The Refolding of Recombinant Human Liver Methylmalonyl-CoA Mutase from Inclusion Bodies Produced in *Escherichia coli*

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ABSTRACT

Human methylmalonyl-CoA mutase (hMCM) is an adenosylcobalamin-dependent enzyme that catalyses the structural rearrangement of (R)-methylmalonyl-CoA to succinyl-CoA as part of the catabolism of the branched chain amino acids valine, leucine and isoleucine, odd chain fatty acids and intermediates of cholesterol metabolism. Reactions that require adenosylcobalamin (AdoCbl) have been intensively studied, and the first step in the catalysis is widely agreed to involve homolytic cleavage of the unusual carbon-cobalt bond in the cofactor. A reliable source of recombinant hMCM would be useful in defining more fully the mechanistic pathway of AdoCbl-dependent enzymes.

Recombinant hMCM overexpressed in *E. coli* forms insoluble aggregates of inactive protein known as inclusion bodies. hMCM inclusion bodies were purified, solubilised and then several different *in vitro* refolding techniques were tested in attempts to produce active recombinant hMCM from purified solubilised inclusion body material. These methods included refolding by rapid dilution, refolding by dialysis, detergent-assisted refolding, refolding by gel filtration chromatography and chaperonin-assisted refolding. Chaperonin-assisted refolding necessitated the purification of recombinant *E. coli* chaperonins GroES and GroEL from the *E. coli* strain DH1/pGroESL.

Refolding by rapid dilution of the GdmHCl-solubilised inclusion bodies into a refolding buffer was judged to be the simplest and most effective method, however the refolding process was extremely inefficient. Refolding by rapid dilution was scaled up to 2 litres to produce as much active hMCM as possible. The refolded protein was concentrated by batch adsorption to and stepwise elution from hydroxyapatite, and further purified using a synthesised 5′adenosylcobalamin-agarose 'affinity' chromatography column. The final refolded hMCM preparation contained a single ~29 kDa contaminant protein, tentatively identified as E. coli branched-chain amino acid aminotransferase (EC 2.6.1.42), present in approximately equal amounts to the hMCM, and had a specific activity of ~3.11 units/mg.

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Extra special thanks must go to Terry and Geordi for their encouragement and understanding while I was writing, and for believing me whenever I said it was "*just about finished*"!

Thank God for methylmalonyl-CoA mutase; it is a stubborn but special little protein.

And finally thanks to anyone who reads this line and has picked up this thesis hoping that they will find the information they need. Good luck!

LIST OF ABBREVIATIONS

5'AdoCbl	5'deoxyadenosylcobalamin
Amp	Ampicillin
Avg	Average
bMCM	Bacterial methylmalonyl-CoA mutase
BSA	Bovine serum albumin
C-terminal	Carboxyl terminal
C_8-β-D Gluc	C_8-β-D Glucopyranoside
CAM	Chloramphenicol
CAPS	3-[Cyclohexylaminol}-1-propanesulfonic acid
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHAPSO	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxypropane-1-sulfonate
CMC	Critical micelle concentration
CNCbl	Cyanocobalamin
CTAB	Cetyltrimethylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
g	Gravitational field, unit of
GdmHCl	Guanidinium hydrochloride
hMCM	Human methylmalonyl-CoA mutase
HTP	Hydroxyapatite
kan	Kanamycin
KP	Potassium phosphate
LDAO	Lauryldimethylamine oxide
LM	Lauryl Maltoside
Lubrol PX	Polyoxyethylene (9) lauryl ether
MAP	Methionine aminopeptidase

MEGA-9	Nonanoyl-N-methylglucamide
MCM	Methylmalonyl-CoA mutase
Mr	Relative molecular mass
mt	Mitochondrial
NADH	Nicotinamide-adenine dinucleotide, reduced
Nonidet P40	Nonaethylene glycol octylphenyl ether
N-terminal	Amino terminal
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein disulphide isomerase
PK	Protein Kinase
PMSF	Phenylmethylsulphonyl fluoride
PPI	Peptidyl-prolyl *cis-trans* isomerase
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
TCA cycle	Tricarboxylic acid cycle
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Nonaethylene glycol octylphenyl ether
Tween 20	Polyoxyethylene sorbitan monolaurate
Z-3-XX	Zwittergent 3-XX series

THREE AND ONE LETTER AMINO ACID CODE

Ala	**A**	Alanine
Arg	**R**	Arginine
Asn	**N**	Asparagine
Asp	**D**	Aspartic acid
Cys	**C**	Cysteine
Gln	**Q**	Glutamine
Glu	**E**	Glutamic acid
Gly	**G**	Glycine
His	**H**	Histidine
Ile	**I**	Isoleucine
Leu	**L**	Leucine
Lys	**K**	Lysine
Met	**M**	Methionine
Phe	**F**	Phenylalanine
Pro	**P**	Proline
Ser	**S**	Serine
Thr	**T**	Threonine
Tyr	**Y**	Tyrosine
Val	**V**	Valine
Trp	**W**	Tryptophan

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CHAPTER ONE

INTRODUCTION

1.1 Methylmalonyl-CoA Mutase

Human methylmalonyl-CoA mutase (MCM) is a homodimeric mitochondrial matrix protein encoded by a nuclear gene. The initial translation product is a precursor protein which contains a leader sequence that directs the protein into the mitochondrial matrix (Leadlay and Ledley, 1990). The leader sequence is then cleaved and the mature protein dimerizes in the matrix. The human MCM cDNA has 2226 nucleotides which encode a protein of 742 amino acids with a predicted molecular mass of 82 283 Daltons. The N-terminal amino acid sequence of the mature protein indicates that it begins at amino acid 33, and the mature protein is predicted to have a molecular mass of 78 489 Daltons (Leadlay and Ledley, 1990). Figure 1.1 shows the crystal structure at 2Å resolution of the α subunit of MCM from *Propionibacterium shermanii* in complex with coenzyme B_{12} (5′AdoCbl) and with the partial substrate analogue desulpho-CoA (lacking the succinyl group and the sulphur atom of the succinyl-CoA substrate) (Mancia *et al.*, 1996). The bacterial form of MCM is a α/β heterodimer. In contrast, the human enzyme is an α_2 homodimer, and is highly homologous (60% amino acid sequence identity) to the active α chain of its bacterial counterpart (Jansen *et al.*, 1989). hMCM belongs to a class of enzymes that uses coenzyme B_{12} (5′deoxyadenosylcobalamin) as a cofactor. 5′deoxyadenosylcobalamin is a vitamin B_{12} (cobalamin) derived cofactor. Cobalamin is absorbed from the intestine. After transport in the blood, free cobalamin is released into the cytosol of cells as hydroxocobalamin, this is then converted in the cytosol to methylcobalamin or enters mitochondria for conversion to 5′deoxyadenosylcobalamin (5′AdoCbl). Enzyme catalysed reactions that use 5′AdoCbl as a cofactor have been the subject of much research. The first step in the catalysis is thought to involve the homolytic cleavage of the unusual carbon-cobalt bond in the cofactor (Finke and Hay, 1984; Halpern, 1985). The enzyme induces the formation of an adenosyl radical from the cofactor.

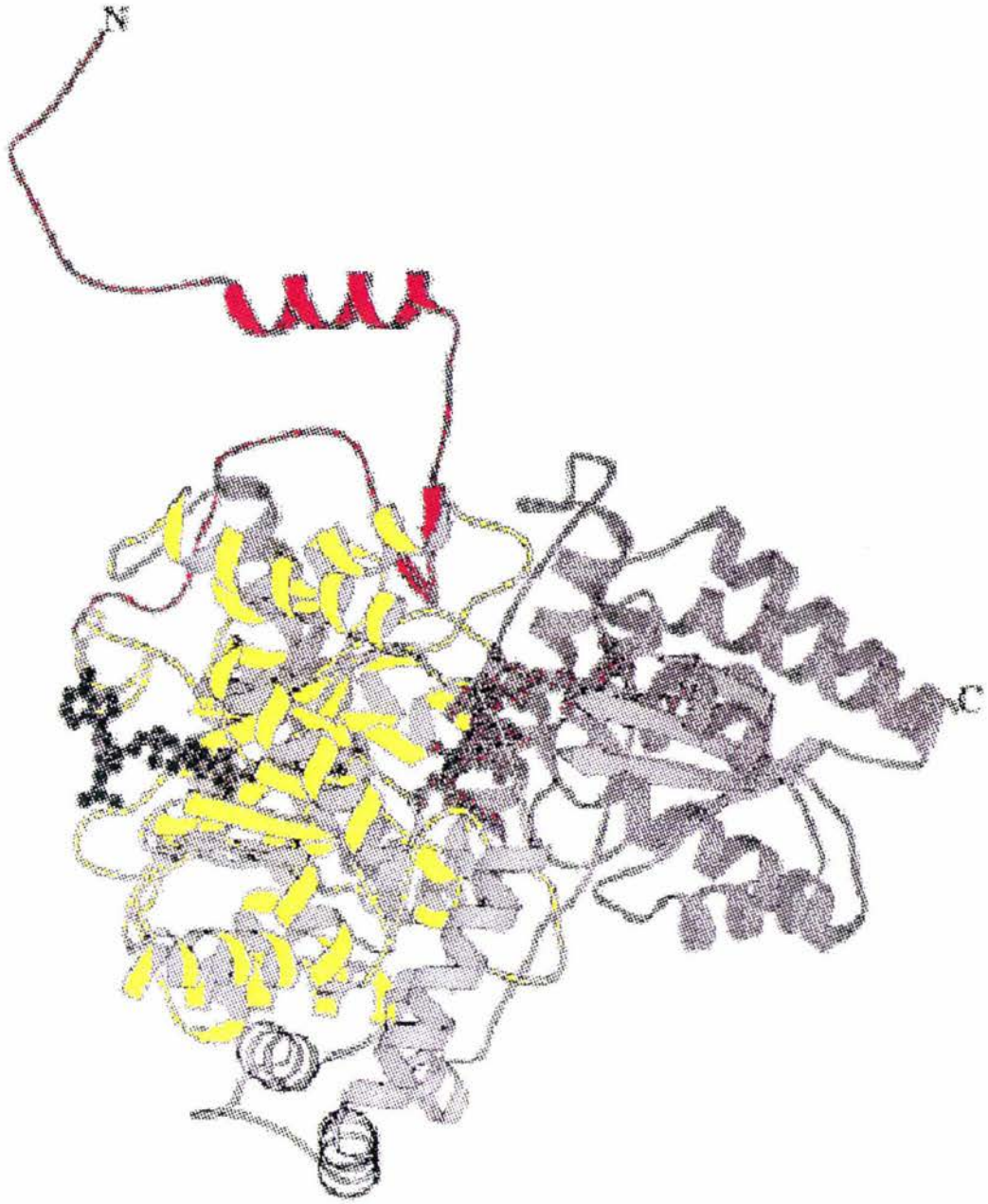


Figure 1.1: Schematic view of the structure of the α chain of methylmalonyl-CoA mutase from *Propionibacterium shermanii*. Drawn with MOLSCRIPT (Kraulis, 1991). The N-terminal 'arm', which wraps around the other subunit, is red; the $(\beta/\alpha)8$ barrel domain, which binds the green desulpho-CoA, partial substrate is yellow with the β sheet strands in orange; the long linker 'arm', which wraps around the $(\beta/\alpha)_8$ -barrel is green; and the C-terminal B_{12} binding domain is blue, with purple β-sheet strands. The B_{12} molecule is dark red. Mancia *et al.* (1996).

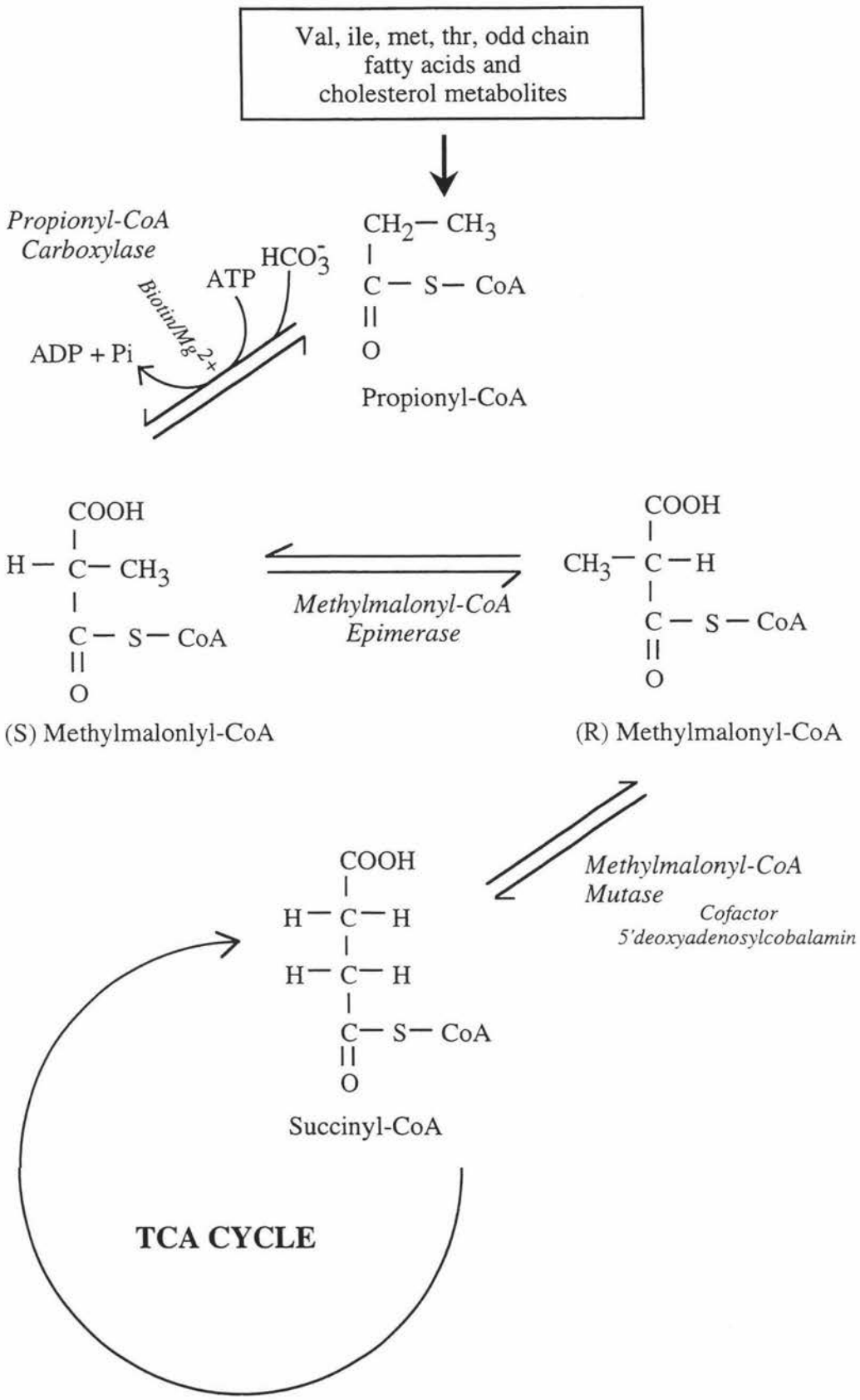


Figure 1.2: The reaction pathway involved in the interconversion of succinyl-Coa and propionyl-CoA. Succinyl-CoA enters the TCA cycle (the cycle for the oxidation of fuel molecules in mitochondria). Alternatively, the (S) methylmalonyl-CoA may be hydrolysed by methylmalonyl-CoA hydrolase (Kovachy *et al.*, 1988) to form methylmalonic acid which is excreted in the urine.

This radical then initiates a free-radical rearrangement of its substrate, (R)methylmalonyl-CoA, to succinyl-CoA (figure 1.3) (Halpern, 1985). This isomerisation is an intermediate step in the catabolism of the branched chain amino acids valine, leucine and isoleucine, and the amino acids threonine and methionine, as well as odd chain fatty acids and intermediates of cholesterol metabolism (figure 1.2).

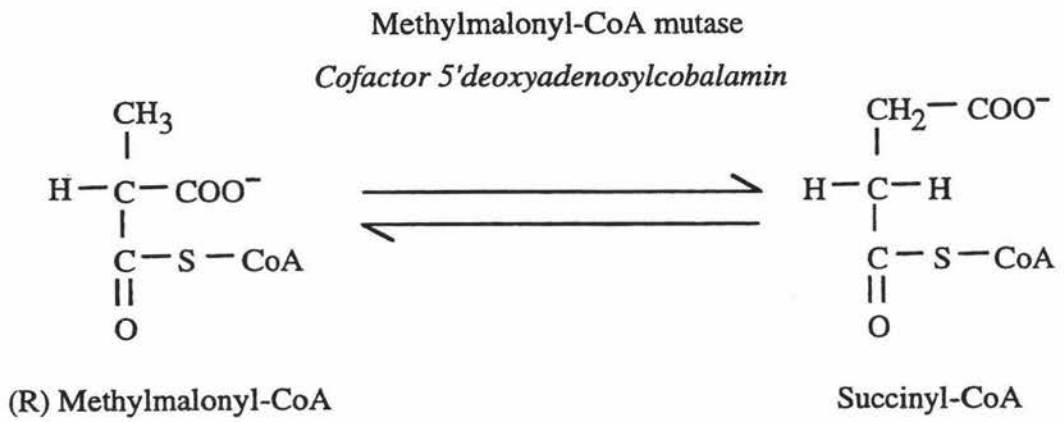


Figure 1.3: hMCM-catalysed reaction. The isomerisation of (R)Methylmalonyl-CoA mutase to succinyl-CoA by the migration of the O=C-SCoA group (thioester group) catalysed by methylmalonyl-CoA mutase.

1.2 The Formation of Inclusion Bodies

Initial attempts by McKie *et al.* (1990) to produce soluble recombinant MCM from *Propionibacterium shermanii* in *E. coli* resulted in the formation of MCM inclusion bodies. Lowering the *E. coli* growth temperature from 37 °C to 28 °C prevented the formation of inclusion bodies. However, recombinant hMCM expressed at high levels in E. coli is invariably found as insoluble inclusion bodies, regardless of growth and induction conditio: ɜ (M. Patchett, pers. Comm).

Recombinant and naturally occurring proteins, both eukaryotic and prokaryotic, can be over-expressed and produced on a large scale in bacterial systems. Most commonly,

recombinant proteins are expressed in various strains of *E. coli*. After purification the proteins produced can be used for research purposes, as well as for clinical and industrial uses. If a protein is expressed in the cytoplasm of *E. coli* then the polypeptide produced has one of three fates (figure 1.4).

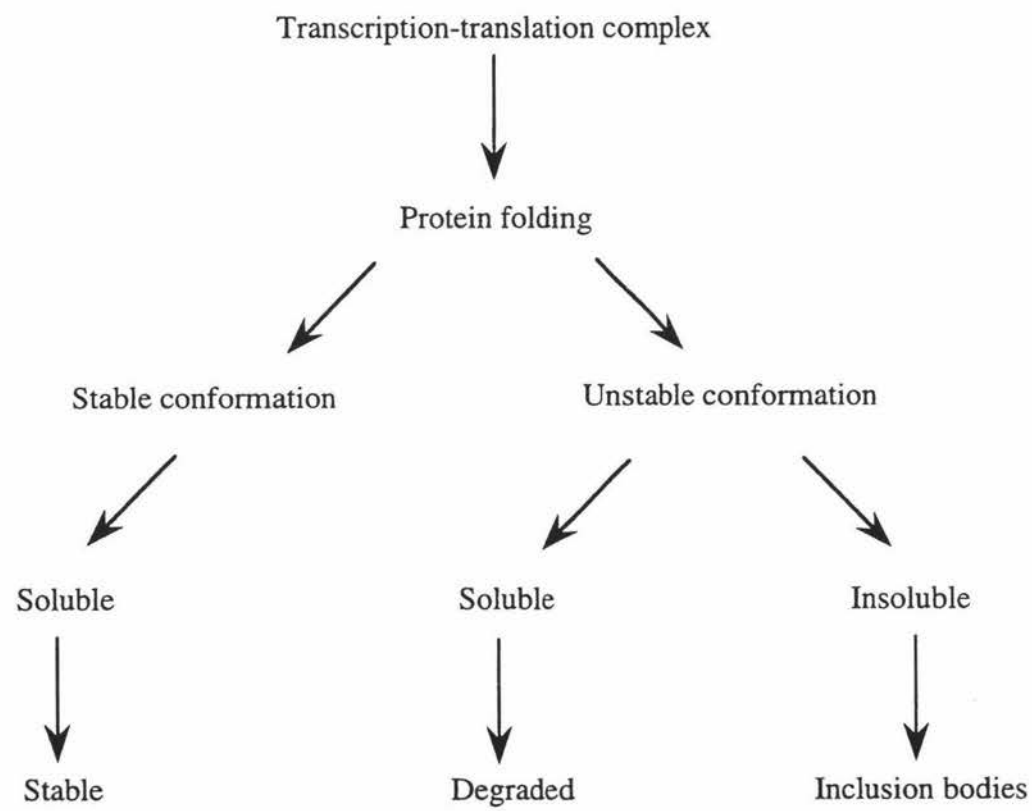


Figure 1.4: Possible fates of an over-expressed protein in *E. coli*.

Firstly, the protein can be soluble, stable and functional. Secondly, the protein can be soluble but unstable and degraded by one of several proteolytic activities in the *E. coli* cytoplasm, which target foreign, misfolded and damaged proteins. The third possible fate of the newly synthesised protein is its incorporation into insoluble and inactive protein

aggregates, known as inclusion bodies. Upon analysis by electron microscopy these inclusion bodies appear as large, spherical particles which are clearly separated from the cytoplasm as distinct structures, but not enclosed by membranes. Provided that the expression level is sufficiently high, the inclusion bodies may occupy a large portion of the host cell, often spanning the entire diameter of the cell. Due to their refractile character, these inclusion bodies can be seen directly within the host cell by phase contrast microscopy ("refractile body" is frequently used as a synonym for inclusion body) (Rudolph, 1990). The foreign gene product is the main component of the inclusion bodies, which are stabilised by non-specific (hydrophobic) interactions. Several lines of evidence indicate that the aggregated polypeptide chains are at least partially structured (Mitraki and King, 1989). In addition to the recombinant polypeptide, inclusion bodies contain host proteins and ribosome components (ribosomal RNA), as well as covalently closed circular and nicked forms of plasmid DNA (Hartley and Kane, 1988). There is evidence which suggests that polypeptides in such insoluble aggregates are protected from the degradative action of host proteases (Cheng *et al.*, 1981).

Genes expressed in the cytoplasm of *E. coli* characteristically accumulate to levels ranging up to 25% or more of total cell protein (Marston, 1986). It is not uncommon for proteins over-expressed in *E. coli* to form inclusion bodies. It is difficult to predict if the expression of a particular protein will result in the formation of inclusion bodies. Kane and Hartley (1988) surveyed 34 recombinant expressed proteins in *E. coli*, primarily strain K12 and its derivatives. Only 11 of these proteins were soluble following cell lysis and centrifugation. Similarly, Marston (1986) reported that 9 out of 23 additional proteins were found in the soluble fraction after cell lysis and low speed centrifugation. The survey Kane and Hartley published in 1988 indicated that there was no correlation between the host strain and inclusion body formation. Various hypothesis have been put forward to explain why certain over-expressed proteins form inclusion bodies while others do not. Possible correlation's include the original source of the gene, whether it is expressed directly or as a fusion protein, the level of its expression and the promoter used, and the molecular weight of the protein. However, none of these hypothesis were confirmed by the data collected by Kane and Hartley (1988) or Marston (1986). It is also known that it is not simply a

result of a foreign protein being expressed as there are examples of overexpressed *E. coli* proteins forming inclusion bodies (Gribskov and Burgess, 1983). Some eukaryotic proteins expressed even at low levels in *E. coli* still form inclusion bodies (Cheng *et al.*, 1983), and some proteins are more soluble in some strains of *E. coli* than others (Kane and Hartley, 1988). These observations show how unpredictable the formation of inclusion bodies is, when a recombinant protein is expressed in *E. coli*. Some other factors which may have an effect on the formation of inclusion bodies are the physiological state of the host cell (phase of growth, temperature, oxygenation etc.), proteolytic activity in the host cell, and properties of the protein being expressed.

There are practical advantages to the formation of inclusion bodies in that they are easily purified as they are dense and sediment readily at low centrifugation speeds. For example, inclusion bodies sediment more rapidly than the bulk of the cell debris and relatively effective purification is achieved. The preparation is not totally pure as some proteins do co-purify with inclusion bodies. These are typically components of membranes or cell wall fragments, but are not necessarily physically associated with the recombinant proteins which constitute the bulk of the inclusions. Solubilisation and one or two more specific purification steps may be needed, but this method of purification can save time and reduce the cost of producing the protein on a large scale by eliminating the need for more costly purification steps.

The main purification problem for inclusion body proteins is the development of techniques to solubilise and refold the purified inclusion bodies into stable and biologically active protein. Formation of inclusion bodies is not simply a precipitation phenomenon resulting from the accumulation of a high concentration of protein, because the solubilisation of protein from inclusion bodies requires strong chemical interactions. Precipitation may be an initial effect in the formation of IB's , but at some stage during inclusion body formation covalent interactions form between the protein molecules (Marston *et al.*, 1986). The only covalent interaction found in inclusion bodies is intermolecular disulphide bonds. Because the cytoplasm of *E. coli* is a highly reduced environment (Fahey *et al.*, 1977), it is unlikely that these disulphide bonds form with the

initial production of the inclusion bodies. Rather these interactions most probably begin upon lysis of the cells in the purification of the inclusion bodies, when they are exposed to the air and oxygenated buffer solutions. Keeping solubilisation conditions highly reduced with a thiol reagent such as dithiothreitol prevents the formation of non-native intermolecular and intramolecular disulphide bonds and aids in the solubilisation of the polypeptides. Chaotropes such as urea and guanidinium hydrochloride are essential components of the solubilisation process. They are used to disrupt non-covalent interactions in the inclusion bodies. Having disrupted the aggregated polypeptides, conditions must then be adjusted to allow refolding. Refolding of a solubilised protein requires a decrease in the concentration of the denaturant keeping the protein unfolded. This requires dilution of the denaturant to a concentration where it can no longer keep the protein in a denatured state. The final protein concentration in the refolding solution is an important factor for optimum refolding (see section 1.3.2). During inclusion body purification and solubilisation a highly reduced environment is required to prevent non-native disulphide bond formation, but some proteins contain disulphide bonds in their native folded state, and this necessitates a less reducing environment for successful refolding. *In vivo* protein folding is assisted by protein disulphide isomerases (PDIs) which are general and non-specific catalysts for disulphide interchange in proteins containing disulphide bonds (Loferer and Hennecke, 1994). To aid with refolding *in vitro* in the absence of PDIs, the redox environment needs to be opportunistic to disulphide bonds forming or not. The crystal structure of the MCM from *P. shermanii* found that there are no disulphide bonds in the α-subunit (Mancia *et al.*, 1996). On the assumption that the α-subunit structures of hMCM and *P. Shermanii* MCM are similar, a reducing agent (DTT) was added to the solubilisation and refolding solutions. This should prevent the formation of presumably unwanted disulphide bonds during refolding. It is worth noting, however, that there is one reported example of a disulphide bonded-refolding intermediate forming during the refolding of a non-disulphide bonded protein (Robinson and King, 1997).

1.3 Protein Refolding From Inclusion Body Material

The protein present in inclusion bodies lacks its native structure and therefore is both insoluble and has no biological activity. This protein must be solubilised and refolded to its native structure to regain biological activity. The ability to correctly fold proteins that have been produced by recombinant DNA technology is of importance since in many cases this is a major obstacle to the cost-effective production of proteins for medical, industrial or research use. Some recombinant therapeutic proteins already produced from inclusion bodies are bovine growth hormone, tissue plasminogen activator, macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), interleukin-2 and insulin (Chaudhuri, 1994). For efficient refolding, each protein requires unique refolding conditions. It has been over 35 years since Anfinsen showed that urea-denatured rhodanese could refold spontaneously *in vitro* without assistance (Anfinsen and Haber, 1961), suggesting that all the information needed for a protein to correctly fold is contained in the amino acid sequence. Subsequent studies have shown that protein folding/refolding *in vitro* is in many cases a very inefficient process. Several different approaches to protein refolding *in vitro* are outlined in this chapter.

1.3.1 Differences Between Refolding *In Vitro* and Folding *In Vivo*

Anfinsens *in vitro* refolding work showed that all the information needed for a protein to fold *in vitro* was held in a proteins amino acid sequence (Sela *et al.*, 1957; Anfinsen, 1973). This postulate did not preclude the existence of cellular substances that would assist in the folding process *in vivo*. The refolding of many proteins *in vitro* is less efficient than *in vivo*. This is due to the absence 'helper' proteins that would normally be present *in vivo*. Three classes of these proteins found so far are the molecular chaperones, protein disulphide isomerase (PDI), and prolyl-peptidyl *cis/trans* isomerase (PPI). All three of these 'helper' proteins are located in the lumen of the endoplasmic reticulum (ER), and molecular chaperones are also present in the cytoplasm and in other organelles of mammalian cells. When refolding proteins *in vitro* it may, depending on the target protein, be necessary to compensate for the absence of these proteins. This can be done to some

extent by optimising the temperature and concentration at which the refolding is attempted and by adding labilizing agents such as detergents or arginine to the refolding buffer (Jaenicke and Rudolph, 1989). At this time there is no obvious way, other than addition of purified PPI to the refolded solution, of replacing the PPI activity *in vitro*. Examples of the use of purified chaperone proteins to assist refolding *in vitro* have been reported, and this is discussed further in section 1.3.6. Satisfactory renaturation results can be obtained when refolding protein *in vitro* in the absence of the catalytic activity of PDI by optimising the redox conditions in the refolding solution for non-enzymatic disulphide bond formation (Rudolph, 1990).

1.3.2 Refolding by Rapid Dilution

Protein refolding will only occur when the denaturant used to solubilise the inclusion bodies is removed. Horowitz and Simon (1986), found that the protein concentration in the refolding solution is a critical variable when optimising refolding conditions. Aggregation is the main competing pathway to refolding (figure 1.5), so refolding is typically carried out at low protein concentrations, in the region of 0.1 to 100 μg/ml, to minimize interactions between folding intermediates that may lead to aggregation. However if a solution is too dilute this can lead to protein loss by adherance of the protein to the sides of the vessel.

Decreasing the concentration of the denaturant can be achieved by several methods including dialysis, diafiltration and rapid dilution. Refolding by rapid dilution into a non-denaturing buffer or refolding buffer, works by diluting the denaturant below the concentration that can keep the protein unfolded. The protein can then either fold up into its native soluble active form, fold up into a non-native and inactive soluble from, or form inactive protein aggregates due to interaction between hydrophobic surfaces on folding intermediates (figure 1.5) (Rudolph, 1990). Patterns of results consistently seen in refolding trials of many different proteins are typified by the refolding of rhodanese (Horowitz and Simon, 1986). Rhodanese is a good model for refolding studies, this protein is a monomeric mitochondrial matrix enzyme found in the matrix of all mammalian

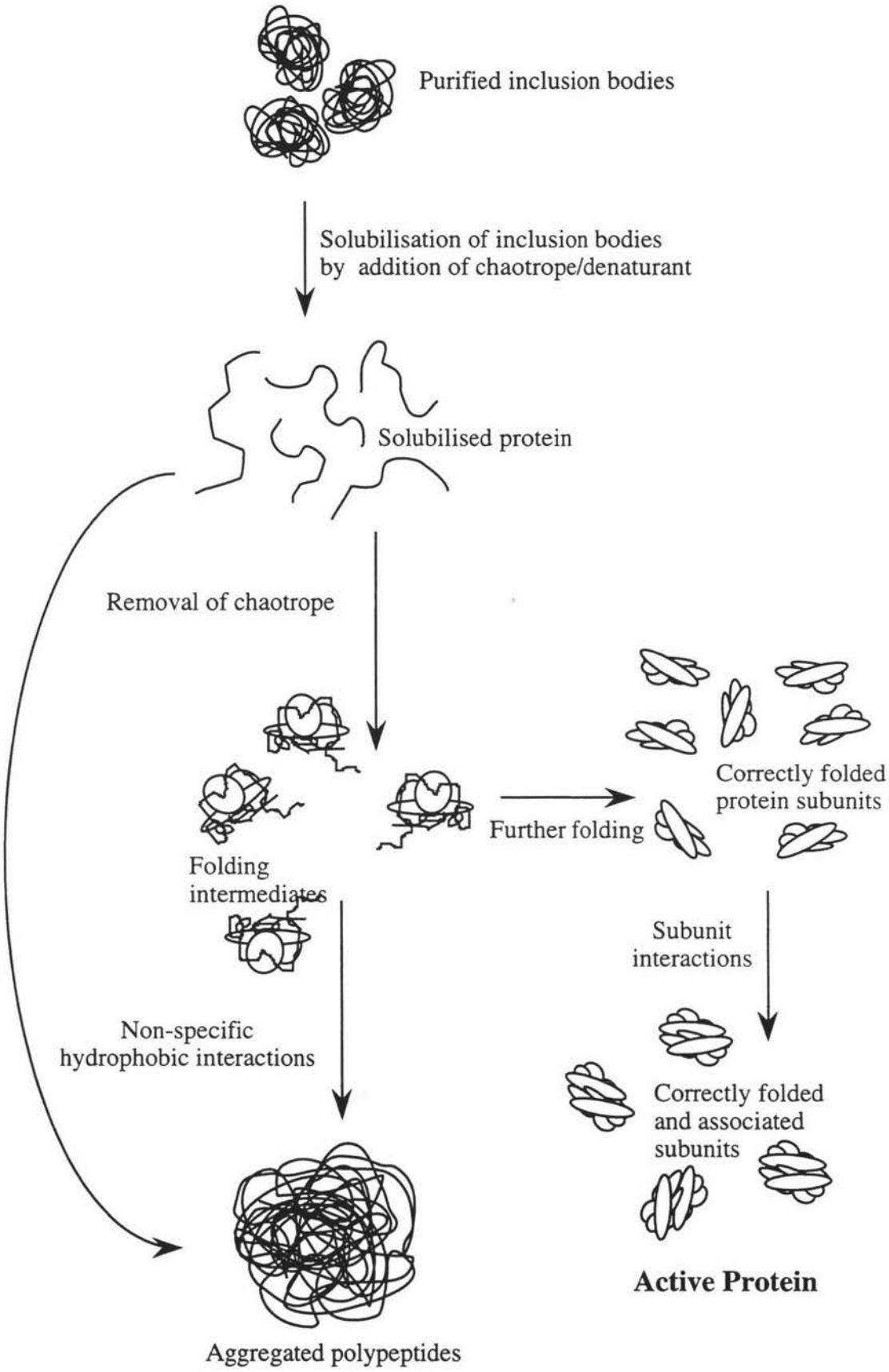


Figure 1.5: Flow chart showing the possible outcomes of *in vitro* refolding.

mitochondria (Mendoza *et al.*, 1991b). Figure 1.6 shows that rhodanese reactivation depends on the final concentration of the enzyme after rapid dilution of the solubilised protein. Rhodanese refolding experiments involve the refolding of active protein which has been solubilised, rather than from an inclusion body source.

