	62.8 ± 0.2	510 ± 15	9 160 ± 210	92.42 ± 17.52	92.42 ± 17.52

Sample	s_i (I_{Trp}/°C)	s_f (I_{Trp}/°C)
pH 6.7, BLG A	0.395 ± 0.007	0.739 ± 0.007
pH 6.7, BLG B	0.320 ± 0.001	0.602 ± 0.001
pH 6.7, BLG C	0.375 ± 0.001	0.705 ± 0.001
pH 7.4, BLG A	0.360 ± 0.005	0.683 ± 0.008
pH 7.4, BLG B	0.543 ± 0.028	1.015 ± 0.052
pH 7.4, BLG C	0.538 ± 0.004	1.012 ± 0.008

* Fluorescence emission intensity extrapolated to 0 °K