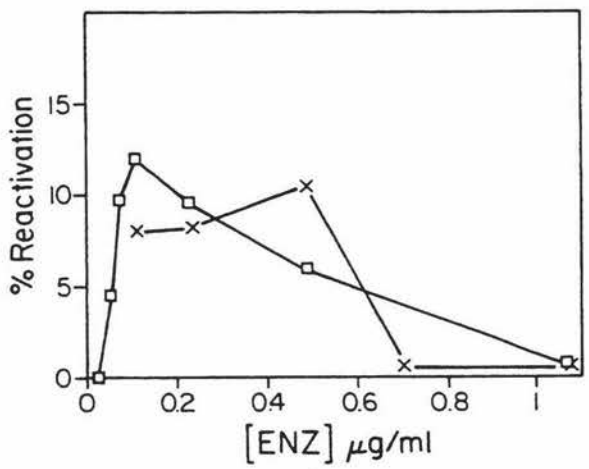


Figure 1.6: Illustration of the effect of protein concentration on the refolding of rhodanese. Percentage reactivation versus finalprotein concentration for rhodanese denatured in 8.4 M Urea (□) or 5.6 M GdmHCl (×) then rapidly diluted to the protein concentrations displayed on the x axis and assayed for rhodanese activity (Horowitz and Simon, 1986).

At concentrations higher than 1.05 μg/ml very little activity is recovered. The data obtained using urea showed a peak at about 0.1 μg/ml, while the activity recovered when GdmHCl was used as the denaturant appears fairly constant at protein concentrations between 0.1 and 0.5 μg/ml.

Another important variable when refolding protein is the temperature. Typically, the lower the temperature at which refolding is attempted the better the recoveries of active protein. When urea-denatured rhodanese is refolded at a final concentration of 3.6 μg/ml the percentage recovery of activity after 60 minutes rose steadily from 3% at 42 °C to 65% at 12 °C. A maximum recovery of 80% at 10 °C after 24 hours was finally achieved (Mendoza *et al.*, 1991a). Similar results were found in the refolding of recombinant F_{ab}-fragments (antibody chains) produced in *E. coli* (figure 1.7; Buchner and Rudolph, 1991).

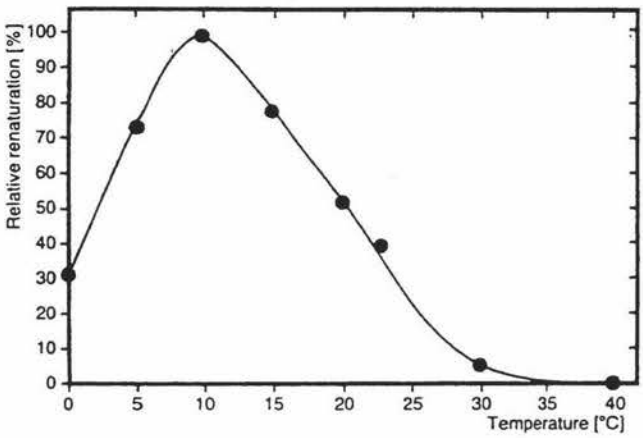


Figure 1.7: Illustration of the temperature dependence of renaturation. Renaturation of recombinant F_{ab}-fragments was carried out by dilution of the denatured and reduced protein into a refolding solution. The concentration of denatured F_{ab} was 40 µg/ml (Buchner and Rudolph, 1991).

In this study temperature also influenced the protein concentration dependence of renaturation; at 20 °C optimum refolding occurred at a final protein concentration of 20 µg/ml, whereas at 10 °C optimum refolding was found at 80 µg/ml of protein (figure 1.8).

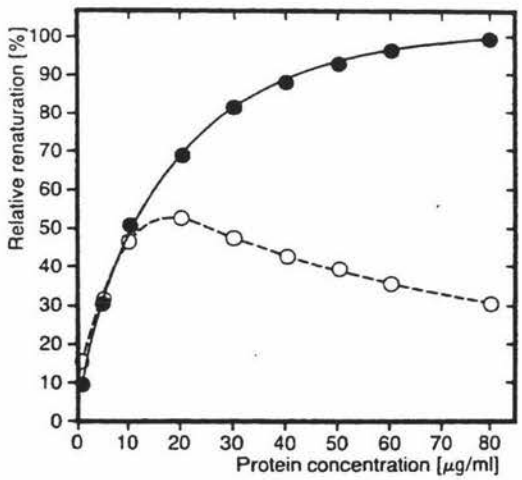


Figure 1.8: Illustration of the concentration dependence of functional renaturation. Renaturation was carried out by dilution of the denatured and reduced protein into refolding solution. The time of renaturation was 170 hours at 10 °C or 30 hours at 20 °C. Maximum renaturation was about 43% of the total recombinant protein. (●) 10°C. (○) 20°C. (Buchner and Rudolph, 1991).

Also, addition of the basic amino acid arginine added to the refolding buffer increased the renaturation yields of some proteins (Buchner and Rudolph, 1991; Buchner *et al.*, 1992). Arginine is added to the refolding solution at a concentration of 0.35 - 0.5 M resulted in ~60% increase in active F_{ab} fragments (figure 1.9). Buchner and Rudolph (1991) suggested that the chaotropic nature of arginine destabilises incorrectly disulfide-bonded structures, allowing the molecules to proceed to the correct folding pathway. A similar trend was seen when arginine was used in the refolding buffer of tissue plasminogen activator (Chaudhuri, 1994).

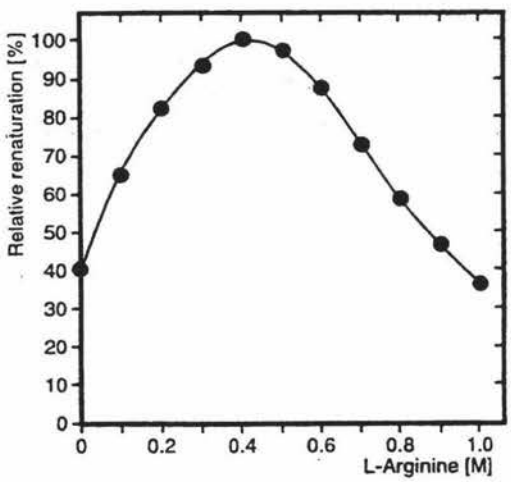


Figure 1.9: The effect of the addition of arginine to the refolding solutions on the yield of renaturation. The concentration of denatured F_{ab} was 60 µg/ml, temperature was 10 °C and time of renaturation was 150 hours (Buchner and Rudolph, 1991).

1.3.3 Refolding by Dialysis

The denaturing solution may be changed for a non-denaturing buffer by dialysis. Dialysis membranes are selectively permeable allowing the movement of small chaotrope molecules across the membrane while retaining the larger protein molecules. The diffusion of small chaotrope molecules decreases the denaturant concentration inside the dialysis bag when dialysed against a large volume of non-denaturing buffer. As the denaturant concentration in the dialysis bag slowly decreases, the protein may fold into its native active conformation. Dialysis has been used to refold several proteins on a small scale, as it is more gradual and relatively high protein concentrations can be used. Examples of proteins

refolded by dialysis are chymotrypsinogen A (Orsini and Goldberg, 1978) and rhodanese (Horowitz and Simon, 1986) as well as citrate synthase and urokinase (Chaudhuri, 1994). Refolding by dialysis is still protein concentration dependent and this is seen in figure 1.10.

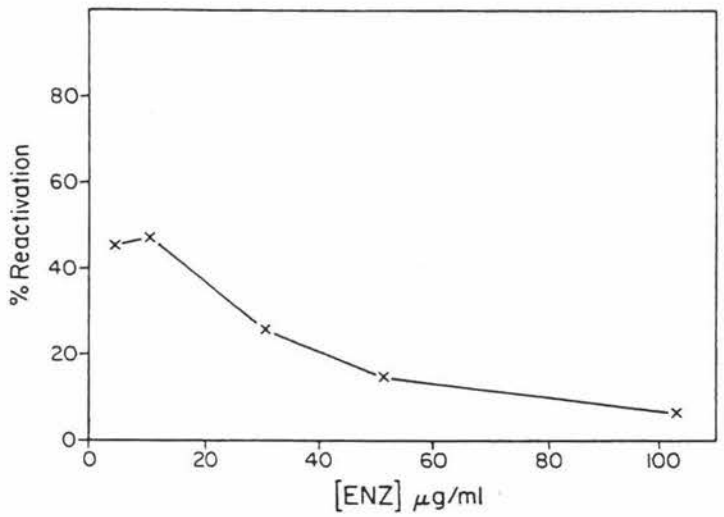


Figure 1.10: Percent reactivation vs rhodanese concentration after denaturation in GdmHCl and subsequent dialysis. Samples at the indicated protein concentrations were dialysed for 20 hours against a non-denaturing buffer (Horowitz and Simon, 1986).

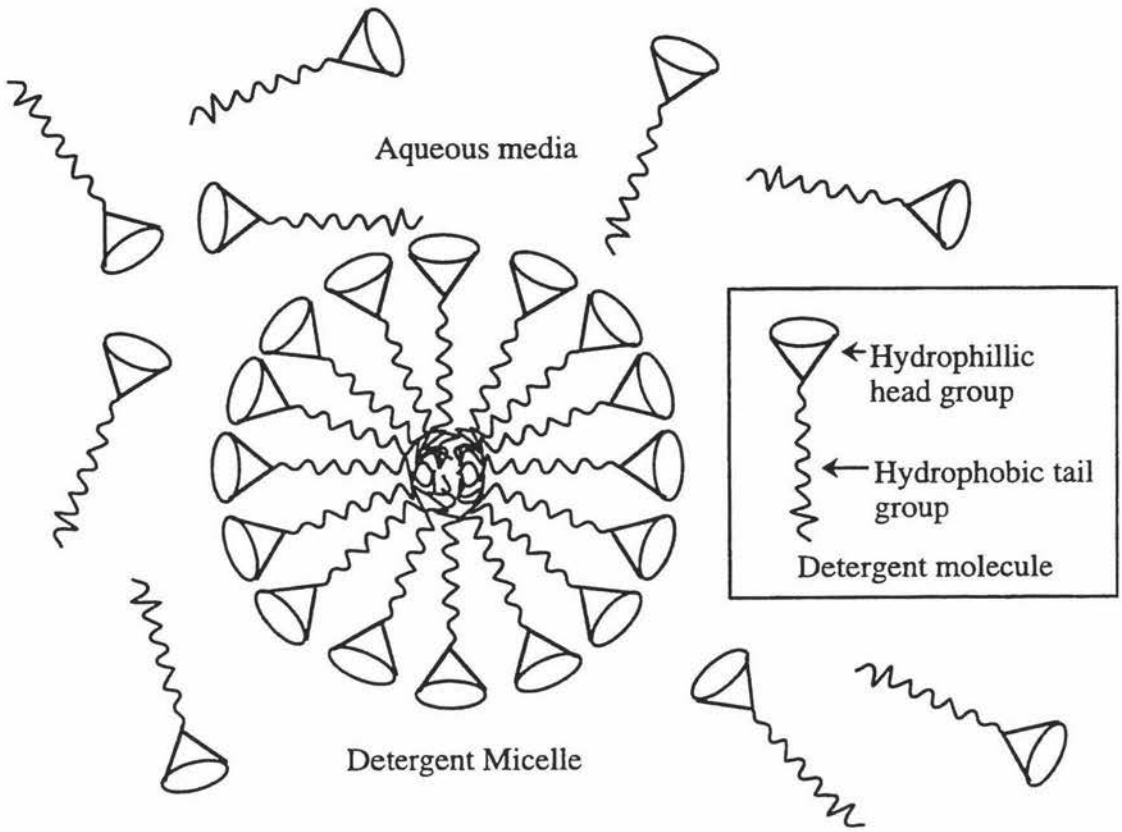
Where renaturation is favoured by low protein concentrations (≤10μg/ml), and where the 42% recovery when refolding GdmHCl denatured rhodanese by dialysis, was considerably higher than the 12% reactivation observed for rapid dilution (see figure 1.6).

Chymotrypsinogen A has not only been refolded by dialysis, but also by a more efficient refolding method. This method involved an initial rapid dilution step which decreased the GdmHCl concentration from 6 M to 1.2 M, followed by a dialysis step to remove remaining GdmHCl (Orsini and Goldberg, 1978). Disadvantages of using dialysis to refold proteins are that it is a relatively slow process which is difficult to scale up due to the large amounts of non-denaturing buffer needed.

1.3.4 Detergent-Assisted Refolding

The main use of detergents in biochemistry is for the solubilisation, purification and characterisation of integral membrane proteins. Detergents are also used in various assays, electrophoresis, protein crystallization, bioseparation and protein refolding. It is thought that detergents may facilitate in the refolding of denatured enzymes by masking the hydrophobic surfaces which appear on the folding intermediates, thus preventing aggregation which reduces the yield of active protein. Each detergent molecule has a hydrophobic portion, which is soluble in hydrocarbon solvents, and a hydrophilic portion, which is soluble in water (figure 1.11). In aqueous solutions detergent molecules are present as monomers, and at detergent concentrations above the cmc, a mixture of monomers and detergent micelles exist in equilibrium (figure 1.11). The micelles are compact aggregates of detergent molecules that are organized in such a way that the hydrophobic portions are in contact with each other in the micellar "core", while the hydrophilic portions form a "shell" which is in contact with the aqueous environment. The hydrophobic portions of detergent monomers would associate with and mask hydrophobic surfaces of the folding protein, while the hydrophilic part of the detergent molecule could protect the folding protein from interactions with transitional hydrophobic surfaces on other folding protein molecules.

Some detergents have been found to assist in refolding of denatured proteins only over a small range of concentrations. For example, adding the non-denaturing detergent lauryl maltoside (LM) at a certain concentration to the refolding solution, increased the efficiency of rhodanese refolding at high protein concentrations (Tandon and Horowitz, 1986; Tandon and Horowitz, 1987; Mendoza *et al.*, 1991a). With an optimal 5 mg/ml LM in the refolding solution 90% reactivation was acheived (Tandon and Horowitz, 1986). Above ~5 mg/ml LM the reactivation started decreasing and at 20 mg/ml LM the reactivation had dropped significantly. The concentration of LM at which significant reactivation begins to be observed is in the concentration range in which the critical micelle concentration of LM lies under the conditions used. The critical micellar concentration (cmc) represents the highest monomeric detergent concentration obtainable for a particular detergent, or



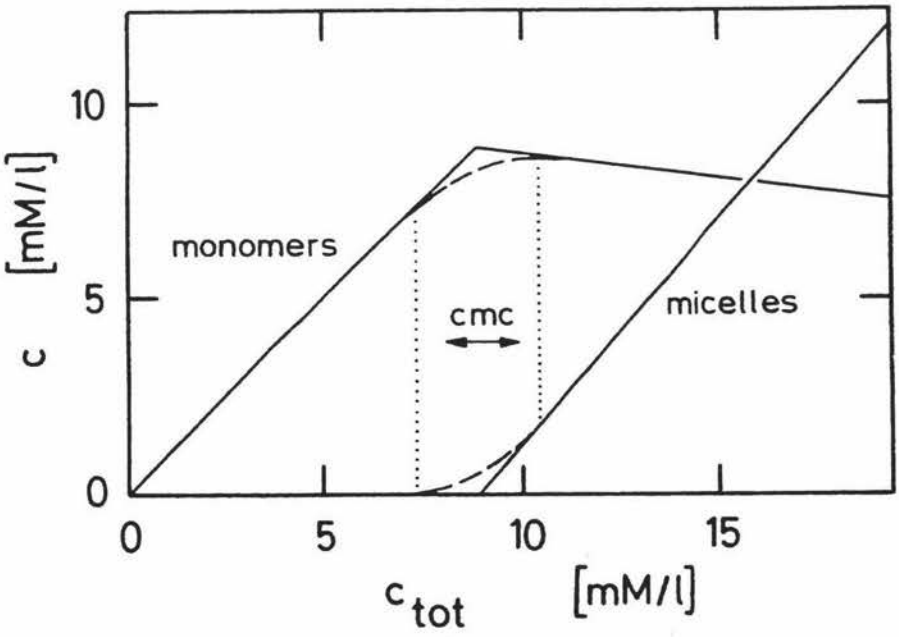


Figure 1.11: Schematic diagram of detergent monomers and micelles in solution. Plot of monomer and micelle concentrations as a function of total detergent concentration, ctot for sodium dodecylsulfate in water at room temperature.

alternatively the detergent concentration above which micelles begin to form. Other non-denaturing detergents were trialled over a range of concentrations to see if they also would aid in the refolding of rhodanese. Not all detergents trialled were useful, and the ones that were useful only aided in refolding over a small range of concentrations that were always being above the detergents cmc (Tandon and Horowitz, 1987). However, none of the detergents trialled were as effective in reactivation as LM.

The fact that for the detergent to aid in refolding it had to be at a concentration higher than its cmc suggested that the micelle structure might be important. However gel filtration experiments determined that the number of detergent molecules actually associated with the folding protein molecules was not enough to form an micelle under the conditions used (Tandon and Horowitz, 1987). It is unclear why detergents have to exceed their cmc before significant reactivation of rhodanese occurs. One theory suggest that at the detergents cmc the detergent monomer just exceeds its solubility limit in aqueous solution. At this point it would be expected that the monomers would have their weakest binding to the protein molecule as the detergent molecules are just beginning to form micelles; i.e. there would be competition between folding protein and micelles for detergent monomers (Tandon and Horowitz, 1986). The weak binding could stabilize folding intermediates without interfering with the formation of the native conformation of the protein. Following the refolding studies on rhodanese, Horowitz and co-workers tested the effects of LM on the reactivation of five other enzymes after treatment with GdmHCl (Tandon and Horowitz, 1988). LM assisted in the reactivation of three of the enzymes investigated, but at concentrations not generally related to its cmc. The need for LM to be at a concentration near its cmc to be effective seems specific to rhodanese (Tandon and Horowitz, 1986). These findings indicate that 'non-denaturing' detergents may assist refolding of enzymes, although the optimum detergent and concentration range will have to be determined for each individual protein. Although not a detergent, PEG has been used during the refolding of bovine carbonic anhydrase B. It was found to inhibit aggregation during refolding through the formation of a nonassociating PEG-intermediate complex (Cleland *et al.*, 1992; Cleland and Wang, 1990).

1.3.5 Refolding by Size-Exclusion Chromatography

This method uses size-exclusion (or gel filtration) chromatography matrices to perform buffer exchange, aggregate removal, and the folding reaction. The reduced diffusion of proteins in gel-filtration media has been shown to reduce the probability of non-specific interactions between partially folded molecules, thus reducing aggregation. The crosslinked structure of gel filtration media offers an ideal environment for protein folding, as proteins passing through the column are partitioned between the stationary and mobile phases. Contacts between protein molecules, although greatly reduced, are not stopped, which, depending on the characteristics of the system, gives some possibility for aggregation. One advantage of this method is that larger protein aggregates that do form are separated from the refolding protein, and so a fraction of pure refolded protein may be obtained.

The following mechanism for size-exclusion chromatography protein folding has been proposed by Batas and Chaudhuri (1996). Initially, the denatured unfolded protein applied to the column has a random coil configuration and thus a large hydrodynamic radius. This hydrodynamic radius is larger than the gel media pores, and so the protein is completely excluded from the beads (figure 1.12). When the eluent (refolding buffer) is applied, there is a decreasing gradient of chaotrope concentration until the mobile phase re-equilibrates the column. As the chaotrope concentration is reduced, the protein starts to fold, and this results in a smaller hydrodynamic radius as the protein develops a more compact and native-like structure. At this stage the protein can move into the bead in a partially folded form (figure 1.12). Within the pores the refolding reaction can occur, and there is minimal likelihood of aggregation with other proteins (the gel pores are mimicking the action of chaperone proteins, see section 1.3.6). When the protein is fully refolded, its hydrodynamic radius is constant and the protein is eluted from the column as an active native protein. The size-exclusion nature of this process ensures that the aggregates that do form in the column are removed from the column first, and the chaotrope species which have very small molecular weights in comparison to the protein are eluted after the refolded protein. Some proteins sucessfully refolded by this relatively new process are

chicken egg white lysozyme and bovine carbonic anyhdrase (Batas and Chaudhuri, 1996), as well as human rETS-1 protein, *E. coli* integral host factor and bovine ribonuclease A, all of which form inclusion bodies when over-expressed in *E. coli* (Werner *et al.*, 1994).

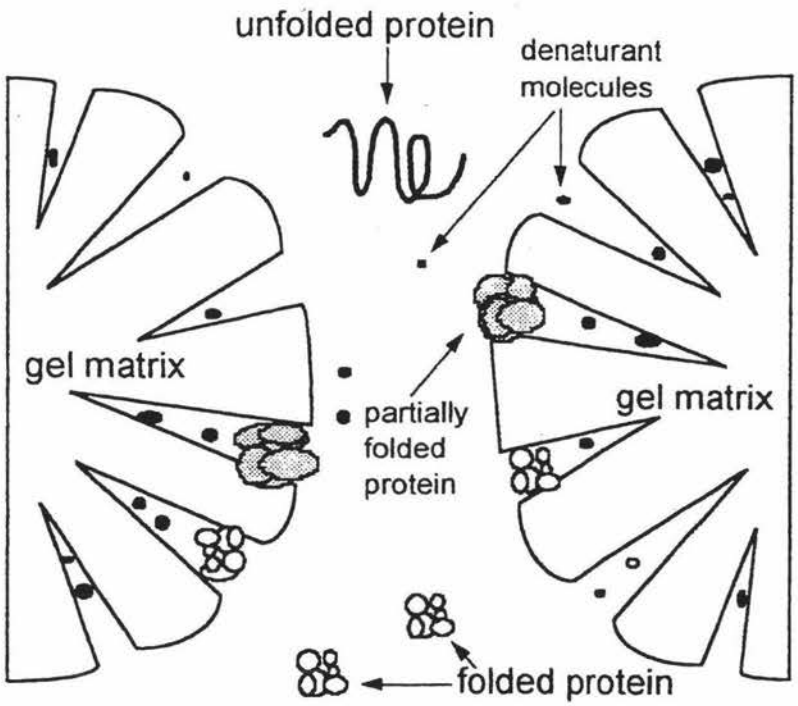


Figure 1.12: Schematic representation of protein refolding within size-exclusion chromatography media. Shown is the transition of the protein structure from an unfolded to folded conformation resulting from interations with the gel filtration media (Batas and Chaudhuri, 1996).

1.3.6 *In Vitro* Chaperonin-Assisted Refolding

Protein folding in the cell is assisted by a class of proteins termed molecular chaperones (Gething and Sambrook, 1992; Morimoto *et al.*, 1994). The chaperonin ring family of molecular chaperones are a group of related proteins found in prokaryotes, mitochondria and plastids that mediate ATP-dependent folding of polypeptides to the native state (Ellis and Hemmingsen, 1989). The best characterised chaperonin, GroEL from *Escherichia coli*, is composed of two heptameric rings of 57 kDa subunits stacked back to back. The full function of GroEL is dependent on the the co-chaperonin GroES, is composed of a

single heptameric ring of 10 kDa subunits, that forms a 1:1 complex with GroEL by binding one end of the GroEL cylinder (Saibil *et al.*, 1991; Ishii *et al.*, 1992; Langer *et al.*, 1992), see figure 1.13. Despite a large number of theoretical and experimental studies, the physical basis for how chaperonins convert the energy of ATP hydrolysis into an increased efficiency of protein folding is poorly understood. GroEL-mediated folding in the absence of GroES is generally a slow process compared with spontaneous folding (e.g. Martin *et al.*, 1991; Viitanen *et al.*, 1991; Corrales and Fersht, 1995). This suggests that the enhanced yield of native protein observed *in vitro* in the presence of GroEL alone (Ayling and Baneyx, 1996), is due to a buffering effect in which the efficiency of folding is increased by lowering the concentration of aggregation-prone folding intermediates in solution (Weissman *et al.*, 1995). By contrast, in the presence of GroEL and GroES folding rates are generally comparable to the rate of spontaneous folding or, in some cases, substantially faster (Todd *et al.*, 1994; Peralta *et al.*, 1994). Weissman *et al.* (1995) found that by adding polypeptide to a solution of GroEL *in vitro* before addition of GroES can result in the sequestering of the peptide bound inside GroEL underneath the GroES which caps the top of the $GroEL_{14}$ cavity when added to the solution. This sequestering of the polypeptide protects it from proteolysis by proteinase K (PK).

Proteolysis experiments were carried out with the polypeptides ornithine transcarbamylase (OTC), rhodanese and methylmalonyl-CoA mutase (MCM) (Weissman *et al.*, 1995), looking at the mechanism of action of GroEL, and the productive release of polypeptides from a sequestered position beneath GroES. To follow quantitatively the proteolysis protection observed, the effect of order of addition of polypeptide and GroES on the rate of proteolysis of substrate was examined (figure 1.13). For these studies, three complexes were formed: a radiolabeled substrate-GroEL complex in the absence of GroES (complex i); a complex in which radiolabeled substrate was bound to a preformed complex between GroEL and GroES (complex ii); a complex in which labeled substrate was first bound to GroEL and then GroES was added (complex iii). For all three complexes, the rate of disappearance of full-length OTC and rhodanese was determined. Both in the absence of GroES (complex i) and when GroES was added prior to substrate (complex ii), all of the OTC or rhodanese was found to be rapidly degraded (figures 1.13A and 1.13B).

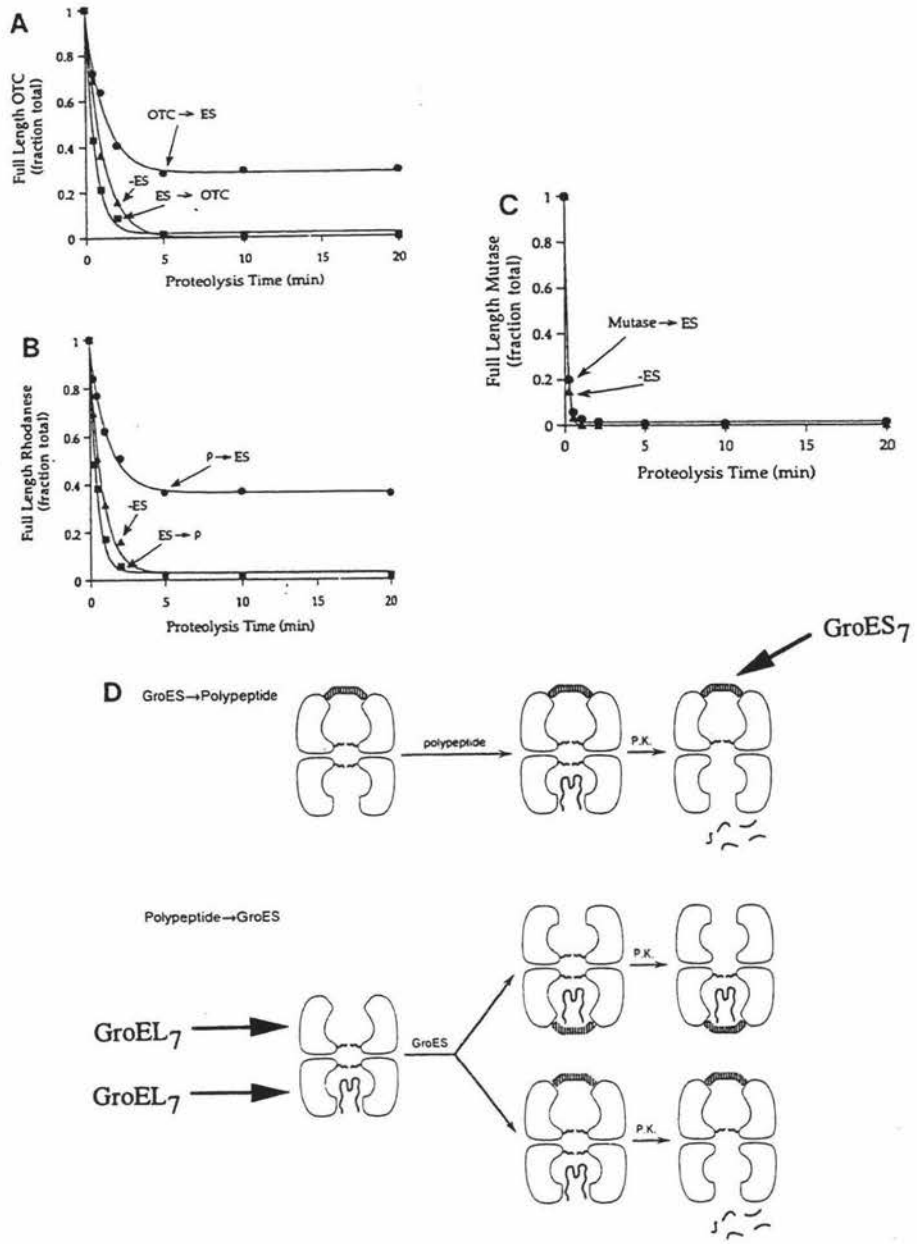


Figure 1.13: Effect of order of addition of GroES and substrate on protection of substrate from proteolysis. Unfolded ^{35}S- labelled polypeptides were diluted into a mixture with GroEL either before or after addition of GroES and MgATP. As a control, polypeptides were added to GroEL alone. The mixtures were treated with PK (200 ng/ml) for varying times. The products were analysed by SDS-PAGE, and the relative amount of full-length polypeptide was quantitated on a phosphorimager. (A) Time course of OTC proteolysis. (B) Time course of rhodanese proteolysis. (C) Time course of methylmalonyl-CoA mutase proteolysis. (D) Schematic illustrating the interpretation of the results. The hatched cap-like structure represents GroES, and the open double ring structure represents GroEL. As noted by Langer *et al.* (1992), PK treatment of the assymmetric GroEL-GroES complex results in removal of 16 residues (curly lines) from the carboxyl terminus of the GroEL subunits of the ring not in contact with GroES, making it possible to distinguish the GroEL ring bound by GroES (uncut) from the unbound ring (cut) by SDS-PAGE.

By contrast, when substrate was added prior to GroES (complex iii), ~30% of bound OTC and ~40% of bound rhodanese molecules were protected from proteolysis (figures 1.13A and 1.13B). No protease resistance was observed with a larger protein, the 79 kDa subunit of methylmalonyl-CoA mutase, even when it was added prior to GroES (figure 1.13C). These observations suggest that the 79 kDa mutase was too large to be accommodated under GroES in the GroEL cavity. Further crosslinking studies by the same group indicated that a fraction of the polypeptide molecules were bound on the same GroEL ring as GroES. This suggests strongly that the proteolysis protection is due to sequestering of peptide under GroES in the central cavity of GroEL (figure 1.13D). The proposal that polypeptides are productively released from a sequestered position under GroES raises the possibility that there is a physical limit to the size of a polypeptide whose folding can be assisted by GroEL in a GroES-dependent manner.

Examination of the crystal structure of unliganded GroEL suggests that, in the absence of GroES, polypeptides up to ~35 kDa can be accomodated within a single ring of GroEL (Braig *et al.*, 1994). Binding of GroES, however, induces a large conformational change in GroEL, leading to an approximate doubling of volume in the central cavity of the GroEL ring to which it is bound (Chen *et al.*, 1994). Notably, a number of proteins that are near or beyond the physical limit predicted to be accomodated under GroES are not assisted in folding by GroEL alone or by GroEL and GroES, even though they can form stable complexes with GroEL. For example, the 60 kDa firefly luciferase (Schroder *et al.*, 1993), the 124 kDa phytochrome photoreceptor (Grimm *et al.*, 1993), and the 72 kDa tailspike protein of phage P22 (Gordon *et al.*, 1994) bind to GroEL but are not assisted in folding by GroES. Likewise, the 79 kDa protein methylmalonyl-CoA mutase, apparently is not accommodated under GroES (figure 1.13C), is not productively folded by GroEL *in vitro*.

The following is a possible mechanism of chaperonin action (Weissman *et al.*, 1995; Weissman *et al.*, 1996; Ranson *et al.*, 1997). At physiological ATP and ADP concentrations, GroEL and GroES form a stable but dynamic complex (Martin *et al.*, 1993; Todd *et al.*,1994). The asymmetric GroES-GroEL complex containing bound ADP

has a high affinity for polypeptide (Burston *et al.*, 1995). The polypeptide is initially bound exclusively to the GroEL ring not occupied by GroES (trans). During the folding cycle, release and rebinding of GroES allows the formation of a cis complex in which GroES and polypeptide bind to the same ring of GroEL. The polypeptide is held rigidly in the cis complex sequestered beneath GroES prior to binding of ATP. Productive folding from the cis ternary complex is induced by the binding of ATP in the GroEL ring opposite that occupied by GroES and polypeptide. Binding of ATP in the trans ring starts the 'timer' for hydrolysis and release. When ATP hydrolysis occurs in the trans ring ($t_{½} \approx 15$ seconds), GroES is released (Todd *et al.*, 1994; Burston *et al.*, 1995; Hayer-Hartl *et al.*, 1995), giving the polypeptide the opportunity to depart and allowing rebinding of ATP. The released polypeptide is either committed to fold (or already folded) or in an uncommitted state, which can rebind to the same or a different GroEL complex and undergo another round of folding upon ATP and GroES binding and polypeptide release (Weissman *et al.*, 1994).

1.3.7 Co-expression of Molecular Chaperonins *In Vivo*

Another potential application of the chaperones in refolding is their co-expression to high levels in *E. coli* to prevent recombinant proteins produced in the same *E. coli* cell from forming inclusion bodies. Activity of recombinant ribulose bisphosphate carboxylase of *Anacystis nidulans* produced in *E. coli* was increased by the co-production of GroEL and GroES (Goloubinoff *et al.*, 1989). Co-expression of the genes of molecular chaperones, dnaK and GroESL together, caused an increase in solubility of human procollagenase produced in *E. coli* (Lee and Olins, 1992). Co-production of human growth hormone with dnaK significantly reduced the formation of inclusion bodies and the extent of aggregation of the hormone protein (Blum *et al.*, 1992). The over-expression of the *murI* (*glr*) gene, which encodes the glutamate racemase of *E. coli*, resulted in the formation of inclusion bodies of the enzyme, and little activity was found in the soluble fraction. The co-expression of the GroES and GroEL genes with *murI* enhanced the *in vivo* folding of glutamate racemase in an active form (Ashiuchi *et al.*, 1995).

1.4 Aims of this Project

The overall aim of this project was to purify hMCM inclusion bodies from *E. coli* and use this inclusion body material to produce soluble and active recombinant hMCM. As discussed previously hMCM is an adenosylcobalamin-dependent enzyme, much work has been carried out on reactions that require this co-enzyme. Further progress in defining the mechanism by which these enzymes work will require detailed information about the structure and active site chemistry of enzymes such as hMCM. After determining the best solubilising conditions for the hMCM inclusion bodies, various refolding conditions mentioned in the introduction were to be trialed in an attempt to optimise *in vitro* refolding of hMCM. Once the most productive method of refolding had been found, this would be scaled up to prepare a larger quantity of refolded hMCM for structural studies. Another aim of this project was to produce a 5'deoxyadenosylcobalamin-agarose affinity chromatography column which would be used to further purify the correctly refolded hMCM from incorrectly refolded hMCM. A parallel experimental route to producing soluble and active hMCM was the co-expression of the molecular chaperonin genes GroES and GroEL in the same strain of *E. coli* as the hMCM gene.

CHAPTER TWO

MATERIALS AND METHODS

Methanol, guanidinium hydrochloride (AnalaR®), and NADH were from BDH. Isopropanol and ethanol were from Ajax Chemicals. Magnesium chloride was from Riedel-de Haën, Tris (basic form) and dithiothreitol (DTT) from Serva and ammonium persulphate from Gibco BRL. All other compounds and chemical reagents used were of the highest grade available. Unless stated otherwise, the water used in all solutions was supplied from a NANOpure II water purification unit (Barnstead). This water is referred to as Milli-Q water in this thesis, and is reagent grade deionized water with a resistivity greater than 16 MΩ.cm. All buffers were adjusted to the required pH at room temperature. Unless otherwise stated procedures were carried out at room temperature (20-25 °C)

2.1 Co-expression of Chaperonins *In Vivo* with hMCM

All *E. coli* growth media were prepared as described in Sambrook *et al.* (1989) and were autoclaved before use.

pMEXHCO (see Appendix 1) was introduced into bacteria by the transformation method described in Sambrook *et al.* (1989), using $CaCl_2$-competent (Sambrook *et al.*, 1989) BL21(DE3)/pGroESL *E. coli* cells. BL21(DE3) is a *lon*⁻ *E. coli* strain in which the T7 RNA polymerase gene is integrated into the *E. coli* chromosome (Studier *et al.*, 1990). pGroESL is a pACYC184-derived expression plasmid for the IPTG-inducible production of GroEL and GroES that confers chloramphenicol resistance, was a kind gift from Dr G. Lorimer, DU PONT. As a negative control and to ensure that the BL21(DE3)/pGroESL $CaCl_2$-competent cells contained no intrinsic ampicillin resistance, they were plated onto 2TY agar containing ampicillin (100 μg/ml). As a positive control to ensure that the BL21(DE3)/pGroESL $CaCl_2$-competent cells were chloramphenicol resistant and were capable of growth they were plated onto 2TY agar

containing chloramphenicol (75 μg/ml). To rule out any contamination of the SOC medium used during the transformation procedure, 150 μl of SOC medium was plated onto 2TY agar containing ampicillin (100 μg/ml) and chloramphenicol (75 μg/ml).

BL21(DE3)/pGroESL *E. coli* cells were prepared for electroporation using the method described by Dower *et al.* (1988). Electroporation cuvettes with a gap of 2 mm were cooled on ice and 2 μl of the pMEXHCO DNA solution was added to the bottom of the cuvette. 40 μl of the BL21(DE3)/pGroESL electroporation-competent *E. coli* cells were added to the cuvette and the solution was mixed by flicking. The cuvette and contents were incubated on ice for 2 minutes. The outside of the cuvette was then wiped dry and electroporation carried out using a BioRad Gene Pulser (Resistance = 800Ω, capacitance = 25 μFD, voltage = 2.5V). Immediately after electroporation, 0.5 ml of room temperature sterile SOC medium was added to the cuvette and mixed up and down with a sterile Pasteur pipette. The cells were transferred to a sterile 1.5 ml Eppendorf tube and incubated on a shaker at 37 °C for 30 minutes. The cells were then plated on 2TY agar containing ampicillin (100 μg/ml) and chloramphenicol (75 μg/ml).

2.2 Expression of hMCM Inclusion Bodies for Refolding

E. coli SRP84/pGP1-2/pMEXHCO (Tabor and Richardson, 1985; see also Appendix 1) has previously been used to express hMCM as inclusion bodies. The expression protocol was very similar to the procedure described in section 2.2.2. These cells had been induced and prepared prior to the start of this project, they were stored as a cell pellet at -70 °C.

2.2.1 Comparison of Two hMCM Expression Systems

Two systems for the expression of hMCM in *E. coli* were tested to see which system produced the most hMCM inclusion body protein.

E. coli strain BL21(DE3)/pMEXHCO (Studier *et al.*, 1990; M.L. Patchett, unpublished) was streaked out on 2TY agar containing 100 μg/ml ampicillin. Single colonies were used to innoculate 20 ml of M1 medium containing 100 μg/ml ampicillin. This innoculum was cultured with shaking at 37 °C to an $O.D_{600}$ of ~2.0. A sample was taken for SDS-PAGE analysis (sampling procedure and sample preparation described in section 2.9.1), and 0.2 mM IPTG (final concentration) was added to the remaining culture to induce hMCM expression. Growth was continued and the culture was sampled for SDS-PAGE analysis at 1, 2, and 4 hours after induction.

The other *E. coli* expression system used was SRP84/pGP1-2/pMEXHCO (Tabor and Richardson, 1985; see also Appendix 1). This system relies on a heat shock step for induction of hMCM, so prior to induction the SRP84/pGP1-2/pMEXHCO cells were grown at 30 °C. M1 medium (20 ml) containing 100 μg/ml ampicillin was innoculated with one colony of SRP84/pGP1-2/pMEXHCO. This was then grown overnight at 30 °C with shaking. After 18 hours at 30 °C the $O.D._{600}$ was 1.95. The cells were induced by a 42 - 43 °C heat shock for 1 minute, and cooled rapidly on ice to 37 °C. Growth continued at 37 °C and the $O.D._{600}$ of the culture was read at 0, 1, 2, and 4 hours after induction and appropriately sized samples were taken and treated as described in section 2.9.1 for SDS-PAGE analysis.

2.2.2 Large-Scale Growth of SRP84/pGP1-2/pMEXHCO and Induction of Recombinant hMCM Expression

The *E. coli* strain SRP84/pGP1-2/pMEXHCO was streaked out on 2TY agar plates containing 100 μg/ml ampicillin and 70 μg/ml kanamycin. The plates were incubated overnight at 30 °C and single colonies were picked and cultured separately in 2 ml of M1 media supplemented with antibiotics and glucose (10 mM). After incubation overnight at 30 °C, one of the 2 ml cultures was used to innoculate 50 ml of M1 media (supplemented with antibiotics and glucose as before) in a 250 ml flask. This culture was then grown at 28 - 30 °C, while shaking at 300 rpm, to an $O.D._{650}$ of 3.9. The 50 ml culture was used to innoculate five 2 litre flasks (9 ml into each flask) each

containing 600 ml of M1 media (supplemented as before). These cultures were grown at 28 - 30 °C until the $O.D._{650}$ reached ~2.0. The cultures were then induced by heat shock; 400 ml of M1 media (without antibiotics, but with 10 mM glucose), preheated to 65 °C, was added to each 2 litre flask. The cultures were immediately swirled vigorously for 1 minute. The culture temperature was monitored carefully with a sterile thermometer and ranged from 42 - 43 °C. The flasks were then swirled vigorously in ice cold water for about 1 minute until the temperature had dropped to 30 °C. Following this heat shock step the cultures were incubated for 14 hours at 28 - 30 °C and then harvested over a period of 2 hours by centrifugation at 10414×*g* (r_{max}) for 10 minutes. Cell pellets were stored at -70 °C.

2.3 Preparation of hMCM Inclusion Bodies for Refolding Experiments

2.3.1 Isolation of Inclusion Bodies by Differential Centrifugation

Lysis Buffer	0.44 M	Sucrose
	2 mM	Dithiothreitol (DTT)
	1 mM	EDTA, pH 7.5
	0.1 %	Nonidet-P40
	0.1 %	Deoxycholate (sodium salt)
	0.1 %	Tris-HCl, pH 8.0
Wash Buffer	10 mM	Potassium phosphate buffer, pH 7.2
	1 mM	DTT
	0.1 mM	EDTA, pH 7.5

Induced SRP84/pGP1-2/pMEXHCO cell pellets (typically 13 g) were thawed at room temperature in a glass beaker. The cells were chilled on ice and 3 volumes of ice cold lysis buffer added with stirring (magnetic flea and stirrer). The solution was stirred continuously at 4 °C for 30 minutes to give a smooth cell suspension, then lysozyme (Grade I from chicken egg white, Sigma Cat. No. L6876) and solid PMSF were added to final concentrations of 1 mg/ml and 0.12 mg/ml respectively. The solution was stirred for a further 25 minutes at 4 °C. Mn(II) chloride and magnesium chloride were

added to final concentrations of 1 mM. DNase I (Type IV DNase I from bovine pancreas, Sigma Cat. No. D5025) and RNase A (Type II-A RNase A from bovine pancreas, Sigma Cat. No. R5000) were added to final concentrations of 0.63 μg/ml and 0.31 μg/ml respectively. Stirring continued for 20 minutes at 4 °C then an equal volume of a room temperature solution of 0.2 M NaCl in 20 mM Tris-HCl pH7.5 was added. This solution was stirred for a further 20 minutes at 4 °C, then chilled on ice. The solution was then centrifuged at 4923×*g* (r_{max}) at 4 °C for 10 minutes, and the centrifugation was repeated on the decanted supernatant. The two pellets were suspended in a total volume of 50 ml 10 mM EDTA pH7.5 with 0.5% Triton X-100 at room temperature, and this was centrifuged at 4302×*g* (r_{max}) at 4 °C for 10 minutes. The pellet was resuspended in the EDTA-Triton buffer and centrifuged two more times, followed by three cycles of resuspension in 50 ml of wash buffer and centrifugation. The final pellet was resuspended in 4-6 ml of wash buffer and stored at -70 °C. The protein concentration of the purified inclusion bodies was ~48.4 mg/ml.

2.3.2 Solubilisation of Inclusion Bodies

Guanidinium hydrochloride solubilisation solution

8 M	Guanidinium hydrochloride
1 mM	EDTA
50 mM	Potassium phosphate buffer, pH 7.4

Urea solubilisation solution

8 M	Urea
1 mM	EDTA
50 mM	Potassium phosphate buffer, pH 7.4

Guanidinium isothiocyanate solubilisation solution

5 M	Guanadinium isothiocyanate
1 mM	EDTA
50 mM	Potassium phosphate buffer, pH 7.4

In small-scale solubilisations 49 μl of the suspension of purified inclusion bodies was added to 150 μl of solubilisation solution and then 1 μl of 1 M DTT was added. This solution was vortexed briefly, incubated at room temperature for one hour and then

centrifuged for 3 minutes at 9650×*g* (r_{max}). The supernatant was then transferred to a Eppendorf tube and stored on ice. For some trials where a larger volumes of inclusion bodies were needed the volumes used in the small scale solubilisation were directly scaled up, i.e. no volume ratios were changed.

2.4 Initial hMCM Refolding Experiments

2.4.1 Initial Small-Scale Rapid Dilution Experiments

Basic Refolding Solution

10 % (v/v)	Glycerol
0.1 M	Potassium fluoride
0.1 M	Arginine-HCl
1 mM	DTT
0.05 mM	EDTA
10 mM	Potassium phosphate buffer, pH 7.3
0.05 %	40 mM PMSF in isopropanol

Small droplets of inclusion bodies, solubilised as described in section 2.3.2, were carefully pippetted on to the inner wall of a 1.5 ml Eppendorf tube containing ice-cold refolding solution. The Eppendorf tube was capped and the contents of the tube were mixed rapidly for 1 second using a vortex mixer. The volume of solubilised inclusion bodies and refolding solution in these experiments depended on the final protein concentration required in the refolding solution. Typically a 100-fold dilution would involve 4 µl of solubilised inclusion bodies being rapidly diluted into 396 µl of ice-cold refolding solution.

2.4.2 Scale-up of Small-Scale Rapid Dilution Experiments

Purified hMCM inclusion bodies in a suspension (775 µl, 48.4 mg/ml) were solubilised as described in section 2.3.2 using 6M GdmHCl (final concentration). 6M GdmHCl was then used to dilute the solution of solubilised inclusion bodies to a final protein concentration of 5 mg/ml (total volume of 7.5 ml) 1M DTT was added to maintain the DTT concentration at 1 mM. The solution of solubilised inclusion bodies was pumped

into 242.5 ml of 4 °C basic refolding solution (stirring rapidly in a conical flask) at a rate of 12 µl/min over ~10 hours, with a peristaltic pump. The resulting 250 ml solution of 'refolded' hMCM solution was then stored at 0 °C and assayed for hMCM activity. Strands of insoluble protein formed on the injection needle during pumping, and wherever possible these strands of aggregated protein were wiped from the needle in an attempt to minimise further aggregation. The top of the conical flask was covered in glad wrap to minimise exposure of the refolding solution to air.

2.4.3 Dialysis of Solubilised Protein

Dialysis tubing (12 000 molecular weight cutoff, Union Carbide) was prepared by heating to 60 °C in 15 mM sodium bicarbonate, 10 mM EDTA. The solution and tubing was then allowed to cool and rinsed thoroughly with Milli-Q H_2O. If the tubing was not to be used straight away it was stored in Milli-Q H_2O at 4 °C.

The inclusion body preparation, with a protein concentration of 48.4 mg/ml, was solubilised at three different final protein concentrations, 1.2 mg/ml, 0.12 mg/ml and 0.012 mg/ml, in 6M GdmHCl/1 mM DTT, for one hour at room temperature. 1 ml of each solubilised protein solution was then dialysed with stirring against 1 litre of refolding solution overnight (~18 hours) at 4 °C. The dialysed solutions were stored on ice for 2 hours and assayed for hMCM activity.

2.4.4 Detergent-Assisted Refolding by Rapid Dilution

Solubilisation of inclusion body material in 6M GdmHCl was carried out as described in section 2.3.2, at a protein concentration of 12 mg/ml, with DTT present at a concentration of 1 mM. After solubilisation at room temperature for 1 hour, 100-fold dilutions of the solubilised inclusion bodies were made into 396 µl of ice-cold refolding solution. This dilution resulted in a protein concentration in the refolding solution of 0.12 mg/ml. Refolding solutions were prepared that were supplemented with appropriate concentrations of the detergents shown in table 2.1. Each detergent was

used in the refolding solutions at concentrations related to its critical micellar concentration (cmc) as listed in Neugebauer (1990). For full details on the detergents listed in table 2.1 see Dawson *et al.* (1986). Detergents were used at three concentrations: 5× lower than their cmc, at their cmc, and finally at a concentration 5× higher than their cmc under the refolding conditions. For each set of activity assays of the refolding solutions with various detergents added, a control assay was carried out in which detergents were omitted from the refolding solution. Solutions were left to refold on ice at 4 °C for 24 hours then assayed for hMCM activity. The Zwittergents referred to in table 2.1 are part of a zwitterionic series where the number of methylene groups linking the sulpfonic acid group to the quaternary amine groups, and the length of the hydrophobic alkyl chain are variable. Molecules in this series have no net change in charge between pH 2 and pH 12.

2.4.5 Time Trial Refolding Experiment Over 24 Hours

hMCM inclusion bodies prepared as described in section 2.3.1, were solubilised as described in section 2.3.2 in a final concentration of 6M GdmHCl. The solubilised protein solution was then stored on ice. At regular intervals over the course of 24 hours an aliquot of solubilised protein was rapidly diluted 85-fold into ice-cold basic refolding buffer (section 2.4.1). The diluted samples were stored on ice until they were assayed for hMCM activity.

2.4.6 Dimerization Experiment

A large-scale rapid dilution experiment was carried out as described in section 2.4.2, except that the solubilised protein was pumped into the refolding solution over 1.7 hours at a rate of 73.3 µl/min. The protein in the 'refolded' hMCM solution was then adsorbed onto hydrated HTP (1.5 g dry weight; BIORAD Hydroxyapatite for column chromatography, BIO-GEL® HTP, Cat No. 130-0420) then packed into a 10 ml disposable column. The hMCM was eluted from the hydroxyapatite as described in section 2.5.3. 1.5 ml fractions were collected during elution at 30% B and a 7 ml

Table 2.1: Table of each detergent concentration used in refolding solutions

Detergent to be added to the refolding solution	cmc of the detergent in the refolding solution mmoles/litre
CTAB	1
Z-3-08	140
Z-3-10	14
Z-3-12	1.4
Z-3-14	0.14
Z-3-16	0.014
Nonidet P-40	0.11
Triton X-100	0.29
Lauryl maltoside	0.12
Tween 20	0.059
CHAPS	4
CHAPSO	4.1
MEGA-9	18
LDAO	0.14
Lubrol PX	0.10
SDS	1.5
C_8-β-D Gluc	21

fraction containing the majority of hMCM was collected during elution with 60% B. As the fractions were collected they were put on ice and stored at 0 °C overnight for hMCM activity assays the next day. When the 60% B ml fraction came off the column a sample was snap-frozen in liquid nitrogen and stored at -70 °C for protein concentration determination. The increase in hMCM activity in the 60% B fraction was followed over a period of 189 hours as it was stored on ice at 0 °C. A 60 µl sample, enough for two hMCM activity assays, and a 50 µl sample, enough for five native gels, were taken at various times. These samples were snap-frozen in liquid nitrogen and immediately stored at -70 °C. Once all the samples had been collected

they were assayed for hMCM activity. Each sample was thawed 1 minute before being assayed.

2.4.7 Refolding by Gel Filtration Chromatography

G-150-40 Sephadex superfine (Sigma) gel filtration beads have an approximate bed volume of 18 - 22 ml per gram of beads. The internal radius of the glass column to be poured was 0.5 cm and the height of the column was 47 cm, a column volume of 37 cm^3. 1.68 g of beads was weighed out and hydrated at 20 °C for 72 hours in 50 ml of a 10 mM KP buffer pH 7.4, 10% (v/v) glycerol adjusted to pH 7.2 with HCl, and 0.02% sodium azide. The swollen beads were stored at 4 °C and fines were removed by decanting the surface liquid. Before the gel slurry was poured into the column, 70 ml of buffer was added to the hydrated beads the slurry was then degassed under vacuum from a water pump at room temperature for 30 minutes. The degassed slurry was allowed to warm to room temperature and the liquid was decanted of the top of the settled beads until ~75% of the volume of the slurry was settled beads. The column was packed at a gravity-fed flow rate of 36.4 µl/minute. Once the gel was completely settled an adaptor was fitted to the top of the column and lowered onto the top of the settled matrix. The direction of flow was then reversed. Three column volumes of eluent buffer (basic refolding buffer) were passed through the column in order to stabilise the bed, wash through any traces of sodium azide and equilibrate the gel beads with the refolding buffer. The inclusion bodies were solubilised as described in section 2.3.2, with 8M GdmHCl solublisation solution. 490 µl of inclusion bodies (48.4 mg/ml) was solublised in a total volume of 2 ml. The solution of solublised inclusion bodies was centrifuged at 4 °C for 10 minutes at 9650×*g* (r_{max}), then loaded from the bottom of the column, and 'chased' on by room temperature basic refolding buffer. The 2 ml sample loaded onto the column was 5% of the column volume, the maximum volume recommended for good resolution. The first 1 ml fraction was collected as soon as the whole sample was loaded and 83 fractions were collected off the column. As the gel filtration column was run a precipitate formed in the first 15 cm of gel which slowed the flow, so the column was running for a lot longer than originally planned. All the

fractions (~1 ml each) collected over the first 16 hours were put on ice. Fractions collected over the next 16 hours were smaller due to the decreased flow rate of the column. Fractions 23 to 36 were pooled with a total volume of ~7 ml. 200 mg (dry weight) of hydroxyapatite was added to these pooled fractions and mixed by inversion, the suspension was then put at 4 °C overnight as the hydroxyapatite settled. The supernatant was then removed and the hydroxyapatite was resuspended in refolding solution to dilute any GdmHCl present. The supernatant was removed and adsorbed protein was eluted from the hydroxyapatite with 0.7 ml of 0.5 M KP buffer pH 7.2. A sample of this solution was run on an SDS-PAGE as described in section 2.9.2. Another sample was assayed for hMCM activity. The hydroxyapatite adsorption and elution was done to concentrate any refolded hMCM to an assayable level. This sample was later prepared for N-terminal sequence analysis as described in sections 2.9.4 and 2.9.5, in an attempt to identify the main protein component of this sample.

2.5 Concentration of Refolded Protein with Hydroxyapatite

2.5.1 Hydration of Hydroxyapatite

BIO-GEL® HTP hydroxyapatite (for column chromatography, BioRad Cat. No. 130-0420) was hydrated by adding 1 g of dried hydroxyapatite to six parts of starting buffer (20 mM potassium phosphate (KP) buffer pH 7.2, 0.5 mM DTT) and mixing occasionally with a stirring rod for 30 minutes. When hydrated the hydroxyapatite occupies ~2-3 ml per dry gram. The hydroxyapatite was left undisturbed for 10 minutes and the supernatant was decanted. The hydroxyapatite was suspended in three volumes of starting buffer and stored at 4 °C.

2.5.2 Adsorption of Protein to Hydroxyapatite

The hydrated hydroxyapatite was added to a refolding solution containing hMCM to be purified and concentrated. The hydroxyapatite was then maintained in suspension for 30 minutes at 4 °C by constant mixing on a magnetic stirrer. The suspension was then

left undisturbed for 15 minutes and the supernatant removed and stored at 4 °C. The settled hydroxyapatite was packed into a column and washed with two column volumes of starting buffer.

2.5.3 Elution of Protein from Hydroxyapatite

The packed hydroxyapatite was washed at a rate of 1 ml/min with starting buffer (buffer A). A pulse of 20% B (0.1 M KP, 0.5 mM DTT, pH 7.2) was then put through the column, followed by a pulse of 60% B (0.3 M KP, 0.5 mM DTT, pH 7.2). The column was then washed with 100% B (0.5 M KP, 0.5 mM DTT, pH 7.2). 2 ml fractions were assayed for hMCM activity and protein. Some fractions were used for affinity chromatography experiments described in section 2.6.3 and 2.6.4.

2.6 Synthesis and Testing of 5'AdoCbl-agarose for Affinity Chromatography

2.6.1 Synthesis of 5'AdoCbl-agarose Resin

The 5'deoxyadenosylcobalamin-agarose (5'AdoCbl-agarose) was synthesised from vitamin B_{12} (1 mg/ml resin) immobilised on 4% beaded agarose (Sigma Cat. No. V3254) using a method similar to that described by Yamada and Hogenkamp (1972). All manipulations of cobalamin solutions and cobalamin-containing compounds were carried out in the dark or in dim red light to minimize photodecomposition (Dawson *et al.*, 1986). All solutions were protected from oxygen by maintaining a continuous stream of oxygen-free nitrogen gas over the surface of the liquid when transferring solutions. 1.5 ml of vitamin B_{12}-agarose (in 0.5 M NaCl with 0.02% thiomersal) was washed thouroughly with 0.1 M EDTA-NaOH, pH 9.5, and resuspended in 3 ml of 0.1 M EDTA-NaOH, pH 9.5. Nitrogen was bubbled gently through the suspension for 30 minutes to deoxygenate the solution. The suspension was then treated with 13 mg of chromous chloride dissolved in 2.8 ml of deoxygenated 0.1 M EDTA, pH 9.5, and gently mixed for 15 minutes by bubbling nitrogen through the solution. 50 µl of 5'-O-

tosyladenosine solution (5 mg of 5′-O-tosyladenosine dissolved in 50 µl of deoxygenated dimethylsulfoxide) was then added under nitrogen. The reaction mixture was mixed end-over-end at room temperature in the dark for 4 hours, then the suspension was transferred to a 10 ml disposable column and allowed to settle, then the liquid decanted off. Milli-Q H_2O was washed through the column until all blue colour due to the chromous chloride had washed through. 1.5 ml of 5′deoxyadenosylcobalamin-agarose was produced, and stored in 0.5 M NaCl with 0.02 % thiomersal (BDH Chemicals Ltd, Product No. 30416). The resin was protected from the light and stored at 4 °C.

2.6.2 Scanning of Prepared 5′AdoCbl-agarose Resin

Before each spectral scan a baseline correction from 250 - 600 nm was carried out on 950 µl sample of 20 mM KP buffer pH 7.2 using the CARY (CARY 1E. UV-Visible double beam spectrophotometer, Varian). The absorption spectrum of this solution was then determined again to confirm a flat baseline between 250-600 nm. Absorption spectra for the control solutions (1 mM 5′AdoCbl, 1 mM cyanocobalamin and 1 mM hydroxocobalamin, all in 20 mM KP buffer pH 7.2) were also measured between 250 and 600 nm. 50 µl of control solution was added to 950 µl of buffer in the cuvette, mixed by inversion for 10 seconds and the adsorption spectrum from 250 - 600nm determined at a scanning rate of 3000 nm/min over a period of 7 seconds. To acidify a sample, 10 µl of concentrated hydrochloric acid was added to the cuvette, mixed by inversion and scanned as described above. The prepared 5′AdoCbl-agarose resin (53 mg) was suspended in 950 µl of 20 mM KP buffer, pH 7.2, mixed by inversion and scanned as described above. The sample was then acidified and scanned again.

2.6.3 Testing Reversible Adsorption of MCM to the Prepared 5′AdoCbl-agarose Resin

200 mg of vitamin B_{12}-agarose and prepared 5′AdoCbl resins were washed with 20 mM KP buffer, pH 7.2, to remove traces of thiomersal preservative. Known weights

of drained resin were transferred to 1.5 ml Eppendorf tubes in dim red light. MCM solutions were then added to the tubes followed by end-over-end mixing for 30 minutes at 4 °C in the dark. The solution was then centrifuged at 4 °C for 5 minutes to pellet the resin and the supernatant was assayed for MCM activity (section 2.10.3). A control for 'zero binding' (i.e. a 100% activity control) in which resin was replaced by a similar volume of buffer, was also carried out. Little or no activity in the 5'AdoCbl-resin supernatant compared to the 'zero binding' control would indicate adsorption of the enzyme to the resin.

Recombinant *P. shermanii* MCM (0.03 units/µl in, 50% (w/w) glycerol, 25 mM Tris-HCl pH 7.3, 0.5 mM DTT, 0.05 mM EDTA, 0.15 M NaCl), partially purified from *E. coli*, was used to test the effectiveness of the 5'AdoCbl-agarose resin. ~50 mg of washed resin was mixed with 20 mM KP buffer, pH 7.0, to a total volume of 495 µl in a 1.5 ml Eppendorf tube, and 5 µl of *P. shermanii* MCM was added to the mix. To test the adsorption of recombinant hMCM to the 5'AdoCbl resin, 450 µl of a hMCM solution (fraction from preparation described in section 2.4.2 and concentrated with HTP as described in sections 2.5.1-2.5.3) and 50 mg of washed resin were mixed. Similar incubations were also carried out for two controls, i.e. with 50 mg of CNCbl resin to test the specificity of binding, and with 50 µl buffer instead of resin to determine 100% activity (i.e. 'zero binding' activity).

The feasibility of using 1 mM 5'AdoCbl to elute bound MCM from 5'AdoCbl-agarose was also tested. Resin that had been incubated with 450 µl hMCM was mixed end over end with ~50 µl of affinity elution buffer (2 mM 5'AdoCbl in 20 mM KP buffer pH 7.0) at 4 °C for 30 minutes. The solution was then centrifuged at 4 °C for 5 minutes at 13135×*g* (r_{max}). The supernatant was assayed for MCM activity (substrate added last to start the assay).

2.6.4 Conditions for Effective hMCM Adsorption to 5′AdoCbl-agarose

400 µl of the hMCM preparation in ~0.3 M KP buffer, pH 7 (described in the previous section), was diluted to 20 mM KP with water to allow binding to 50 mg of 5′AdoCbl-agarose resin. After binding, the pelleted resin was mixed with an equal volume of affinity elution buffer (2 mM 5′AdoCbl in 20 mM KP buffer, pH 7.0) and the supernatant was assayed for hMCM activity (due to 5′AdoCbl in the sample, no additional 5′AdoCbl was added and the assay was started by adding substrate after a steady basal rate had been obtained). Appropriate controls with vitamin B_{12} resin and no resin were also carried out.

2.7 Large-Scale Refolding and Purification of hMCM

2.7.1 Inclusion Body Purification, Solubilisation and Refolding

Inclusion bodies were purified as described in section 2.3.1 from 13.9 g of cells. 1552 µl of purified inclusion bodies with a protein concentration of 171.1 mg/ml was diluted to a final volume of 5878 µl with wash buffer, to give a final protein concentration of 45 mg/ml. This suspension of purified inclusion bodies was solubilised for 1 hour at room temperature using 18367 µl of 8 M GdmHCl solubilising solution and 24.5 µl of 1 M DTT (final concentration 1 mM DTT). The solubilised inclusion body solution (24.5 ml, 10.8 mg protein/ml) was centrifuged at 26891×*g* (r_{max}) for 30 minutes at 4 °C. The solubilised inclusion bodies were refolded by rapid dilution into a final volume of 2 litres of the basic refolding solution. The solubilised inclusion bodies were pumped into the refolding solution at 3 °C at a rate of ~0.04 ml/min over 10.5 hours. The entire refolded solution was maintained at 3 °C and centrifuged at 8281x*g* (r_{max}) for 20 minutes at 4 °C to pellet any aggregated incorrectly folded proteins. The supernatant of refolded hMCM (protein concentration of ~0.1 mg/ml) was stored at 0 °C.

2.7.2 Batch Adsorption to and Stepwise Elution from Hydroxyapatite

As a direct scale up of the hydroxyapatite adsorption described in section 2.5.2, 12 g of hydroxyapatite was hydrated, added to the refolded hMCM solution and treated as described in sections 2.5.2. The slurry of hydroxyapatite-protein complex was packed into a 2.5 × 7.5 cm column. The protein was eluted, using a peristaltic pump, from the hydroxyapatite at a flow rate of 1 ml/min. An initial 30 minute wash with buffer A was followed by three 45 minute pulses with 20% B, 60% B and 100% B (see section 2.5.3, with the exception that the elution buffers contained 10% (w/v) glycerol). Collection of 5 ml fractions began as the 60% B buffer was applied to the column. Fractions were placed on ice as soon as they were collected and were assayed for hMCM activity and protein within 24 hours. The most active fractions were pooled and purified further by affinity chromatography as described in the next section.

2.7.3 5′AdoCbl-agarose 'Affinity' Chromatography

The prepared 5′AdoCbl-agarose column (approximately 1.5 ml of resin) was washed with 10 ml of 4 °C 1 M NaCl and equilibrated in 20 mM KP buffer, pH 7.2. All buffers were equilibrated at 4 °C before addition to the column. Pooled fractions from the previous large-scale hydroxyapatite step (22.4 ml in 0.3 M KP buffer, 1 mM DTT, pH 7.2) was centrifuged at 30596×*g* (r_{max}) for 20 minutes at 4 °C to remove any precipitate that had been found. The supernatant was diluted to a total volume of 134.4 ml with Milli-Q H_2O, a final potassium phosphate concentration of 50 mM. The sample was loaded by gravity (0.75 ml/min) at 4 °C in the dark. The rate of loading slowed as the last of the sample was draining into the column. As the sample was loading the wash through was collected and assayed for hMCM activity. By comparing the activity in the sample that was loaded with the activity in the wash through during loading, it was estimated that 80% of the hMCM activity in the sample had bound to the resin. The wash through was fed by gravity through the column overnight to ensure that the maximum amount of active hMCM bound to the resin, but no further active hMCM was bound by the resin. The resin was washed with 3 ml of 20 mM KP buffer pH 7.2

then left undisturbed (with no flow) for 10 minutes. To elute the hMCM from the resin a stepwise elution was started with 3 ml of 0.2 M NaCl in 20 mM KP buffer pH 7.2 with 1 mM DTT. This was drained into the resin and then left undisturbed for 10 minutes (1 ml fractions were collected from this step onwards). Each step in the elution was performed this way, with 0.4, 0.6, 0.8 and 1 M NaCl steps. All NaCl solutions were made up in 20 mM KP buffer pH 7.2, which included 1 mM DTT. Finally, 2 ml of 0.2 M NaCl solution was drained through the column, and the column was stored in this solution until all the fractions collected had been assayed for hMCM activity, adding the substrate last to start the assay. This change in the assay was made necessary due to traces of 5′AdoCbl in the flow through from the column causing a high basal rate when the assay was initiated by adding the 5′AdoCbl. This suggests that some of the 5′AdoCbl may be gradually leaching from the agarose during use of the 5′AdoCbl-agarose resin.

2.7.4 Concentration of the Pooled 'Affinity' Chromatography Fractions

In an attempt to separate the 29 kDa contaminating protein from the hMCM protein the two pools from the affinity chromatography purification were concentrated separately using a 50 kDa cut-off Centricon concentrator's in a SORVALL® SS34 rotor at 4500rpm (2420×*g* at r_{max}). The filtrates from both pools were combined and concentrated, using a 10 kDa cut-off microsep concentrator. The three concentrated samples were analysed by SDS-PAGE. During the concentration of the first pool the concentrate was diluted 10-fold with Milli-Q H_2O to reduce the NaCl concentration in the final sample. The concentrate of the second pool was diluted 100-fold with Milli-Q H_2O.

2.8 Purification and Use of Recombinant GroEL and GroES

2.8.1 Ammonium Sulphate Precipitation Method

At 20 °C, the weight of ammonium sulfate to be added to 1 litre of a solution at S_1% saturation, to take it to S_2% saturation was calculated using the following formula (Scopes, 1994) :

$$g = (533 (S_2 - S_1)) \div (100 - 0.3S_2)$$

Solid ammonium sulfate (UltraPure™, Bethesda Research Laboratories enzyme grade, Cat. No. 5501UA) was added to the solution to be precipitated over 10-15 minutes. At first the salt was added and dissolved quite quickly, but as the S_2 saturation is approached the rate of salt addition was slowed. After the last of the salt had dissolved, gentle stirring continued for 10-30 minutes to allow complete equilibration between soluble and aggregated proteins. The solution was then centrifuged at 13 182×*g* (r_{max}) for 15 minutes at 4 °C.

2.8.2 Cell Lysis, Clarification of Extract and First DEAE-Sephacel Step

The chaperonins GroES and GroEL were purified using a modified and scaled down version of a method kindly supplied by George Lorimer of the Du Pont Company. Lorimers method was derived from purification schemes described by Hendrix. (1979) and Chandrasekhar *et al.* (1986).

Buffer A: 0.1 M Tris-HCl, pH 8.1, 0.1 mM EDTA, 1 mM DTT

Buffer B: 0.05 M Tris-HCl, pH 7.2, 0.1 mM EDTA, 1 mM DTT

Buffer C: 5 mM Potassium phosphate, pH 7.6, 0.1 mM EDTA, 1 mM DTT

The starting material of this purification was frozen *E. coli* DH1/pGroESL cells that had been induced with lactose to express GroEL and GroES (M.L.Patchett, pers. comm.). 126.3 g of frozen cell pellet was thawed by suspension in 250 ml of room

temperature buffer A. The cells and buffer were stirred with a glass rod and sucked up and down with a 5 ml pipettor until a smooth suspension was obtained. 125 mg of lysozyme (Grade I from chicken egg white, Sigma Cat. No. L6876), 15 mg of DNase (Type IV from bovine pancreas, Sigma Cat. No. D5025) and 44 mg of PMSF were added. The cell suspension was quickly bought to 30 °C and stirred for 10 minutes to allow lysis of the cells. The crude cell lysate was rapidly chilled on ice and then centrifuged at 11 758×*g* (r_{max}) at 4 °C for 30 minutes. The supernatant was passed (very slowly) through a 0.2 µm filter using a 50 ml syringe and the pellet was resuspended in 125 ml of buffer B, centrifuged, and the supernatant filtered as before. The filtered supernatants were combined and ultracentrifuged at 107000×*g* (r_{max}) for 60 minutes at 4 °C. 50 ml that did not fit in the ultracentrifuge tubes was filtered through a 0.2 µm filter as before. The supernatant from the ultracentrifugation was then filtered through a 0.2 µm filter again. The combined and filtered supernatants were then concentrated to 62 ml using a YM30 ultrafiltration membrane mounted in a 400 ml Amicon stirred ultrafiltration cell. The concentrated sample was diluted to 620 ml in buffer B. This effectively changed the sample buffer from buffer A to buffer B, but for future chaperonin purifications large-scale dialysis would be an easier way of achieving this buffer exchange. The 620 ml sample was then loaded on to a 5 x 15 cm (300 ml) column of DEAE-Sephacel equilibrated in buffer B, using an Econo system (BioRad). The column was washed with 2 column volumes of buffer B at 2 ml/minute. The bound proteins were eluted with a 2.4 litre linear salt gradient (0 to 0.5 M NaCl in buffer B) at 1.5 ml/minute. Fractions containing GroEL and GroES were identified by SDS-PAGE analysis. The fractions containing GroEL were pooled and taken to 67% saturation with ammonium sulphate as described in the previous section. The precipitate was stored at -70 °C. The GroES fractions were pooled and purified further (see next section).

2.8.3 Continued Purification of GroES

The buffer in the 125 ml pooled GroES fractions was exchanged for buffer C by dialysed overnight against 4 litres of buffer C at 4 °C. The sample was then applied to a

5 x 15 cm (300 ml) column of DEAE Sephacel equilibrated in buffer C, at 1.5 ml/min. The column was washed with two column volumes of buffer C at 0.9 ml/min overnight. The column was eluted with a 1.8 litre gradient of potassium phosphate (0.02 M to 0.5 M, pH 7.6). Fractions containing the purest GroES were identified by SDS-PAGE and pooled. The buffer was again exchanged by dialysis of the pooled fractions against 4 litres of buffer B, and insoluble material was removed by centrifugation for 15 minutes at 9650×*g* (r_{max}) at room temperature. The 103 ml supernatant was then applied at 1.5 ml/min to a HiLoad 26/10 Q-Sepharose high performance column, equilibrated in buffer B using an Econo system (BioRad). The column was washed with 2 column volumes (100 ml) of buffer B at 2 ml/min using an FPLC at room temperature. All buffers used with the FPLC were filtered through a 0.2 μm filter by vacuum filtration, to remove any particulate material and to partially degass the buffers. The column was eluted at 2 ml/minute with an 800 ml linear gradient from 0 to 0.5 M NaCl in buffer B. A sample of each fraction was analysed by SDS-PAGE. The purest GroES fractions were pooled and concentrated to 7.37 ml using a 10K Filtron Macrosep centrifugal concentrator centrifuged at 4068×*g* (r_{max}) in an SS34 rotor. DTT (1 mM) was added to the concentrated sample. The sample was transferred out of the Macrosep centrifugal concentrator, mixed and then centrifuged for 5 minutes at 1307×*g* (r_{max}) at 4 °C to sediment a precipitate present in the sample. Glycerol was added to a final concentration of 15% (w/v), and the sample was frozen and stored at -70 °C in 1 ml aliquots.

2.8.4 Continued Purification of GroEL

The frozen ammonium sulphate precipitate containing GroEL was thawed and dissolved in 15 ml of buffer B. This sample was centrifuged at 17 210×*g* (r_{max}) for 15 minutes. The maximum volume that could be loaded onto a 2.5 x 80 cm (400 ml) Sephacryl S-300 column was determined, as good resolution in a gel filtration experiment depends on low ratios of sample volume to column volume. A sample volume of ~4 % of the column volume was used, i.e a 15 ml sample. In practice the relative viscosities of sample and eluent should not differ by more than a factor of ~ 2,

which corresponds to a protein concentration in the sample of about 70 mg/ml when a dilute aqueous buffer is used as the eluent. The protein concentration in the supernatant of the dissolved GroEL ammonium sulfate precipitate was ~50 mg/ml in a total volume of 25 ml. The sample was then diluted to 30 ml and the column was run twice, each time loading 15 ml of sample with a protein concentration of ~44 mg/ml. The column was equilibrated in buffer B and the sample was loaded each time by gravity at a rate of 0.3 ml/minute followed on by buffer B. 10 ml fractions were collected as soon as all the sample was loaded. Because of its large molecular mass (420 kDa), $GroEL_{14}$ is resolved from smaller contaminating proteins. Purity was assessed by SDS-PAGE analysis and the fractions which contained the purest GroEL from the two runs were pooled, and precipitated by bringing the solution to 67 % ammonium sulphate saturation as described in section 2.8.1. The pellet of precipitated protein was then dissolved in buffer B to a final volume of 20 ml. This was then centrifuged for 15 minutes at 17210×*g* (r_{max}) at 4 °C. The 20 ml of supernatant was dialysed against 5 litres of buffer B containing 15 % (w/v) glycerol. DTT (1 mM final concentration) was added to the 22.5 ml dialysed solution which was then centrifuged for 15 minutes at 20198×*g* (r_{max}) at 4 °C. 1.5 ml aliquots of the supernatant were stored at -70 °C.

2.8.5 Chaperonin-Assisted Refolding of β-Galactosidase

Solution I:	100 mM	Tris-HCl pH 7.4
	0.1 mM	EDTA
	0.01%	Tween 20
	1 mM	DTT

Denaturation solution I:		
	5 M	GdmHCl
	100 mM	Tris-HCl pH 7.4
	0.1 mM	EDTA
	0.01%	Tween 20
	1 mM	DTT

Refolding solution II:	100 mM	Tris-HCl pH 8.0
	10 mM	$MgCl_2$
	10 mM	KCl
	0.1 mg/ml	BSA
	2.5 mM	DTT
	0.01%	Tween 20

Denatured *E. coli* β-galactosidase was prepared by the addition of 4 µl of 10 mg/ml *E. coli* β-galactosidase (Grade VI, Sigma Cat. No. G6008) to 21 µl of room temperature denaturation solution I. The control solution was prepared by addition of 4 µl of 10 mg/ml *E. coli* β-galactosidase to 21 µl of room temperature solution I. Both solutions were vortexed briefly and incubated for 30 minutes at room temperature. 1 µl of the denatured *E. coli* β-galactosidase solution and the control β-galactosidase solution were rapidly diluted into 99 µl of the following refolding solutions:

Refolding solution II

Refolding solution II + $GroEL_{14}$

Refolding solution II + $GroES_7$

Refolding solution II + $GroEL_{14}$ + $GroES_7$.

Ayling and Baneyx (1996) suggested a 2-fold molar excess of $GroEL_{14}$ complex over β-galactosidase monomers. With a final concentration of β-galactosidase monomers of 136 nmoles/litre in the 100 µl of refolding solution, a concentration of 272 nmoles/litre of the $GroEL_{14}$ complex was needed. GroEL has a subunit molecular mass of 60 kDa, so the 14-mer complex would have a molecular mass of 840 kDa. For a protein of molecular mass 840 kDa to have a final concentration of 272 nmoles/litre in 100 µl of solution, there would be 23 µg of protein in that 100 µl. The purified GroEL preparation contains 36 mg/ml in 15% glycerol. So 0.64 µl of the GroEL preparation was added to the refolding solutions where it was required. $GroES_7$ was similarly added to the refolding solutions at a 2-fold molar excess over the β-galactosidase monomers. 0.5 µl of the GroES preparation was added to the refolding solutions when it was required.

2.8.6 Chaperonin-Assisted Refolding of hMCM

hMCM inclusion bodies in suspension (48.4 mg/ml) were solubilised as described in section 2.3.2 in 6M GdmHCl. Refolding was carried out at a final hMCM concentration of 0.12 mg/ml (1.5 μM α-subunit concentration). This involved a rapid dilution of the solubilised inclusion bodies into ice cold refolding solution containing additions of different combinations of chaperonins. A 2-fold molar excess of $GroEL_{14}$ and $GroES_7$ complexes over the hMCM α-subunit concentration was used, i.e. 3 μM for each. 7 ATP molecules are needed for every $GroEL_{14}$ - $GroES_7$ complex (Langer *et al.*, 1992; Fenton and Horwich, 1997) and ATP was added to the refolding solution at a concentration of 30 μM. Mg^{2+} was needed in equivalent amounts to the ATP (Stryer, 1995) so this was also added to the refolding solution at a concentration of 30 μM. ATP and $MgCl_2$ were added to the refolding solution from 3 mM stock solutions, and the GroEL at 42.9 μM and GroES at 543 μM were added from stocks in 15% glycerol. 3 μl of solubilised inclusion bodies were diluted 134-fold into 399 μl of ice-cold refolding solutions containing different combinations of the above components. When different components of the basic refolding solution (used previously in hMCM rapid dilution experiments) were omitted, the refolding solutions were made up separately with each one missing one of the components, and 3 μM $GroES_7$, 3 μM $GroEL_{14}$, 30 μM ATP and 30 μM $MgCl_2$ were added to each of the depleted refolding solutions.

2.9 Protein Electrophoresis, Electroblotting and N-terminal Sequencing

2.9.1 Treatment of Samples Taken from Cultures for SDS-PAGE

Samples were taken from *E.coli* cultures of known $O.D._{600}$ and centrifuged at 13136×*g* (r_{max}) in a bench top centrifuge for 1 minute. The following equation was used to estimate the volume of culture to centrifuge:

volume to centrifuge (µl) = 500 ÷ $O.D._{600}$

All the supernatant liquid was then removed from the cell pellet by aspiration. To ensure as much liquid as possible was removed the sample was centrifuged again and any more liquid removed. The pellet was then frozen at -70 °C. When the gel was to be run the pellets were thawed and suspended in 100 µl of 1×SDS-PAGE sample buffer, boiled for 3 minutes, and 10 µl loaded onto a SDS-polyacrylamide 10 well mini-gel.

2.9.2 Discontinuous SDS-Polyacrylamide Gel Electrophoresis

Resolving Gel Acrylamide Solution

32 g	Acrylamide
0.2 g	Bisacrylamide
Made up to 100 ml with Milli-Q water	

Stacking Gel Acrylamide Solution

5 g	Acrylamide
0.105 g	Bisacrylamide
Made up to 50 ml with Milli-Q water	

Add 0.25 % (w/v) of analytical grade mixed ion-exchange resin (e.g. AG 501-X8(D), 20-50 mesh, BioRad) and store solutions in brown bottles at 4 °C. This resin will settle to the bottom of the bottle, so liquid should be pipetted from the top of the solution.

Resolving Gel Buffer Solution (4×)

1.5 M	Tris-HCl, pH 8.7, 20 °C
0.4 %	Sodium dodecyl sulphate (SDS)

Stacking Gel Buffer Solution (8×)

1 M	Tris-HCl, pH 6.7, 20 °C
0.8 %	SDS

To prepare 10 ml of a 20 % resolving gel mix, mix (20 % / 32 %) × 10 ml = 6.25 ml of resolving gel acrylamide solution and 2.5 ml of the resolving gel buffer solution and make up to 10 ml with Milli-Q H_2O. To prepare 10 ml of 5% stacking gel mix, use 5

ml of stacking gel acrylamide solution, 1.25 ml of the stacking gel buffer solution and make up to 10 ml with Milli-Q H_2O. Allow the gel mix to come to room temperature before polymerizing. To polymerize the resolving gel mix use 7 µl TEMED and 40 µl 10 % (w/w) ammonium persulphate solution per 10 ml of gel mix. Pour the resolving gel mix into the gel mould, overlay with water-saturated butanol, and allow the gel to polymerize for 60 minutes. To polymerize the stacking gel mix use 12 µl TEMED and 60 µl 10 % (w/w) ammonium persulphate solution per 10 ml of gel mix. Remove the layer of water-saturated butanol from the surface of the resolving gel before pouring the stacking gel and inserting the well comb. The "Instruction manual for mighty small II slab gel electrophoresis unit SE 250" (Hoefer) was followed to prepare and run all SDS-PAGE mini-gels. To prepare a sample for SDS-PAGE analysis an equal volume of 2×SDS-PAGE sample buffer was added to the sample and the mixture was boiled for 3 minutes. After electrophoresis, SDS-PAGE gels were stained in Coomassie blue R-250 stain for 15-20 minutes, then destained in 5:1:5 (strong) destain. SDS-PAGE gels were stored in Welcome destain until they were dried down or photographed.

2× SDS-PAGE Sample Buffer, stored at -20 °C

15 % (v/v)	Glycerol
2 % (w/v)	DTT
0.005 %	Bromophenol blue
6 % (w/v)	SDS
0.125 M	Tris-HCl, pH 6.7

Electrode/Tank Buffer

72 g	Glycine
15 g	Tris
2.5 g	SDS

Made up to 2.5 L with Milli-Q water

Coomassie Blue Stain

0.625 g	Coomassie brilliant blue R-250
225 ml	Methanol
25 ml	Glacial acetic acid

Made up to 500 ml with Milli-Q water

5:1:5 Strong Destain

500 ml	Methanol
100 ml	Glacial Acetic acid
Made up to 1.1 L with Milli-Q water	

Welcome Destain (weak destain for storage)

50 ml	Methanol
50 ml	Glacial Acetic acid
Made up to 1 L with Milli-Q water	

2.9.3 Drying of SDS-Polyacrylamide Gels

The gel to be dried was placed on a piece of Whatman 3-MM filter paper cut slightly larger than the gel. The gel was then covered in a thin film of glycerol to prevent cracking, followed by a sheet of glad wrap plastic film and dried under vacuum using a BioRad model 583 gel drier for 40 minutes at 80 °C.

2.9.4 Electroblotting for N-terminal Sequence Analysis

Transfer buffer

10 mM	CAPS (free acid)
150 ml	Methanol
Make up to 1.5 litres with Milli-Q H_2O	
Adjust to pH 11 with NaOH	

Running buffer containing thioglycollic acid

100 ml	SDS-PAGE running buffer
2 mM	Thioglycollic Acid

Membrane stain solution

0.1g	Coomassie brilliant blue R-250
50 ml	Methanol
50 ml	Milli-Q H_2O

Membrane destain solution

200 ml	Methanol
40 ml	Glacial Acetic Acid
160 ml	Milli-Q H_2O

There were several bands observed on SDS-polyacrylamide gels during this project that needed to be identified. This was done by N-terminal sequence analysis. The 20% gels were cast the day before use so that the polymerisation could go to completion, as free acrylamide can block the N-terminus of some proteins and prevent accurate N-terminal sequence analysis (Walker and Gaastra, 1987). Samples were loaded generously onto an SDS-polyacrylamide gel so that ~ 9 µg of protein was loaded onto each lane. Electrophoresis was carried out with thioglycollic acid in the upper electrode buffer, in a fumehood. Thioglycollic acid is included in the upper electrode buffer as the mobile thiol runs ahead of protein and scavenges free-radicals that may block the N-terminii of proteins (Walker and Gaastra, 1987). Each sample was loaded twice onto the SDS-polyacrylamide gel in a symmetrical pattern so that once the gel was run it could be cut in half down the middle (between the fifth and sixth lanes), and one side would be the mirror image of the other. Half the gel was soaked in transfer buffer for 20 minutes, and the other half of the gel was Coomassie-stained, destained and dried down as described in the previous sections. N-terminal Immobilon^{TM}-P (Millipore Corp.) blotting membrane was cut, wearing gloves at all times, to the size of the gel strip to be blotted. The membrane was immersed in AR methanol for 10 seconds then equilibrated in transfer buffer for 15 minutes. Following equilibration of the blot membrane, a blotting paper / gel / blot membrane / blotting paper sandwich was assembled and the proteins electroblotted using a "Hoefer TE22 Mighty Small Transphor Tank Transfer Unit (Pharmacia Biotech)" at 150 mA for $1^1/_2$ hours. When blotting was finished the blot membrane was removed and soaked immediately in water for 10 minutes with shaking. At the same time the blotted gel was Coomassie-stained to make sure the transfer of protein was complete. After soaking in water the membrane was stained for 1 minute with membrane stain solution, then destained with several changes (2-5 minutes each) in membrane destain solution. Finally, the membrane was rinsed in Milli-Q H_2O for 5 minutes and air dried thoroughly. The membrane was wrapped in plastic and stored at -20 °C until the N-terminus of specific bands could be sequenced.

2.9.5 N-terminal Sequencing

N-terminal sequencing done by J. Mudford, Biochemistry Department (Massey University, Palmerston North, New Zealand). Automated N-terminal sequencing was by the Edman degradation method (Hewick *et al.*, 1981), followed by RP-HPLC separation of the phenylthiohydantoin derivatives of the amino acids on an automatic protein sequencer (Applied Biosystems).

2.10 Protein Determination and Activity Assay Methods

2.10.1 Determination of Protein Concentration

Biuret reagent

1.5 g	Copper sulphate
6.0 g	Sodium potassium tartrate

Dissolved in 500 ml of Milli-Q water, to which was added

300 ml	10 % Sodium hydroxide
1 g	Potassium iodide

Made up to 1 L with Milli-Q water

Coomassie Blue dye-binding reagent

100 mg	Coomassie Brilliant Blue G-250

Dissolved in 50 ml Ethanol to which

100 ml	88 % Orthophosphoric acid

was added. Made up to 1 L with Milli-Q water.
Filtered through Whatman No.1 paper.

Protein concentrations were determined by the Biuret assay, and also by Coomassie blue dye-binding assay (Scopes, 1994). In both of these protein determination methods the standard curve was produced using bovine albumin Fraction V, from Gibco BRL, Cat No. 810-1018IM.

2.10.2 Succinyl-CoA Preparation for use in hMCM Activity Assay

Because CoA (coenzyme A) is expensive, the succinyl-CoA substrate was prepared in 1 ml batches. 10 mg of coenzyme A (CoA trilithium salt from Boehringer Mannheim,

Grade I, Cat No. 103497), 9.0 mg $NaHCO_3$ (sodium bicarbonate), and 8 mg succinic anhydride were weighed into a 1.5 ml screw-capped Eppendorf tube. 0.99 ml of ice cold, vacuum degassed Milli-Q H_2O was added and vortexed immediately for 10 seconds, then returned to ice. The solution was vortexed for less than 1 second, to keep the succinic anhydride crystals in suspension, at 20 second intervals for 30 minutes. The tube was returned to ice when not being vortexed. The pH was measured while keeping the solution cold, and typically was found to be between pH 6 - 6.2. The supernatant was then transfered to a fresh flip-top 1.5 ml Eppendorf tube and 30 µl of 2 M HCl was added. The pH of the solution was checked to make sure it was below pH 4.5, and 250 µl aliquots were stored at -70 °C. The concentration of succinyl-CoA in the preparation can be determined by an enzyme-based method, and is typically between 9 and 10 mM. 9.9 mM was a typical yield and this represents ~99% conversion of Coezyme A to Succinyl-CoA.

0.7 ml of assay mix (as described for the hMCM assay in the next section) was added to a 1 ml quartz cuvette and the A_{340} measured. 1 µl of a 1 mM solution of 5'AdoCbl and 0.5 µl of partially purified recombinant *P. shermanii* MCM (0.03 U/ml) (E. Saafi, MSc.Thesis, 1994) was added and mixed well by inverting the cuvette several times with parafilm over the top. The A_{340} was recorded for 1.5 minutes to obtain a control rate for NADH oxidation. 3 µl of the succinyl-CoA preparation was added to the cuvette, mixed well, and the final absorbance was recorded when the rate of NADH oxidation had returned to the control rate, i.e. when all the succinyl-CoA substrate was used up. To calculate the concentration of succinyl-CoA in the 3 µl added, the change in absorbance at 340nm was calculated by subtracting the final from the initial absorbance value and the answer was divided by $\varepsilon = 6200$ (molar absorption coefficient of NADH) to give the concentration in moles/litre. This was then multiplied by 0.0007 litres (0.7 ml volume of assay mix) to give the moles of succinyl-CoA used, and divided by 3×10^{-6} L (3 µl volume of succinyl-CoA added in this assay) to give the molar concentration of the succinyl-CoA per litre of substrate stock. This stock concentration was then corrected by multiplying by 1000 to give the millimolar amount of succinyl-

CoA per litre of stock. In most 1 ml preparations the succinyl-CoA concentration was 10 mM, making the final concentration in the MCM assays approximately 0.1 mM.

2.10.3 Enzyme-Coupled Assay for Methylmalonyl-CoA Mutase Activity

Assay buffer	0.5 M	Potassium phosphate buffer, pH 7.2
Sodium pyruvate and NADH solution		
	0.4 M	Sodium pyruvate
	4 mM	NADH
Assay mix	4000 µl	Assay buffer
	250 µl	Sodium pyruvate and NADH solution
	28 µl	Epimerase enzyme 50 mMTris-HCl, pH 7.5 (~2U/µl) (E. Saafi, MSc.Thesis, 1994)
	5 µl	L-Malate dehydrogenase solution in 50% Glycerol, Boehringer Mannheim Cat. No. 127248 6 units/µl
	Made up to 10 ml with Milli-Q water	

Early on in the project the hMCM enzyme was assayed at 30 °C. Later for the final large-scale refolding experiment, chaperonin-assisted refolding and batch adsorption and elution trials with the prepared 5′AdoCbl-agarose the assay temperature was increased to 37 °C. At 37 °C the hMCM enzyme is more active, so lower levels of activity can be measured. For each set of assays, just enough assay mix was prepared for the number of assays that were intended to be carried out that day. To perform the assay, 0.8 ml of the assay mix was placed in an Eppendorf tube in a 30 °C waterbath for 2-3 minutes to pre-equilibrate the assay mix. 750 µl of the assay mix was transfered to a pre-warmed 1 ml quartz cuvette, followed by the addition of 8 µl of succinyl CoA, 0.5 µl of transcarboxylase coupling enzyme (methylmalonyl-CoA carboxyltransferase, EC 2.1.3.1) in 50% glycerol (~0.12 U/µl), and 1.5 µl of 1 mM 5′deoxyadenosylcobalamin dissolved in 20 mM potassium phosphate buffer pH 7.0 (protected from light). All these components were mixed by inversion of the cuvette

with parafilm over the top. The cuvette was then placed in the temperature controlled cuvette holder, set at 30 °C, of the CARY 1E, UV-visible double beam spectrophotometer (Varian). The rate of NADH oxidation was then monitored by recording the decrease in absorbance at 340nm over a period of 2 minutes. The background rate of oxidation is probably due to any contaminating NADH oxidase and *P. shermanii* MCM in the coupling enzyme preparations used in the assay. After the background rate is obtained, 2 - 80 µl of hMCM sample is added, depending on the concentration of hMCM in the sample. The assay solution is mixed by inversion as before and placed back in the cuvette holder for a further 2 minute recording of A_{340}. The amount of hMCM sample required to give a measurable rate was determined by conducting trial assays. The difference between the basal rate and the reaction rate can be used to calculate the units of hMCM per µl of sample added to the cuvette. A unit of hMCM activity is defined as the amount of hMCM that results in the oxidation of 1 µmole of NADH per minute in the enzyme-coupled assay. The assay mix was tested before every batch of assays to ensure that it would detect and measure the hMCM present in the sample. This was done by adding 0.5 µl of partially purified recombinant *P. shermanii* MCM (0.03 U/ml) to the reaction mix and following the assay procedure described above.

Later on in this project the assay was changed slightly to more effectively characterise the contribution of the background rate to the total activity. The basal rate was obtained with all components of the assay, including the sample to be assayed, present except for either succinyl-CoA or 5′AdoCbl (depending on the circumstances of the assay), so that after the background rate had been obtained, addition of either compound would complete the assay mix and initiate the assay. This change in the assay protocol would correct for any decrease in absorbance at 340 nm that was not due to hMCM, but rather due to a contaminant in the sample which may also use up NADH in the assay cuvette.

2.10.4 β-Galactosidase Activity Assay

β-galactosidase activities were determined as described in Herbomel *et al.*, 1984.

CHAPTER THREE

RESULTS

3.1 Purification of hMCM Inclusion Bodies

hMCM inclusion bodies were purified from previously induced and harvested *E. coli* strain SRP84/pGP1-2/pMEXHCO (see section 2.2) as described in section 2.3.1. The protein concentration of the final inclusion body preparations (i.e. the suspension of purified insoluble inclusion bodies in wash buffer) was determined by the Biuret method, as the Coomassie blue dye-binding method gave unreliable results (both methods described in section 2.10.1). The Coomassie blue dye-binding protein assay is more sensitive than the Biuret assay (Scopes, 1994), but coomassie blue assay reagent is acidic and so is less likely to solubilise all the insoluble protein in the sample than is the alkaline Biuret reagent. This was apparent when the Coomassie blue dye-binding method was used to determine the protein concentration of a suspension of purified inclusion bodies; small particles of Coomassie blue-stained protein settled to the bottom of the assay tube. The protein concentration of the suspension of purified inclusion bodies was 48.4 mg/ml, with a total yield of 339 mg protein from 13 g (wet weight) of *E. coli* cells. Various purification samples were treated with 2×SDS-PAGE sample buffer and analysed by SDS-PAGE (figure 3.1). A series of centrifugation and washing steps resulted in a preparation that contained partially purified inclusion bodies of hMCM. It can be seen in lanes 5, 6, 7 and 8 of figure 3.1 that some hMCM was solubilised in the washes of the inclusion bodies with Triton-X100 The main band in lane 9 of figure 3.1 migrated slightly slower than the 66 kDa molecular mass marker in lane 10. This is similar to the molecular mass of ~79 kDa for the mature hMCM α subunit (as predicted from the amino acid sequence in Appendix 2). There are contaminating proteins (Hart *et al.*, 1990) present in the final hMCM inclusion body preparation (figure 3.1, lane 9), but these proteins are present in small amounts compared to the amount of hMCM in the final preparation.

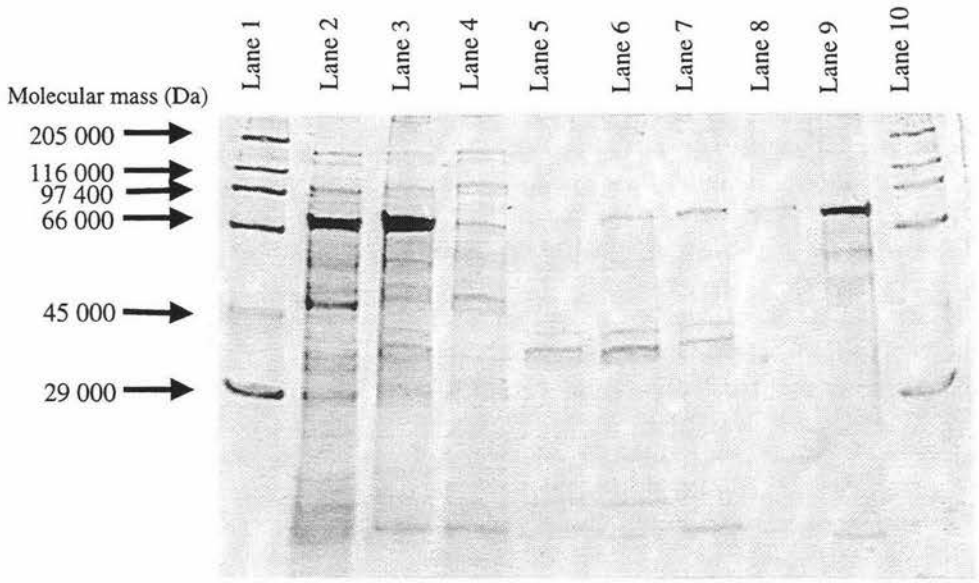


Figure 3.1: SDS-PAGE analysis of samples taken during the small-scale preparation of inclusion bodies.

Lane 1: SDS-6H molecular mass markers (Sigma).

Lane 2: Whole cell lysate.

Lane 3: Low-speed pellet from cell lysate, resuspended.

Lane 4: Supernatant from low-speed centrifugation of cell lysate.

Lanes 5-8: Supernatants from IB washing and centrifugation steps.

Lane 9: Purified hMCM inclusion bodies.

Lane 10: SDS-6H molecular mass markers (Sigma).

3.2 Comparison of Two hMCM Expression Systems

Two systems for the expression of hMCM in *E. coli* were tested to see which system produced the most hMCM inclusion body protein under the conditions used. *E. coli* strain BL21(DE3)/pMEXHCO (Studier *et al.*, 1990) was grown up and induced with IPTG as described in section 2.2.1. The second *E. coli* expression system used was SRP84/pGP1-2/pMEXHCO (Tabor and Richardson, 1985; see Appendix 1). Figure 3.2 shows the SDS-PAGE analysis of samples taken after the induction of expression in both systems. Lane 1 contains a sample from the purified inclusion bodies described in section 3.1, but more protein loaded than in lane 9 of figure 3.1. Very little hMCM was expressed in the BL21(DE3)/pMEXHCO system. However SRP84/pGP1-2/pMEXHCO sample (lane 5) contained a band corresponding to the hMCM α-subunit, indicating successful induction of hMCM expression. By comparing the levels of hMCM in lane 5 of figure 3.2 with lane 2 of figure 3.1, which are two separate inductions of the same SRP84/pGP1-2/pMEXHCO *E. coli* strain, it can be seen that the yield of hMCM in this expression system varies considerably between cultures.

3.3 Large-Scale Growth of SRP84/pGP1-2/pMEXHCO and Induction of Recombinant hMCM Expression

The *E. coli* strain SRP84/pGP1-2/pMEXHCO was grown and induced as described in section 2.2.2. The $O.D._{650}$ at harvesting was 5.3, and a sample was taken and treated as described in section 2.9.1 for SDS-PAGE analysis. 41 g of cells (wet weight) were harvested and frozen at -70 °C. It was apparent that only a small amount of hMCM was produced, compared to the amounts of other proteins expressed at normal levels also present in the cell lysate. Comparing lanes 3 and 4 of figure 3.3 showing induced SRP84/pGP1-2/pMEXHCO *E. coli* cells 14 hours after induction, to lane 5 of figure 3.2 showing induced SRP84/pGP1-2/pMEXHCO *E. coli* cells 4 hours after induction, indicates that the extra 10 hours of growth after induction may be responsible for the higher levels of hMCM

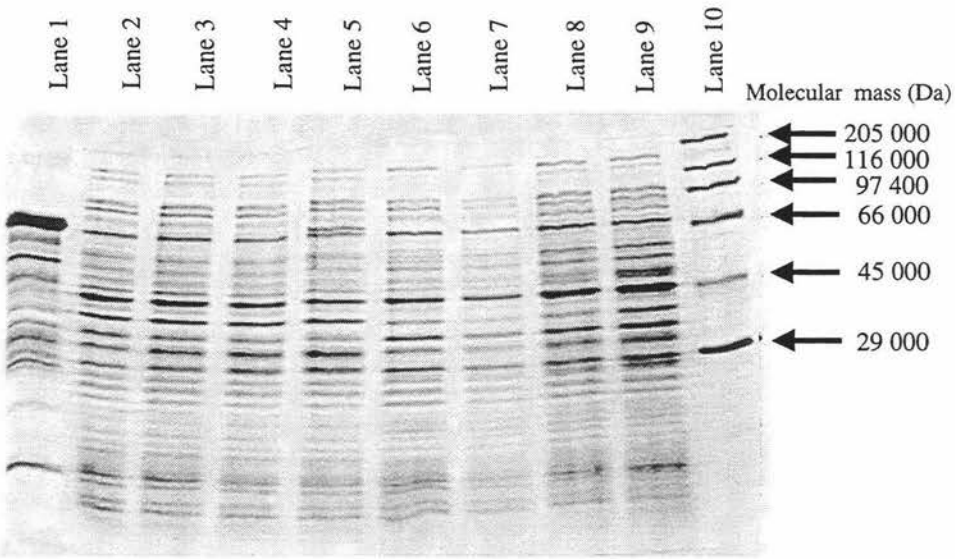


Figure 3.2: SDS-PAGE of samples taken after induction of two hMCM *E. coli* expression systems.

Lane 1: Purified hMCM inclusion bodies.

Lane 2: SRP84/pGP1-2/pMEXHCO at 0 hours after induction.

Lane 3: SRP84/pGP1-2/pMEXHCO at 1 hour.

Lane 4: SRP84/pGP1-2/pMEXHCO at 2 hours.

Lane 5: SRP84/pGP1-2/pMEXHCO at 4 hours.

Lane 6: BL21(DE3)/pMEXHCO at 0 hours after induction.

Lane 7: BL21(DE3)/pMEXHCO at 1 hour.

Lane 8: BL21(DE3)/pMEXHCO at 2 hours.

Lane 9: BL21(DE3)/pMEXHCO at 4 hours.

Lane 10: SDS-6H molecular mass markers (Sigma).

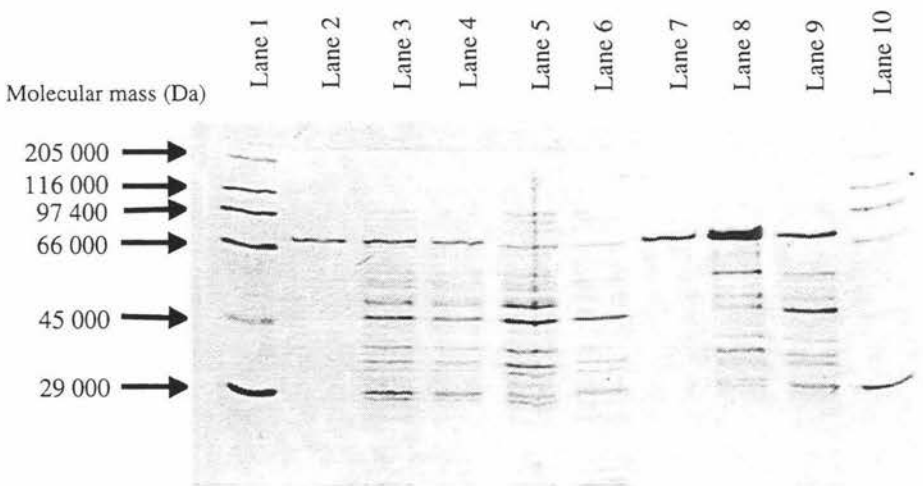


Figure 3.3: SDS-PAGE of samples taken during large-scale growth and induction of *E. coli* SRP84/pGP1-2/pMEXHCO.

Lane 1:	SDS-6H molecular mass markers (Sigma).
Lane 2:	3µg previously purified hMCM inclusion bodies.
Lanes 3 and 4:	SRP84/pGP1-2/pMEXHCO cells 14 hours after induction.
Lanes 5 and 6:	Not applicable.
Lane 7:	6µg previously purified hMCM inclusion bodies.
Lanes 8 and 9:	Not applicable.
Lane 10:	SDS-6H molecular mass markers (Sigma).

present in the large scale growth of SRP84/pGP1-2/pMEXHCO.

3.4 Large-Scale hMCM Inclusion Body Purification

Purification started with 41 g of induced SRP84/pGP1-2/pMEXHCO cells prepared as described in section 2.2.2. The isolation of hMCM inclusion bodies was carried out immediately after harvesting the cells, as described in section 2.3.1. Figure 3.4 shows an SDS-PAGE analysis of samples taken during this large-scale hMCM inclusion body purification. Comparing the initial cell lysate (lane 10) with the final inclusion body preparation (lane 2), it can be seen that the washing procedures have removed a substantial amount of the contaminating proteins. These contaminating proteins can be seen in the supernatant lanes (lanes 3, 4 and 5, figure 3.4), these lanes also contain small amounts of solubilised hMCM. The final preparation is not as pure as in the first inclusion body purification (section 3.1) that was done on a smaller scale, but still consisted predominantly of hMCM.

This large scale inclusion body preparation was to have been used in the large scale refolding experiments described in section 2.7, but because the small scale inclusion body hMCM preparations were of higher purity, and there was sufficient material for the experiments, the inclusion bodies produced on the smaller scale were used for the large scale refolding experiments.

3.5 Determination of the Best of Three Different Solubilisation Solutions

Three different solubilising agents were tested, 6M guanidinium hydrochloride, 5M guanidinium isothiocyanate and 8M urea. Inclusion bodies were solubilised by mixing a suspension of inclusion bodies with the three different solubilisation solutions (section 2.3.2). Each of the solubilised inclusion body solutions were diluted 75, 100 and 130-fold into basic refolding solution. After being diluted into basic refolding solution and allowed to refold for 24 hours at 0 °C the solutions were assayed for hMCM activity. The results obtained are shown in table 3.1.

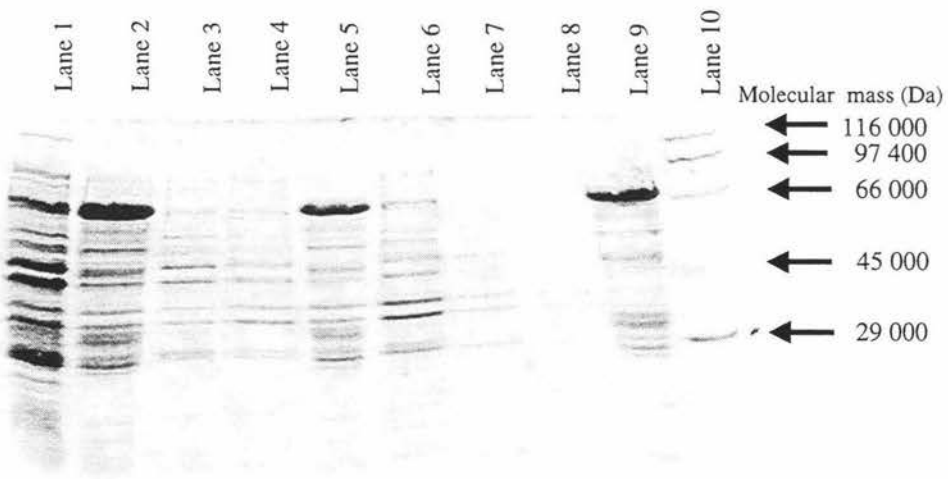


Figure 3.4: SDS-PAGE analysis of large-scale inclusion body purification.

Lane 1:	Initial cell lysate before centrifugation and washing steps.
Lane 2:	First resuspended pellet after low speed centrifugation.
Lane 3 and 4:	Two more supernatants from washes of the inclusion bodies.
Lane 5:	A sample of the inclusion bodies before the washes.
Lanes 6, 7 and 8:	Supernatants from washes of hMCM inclusion bodies.
Lane 9:	Final hMCM inclusion body preparation
Lane 10:	SDS-6H molecular mass markers (Sigma)

Table 3.1: Refolding by rapid dilution of hMCM inclusion bodies solubilised with three different denaturants

Solubilising agent (final concentration)	Final concentration of protein in diluted solution mg/ml	Dilution factor	hMCM activity μmoles/ml /min	Specific hMCM activity μmoles/mg /min
4.5M GdmHCl	0.158	75-fold	7.9×10^{-3}	0.05
4.5M GdmHCl	0.119	100-fold	4.8×10^{-3}	0.04
4.5M GdmHCl	0.091	130-fold	8.2×10^{-3}	0.09
3.75M GdmHSCN$^-$	0.158	75-fold	0	0
3.75M GdmHSCN$^-$	0.119	100-fold	0	0
3.75M GdmHSCN$^-$	0.091	130-fold	0	0
6M Urea	0.158	75-fold	0	0
6M Urea	0.119	100-fold	0	0
6M Urea	0.091	130-fold	0	0

Comparing the activities in table 3.1 to the same dilutions in table 3.2, the specific activities are higher when a final concentration of 6M GdmHCl was used compared to 4.5M GdmHCl. Each value in table 3.2 was an average of two assays. The data in table 3.2 were collected on the same day. Two solubilised samples were rapidly diluted into a basic refolding solution and were assayed twice, after 24 and 26 hours at 0 °C. There appeared to be more activity recovered when the higher concentration of GdmHCl was used to solubilise the inclusion bodies before rapid dilution. Results with the lower concentration of denaturant were more variable. Whether this is a result of the lower concentration of denaturant, or a result of the variability in the assay it is not clear. It was decided to use GdmHCl at a final solubilisation concentration of 6 M to solubilise the inclusion bodies.

Table 3.2: 100-Fold rapid dilutions of hMCM inclusion bodies solubilised at two different final GdmHCl concentrations and assayed in duplicate for hMCM activity

Final GdmHCl concentration when solubilising and time of activity assay after rapid dilution	hMCM activity μmoles/ml/min	hMCM specific activity μmoles/min/mg
4.5 M GdmHCl, 24 hours	4.88×10^{-3}	0.041
4.5 M GdmHCl, 26 hours	7.26×10^{-3}	0.061
6 M GdmHCl, 24 hours	8.45×10^{-3}	0.071
6 M GdmHCl, 26 hours	8.45×10^{-3}	0.071

3.6 The Effect of Protein Concentration on Refolding by Rapid Dilution

Rapid dilution trials were carried out as described in section 2.4.1. The results shown in table 3.3 and graphed in figure 3.5 show a definite optimum range of final protein concentrations, over which there is a greater recovery of hMCM activity after rapid dilution into a refolding solution.

3.7 Scale up of Rapid Dilution Experiments

The solubilisation and small-scale rapid dilution experiments were directly (except for the speed of dilution) scaled up to a level where the final volume of the refolded solution was 250 ml (see section 2.4.2 for details). The final protein concentration in the refolding solution was calculated, based on the starting concentration of inclusion bodies used, to be 0.15 mg/ml. The final 250 ml refolded hMCM solution was assayed for hMCM activity several times and the

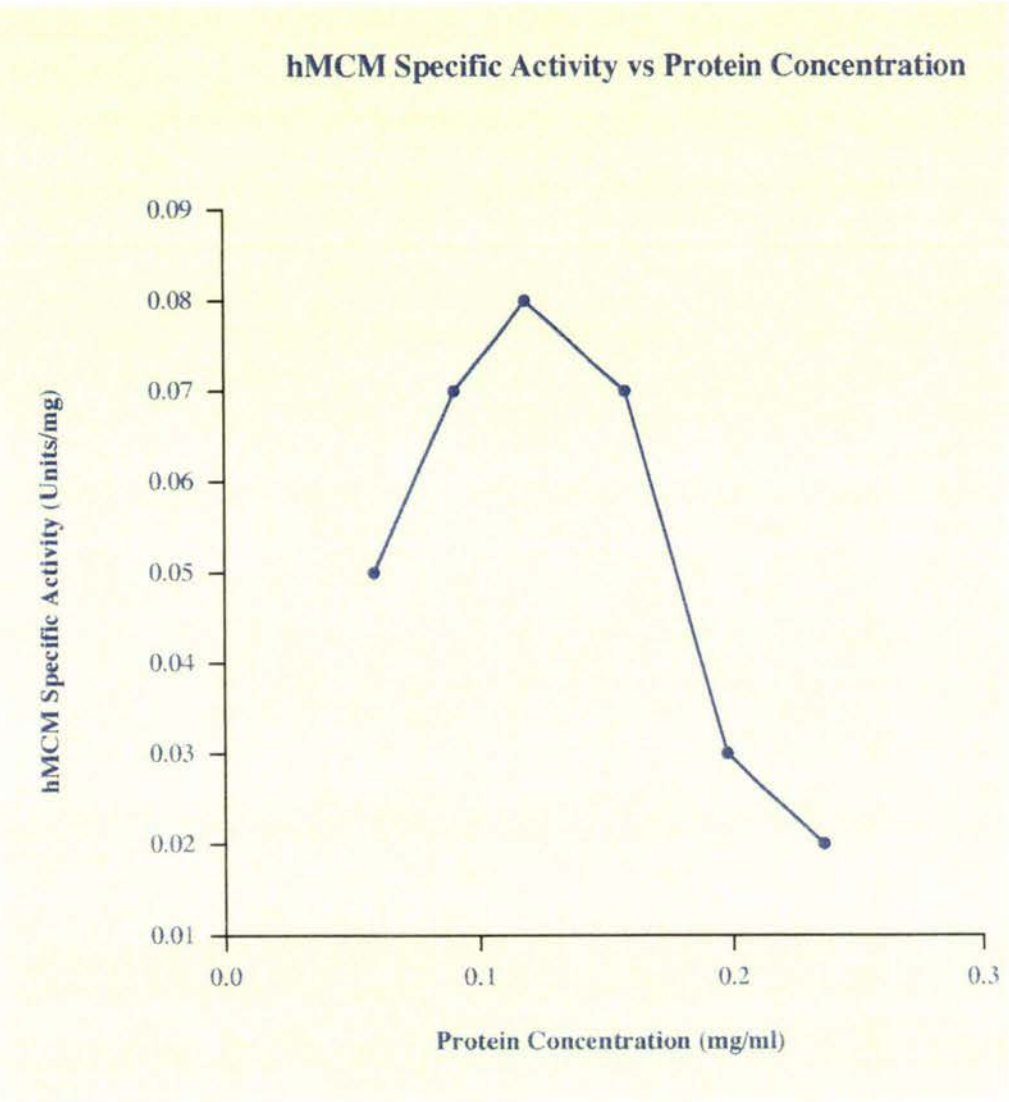


Figure 3.5: Graph showing the hMCM activity recovered after rapid dilution experiments over a range of final protein concentrations.

Table 3.3: Effect of protein concentration on activity regain when refolding by rapid dilution into a non-denaturing buffer or refolding solution

Protein concentration in dilution mg/ml	Dilution factor	hMCM activity in refolded solution μmoles/ml/min	hMCM specific activity μmoles/min/mg
0.237	50-fold	4.7×10^{-3}	0.02
0.198	60-fold	5.9×10^{-3}	0.03
0.158	75-fold	11×10^{-3}	0.07
0.119	100-fold	9.5×10^{-3}	0.08
0.091	130-fold	6.4×10^{-3}	0.07
0.059	200-fold	3.0×10^{-3}	0.05

results were averaged. The solution had an average specific activity of 0.044 units/mg of calculated protein. Because all the protein in the refolded solution would not have been soluble, the concentration of soluble protein would have been less than 0.15 mg/ml and so the hMCM specific activity would have been >0.044 units/mg.

3.8 Time Trial Experiment

An experiment was carried out as described in section 2.4.5 to track the refolding of hMCM, by measuring the gradual increase in hMCM activity, over a 24 hour period following rapid dilution into refolding buffer and subsequent storage on ice. A final protein concentration of 0.14 mg/ml was used, as that had given optimal refolding in previous rapid dilution experiments. Despite the poor reproducibilty of the MCM assay at these low activity levels, figure 3.6 clearly shows a definite increase in hMCM activity over 24 hours.

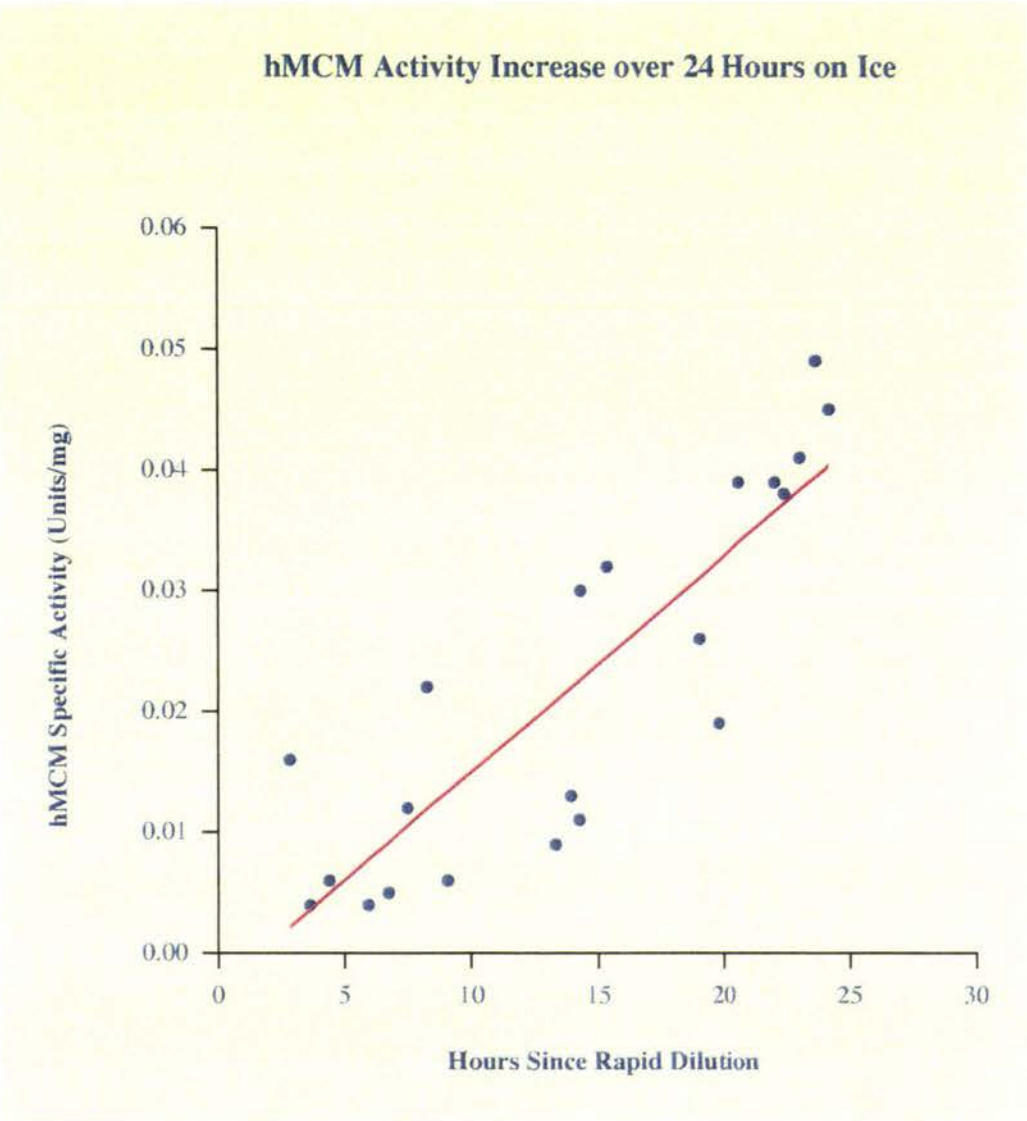


Figure 3.6: Plot of the gain in hMCM activity during 24 hours refolding on ice.

3.9 Dimerization Experiment

A separate hydroxyapatite adsorption and elution of a 250 ml refolded solution of hMCM was carried out as described in section 2.4.6. This experiment was designed to confirm a previously observed increase in hMCM activity over time while in solution at 4 °C after being eluted from hydroxyapatite. It was postulated that an apparent increase in activity in HTP concentrated refolded solutions of hMCM might be due to the gradual association of already refolded hMCM α-subunits, which are most likely inactive until dimerization occurs (Thoma and Leadlay, 1996). The shortened pumping time compared to other large scale rapid dilution experiments resulted in a cloudy refolded solution, indicating that there was substantial aggregation and precipitation of folding intermediates and denatured protein in this solution. In earlier large scale rapid dilution experiments where the solubilised inclusion bodies were pumped into the refolding solution over a longer period of time there was some aggregation but much less than the amounts seen here.

Table 3.4 shows the hMCM activity over time in each of the fractions that were eluted from the hydroxyapatite column. From these results it was apparent that the majority of the hMCM activity eluted from the HTP column was contained in fraction 12, in ~0.3 M potassium phosphate buffer, 0.5 mM DTT, pH 7.2. Fraction 12 was stored at 0 °C and sampled as described in section 2.4.6. The increase in specific activity of fraction 12 over the 189 hours can be seen from the data in table 3.5 and more clearly in figure 3.7. There was only a ~20% increase in activity in total which, given the variation in the activity assay, may not be significant, however, the increase seems real.

Samples were also taken over 189 hours to be run on a native-PAGE gel. One 7% native gel was run containing three native PAGE molecular weight markers and 7 samples that were taken during the 189 hours, but an unsatisfactory result was obtained because the amount of protein in each sample which was too low to show up on the gel, and the concentration of salt in the samples was too high to run with the running buffer of the native gel. The size markers (Sigma, non-denaturing protein, Molecular

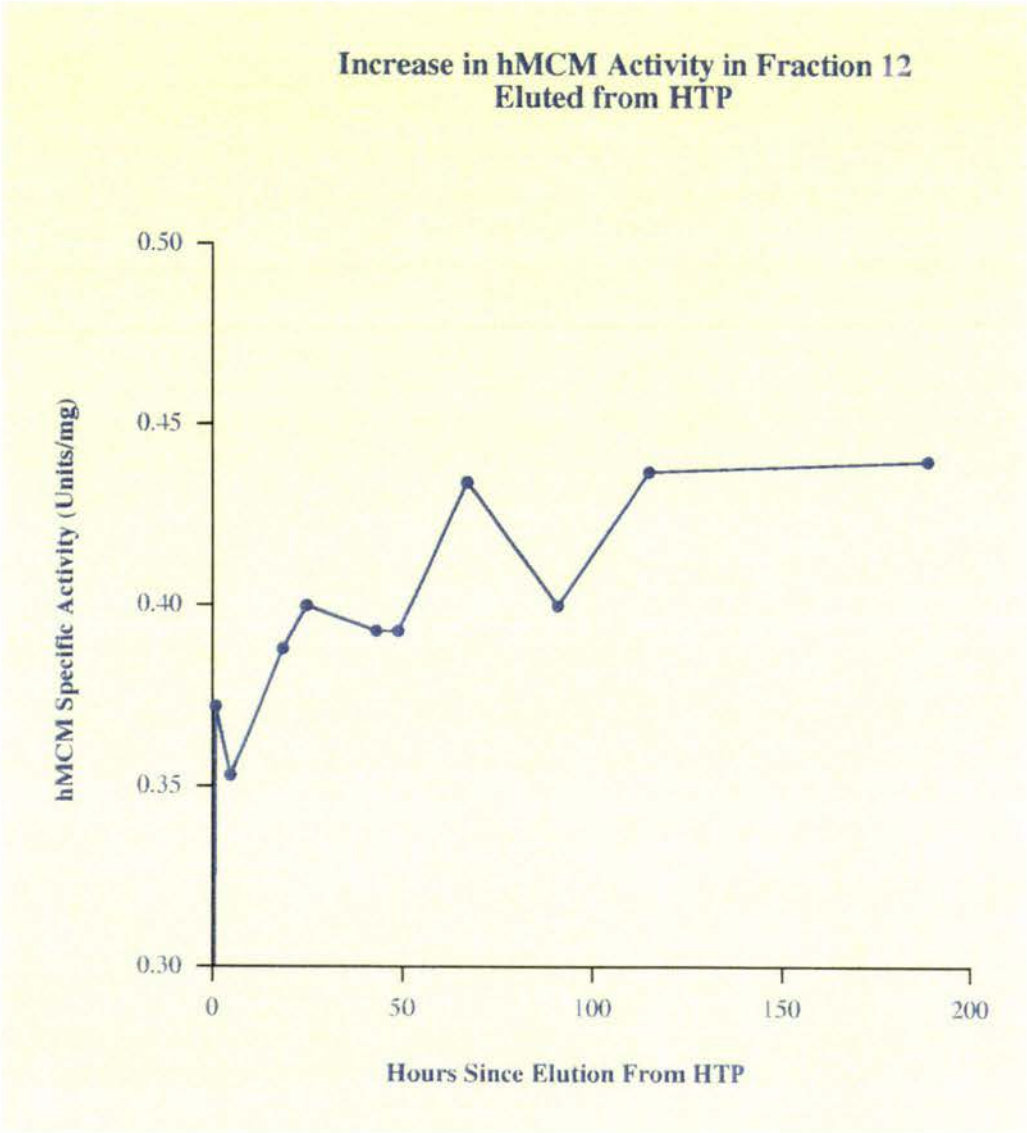


Figure 3.7: Graph of hMCM specific activity (Units/mg) in fraction 12 vs the number of hours since fraction 12 was eluted from hydroxyapatite.

Table 3.4: hMCM activity assays of fractions eluted from hydroxyapatite

Fraction number	Hours after elution	μmoles/ml/min
Control	24	0
8	48	0
9	48	0
10	48	0.019
10	216	0.018
11	24	0.009
11	48	0.026
11	216	0.020
12	24	0.228
12	48	0.311
12	48	0.351
12	48	0.364
12	216	0.297
13	24	0.077
13	48	0.090
13	216	0.095
14	48	0.043
14	216	0.049
15	48	0.022
15	216	0.021
16	48	0.016
17	48	0.025
17	216	0.028
18	48	0.021
19	48	0.020
19	216	0.019
20	48	0.020
21	48	0.030
21	216	0.032
22	48	0.013

Table 3.5: hMCM activity assays of HTP fraction 12 over 189 hours

Hours after elution from hydroxyapatite	hMCM activity mmoles/ml/min	hMCM specific activity mmoles/min/mg of protein
0	0	0
1	0.205	0.372
5	0.194	0.353
19	0.213	0.388
25	0.220	0.400
43	0.216	0.393
49	0.216	0.393
67	0.239	0.434
91	0.220	0.400
115	0.240	0.437
189	0.242	0.440

weight marker kit) were clearly visible on the native gel, but the samples from the dimerization experiment were not. Repitition of this native-PAGE gel was necessary but due to time constraints analysis of these samples by this method was not attempted again. Another possible approach to investigating the putative dimerization process would have been to treat samples with protein crosslinking reagents under conditions favouring intermolecular subunit crosslinking, and examine the reaction products by SDS-PAGE.

A 'side' experiment was carried out to give some indication of what effect the freezing and thawing of the samples had on the stability of the refolded hMCM in fraction 12. The results indicated a decrease in hMCM activity , but due to the variability of the

assay it was difficult to estimate how much hMCM activity was lost. Active hMCM, stored at 4 °C in ~0.3 M potassium phosphate buffer pH 7.2 after elution from hydroxyapatite, has been seen to maintain its full activity for at least 9 months. These two observations suggest that storage on ice is a better means of short term storage to preserve hMCM activity rather than subjecting the sample to freezing and thawing.

3.10 Detergent-Assisted Refolding

Detergent-assisted refolding was carried out as described in section 2.4.4. Tables 3.6a and 3.6b show how variable the hMCM activity assay was. The average control specific activity was 0.063 μmoles/min/mg of protein, but this was over a number of days and would vary from day to day. On days when relatively high specific activities were obtained with a detergent present, this specific activity was never higher than the specific activity of the control prepared and assayed on the same day. None of the detergents tested were effective in increasing refolding by rapid dilution. During hMCM refolding the presence of some detergents in the refolding solution e.g. Z-3-10, Z-3-12, Nonidet P40, lauryl maltoside, Lubrol PX and SDS appeared to prevent refolding. This may be due to an interaction between the detergent molecules and the folding protein, preventing folding to its native conformation. Another possibility is that the protein has refolded correctly but the detergent molecules are still associated with the folded protein in such a way as to prevent the binding of the coenzyme or substrate, or even to prevent subunit association. Also, the presence of some of the detergents in the sample being assayed may have interfered with one or more of the coupling enzymes in the hMCM activity assay, preventing detection of active hMCM. These values were all obtained before the hMCM activity assay was changed, by the addition of one of the components of the assay other than the sample to start the assay, as described in section 2.10.3, so the measurement of these small amounts of activity would not have been very accurate.

Table 3.6a: Table of hMCM refolding by rapid dilution using detergent-supplemented refolding buffers

Detergent added to refolding solution	Concentration of detergent in refolding solution mM	hMCM activity mmole/ml/min	hMCM specific activity mmole/min/mg
No Detergent Avg	0	7.56×10^{-3}	0.063
Z-3-10	2.8	3.36×10^{-3}	0.028
	14	-1.56×10^{-3}	-0.013
	70	-4.56×10^{-3}	-0.038
Z-3-12	0.28	7.20×10^{-4}	0.006
	1.4	-3.60×10^{-4}	-0.003
	7.0	-8.40×10^{-4}	-0.007
Z-3-14	0.028	1.68×10^{-3}	0.014
	0.14	9.60×10^{-4}	0.008
	0.7	0	0
Z-3-16	0.0028	5.16×10^{-3}	0.043
	0.014	9.48×10^{-3}	0.079
	0.07	5.88×10^{-3}	0.049
Nonidet P-40	0.022	9.24×10^{-3}	0.077
	0.11	5.88×10^{-3}	0.049
	0.55	-2.88×10^{-3}	-0.024
Triton X-100	0.058	-1.08×10^{-3}	-0.009
	0.29	-1.44×10^{-3}	0.012
	1.45	4.20×10^{-3}	0.035
Lauryl Maltoside	0.024	5.52×10^{-3}	0.046
	0.12	4.80×10^{-4}	0.004
	0.6	-2.64×10^{-3}	-0.022

Table 3.6b: Table of hMCM refolding by rapid dilution using detergent-supplemented refolding buffers

Detergent added to refolding solution	Concentration of detergent in refolding solution mM	hMCM activity mmole/ml/min	hMCM specific activity mmole/min/mg
Tween 20	0.0118	6.48×10^{-3}	0.054
	0.059	5.52×10^{-3}	0.046
	0.295	6.48×10^{-3}	0.054
CHAPS	0.8	6.12×10^{-3}	0.051
	4.0	0	0
	20	-1.20×10^{-3}	-0.010
CHAPSO	0.82	9.12×10^{-3}	0.076
	4.1	5.04×10^{-3}	0.042
	20.5	-2.64×10^{-3}	-0.022
MEGA-9	3.6	7.80×10^{-3}	0.065
	18	1.80×10^{-3}	0.015
	90	-	-
LDAO	0.028	-3.00×10^{-3}	-0.025
	0.14	5.76×10^{-3}	0.048
	0.7	6.36×10^{-3}	0.053
Lubrol PX	0.02	3.00×10^{-3}	0.025
	0.10	-2.40×10^{-3}	-0.020
	0.5	-1.20×10^{-3}	-0.010
SDS	0.3	0	0
	1.5	-3.60×10^{-3}	-0.030
	7.5	-3.36×10^{-3}	-0.028
C_8-β-D Gluc	4.2	3.60×10^{-4}	0.003
	21	8.04×10^{-3}	0.067
	105	8.76×10^{-3}	0.073

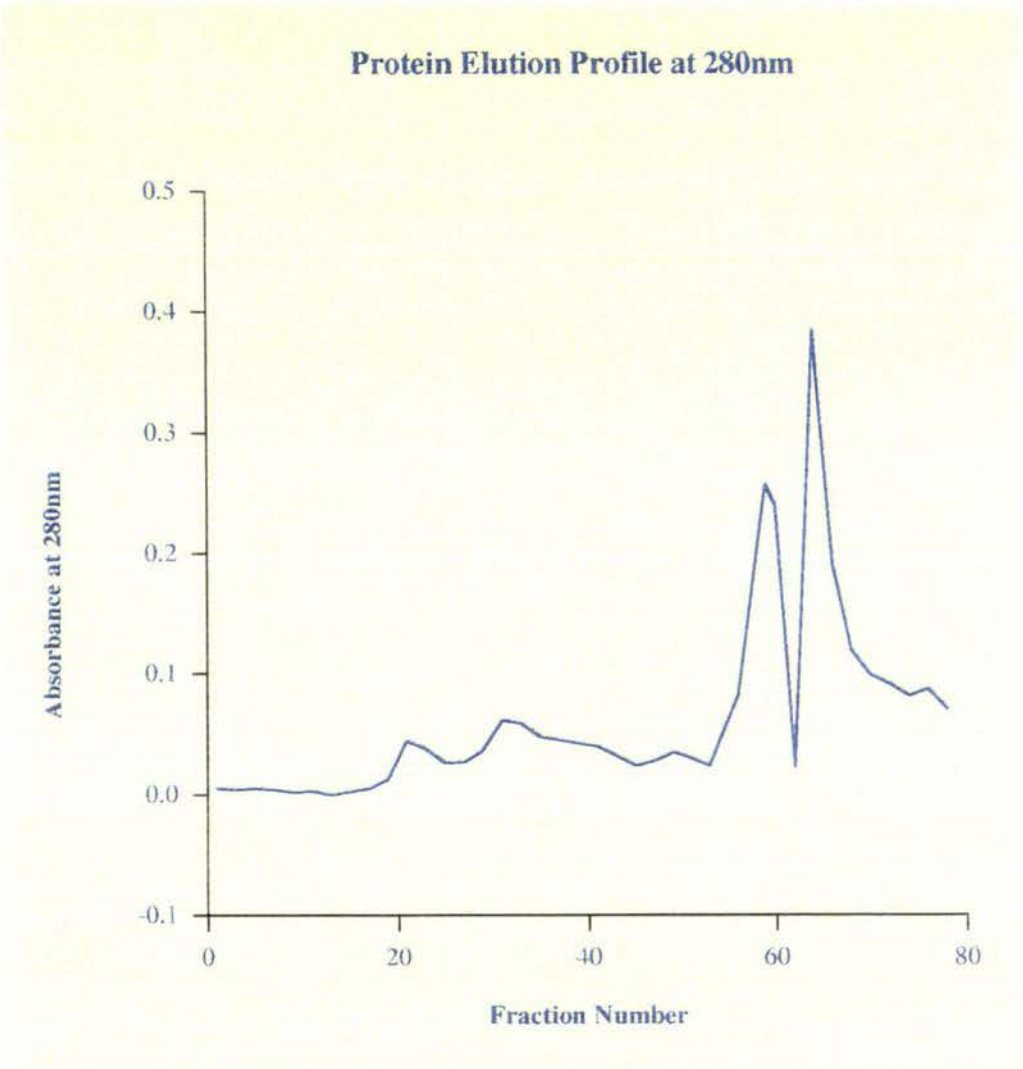


Figure 3.8: A_{280} nm profile of fractions collected during hMCM refolding by gel filtration chromatography.

This graph shows the protein elution profile over the course of the chromatography from fraction 1 which was collected as soon as the sample was completely loaded onto the column.

3.11 Protein Refolding by Dialysis Against a Refolding Buffer

This method was trialled using three different protein concentrations as described in section 2.4.3. The results obtained are shown in table 3.7 the hMCM activity recovered increased as the solubilised protein concentration in the dialysis bag decreased. At very low concentrations of protein compared to the small-scale rapid dilution experiments, the specific activity was higher than in any of the rapid dilution experiments on either the small or larger-scales.

Table 3.7: Refolding by dialysis at three different protein concentrations

Initial protein concentration in dialysis bag mg/ml	hMCM activity recovered after dialysis and storage on ice µmoles/min/ml of sample	hMCM specific activity after dialysis µmoles/min/mg
1.2	4.2×10^{-3}	0.004
0.12	4.1×10^{-3}	0.034
0.012	1.8×10^{-3}	0.150

3.12 Refolding by Gel Filtration Chromatography

Refolding by gel filtration chromatography was attempted as described in section 2.4.7. The A_{280} nm values for the 83 fractions eluted from the gel filtration column are plotted in figure 3.8. 50 µl samples of every third fraction (i.e. 1, 4, 7, 10 etc) were assayed for hMCM activity, but no substantial hMCM activity was found in any fraction. The absence of detectable hMCM activity suggests that refolding on the column, if it occurred at all, was very inefficient. An SDS-polyacrylamide gel was run to analyze the proteins present in the sample loaded and in several fractions (figure 3.9). From the protein elution profile (figure 3.8) fractions 65 and 66 had quite high absorbances at 280 nm but very little protein was evident in the SDS-PAGE analysis. This high

absorbance could not have been due to GdmHCl in the samples as GdmHCl does not absorb at 280 nm, a 6 M solution of research-grade GdmHCl should have an absorbance of <0.05 at 280 nm. This was confirmed by the reaction of fraction 65 with the Coomassie blue G-250 dye-binding reagent which gave a dark blue colour compared to no colour development in a GdmHCl-only control, suggesting high concentrations of protein were present. Lane 6 of figure 3.9, containing the HTP concentrated fractions 23 to 36, contains a faint band corresponding to the hMCM α-subunit. This confirmed that some hMCM had passed through the column. More fractions should have been concentrated as the hMCM may have eluted suddenly in fractions that were not concentrated with HTP, and this may account for the low amounts visible on the SDS-PAGE (figure 3.9).

Two occurrences which could explain the absence of substantial hMCM activity in any of the fractions assayed were the precipitation of protein which occurred on the column during gel filtration, and possibly also a lack of efficient refolding of the hMCM. Whether the hMCM seen faintly in lane 6 of figure 3.9 is correctly folded or still denatured is unknown as it is too dilute to assay even if properly refolded. The concentration of these pooled fractions has also made obvious the presence of a band more dominant than the hMCM band which corresponds to a molecular weight of ~29 kDa (lane 6, figure 3.9). Given the relative abundance of this protein, and its apparent absence in the sample loaded onto the column (lane 9, figure 3.9), it was thought likely that this band represented a degradation product of hMCM. The 29 kDa band in lane 6 of figure 3.9 was later prepared for N-terminal sequencing as described in section 2.9.4 to confirm its identity (see section 3.21).

3.13 *E. coli* GroEL and GroES Chaperonin Purification

The *E. coli* chaperonins GroES and GroEL were purified from 126.3 g of frozen induced DH1/pGroESL cells as described in sections 2.8.2 to 2.8.4. After lysis of the cells and initial centrifugation and filtration steps a DEAE-Sephacel column was run to separate GroEL from GroES prior to further purification of each chaperonin. Part

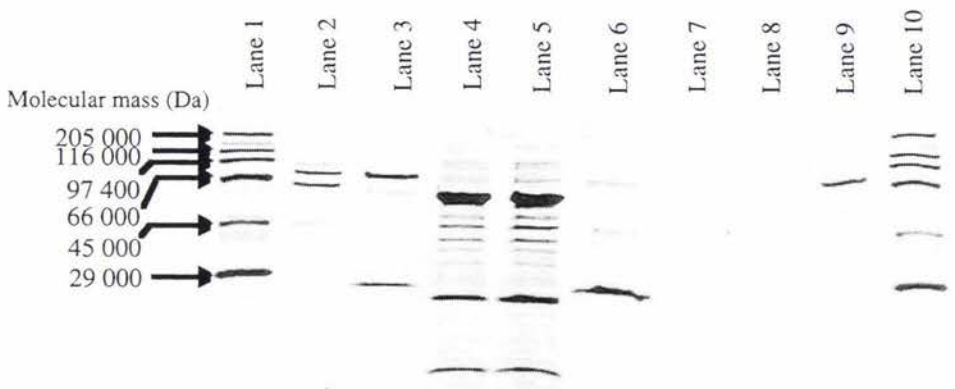


Figure 3.9: SDS-PAGE analysis of fractions collected during refolding by gel filtration chromatography.

Lane 1: SDS-6H molecular mass markers (Sigma).

Lane 2: Partially purified recombinant MCMα and β subunits (McKie *et al.*, 1990).

Lane 3: HTP concentrated sample from 250 ml scale refolding experiment.

Lane 4: Not applicable.

Lane 5: Not applicable.

Lane 6: Concentrated pooled fractions (F23-36) from gel filtration chromatography.

Lane 7: Fraction 65 from refolding by gel filtration chromatography.

Lane 8: Fraction 66 from refolding by gel filtration chromatography.

Lane 9: Sample loaded onto gel filtration column.

Lane 10: SDS-6H molecular mass markers (Sigma).

of the initial clarification of the cell extract involved filtration through 0.2 μM filters, but the composition of the extract made this step difficult. An ultrafiltration step was added prior to filtration to remove excess cell debris that was preventing efficient filtration.

A_{280} was measured continuously during elution of the 165 fractions collected during elution from the DEAE-Sephacel column. Fractions containing GroEL and GroES were identified by analysis of selected fractions on 20% SDS-PAGE gels (figures 3.10 and 3.11). The SDS-PAGE analysis (figures 3.11 and 3.12) showed that fractions 85 to 97 contained the majority of the GroEL in a fairly pure state. GroEL has a subunit molecular mass of ~60 kDa and migrates as expected slightly faster than the 66 000 Da marker in the SDS-7 molecular weight standard. Lane 2 of figure 3.10 shows that not all of the GroEL in the sample loaded bound to the DEAE-Sephacel, as it is a major component of the wash-through. No GroES is apparent in the wash-through, so it was assumed that all of the sample GroES bound to the DEAE-Sephacel column. This loss of GroEL in the wash-through may be due to the high sample protein concentrations (20 mg/ml), but it is more likely to be due to the large size of $GroEL_7$ (~420 kDa), as the capacity of DEAE-Sephacel drops as the size of the protein approaches the 1 MDa exclusion limit of the matrix. Fractions 85 to 97 were pooled for further GroEL purification. GroES has a subunit molecular mass of ~10 kDa, but migrated at a higher than expected electrophoretic mobility, i.e. more slowly than the 14 200 Da SDS-7 molecular weight standard, at ~16 kDa. Amrein *et al.*, (1995) also observed this anomalous migration for GroES in SDS-polyacrylamide gels (see figure 3.13). Fractions 57 to 66 contained large amounts of GroES (figures 3.12 and 3.14) and were pooled.

After a buffer exchange step, GroES was purified further by two anion exchange steps (section 2.8.3). First a second DEAE-Sephacel column chromatography step was carried out (figure 3.15). Detailed analysis of the fractions from this step by SDS-PAGE (figures 3.16 and 3.17) showed that fractions 47 to 53 contained fairly pure GroES, and these fractions were pooled (total volume 95 ml).

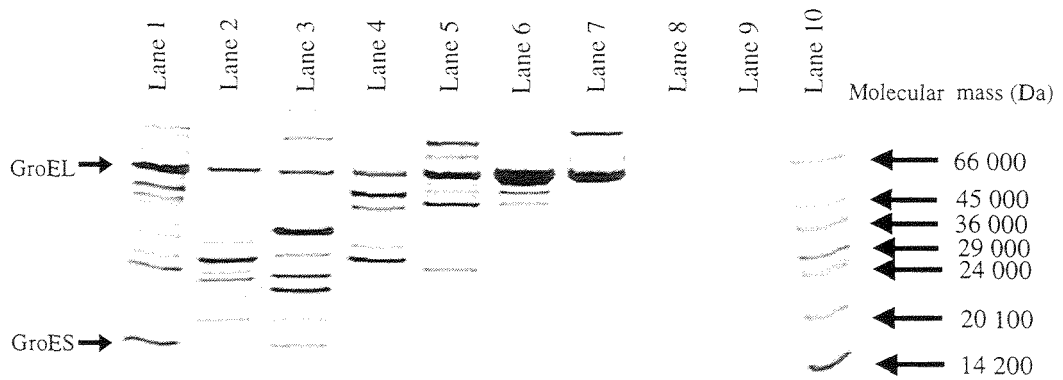


Figure 3.10: SDS-PAGE analysis of sample collected during purification of GroES and GroEL on a DEAE-Sephacel column.

Lane 1: Sample loaded onto DEAE-Sephacel column.

Lane 2: Sample of wash-through collected during loading.

Lane 3: Fraction 38.

Lane 4: Fraction 48.

Lane 5: Fraction 78.

Lane 6: Fraction 89.

Lane 7: Fraction 100.

Lane 8: Fraction 144.

Lane 9: Fraction 151.

Lane 10: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

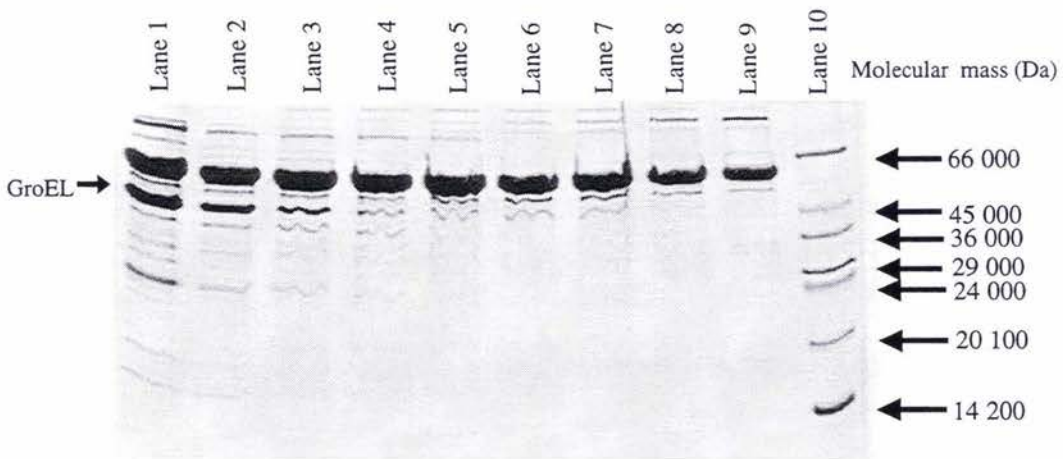


Figure 3.11: SDS-PAGE analysis of fractions 81 to 97 collected during purification of GroES and GroEL on a DEAE-Sephacel column.

Lanes 1 to 9: Fractions 81, 83, 85, 87, 89, 91, 93, 95 and 97 respectively.

Lane 10: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

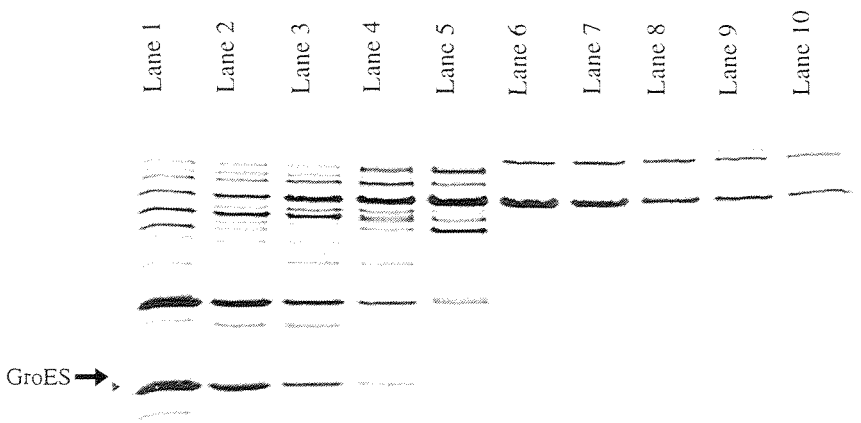


Figure 3.12: SDS-PAGE analysis of fractions 66 to 78 and 100 to 112 collected during purification of GroES and GroEL on a DEAE-Sephacel column.

Lanes 1 to 5: Fractions 66, 69, 72, 75 and 78 respectively.

Lanes 6 to 10: Fractions 100, 103, 106, 109 and 112 respectively.

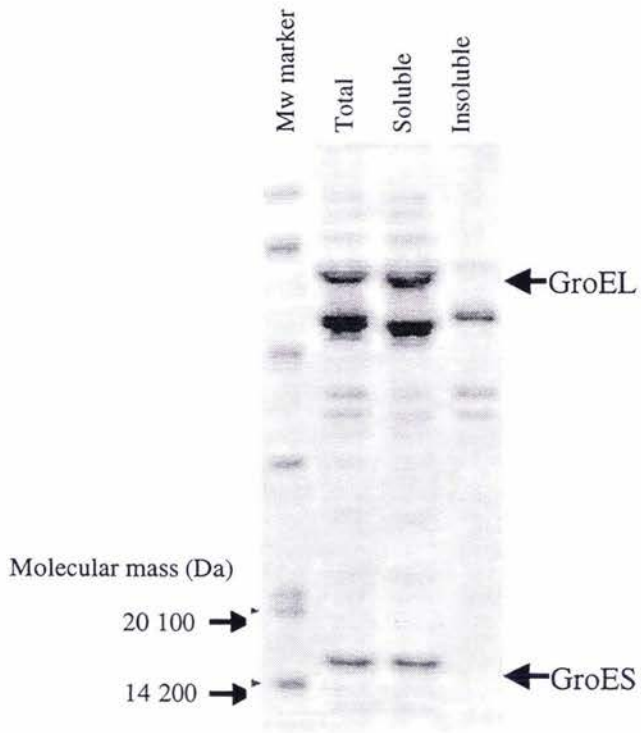


Figure 3.13: SDS-PAGE analysis of the *E. coli* chaperonin GroES (Amrein *et al.*, 1995).

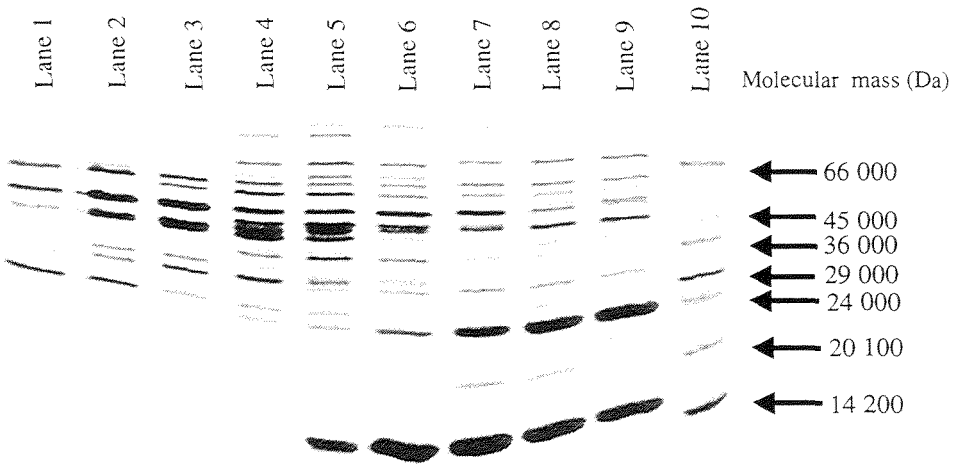


Figure 3.14: SDS-PAGE analysis of fractions 48, 50, 52, 54, 56, 58, 60, 62, and 64 collected during the purification of GroES and GroEL on a DEAE-Sephacel column.

Lanes 1 to 9: Fractions 48, 50, 52, 54, 56, 58, 60, 62 and 64 respectively.

Lane 10: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

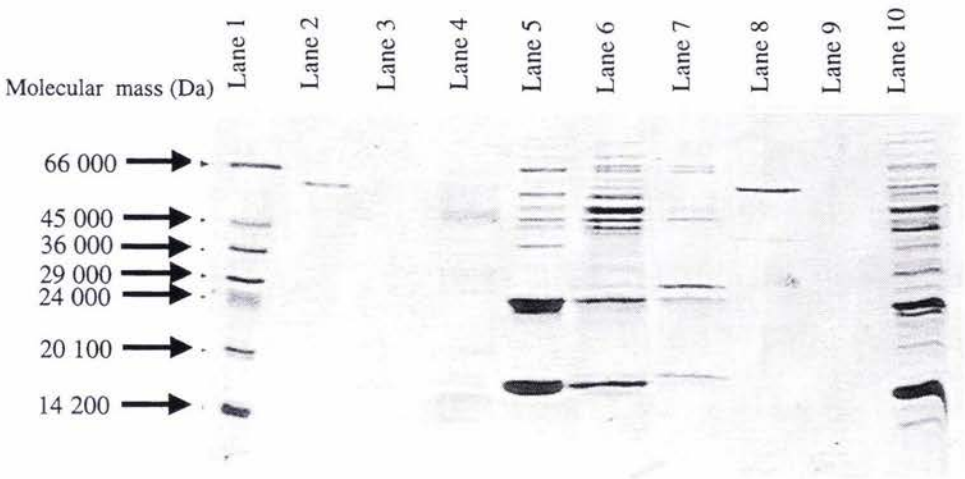


Figure 3.15: SDS-PAGE analysis of fractions collected during the second DEAE-Sephacel chromatography step in the GroES purification.

Lane 1: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

Lane 2: Not applicable.

Lane 3: Fraction 30.

Lane 4: Fraction 40.

Lane 5: Fraction 50.

Lane 6: Fraction 62.

Lane 7: Fraction 68.

Lane 8: Not applicable.

Lane 9: Wash collected while loading the GroES pool onto DEAE-Sephacel.

Lane 10: GroES pool loaded onto DEAE-Sephacel column.

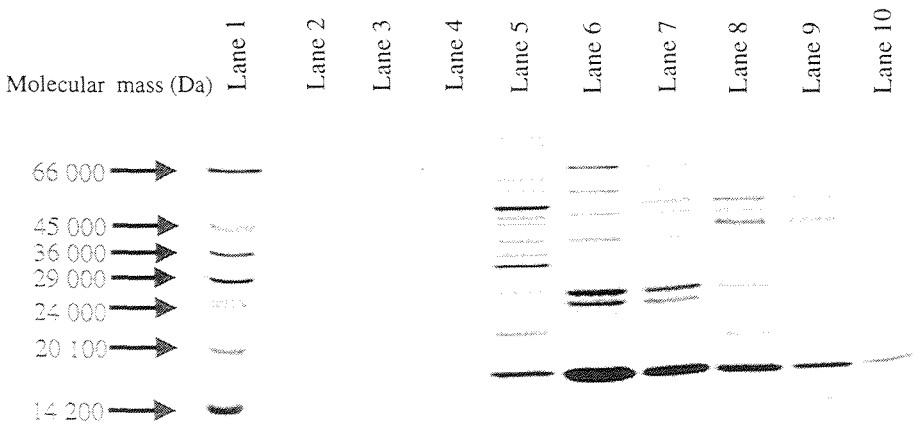


Figure 3.16: SDS-PAGE analysis of more fractions collected during the second DEAE-Sephacel chromatography step in the GroES purification.

Lane 1: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

Lanes 2 to 10: Fractions 37, 40, 43, 46, 49, 52, 55, 58 and 61 respectively.

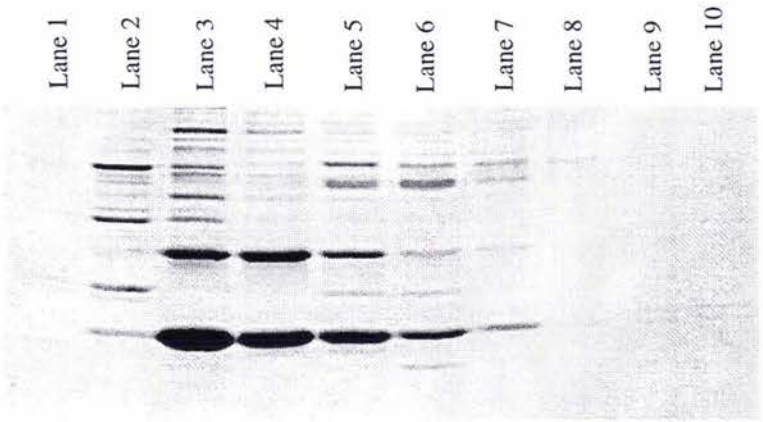


Figure 3.17: Further SDS-PAGE analysis of fractions collected during the second DEAE-Sephacel chromatography step in the GroES purification.

Lanes 1 to 10: Fractions 42, 45, 48, 51, 54, 57, 60, 63, 66 and 68 respectively.

The buffer of the GroES pool was exchanged again and the sample was loaded onto the final anion exchange column, a HiLoad 26/10 Q-Sepharose high performance ion exchange column, and eluted with a salt gradient. A number of fractions contained highly purified recombinant GroES (lanes 3, 4, 5 and 6 of figure 3.18). Fractions 39, 40 and 41 were pooled, concentrated, and stored at -70 °C in 1 ml aliquots. The final GroES preparation had a protein concentration of 38 mg/ml in 8.7 ml. The putative GroES band was blotted and the N-terminus was sequenced (sections 2.9.4 and 2.9.5). The determined N-terminal sequence was compared with the known N-terminal sequence for GroES:

Determined sequence:	MNIR-
Known sequence of GroES:	MNIRPLHDRV-

This result confirmed the identity of this band as GroES. The protocol that this purification was based on (Hendrix, 1979; Chandrasekhar *et al.*, 1986) had further steps in the purification of GroES, but for the purposes of this study it was decided that the GroES was of adequate purity.

The GroEL pool from the first DEAE-Sephacel chromatography step was divided into two aliquots and loaded onto a 2.5 × 80 cm (400 ml) Sephacryl S-300 column. Fractions collected during the chromatography runs were assessed for purity by SDS-PAGE on 15 % polyacrylamide gels. Figure 3.19 shows the pooled fractions 22 to 26 collected from the first 15 ml of sample run through the gel filtration column. Figure 3.20 shows the pooled fractions 67 to 71 collected from the second 15 ml of sample run through the gel filtration column. The final GroEL preparation in 15% (w/v) glycerol had a total volume of 23 ml and a protein concentration of 36 mg/ml. 1 ml aliquots were stored at -70 °C. The putative GroEL protein band was electroblotted and the N-terminus was sequenced (sections 2.9.4 and 2.9.5). The determined N-terminal sequence was compared with the known N-terminal sequence for GroEL:

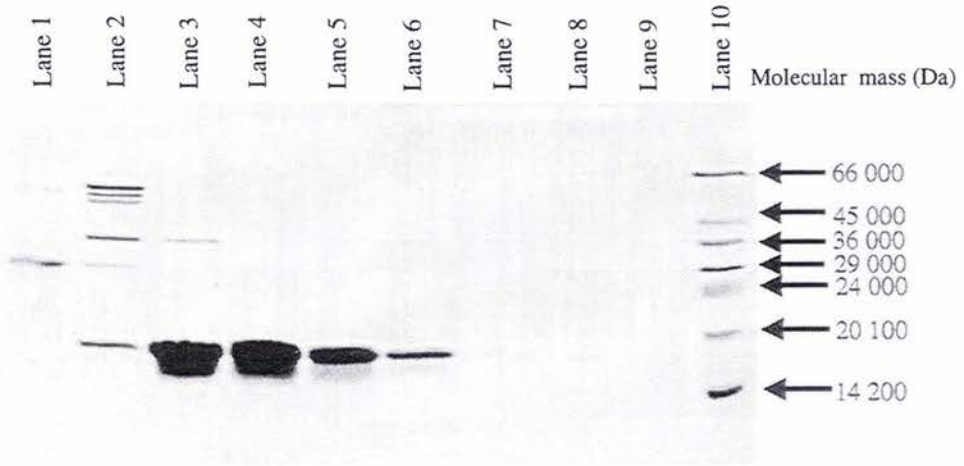


Figure 3.18: SDS-PAGE analysis of fractions collected during elution of the HiLoad 26/10 Q-Sepharose high performance column, during the GroES purification.

Lanes 1 to 9: Fractions 37 to 45 respectively.

Lane 10: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

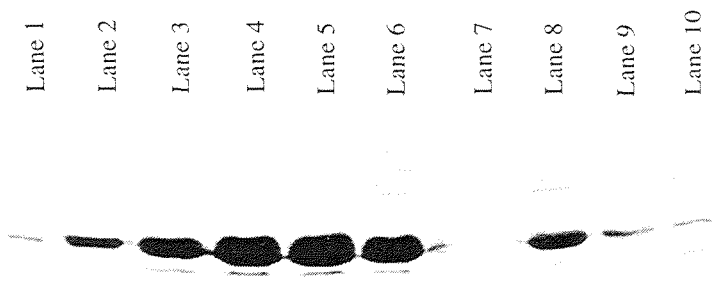


Figure 3.19: SDS-PAGE analysis of fractions collected during Sephacryl S300 chromatography of the first half of the GroEL sample.

Lanes 1 to 10: Fractions 20 to 29 respectively.

Note: Lane 7 contains a sample which accidently became diluted during its prepation for SDS-PAGE. Given the intensity of the bands in lane 6 and lane 8 it was decided that fraction 26 in lane 7 was appropriate to include in the pool.

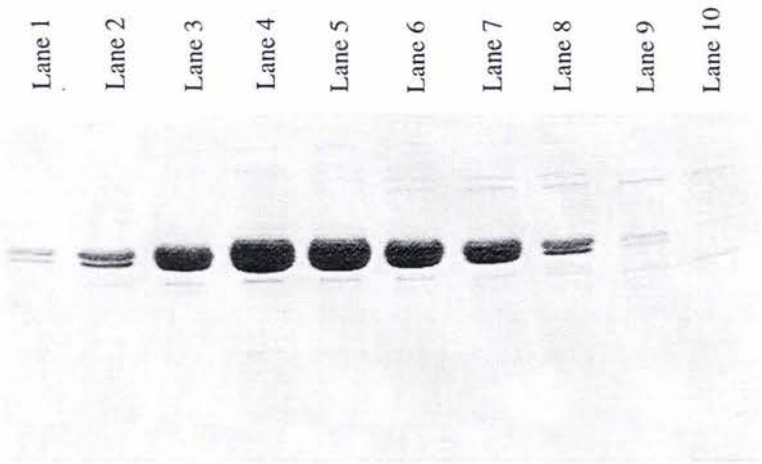


Figure 3.20: SDS-PAGE analysis of fractions collected during Sephacryl S300 chromatography of the second half of the GroEL sample.

Lanes 1 to 10: Fractions 65 to 74 respectively

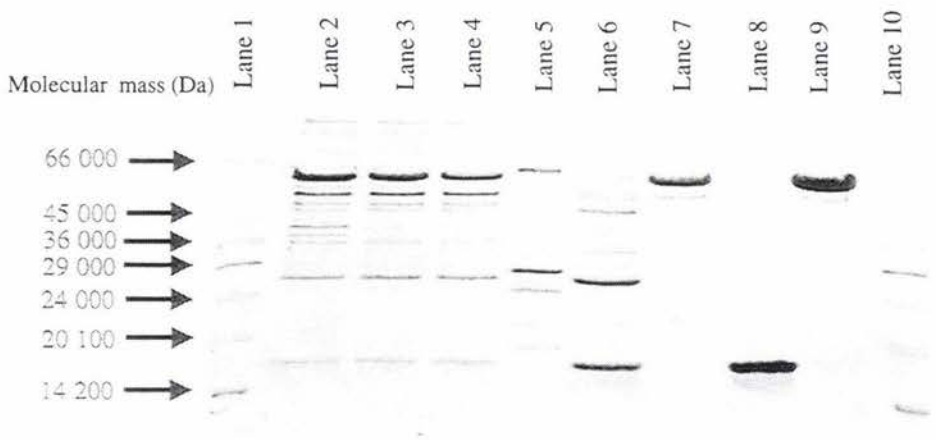


Figure 3.21: Combined GroES and GroEL purification; SDS-PAGE analysis

Lane 1: SDS-PAGE low molecular weight markers (Sigma Cat. No. SDS-7).

Lane 2: DH1/pGroESL cell lysate.

Lane 3: Supernatants from centrifugations of the cell lysate.

Lane 4: Sample loaded onto the first DEAE-Sephacel column.

Lane 5: Wash collected during loading.

Lane 6: 1st GroES pool after initial purification on DEAE-Sephacel column.

Lane 7: 1st GroEL pool after initial purification on DEAE-Sephacel column.

Lane 8: Final GroES preparation in 15% (w/v) glycerol.

Lane 9: Final GroEL preparation in 15% (w/v) glycerol.

Lane 10: SDS-PAGE low molecular weight markers (Sigma Cat. No SDS-7).

Determined sequence:	AAKDVK-
Known sequence of GroEL:	MAAKDVKFG-

This result confirmed the identity of this band as the *E. coli* chaperonin GroEL. The observation that the N-terminal methionine of the GroEL protein was cleaved off and the N-terminal methionine of GroES remained on the protein, illustrates the substrate preference of the *E. coli* methionine aminopeptidase (MAP) protein. It has been shown that the residues adjacent to the initiation methionine significantly influence the methionine cleavage process (Ben-Bassat *et al.*, 1987). In most instances as is illustrated here an adjacent residue with a side chain size smaller than 0.129 nm (e.g. alanine, A) is a preferred substrate for MAP; for side chain sizes larger than 0.143 nm (e.g. asparagine, N, as in the case of GroES), MAP is unable to cleave the methionine.

Figures 3.19 and 3.20 indicate that there may be some proteolysis of the GroEL. Lanes 1 and 9 of figure 3.20, where lower concentrations of protein in these lanes allows the doublet pattern in the GroEL band to be seen. Proteolysis may have occurred at the C-terminal end of the protein, as the results of N-terminal sequencing of this doublet showed the N-terminus to be intact. Samples taken at various stages during the purification of GroEL and GroES were analysed on a 20% SDS-PAGE gel (figure 3.21), as a summary of the purification. Purity of the GroES and GroEL were assessed by examination on SDS-PAGE (figure 3.21) and was determined that both GroES and GroEL were ≥90% pure. The yield obtained in this current study from 126 g of cells, of 330 mg of GroES and 828 mg of GroEL are higher than those from the longer method used by George Lorimer, (DU PONT, Pers. Comm.), which gave yields of 250 mg of GroES, and 1 g of GroEL, from 500 g of cells.

3.14 Testing Purified GroEL and GroES for Biological Activity

The activity of the purified recombinant *E. coli* chaperonins was tested by using them to refold *E. coli* β-galactosidase. The *E. coli* chaperonin $GroEL_{14}$ complex aids in the

refolding of denatured *E. coli* β-galactosidase *in vitro* (Ayling and Baneyx, 1996).Active *E. coli* β-galactosidase (Grade VI, Sigma Cat. No. G-6008) was denatured and refolded, with and without the presence of the purified $GroEL_{14}$ and $GroES_7$, as described in section 2.8.5. The samples and control solutions set up in duplicate were assayed for β-galactosidase activity then averaged to give the absorbances in table 3.8.

Table 3.8: Refolding of *E. coli* β-galactosidase in the presence of GroES and GroEL

Result	Description of sample	Average absorbance at 420nm
1	Denatured β-galactosidase diluted into refolding solution II	0.089
2	Denatured β-galactosidase diluted into refolding solution II + $GroEL_{14}$	0.919
3	Denatured β-galactosidase diluted into refolding solution II + $GroES_7$	0.114
4	Denatured β-galactosidase diluted into refolding solution II + $GroEL_{14}$ + $GroES_7$	1.042
5	Control β-galactosidase diluted into refolding solution II	1.441
6	Control β-galactosidase diluted into refolding solution II + $GroEL_{14}$	2.164
7	Control β-galactosidase diluted into refolding solution II + $GroES_7$	1.303
8	Control β-galactosidase diluted into refolding solution II + $GroEL_{14}$ + $GroES_7$	2.265

Results 1 and 5 from table 3.8 show a 6% recovery of β-galactosidase activity due to rapid dilution of the denatured protein into basic refolding solution. Results 2 and 6 represent a 42% recovery of β-galactosidase due to rapid dilution into refolding solution containing the purified GroEL chaperonin. The presence of $GroES_7$ in the refolding solution on its own did not aid in the refolding and subsequent recovery of activity of the denatured β-galactosidase. These results were promising as the GroEL

appeared to be active after a lengthy purification process and could therefore be tested to see if it could aid in the refolding of hMCM from solubilised inclusion body material.

Results 6 and 8 in table 3.8 are quite high absorbances, it appears as though the presence of GroEL in the refolding solution has increased the amount of β-galactosidase activity, so it is possible that the β-galactosidase activity in result 2 is due to contamination of GroEL by β-galactosidase. Such contamination is possible, given that the DH1/pGroESL cells were induced with lactose (M.L. Patchett, Pers. Comm.), which would also have induced the expression of β-galactosidase. One control which was not carried out was a β-galactosidase assay of refolding solution containing GroEL and GroES but no *E. coli* β-galactosidase. This would have ruled out any background β-galactosidase activity due to contamination of the GroEL preparation. In hindsight, it might have been better to choose a test protein with an activity not found in *E. coli*, e.g. carbonic anhydrase (Persson *et al.*, 1997). Another control which should have been carried out was a refolding solution containing a large protein such as BSA, which would account for any refolding that may occur due to the presence of a large protein in solution with the folding protein.

3.15 Chaperonin-Assisted Refolding of Solubilised hMCM Inclusion Body Material

The use of purified *E. coli* chaperonins GroEL and GroES to aid in the refolding of solubilised hMCM inclusion bodies was based on the experiments described in section 2.8.5 where the chaperonins appeared to be successfully used to assist in the refolding of GdmHCl-denatured β-galactosidase. The chaperonin multimers/oligomers were added in different combinations to the basic refolding solution at a 2-fold molar excess over the hMCM α-subunit monomers (section 2.8.6). ATP and Mg^{2+} were also added in different combinations to the refolding solution as they are needed for the $GroEL_{14}$ and $GroES_7$ complexes to exhibit refolding activity (Langer *et al.*, 1992). When initial chaperonin-assisted *in vitro* refolding experiments failed to yield any hMCM activity it was decided to test whether a component of the basic refolding solution might be interfering with the refolding activity of the chaperonins. This required rapid dilution

of the solubilised inclusion bodies into refolding solutions containing the purified chaperonins, but systematically omitting components of the basic refolding solution described in section 2.4.1. As well as indicating that arginine and glycerol are essential components of the refolding solution, this experiment suggested that no one component of the refolding solution on its own was preventing the chaperonins from refolding hMCM (table 3.9).

Table 3.9: Chaperonin-assisted refolding with incomplete basic refolding solutions

Tube	Description of refolding solution components	Specific activity µmoles/min /mg
1	Basic refolding solution	0.082
2	Basic refolding solution with glycerol left out	0.026
3	Basic refolding solution with arginine left out	0.036
4	Basic refolding solution with potassium fluoride out	0.087
5	Basic refolding solution with EDTA left out	0.087
6	Basic refolding solution with PMSF left out	0.087
7	Basic refolding solution + Chaperonins*	0.026
8	Basic refolding solution with glycerol out + Chaperonins*	0.015
9	Basic refolding solution with arginine out + Chaperonins*	0.000
10	Basic refolding solution with KF out + Chaperonins*	0.026
11	Basic refolding solution with EDTA out + Chaperonins*	0.000
12	Basic refolding solution with PMSF out + Chaperonins*	0.010
13	Basic refolding solution, as a no protein control	0.000

*3 µM $GroES_7$, 3 µM $GroEL_{14}$, 30 µM ATP and 30 µM $MgCl_2$

Refolding of hMCM was also attempted with different combinations of chaperonins

added to refolding solution II described in section 2.8.7 which apparently refolded denatured β-galactosidase, but again there was no further increase in the hMCM activity recovered from the solubilised inclusion body material (results not shown). Finally, different combinations of the chaperonins GroEL and GroES, as well as ATP and Mg^{2+}, were added to the basic refolding solution in an attempt to find conditions that would assist in hMCM refolding by rapid dilution of solubilised inclusion body material (experimental details are described in section 2.8.6). BSA was added to one of the refolding solutions as a negative control to determine to if any assisted refolding in the presence of the chaperonins might simply be due to the presence of another protein in the refolding solution. Table 3.10 gives the results of these rapid dilution experiments and shows that there was still no success in the chaperonin assisted

Table 3.10: Chaperonin-assisted hMCM refolding by rapid dilution

Tube	Additions to basic refolding solution	Specific activity in refolded hMCM solution mmoles/min/mg
1	$GroEL_{14}$	0.046
2	$GroEL_{14}/GroES_7$	0.008
3	$GroES_7$	0.036
4	$GroEL_{14}$/ATP	0.005
5	$GroEL_{14}/GroES_7$/ATP	0.005
6	$GroES_7$/ATP	0.046
7	$GroEL_{14}/ATPMg^{2+}$	0.005
8	$GroEL_{14}/GroES_7/ATPMg^{2+}$	0.003
9	$GroES_7/ATPMg^{2+}$	0.049
10	BSA	0.031
11	Basic refolding solution with no additions	0.092
12	'No hMCM'-control, basic refolding solution	0.008

refolding of hMCM by rapid dilution of solubilized inclusion bodies. The best recovery of hMCM activity gained by rapid dilution of the solubilised inclusion bodies was when the basic refolding solution was used. The presence of different combinations $GroEL_{14}$, $GroES_7$, ATP and Mg^{2+} appeared to inhibit refolding by rapid dilution. If one combination did result in assisted refolding by rapid dilution, the presence of the other components masked any extra hMCM activity. Had a combination of chaperonins been found that assisted in folding *in vitro* then more trials would have been done to minimise the amount of the chaperonin needed to obtain adequate levels of refolding.

3.16 Co-expression of Chaperones *In Vivo* with hMCM

The aim of this experiment was to express the chaperones GroEL and GroES at elevated levels in the cells expressing hMCM. pMEXHCO contains the cDNA which codes for hMCM and also confers ampicillin resistance. Transformation of this plasmid into $CaCl_2$-competent BL21(DE3)/pGroESL *E. coli* cells was attempted with the appropriate controls as described in section 2.1. The pGroESL plasmid confers chloramphenicol resistance and contains the genes for the *E. coli* chaperones GroEL and GroES under the control of an IPTG-responsive promoter (Colandene and Garrett, 1996). No BL21(DE3)/pGroESL/pMEXHCO transformants were generated. The transformations were repeated with higher concentrations of pMEXHCO plasmid DNA, and with freshly made $CaCl_2$-competent cells, still with no success, whereas all the controls gave the expected results. Further controls were carried out and pT7-7 was successfully transformed into the $CaCl_2$-competent BL21(DE3)/pGroESL *E. coli* cells. pT7-7 is the parent plasmid of pMEXHCO (i.e. the plasmid into which the cDNA of hMCM was subcloned). pCMV, which contains the hMCM cDNA and also confers ampicillin resistance, was successfully transformed into the $CaCl_2$-competent BL21(DE3)/pGroESL *E. coli* cells. This transformant grew on 2TY agar containing ampicillin (10υ μg/ml) and chloramphenicol (100 μg/ml). Attempts to transform $CaCl_2$-competent BL21(DE3)/pGroESL *E. coli* cells with pMEXHCO plasmid DNA prepared by different methods were unsuccessful. Transformation of pMEXHCO, and pT7-7 as a control, by electroporation was also tried as described in section 2.1, but

again only the transformation with pT7-7 was successful. From this work it seems that there was either some problem with the pMEXHCO plasmid DNA and it was unable to be transformed, or the plasmid DNA was transformed and for some reason cells with both the pGroESL plasmid and the pMEXHCO plasmid inside were unable to grow.

3.17 Synthesis of 5′deoxyadenosylcobalamin-agarose for Affinity Chromatography

The 5′deoxyadenosylcobalamin-agarose affinity resin was prepared as described in section 2.6.1. Assuming that all of the cyanocobalamin groups attached to the vitamin B_{12} resin were successfully converted to 5′deoxyadenosylcobalamin, and that each of these groups can bind one hMCM monomer then the capacity of the resin would be ~59 mg of hMCM per ml of resin. The affinity resin was stored at 4 °C in buffer containing 0.02% thiomersal as a preservative. Thiomersal is an antibacterial agent that contains organic mercury, so it may interfere with the action of some enzymes. Given the large number of enzymes required for the hMCM activity assay it was important to remove all traces of thiomersal so there was no chance of it interfering with the activity of the bacterial and human MCM used in this experiment, and also with any other coupling enzymes in the MCM activity assay.

Batch adsorption experiments (see section 2.6.3) were carried out soon after the 5′AdoCbl-agarose was synthesized. These batch adsorption experiments were carried out three separate times with the same conditions, in an attempt to gain reproducible results. Batch elution experiments were carried out at the same time as these batch adsorption experiments. The 5′AdoCbl-agarose resin was stored at 4 °C for ~4 months before it was to be used in the large-scale refolded hMCM purification described in section 2.7.3. Due to this long storage time, more batch adsorption and elution experiments were carried out on the 5′AdoCbl-agarose prior to its use in the large-scale hMCM purification, to ensure that there was no degradation of the agarose.

The first three batch adsorption trials with the prepared 5′AdoCbl affinity resin and

bacterial MCM indicated that bMCM had bound to the resin, but it appeared that bMCM was also partially bound to the control resin (cyanocobalamin-agarose) from which 5'AdoCbl-agarose was synthesized (trials 1-3, table 3.11). The results of the hMCM batch adsorption trials were unclear: hMCM bound to the 5'AdoCbl-agarose strongly in trial 1 (>90% activity bound), but poorly in trials 2 and 3. These inconsistent results may be

Table 3.11: Batch adsorption results

Sample	Activity in supernatant after adsorption μmoles/ml of sample/min		
	1st trial	2nd trial	3rd trial
Bacterial control (no resin)	0.145	0.150	0.148
Prepared 5'AdoCbl-agarose + bMCM	0	0.001	0.002
Control cyanoCbl-agarose + bMCM	0.053	0	0.009
Human control (no resin)	0.064	0.065	0.098
Prepared 5'AdoCbl-agarose + hMCM	0.002	0.019	0.084
Control cyanoCbl-agarose + hMCM	0.035	0.029	0.085

due to the practical problems encountered while working with the light sensitive suspended resins, e.g. inacurate weighing of resins in low light conditions, and incomplete removal of resins from supernatant fractions.

One factor not taken into account with the trials in table 3.11 was that as the hMCM test samples used were fractions eluted from hydroxyapatite, they were all in ~0.3 M KP buffer pH 7.2. This compares to 25 mM Tris-HCl buffer, pH 7.3, for the bMCM test sample. To determine if the hMCM would bind more efficiently to the 5'AdoCbl-

agarose at lower KP concentrations, the hMCM preparation in 0.3 M KP was diluted with water to a buffer concentration of 20 mM KP. This change resulted in binding of the hMCM to the prepared 5'AdoCbl-agarose (table 3.12). The bound active hMCM was eluted with 2 mM 5'AdoCbl in 20 mM KP buffer, pH 7.2, into a smaller volume (50 µl), effectively concentrating the active hMCM from the diluted 5950 µl sample in 20 mM KP. A slight concentrating effect was also seen with cyanoCbl-agarose.

Table 3.12: Elution of hMCM from 5'AdoCbl-agarose

Sample	hMCM Activity µmoles/min/ml of sample
Control, hMCM preparation after dilution	1.6×10^{-3}
Elution of hMCM from cyanoCbl-agarose	4.7×10^{-3}
Elution of hMCM from 5'AdoCbl-agarose	0.139

Another lot of batch adsorption trials were carried out prior to the use of the 5'AdoCbl-agarose in the large-scale hMCM purification. These trials were carried out as described in section 2.6.3, the same as the batch adsorption and elution experiments carried out when the 5'AdoCbl-agarose was first synthesized. This was to check that there had been no degradation or change, in column performance during ~4 months storage at 4 °C. An HTP-concentrated fraction (eluted in 0.1 M KP buffer) from a previous 250 ml refolding trial was used to reproduce batch adsorption and elution experiments (absorption experiments in table 3.11, and elution experiments in table 3.12) carried out when the 5'AdoCbl-agarose was first synthesized. Results from the batch adsorption confirmed that hMCM would only bind to the 5'AdoCbl-agarose resin in a low salt buffer. The HTP-concentrated fraction had to be diluted to a buffer concentration of 20 mM potassium phosphate before the hMCM would bind to the resin. Elution of the hMCM from the 5'AdoCbl-agarose was problematic. Table 3.13 shows the results with various elution conditions.

3.18 UV-visible Spectroscopy of Control Cobalamin Solutions and the Prepared 5'AdoCbl-agarose Resin

To confirm that the conversion of cyanocobalamin-agarose to 5'deoxyadenosylcobalamin was successful UV-visible spectroscopy was carried out on the prepared 5'AdoCbl-agarose and the cyanocobalamin-agarose. Some characteristic

Table 3.13: Different elution conditions trialled to elute hMCM from the prepared 5'AdoCbl-agarose

Conditions of elution	hMCM activity after elution
0.25 M MES buffer, pH 5.5	No Activity Regain
0.5 M 5'deoxyadenosylcobalamin	No Activity Regain
20 mM 5'deoxyadenosylcobalamin	No Activity Regain
0.5 M Potassium phosphate buffer	No Activity Regain
1 M Lithium chloride	Total Activity Regain
1 M Sodium chloride	Total Activity Regain

features of the 5'deoxyadenosylcobalamin UV-visible absorption spectrum are prominent absorbance maxima at 263 and 525 nm and lesser maxima at 303 and 375 nm. Upon acidification of a 5'AdoCbl solution the absorbancy peak at 525 nm is shifted to ~460 nm and an increase in absorbance is seen around ~303 nm. A 0.5 mM control solution of 5'AdoCbl was scanned from 250 to 600 nm over a period of 7 seconds (described in section 2.6.2). Figure 3.22 illustrates the characteristic spectral features of acidified and neutral 5'AdoCbl solutions. The absorption spectrum of the prepared 5'AdoCbl-agarose resin was also determined in neutral and acidified solutions, and compared to the spectra of the neutral and acidified control solutions (figures 3.22 and 3.23). In both the resin spectra and the control solution spectra the expected changes upon acidification were apparent, but due to difficulties in scanning a

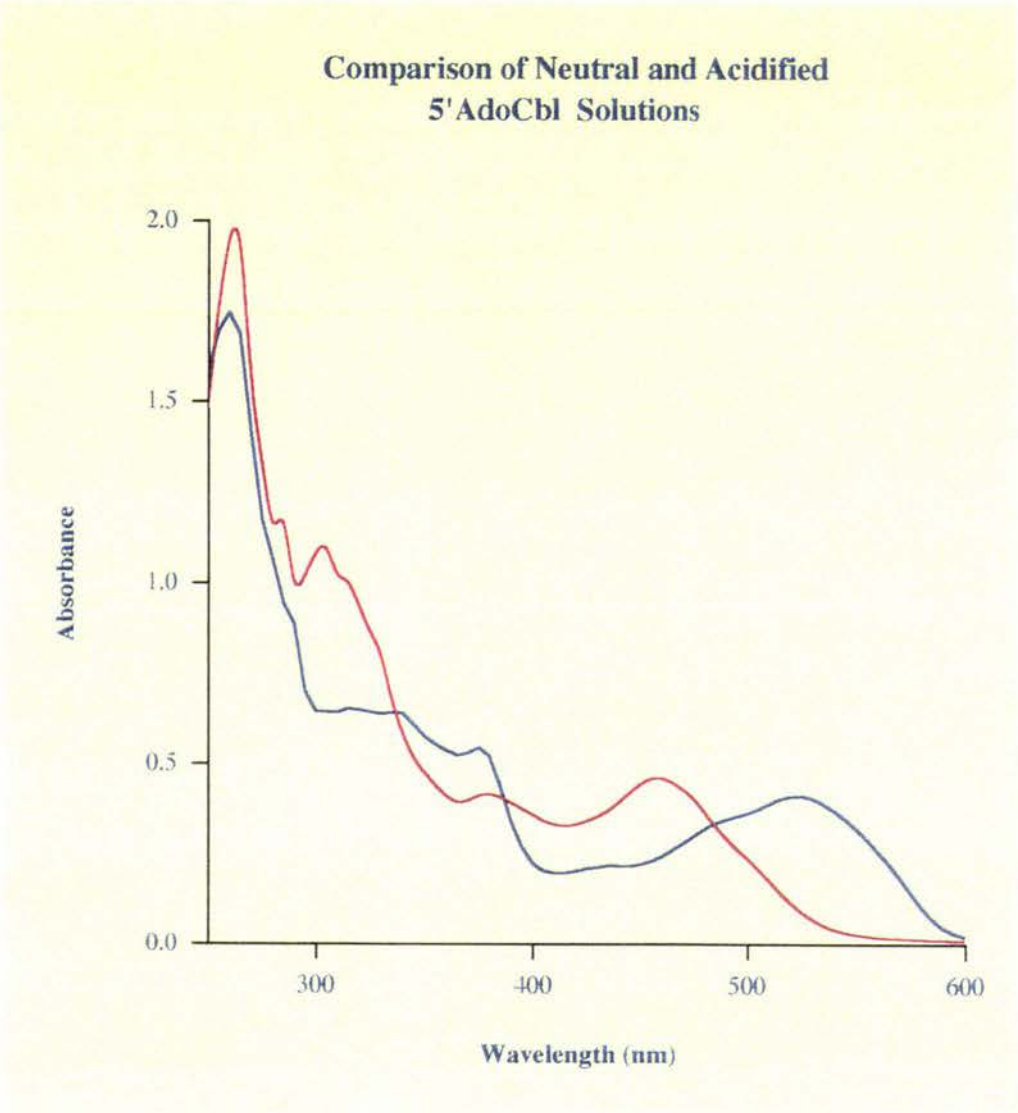


Figure 3.22: Absorbance Spectra of a 0.5 mM control solution of 5'AdoCbl.

The blue line ——— is the spectrum of the 0.5 mM control solution, and the red line ——— is the same solution after acidification with concentrated hydrochloric acid.

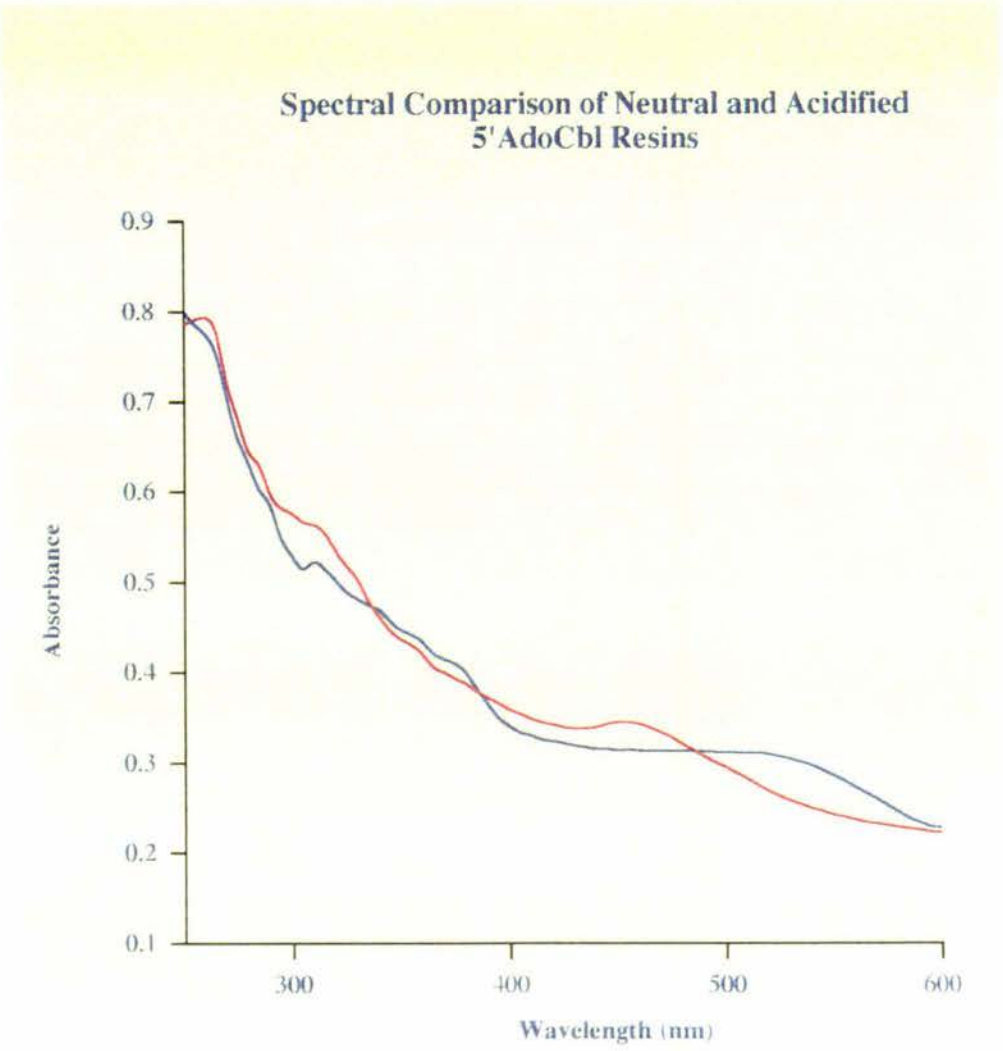


Figure 3.23: Absorbance Spectra of the prepared 5'AdoCbl-agarose resin.

The blue line ——— is the spectrum of the suspended 5'AdoCbl-agarose and the pink line ——— is the spectrum of the same suspended rein after acidification.

suspended agarose resin the shifts in resin absorbance maxima were less obvious than for the control solutions. In figure 3.23 the shift in the 525 nm maxima to ~460 nm upon acidification of the solution is apparent, also a slight increase in the absorbance at 303 nm is seen upon acidification of the 5'AdoCbl-agarose suspension. The change in absorbance due to the settling of resin to the bottom of the cuvette over 30 seconds of absorbance measurement at various wavelengths was never more than 0.05, so over a 7 second scan the effect of this on absorbance would be negligible. This drop in absorbance can be attributed to the decrease in light scattering as the large resin particles drop to the bottom of the cuvette, and to the removal of cobalamin attatched to the resin from the light path of the spectrophotometer.

Additional confirmation of the successful conversion of cyanocobalamin-agarose to 5'AdoCbl-agarose was provided by visual inspection of the colour of the CNCbl resin and the prepared 5'AdoCbl resins in neutral and acidic solutions. In neutral solution a 5'AdoCbl solution is red, whereas an acidic solution (0.12 M HCl) is yellow (Ladd *et al.*, 1961). When a control solution of 5'deoxyadenosylcobalamin was acidified the red to yellow colour change was seen, whereas a cyanocobalamin control solution remained red on acidification. The red-to-yellow colour change was also seen clearly upon acidification of a suspension of prepared 5'AdoCbl-agarose resin. The yellow colour of the acidified 5'AdoCbl-agarose resin was lost upon exposure to light. This is an indication of the photodecomposition of the 5'deoxyadenosylcobalamin moiety to aquocobalamin. All the colours observed are summarised in table 3.14.

3.19 Solubilisation and Refolding of hMCM Inclusion Bodies on a Large-Scale

hMCM inclusion bodies were solubilised and refolded on a 2 litre scale as described in section 2.7.1. The resulting solution of refolded hMCM was further purified and concentrated by batch adsorption onto hydroxyapatite and stepwise elution with potassium phosphate (see section 2.7.2). Table 3.15 shows the hMCM activity of fractions eluted from the hydroxyapatite column. Fractions 8 to 13 were stored on ice and pooled on the basis of their specific activities and further purified by 'affinity'

chromatography (section 2.7.3). Fractions 7 to 15 were also analysed by SDS-PAGE. Two major protein bands could be seen (figure 3.24) in fractions 8, 9 and 10. During the 48 hour storage of fractions on ice a white precipitate formed in fractions 8 to 11. Fraction 8 had both the heaviest precipitate and the highest specific activity of hMCM. A small amount of the precipitate from the pooled fractions was resuspended in 20 mM KP buffer, pH 7.2, and assayed for hMCM activity but none was found.

Table 3.14: Colour of neutral and acidified cobalamin control solutions, prepared 5′AdoCbl-agarose and cyanocobalamin-agarose

Sample	Colour of solution at pH 7.2 (Neutral solution)	Colour of solution in 0.12 M HCl (Acid solution)
1 mM cyanocobalamin control solution	Red	Red
1 mM hydroxocobalamin control solution	Red	Red
1 mM 5′AdoCbl control solution	Red	Yellow
Prepared 5′AdoCbl-agarose	Red	Yellow
Cyanocobalamin-agarose	Red	Red

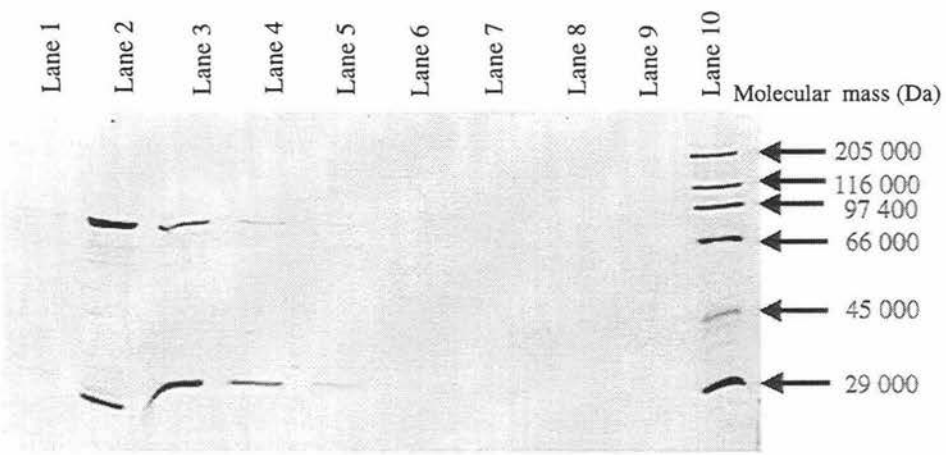


Figure 3.24: SDS-PAGE analysis of fractions collected during stepwise elution from hydroxyapatite.

Lanes 1 - 9: Fractions 7 to 15 respectively.

Lane 10: SDS-6H molecular mass markers (Sigma).

Table 3.15: Fractions collected during elution of protein from hydroxyapatite

Fraction number and conc. of elution buffer	Activity in sample units/ml	Protein concentration in sample mg/ml	Specific activity of fraction units/mg
1, 0.1 M KP	0.007	-	-
2, 0.1 M KP	-	-	-
3, 0.1 M KP	0.011	-	-
4, 0.1 M KP	-	-	-
5, 0.1 M KP	0.015	-	-
6, 0.3 M KP	-	-	-
7, 0.3 M KP	0.013	0.039	0.3
8, 0.3 M KP	0.426	0.225	1.89
9, 0.3 M KP	0.682	0.565	1.21
10, 0.3 M KP	0.480	0.330	1.45
11, 0.3 M KP	0.364	0.180	1.92
12, 0.3 M KP	0.165	0.100	1.65
13, 0.3 M KP	0.108	0.070	1.54
14, 0.3 M KP	0.039	0.052	0.75
15, 0.3 M KP	-	0.042	-
16, 0.5 M KP	0.014	0.026	0.54
17, 0.5 M KP	-	-	-
18, 0.5 M KP	0.008	-	-
19, 0.5 M KP	-	-	-
20, 0.5 M KP	0.006	-	-

Table 3.16: Fractions collected during purification by affinity chromatography of large-scale refolded hMCM

Fraction number	NaCl concentration in fraction moles/litre	Protein concentration mg/ml	hMCM activity units/ml	Specific activity units/mg
1	0	0.013		
2	0	0.006		
3	0	0.006	0.002	0.35
4	0	0.137	0.621	4.53
5	0.2	0.425	1.342	3.16
6	0.2	0.215	1.004	4.67
7	0.2	0.205	0.716	3.49
8	0.4	0.160	0.484	3.03
9	0.4	0.134		
10	0.4	0.108	0.353	3.27
11	0.6	0.065		
12	0.6	0.078	0.337	4.32
13	0.6	0.056		
14	0.8	0.030		
15	0.8	0.066	0.232	3.52
16	0.8	0.031		
17	1.0	0.022		
18	1.0	0.039		
19	1.0	0.027		
20	1.0	0.031		
21	1.0	0.028		

This material was also analysed by SDS-PAGE and found to consist predominantly of hMCM (results not shown). The white precipitate presumably consisted largely of incorrectly folded inactive hMCM protein which had aggregated and precipitated upon concentration by the hydroxyapatite adsorption step. This precipitate interferred with the hMCM assays causing a very unstable basal rate before the assay was started by

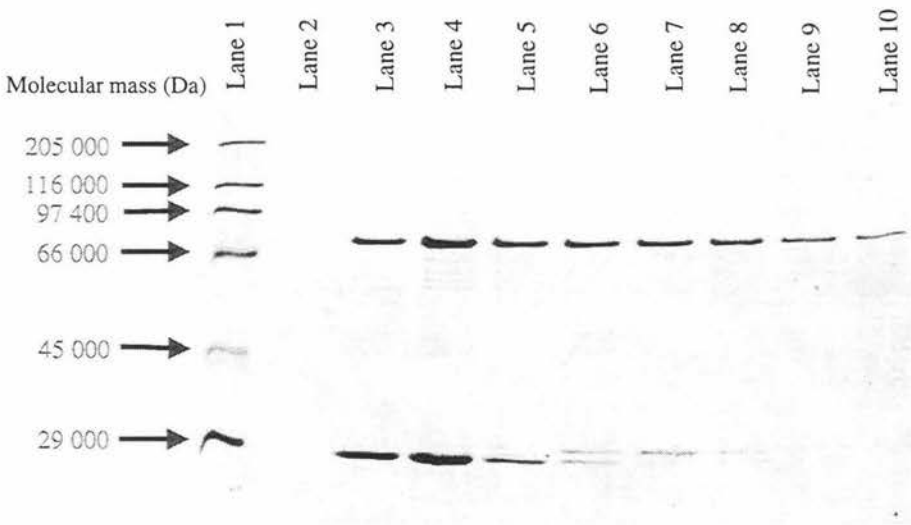


Figure 3.25: SDS-PAGE analysis of fractions collected during stepwise salt elution of the 5'AdoCbl-agarose affinity chromatography resin.

Lane 1: SDS-6H molecular mass markers (Sigma).

Lanes 2 - 10: Fractions 3 to 11 respectively.

addition of 5'AdoCbl. After centrifugation of the fractions, subsequent hMCM activity assays of the supernatants gave basal rates that were acceptably stable for the determination of hMCM activity (table 3.15).

3.20 hMCM Purification by Affinity Chromatography

The HTP-concentrated hMCM preparation from the large-scale inclusion body solubilisation and refolding described in sections 2.7.1 and 2.7.2 was further purified by affinity chromatography using the prepared 5'AdoCbl-agarose resin (section 2.7.3). Table 3.16 shows the hMCM activity of each fraction collected during salt elution from the column. All fractions were also analysed by SDS-PAGE (figure 3.25). Fractions 4 to 11 contained a protein of ~79 kDa, the same molecular mass as the hMCM α-subunit. A second prominent band, seen clearly in SDS-PAGE analysis of fractions 4, 5 and 6, corresponded to a molecular mass of ~29 kDa. Because of the contaminating protein in fractions 4, 5 and 6, the fractions containing the 79 kDa protein were divided into two pools. The low salt pool (5 ml, fractions 4 - 8) contained the two proteins in approximately equal amounts (by band intensity). The high salt pool (11.5 ml, fractions 9 - 21) contained a lower concentration of the 79 kDa protein, and only trace amounts of the 29 kDa contaminating protein compared to the low salt pool.

3.21 Further Purification and Concentration of the Two hMCM Pools

The two pools were concentrated as described in section 2.7.4. A 50 kDa cut-off ultrafiltration membrane was used in an attempt to separate the 79 kDa protein from the 29 kDa contaminating protein during the concentration step. The filtrate from the ultrafiltration of the first pool (fractions 4 - 8) was concentrated using a 10 kDa cut-off microsep concentrator; it was hoped that any 29 kDa protein present in the filtrate would be concentrated by this procedure. An SDS-polyacrylamide gel was run of the concentrated first and second hMCM pools, and of the concentrated filtrate from the first pool (figure 3.26). Both concentrated pools were assayed for protein using the Coomassie blue dye-binding method, and for hMCM activity. The first hMCM pool

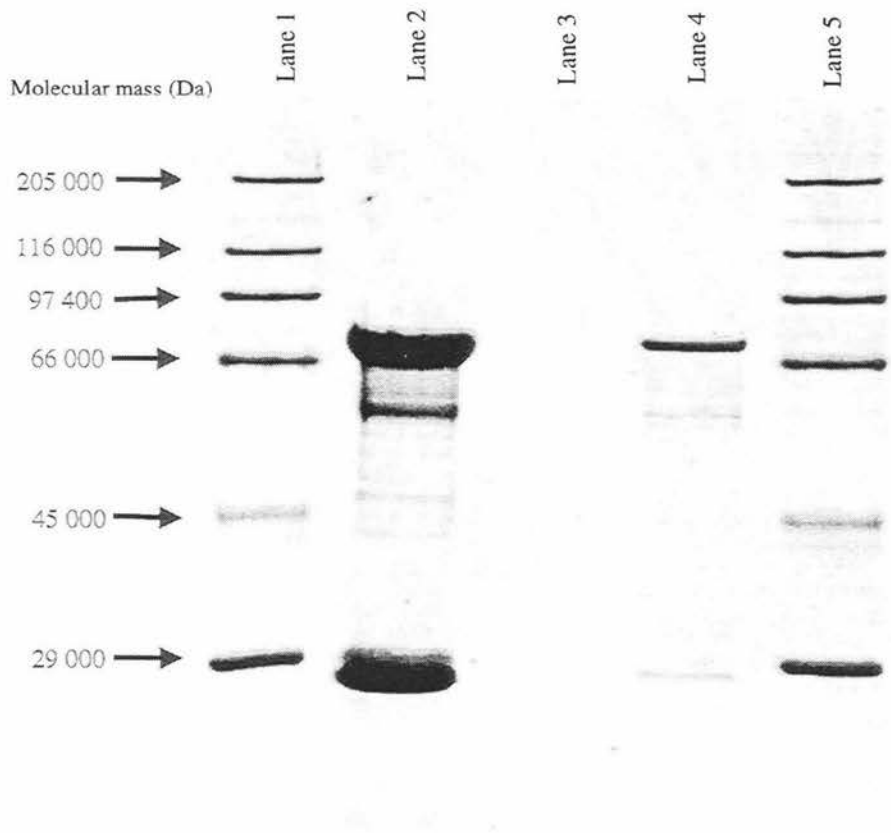


Figure 3.26: SDS-PAGE analysis of the concentrated hMCM pools and the concentrated low salt pool filtrate.

Lane 1:	SDS-6H molecular mass markers (Sigma).
Lane 2:	Concentrated first hMCM pool.
Lane 3:	Concentrated filtrate from first hMCM pool.
Lane 4:	Concentrated second hMCM pool.
Lane 5:	SDS-6H molecular mass markers (Sigma)

had a specific activity of 3.11 units/mg. The second hMCM pool contained 0.23 units/mg.

Figure 3.26 shows that the contaminating 29 kDa protein in the first pool was not separated from the hMCM by ultrafiltration through a membrane with a 50 kDa molecular mass cut-off. It is possible that the 29 kDa protein is forming multimers, making it larger than 50 kDa in solution. A possible identity for the 29 kDa protein is branched chain amino acid aminotransferase (see later in this section), which is a hexamer in solution. Another possibility arising from the co-purification of this contaminating protein in similar amounts to the hMCM protein is that the 29 kDa protein is somehow associated with the 79 kDa hMCM protein in solution. A protein similar in molecular mass to this contaminating protein was apparent in fractions from the refolding by gel filtration chromatography experiment described in section 3.13, and can be seen as the dominant band in lane 6 of the SDS-PAGE gel in figure 3.9. This contaminating protein was electroblotted from an SDS-polyacrylamide gel onto an Immobilon™-P membrane as described in section 2.9.4 and the N-terminal amino acid sequence of the blotted protein band was determined as described in section 2.9.5. Upon analysis this band had an N-terminal sequence of

(X, G, S) T K K (X, K),

X is an unknown, giving a peak between A and R (very close to R). Only 5 cycles of sequencing were requested, as it was assumed that this particular band would be a degradation product of hMCM, whereas this sequence does not match any peptide in the hMCM amino acid sequence. The fact that the peptide sequence was not very clear did not aid in identifying the protein that it was a part of.

Samples were taken throughout the large-scale inclusion body purification, solubilization, refolding and further purification, and were analysed by SDS-PAGE (figure 3.27). Lane 3 of figure 3.27 shows that we were not successful in preparing a totally pure solution of the recombinant hMCM from the purified inclusion bodies.

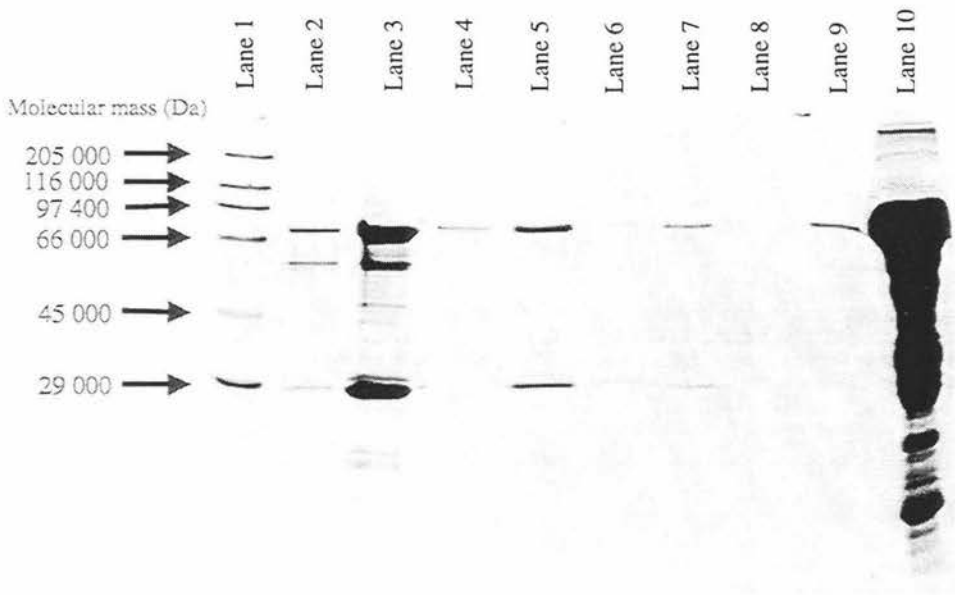


Figure 3.27: SDS-PAGE analysis of samples taken throughout the large-scale hMCM inclusion body purification solubilisation refolding and further purification.

Lane 1: SDS-6H molecular mass markers (Sigma).

Lane 2: Second pool after concentration.

Lane 3: First pool after concentration.

Lane 4: Unconcentrated high salt pool.

Lane 5: Unconcentrated low salt pool.

Lane 6: Proteins not bound by the 5'AdoCbl-agarose resin during loading.

Lane 7: Pooled fractions eluted from hydroxyapatite.

Lane 8: Supernatant after adsorption of protein in refolded solution to HTP.

Lane 9: Sample after refolding by large-scale rapid dilution.

Lane 10: Purified inclusion bodies before solubilisation.

The unknown protein with a molecular mass of ~ 29 kDa was still present at approximately the same levels as the hMCM. After taking this contamination into account, given that this protein is inactive in the hMCM assay, and represents about 50% of the total protein in the sample, the specific activity of the pure refolded hMCM in the heavily contaminated low salt pool would be 6.2 units/mg. The specific activities of other native mammalian MCM's (see discussion) are comparable to the specific activity values deduced for pure refolded recombinant hMCM. This suggests that while the efficiency of refolding, and hence recovery, was very low, the small amount of protein that was successfully refolded has refolded to a native, or near native, state.

CHAPTER FOUR

DISCUSSION

4.1 Preparation of hMCM Inclusion Bodies

Two systems for the expression of hMCM in *E. coli* were tested to see which produced the most hMCM inclusion body protein. BL21(DE3)/pMEXHCO and SRP84/pGP1-2/pMEXHCO were both grown up on a small scale and induced to express hMCM, but only the SRP84/pGP1-2/pMEXHCO was successfully induced. Large-scale (3 litres) growth of SRP84/pGP1-2/pMEXHCO was undertaken and induced to express hMCM. 41 g (wet weight) of cells were harvested and the inclusion bodies were purified. The increased scale of the inclusion body purification may have caused a decrease in the efficiency of the purification so that the inclusion bodies prepared were not as pure as inclusion bodies purified on a smaller scale (i.e. from 13 g wet weight of cells). This decreased purity may have been due to the many pellet resuspensions involved in the inclusion body purification, the smaller pellets from centrifugation would be resuspended more efficiently and so the wash steps would be more efficient in removing contaminating proteins. Another possibility for the lower purity of the inclusion bodies purified on a large-scale compared to those purified on a small-scale, was the fact that the *E. coli* cells producing the inclusion bodies which were purified on a small-scale had a higher level of expression of hMCM than the 41 g of cells grown for the large-scale inclusion body purification. Because of the low purity of the inclusion bodies prepared on the large-scale compared to those prepared on the small-scale, they were not used in any refolding experiments.

4.2 Refolding of hMCM by Rapid Dilution

Initial small-scale (400 µl) rapid dilution experiments were carried out at various final concentrations of hMCM protein. The highest hMCM specific activities of ~0.08 units/mg were obtained at final protein concentrations between of between 0.09 and

0.16 mg/ml in the refolding mix. A time trial experiment was carried out to determine the optimum duration for protein refolding at 0 °C after rapid dilution. Maximum activity was reached after 24 hours at 0 °C. These rapid dilution experiments were scaled up to 250 ml and the solubilised inclusion body material was pumped slowly into well stirred refolding solution. Theoretically, this should have resulted in a better yield of refolded hMCM protein as the first solubilised protein pumped into the refolding solution would be greatly diluted and separated from other refolding hMCM molecules, thus minimising the aggregation of folding intermediates. As more solubilised protein was pumped into the refolding solution the protein pumped in earlier would theoretically have had time to fold and so would not interact with exposed hydrophobic surfaces of folding proteins pumped in more recently. However, the hMCM specific activity of 0.044 units/mg obtained was about half that found in the small scale rapid dilution experiments.

4.3 hMCM Refolding by Dialysis Against a Refolding Solution

The efficiency of refolding by dialysis was affected by the hMCM protein concentration. The lowest protein concentration tested gave the best yield of refolded protein, i.e. 0.012 mg/ml gave a hMCM specific activity of 0.150 units/mg, compared to 1.2 mg/ml, which gave a hMCM specific activity of 0.004 units/mg. The gradual decrease in the denaturant concentration surrounding the hMCM polypeptide was condusive to hMCM refolding as long as the protein molecules were suffiently dilute to refold in isolation. This same trend was seen by Horowitz and Simon (1986) when refolding GdmHCl-denatured rhodanese by dialysis against a non-denaturing buffer over 20 hours.

4.4 Detergent-Assisted Refolding of hMCM

Rapid dilution experiments were carried out using detergent-supplemented refolding solutions. Fourteen different detergents were trialed, each at three different concentrations. None of the detergents increased refolding to active hMCM, but hMCM activity measured in the refolding solutions were always at or near the

detection limit of the assay. In the reported examples of detergents aiding *in vitro* refolding, the type of detergent and the concentration at which it assisted refolding were specific to the protein. For example, lauryl maltoside (LM) assisted the refolding of rhodanese at a concentration just above its cmc (Tandon and Horowitz, 1986) and was able to assist refolding of three out of five different proteins, but at concentrations unrelated to the cmc of LM (Tandon and Horowitz, 1988). If detergent-assisted refolding of hMCM was to be investigated further, one possible approach would be to select three or four detergents and use them over a wider range of concentrations in the refolding solutions. This would be a more efficient use of time than testing 14 detergents at only three concentrations each.

To rule out the effect of detergent on hMCM activity and the activity of coupling enzymes in the activity assay, a good control would have been to assay previously refolded hMCM diluted in detergent-supplemented refolding solution.

4.5 Refolding by Gel Filtration Chromatography

Refolding by gel filtration chromatography has proven successful in the refolding of several proteins (Werner *et al.*, 1994; Batas and Chaudhuri, 1996; Muller-Newen *et al.*, 1997). This method was used in a single unsuccessful attempt to refold hMCM. There was no detectable hMCM activity in any of the fractions eluted from the gel filtration column. Because the experiment was carried out only once it still has the potential to be a efficient method for refolding solubilized hMCM inclusion body material. The major problem encountered was precipitation of protein in the gel filtration column. Although one of the advantages of this method is that small protein aggregates are excluded from the gel matrix and are eluted from the column first, the magnitude of the hMCM aggregation prevented this.

A number of options are worth considering for improving this procedure for hMCM refolding. For example, a possible cause of the precipitation could have been the low flow rate at which the column was run. The flow rate used should be adjusted to satisfy two roles. Initially the flow rate should be fast to separate the protein from the

denaturant as soon as possible, to minimize the formation of irreversible aggregates. This being similar to a rapid dilution of the protein causing rapid removal of the chaotrope preventing the protein from folding. Subsequently a slower rate should be used to give the protein time to fully refold and to increase the column resolution (Batas and Chaudhuri, 1996). For hMCM refolding by this method a gravity fed linear flow rate of 0.046 cm/min was acheived. Successful refolding of bovine carbonic anhydrase and hen egg white lysozyme by this method required flow rates of 0.47 and 0.22 cm/min respectively (Batas and Chaudhuri, 1996). Also the column was run at room temperature, whereas refolding at a cooler temperature may have helped to stabilize any refolded hMCM. Another variable which needs to be optimised for every protein refolded by this method is the gel filtration medium. An appropriate match between an unfolded protein's hydrodynamic radius and the matrix pore size is critical to the effectiveness of this technique. The gel filtration bead pores should allow the partially folded protein into the pores as soon there is a reduction in the hydrodynamic radius. The matching of media with hMCM would have to be determined by a series of experiments given the complexity of folding pathways.

4.6 Dimerization Experiment

The specific activity of hMCM eluted from hydroxyapatite in ~0.3 M potassium phosphate buffer and stored at 4 °C increased over time. This may be due to the gradual association over time of already folded hMCM subunits, which are inactive until dimerization occurs.

It is possible that formation of active hMCM may be rate limiting the dimerization of folded α-subunits. This has been found to be the case for the refolding pathways of several dimeric proteins. For example, mitochondrial aspartate aminotransferase refolds by a very fast collapse to an intermediate with 80% of the secondary structure of the active dimer. This is followed by a slow isomerisation to form assembly-competent monomers that rapidly associate to form an inactive dimer and a final structural rearrangement of the dimer to the native conformation (Artigues *et al.*, 1997). Recombinant human brain-derived neurotrophic factor when refolded by rapid

dilution quickly forms (<1min) a partially folded non-native intermediate with extensive secondary structure but no well defined tertiary structure. This non-native intermediate disappears with a half-time of approximately 30 minutes, with the appearance of native dimers, and without accumulation of monomeric species with native tertiary structure. A significant conformational change in the non-native intermediate is necessary to re-expose the hydrophobic groups to form the very large hydrophobic surface present at the dimer interface, making this the rate-limiting step in reaching the native conformation (Philo *et al.*, 1993). Refolding studies of luciferase (Ziegler *et al.*, 1993) and *Clostridium symbiosum* glutamate dehydrogenase (Aghajanian and Engel, 1997) have shown that dimerization is also the rate limiting step in the reactivation of both these proteins.

4.7 Chaperonin-Assisted Refolding

4.7.1 *In Vitro* Chaperonin Assisted Refolding

The molecular chaperonins GroEL and GroES have been reported to assist the *in vitro* refolding of many proteins when supplemented into a rapid dilution refolding solution. Work done by Weissman *et al.*, (1995) suggested that the hMCM protein, with a subunit molecular mass of 79 kDa, is physically too large to be sequestered in the cavity of GroEL beneath GroES (see section 1.3.6). However, proteins such as *E. coli* β-galactosidase, with a molecular mass of 116 kDa (Ayling and Baneyx, 1996), and the 124 kDa protomer from plant phytochrome (Grimm *et al.*, 1993), have had their folding apparently assisted *in vitro* by the presence of GroEL alone. Due to the ease of the β-galactosidase assay, the purified *E. coli* chaperonin GroEL was used to refold *E. coli* β-galactosidase *in vitro*, to confirm that the purified GroEL chaperonin was biologically active.

Although the final refolding concentrations of β-galactosidase (0.034 μM, Ayling and Baneyx, 1996) and hMCM (0.02 μM, Weissman *et al.*, 1995) were similar, Weissman *et al*, (1995) refolded hMCM in the presence of a ~20-fold molar excess of $GroEL_{14}$ (0.397 μM) over hMCM monomers. This ratio of $GroEL_{14}$ to folding protein are large

compared to the 2-fold molar excess of $GroEL_{14}$ to β-galactosidase monomers successfully used by Ayling and Baneyx (1996) to fold β-galactosidase (116 kDa). This high concentration of $GroEL_{14}$ might have prevented refolding as outlined below. In addition, the refolding buffers used by Weissman *et al.* (1996) may not have been conducive to hMCM refolding with or without $GroEL_{14}$.

The purified chaperonins, $GroEL_{14}$-complex and $GroES_7$-complex, were added to a basic refolding solution at a 2-fold molar excess to the hMCM α-subunits, in different combinations with ATP and Mg^{2+}. None of the combinations used refolded the hMCM with any better efficiency than rapid dilution into the basic refolding solution, confirming the results of Weissman *et al.* (1995). The concentration of $GroEL_{14}$ in the hMCM refolding solution in the current study (3 μM) was relatively high compared to that in the GroEL-assisted refolding of β-galactosidase (Ayling and Baneyx, 1996), and may have hindered the GroEL-assisted refolding of hMCM. β-galactosidase refolding was assisted by GroEL at a final monomer concentration of 0.14 μM, and higher recoveries were obtained at lower concentrations (Ayling and Baneyx, 1996). In this current study hMCM refolding was attempted using a ~10-fold higher final monomer concentration of 1.5 μM hMCM. This resulted in a more crowded solution compared to β-galactosidase refolding conditions (Ayling and Baneyx, 1996), increasing the probability of aggregation of the hMCM folding intermediates even in the presence of GroEL. As yet there is no suggested mechanism for the GroEL-assisted refolding of large molecules such as β-galactosidase. It has been shown for several systems that GroEL can effectively block 'off-pathway' folding reactions, thus preventing folding peptides from aggregating (Buchner *et al.*, 1991; Holl-Neugebauer *et al.*, 1991). However, this prevention of protein aggregation typically arrests refolding (Hartl *et al.*, 1994). These observations are contradicted by the observed GroEL-assisted refolding of β-galactosidase, but confirmed by the absence of hMCM refolding in the presence of GroEL. In hindsight, a possible addition to this series of experiments would have been the rapid dilution of hMCM into refolding buffer containing $GroEL_{14}$-complex at a 2-fold molar excess over hMCM at a lower hMCM subunit concentration of approximately 0.2 μM. This may prevent any aggregation occurring, and upon the addition of GroES and ATP, could result in release of partially folded hMCM

molecules with the potential to fold to a native state. Although the results of Weissman *et al.* (1995) were not encouraging in that purified chaperonins were unable to assist the refolding of hMCM, the fact that β-galactosidase, another large protein, was able to be refolded with different ratios of GroEL to folding protein, justified trying these ratios to refold hMCM. The successful purification of the *E. coli* chaperonins, and the successful refolding of denatured *E. coli* β-galactosidase, meant that this line of experimentation was worth pursuing.

4.7.2 *In Vivo* Chaperonin-Assisted Refolding

The aim of this experiment was to prevent the formation of hMCM inclusion bodies by co-expressing the *E. coli* molecular chaperonins GroEL and GroES. To acheive this co-expression, attempts were made to transform the hMCM expression plasmid pMEXHCO into BL21(DE3)/pGroESL, a strain of *E. coli* already transformed with an IPTG-inducible GroES/GroEL expression plasmid. Although it was possible to establish the *E. coli* strain BL21(DE3)/pGroESL/pT7-7 in which the ampicillin resistance plasmid pT7-7 was the 'parent' plasmid for pMEXHCO, the pMEXHCO plasmid itself yielded no BL21(DE3)/pGroESL/pMEXHCO transformants. The reason(s) why the parent pT7-7 plasmid, but not the pMEXHCO plasmid, can be established in *E. coli* BL21(DE3)/pGroESL have not been determined. It seems unlikely that hMCM could be toxic to *E. coli*, even in a soluble active form. One possible control experiment that was not attempted would be to try to introduce another pT7-7-based expression vector, e.g. pTEEX (E. Saafi, MSc Thesis, 1994), into BL21(DE3)/pGroESL. As a mitochondrial matrix protein, hMCM must be unfolded by cytosolic chaperones prior to translocation across the mitochondrial membranes, and refolded by mitochondrial chaperones in the matrix (Schneider *et al.*, 1996). Although the *E. coli* chaperonins GroEL and GroES might assist in folding hMCM by mimicking the actions of the matrix chaperones, the GroEL/ES chaperonins are not *E. coli* homologues of mitochondrial hsp70 but they are homologues of mitochondrial hsp60. The GroEL/ES chaperonins might be unable to mimic/take over all the roles of the mitochondrial chaperones, such as mitochondrial hsp70, involved in processing/folding of hMCM, although other studies suggest that they can for at least some mitochondrial

matrix proteins, both *in vitro* (Mendoza *et al.*, 1992; Mattingly *et al.*, 1995) and *in vivo* (Wynn *et al.*, 1992; Kelson *et al.*, 1996).

4.8 Production of Active Recombinant hMCM From Inclusion Bodies in *E. coli*

A large-scale preparation of hMCM was undertaken based on the smaller scale rapid dilution refolding experiments. The simplest method of hMCM refolding was rapid dilution of GdmHCl solubilised hMCM inclusion bodies into a well-stirred refolding solution. Scaling up the rapid dilution procedure from 400 µl to 250 ml decreased the active protein produced per ml of refolding solution by ~50%, but the much larger volume of refolded solution resulted in a larger total yield of refolded protein.

The rapid dilution experiments were scaled up to 2 litres. Concentration of the refolded active hMCM by batch adsorption and stepwise elution from hydroxyapatite resulted in fractions with hMCM specific activities ranging from 1.2 to 1.9 units/mg. Further purification by affinity chromatography on the prepared 5′AdoCbl-agarose resin resulted in fractions with hMCM specific activities ranging from 3.0 to 4.5 units/mg. The 29 kDa contaminating protein and most of the hMCM co-eluted in the low salt fractions (0.2M NaCl). The remaining hMCM was eluted at higher salt concentrations (0.4-1.0M NaCl); these fractions contained only trace amounts of the contaminating protein. The fractions were combined to give a low salt pool and a high salt pool. From 1.5 ml (255 mg) of purified hMCM inclusion bodies solubilised in GdmHCl and refolded in 2 litres, the final low salt hMCM preparation had a specific activity of 3.1 units/mg. The final high salt hMCM preparation had a specific activity of 0.23 units/mg. The pool of the low salt-eluted fractions contained higher concentrations of hMCM (as assessed by SDS-PAGE), but were more heavily contaminated with the 29 kDa protein and so should have had a higher specific activity. This as well as the fact that this protein eluted at higher salt concentrations, suggests that these fractions contained mostly unfolded/inactive hMCM, which interacted with the resin with

stronger interactions than the correctly folded/active hMCM. By comparison, the high salt fractions contained a lower concentration of hMCM but were less heavily contaminated with the 29 kDa protein. The very limited N-terminal sequence data, (X) TKK was used to search *E. coli* proteins in release 34.0 of the SWISS-PROT protein sequence database using the GCG programme FINDPATTERNS (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc.). The best match was to branched chain amino acid aminotransferase (EC 2.6.1.42) which has an N-terminal sequence TTKKADYIW-, and a predicted M_r of ~34 kDa. It is interesting that an enzyme involved in the biosynthesis of branched chain amino acids in *E. coli* co-purifies with the hMCM, an enzyme involved in the catabolism of branched chain amino acids e.g. val and ile. The contaminating protein was also abundant in hMCM-containing fractions collected during the refolding by gel filtration chromatography experiment. The hMCM amino acid sequence was searched for the TKK motif using the FINDPATTERNS programme. No matches were found, confirming that the contaminating protein was not a proteolytic fragment of hMCM.

The refolded recombinant hMCM preparation (concentrated low salt pool from the 'affinity' chromatography step) had a specific activity of 3.11 units/mg. Other purification procedures of MCM from native sources produced the following specific activities:

Human liver	14 units/mg	(Fenton *et al.*, 1982)
Human placenta	1.33 units/mg	(Kolhouse *et al.*, 1980)
Sheep liver	7.4 units/mg	(Cannata *et al.*, 1965)
Propionibacteria	14.4 units/mg	(Kellermeyer *et al.*, 1964)

Compared to these purifications the refolded hMCM with a specific activity of 3.11 units/mg was comparable, especially considering that about half of the protein in the preparation was an inactive 29 kDa contaminant.

4.9 Affinity chromatography

It was hoped that an affinity chromatography step in the large-scale hMCM purification scheme would act to separate soluble, correctly refolded hMCM from incorrectly folded soluble hMCM protein by interaction of native 5'AdoCbl binding sites with immobilised 5'AdoCbl molecules. Although initial adsorption and elution tests had indicated that the hMCM could be affinity eluted with 3 mM 5'AdoCbl, this behaviour was not reproducible. A possible explanation for the irreproducible elution behaviour could be deterioration of the column during storage, although Yamada and Hogenkamp (1972) used 5'AdoCbl-agarose for at least 20 purification experiments without any detectable loss of function. Despite the irreproducibility of affinity elution, it was possible to elute hMCM from the prepared 5'AdoCbl-agarose using stepwise salt elution. This suggests that hMCM was bound by ionic interactions rather than affinity interaction. Refolded hMCM eluted from the 'affinity' chromatography column was collected over 12 separate 1 ml fractions, at two different NaCl elution concentrations. This result suggests some heterogeneity of binding, due to slightly different interactions of the active hMCM with the 5'AdoCbl-agarose. This may indicate that some active hMCM molecules folded slightly differently from each other and so interact with the resin in different ways. Kolhouse *et al.* (1980,1988) used a 5'AdoCbl-agarose resin prepared as described in this current study to purify hMCM from human placenta, with an affinity elution buffer containing 3 mM 5'AdoCbl and 1 M NaCl. The elution was shown to be specific to 3 mM 5'AdoCbl; identical concentrations of hydroxocobalamin or cyanocobalamin were ineffective. The only difference between the 5'AdoCbl-agarose used in the purification of human placenta MCM and 5'AdoCbl-agarose used in this study was the length of the spacer arm attaching the 5'AdoCbl to the agarose. Yamada and Hogenkamp (1972) prepared a 5'AdoCbl agarose with a 10 atom spacer. The cyanocobalamin-agarose obtained for conversion to 5'AdoCbl in this study had an 8 atom spacer. This shorter spacer arm may have made the difference betweeen the hMCM being able to bind tightly to the immobilised 5'AdoCbl and being able to bind only weakly or not at all. In

the crystal structure of MCM from *P. shermanii* (Mancia *et al.*, 1996) the α chain is responsible for cofactor binding, with the adenosine group of the 5'AdoCbl bound in a deep pocket between the β sheet and the C-terminal helix of the α subunit. If, like the bacterial MCM, the 5'AdoCbl binding site of hMCM is in a cavity, then the 8 atom spacer may be too short for efficient coenzyme-enzyme binding, possibly allowing another type of interaction between hMCM and the resin.

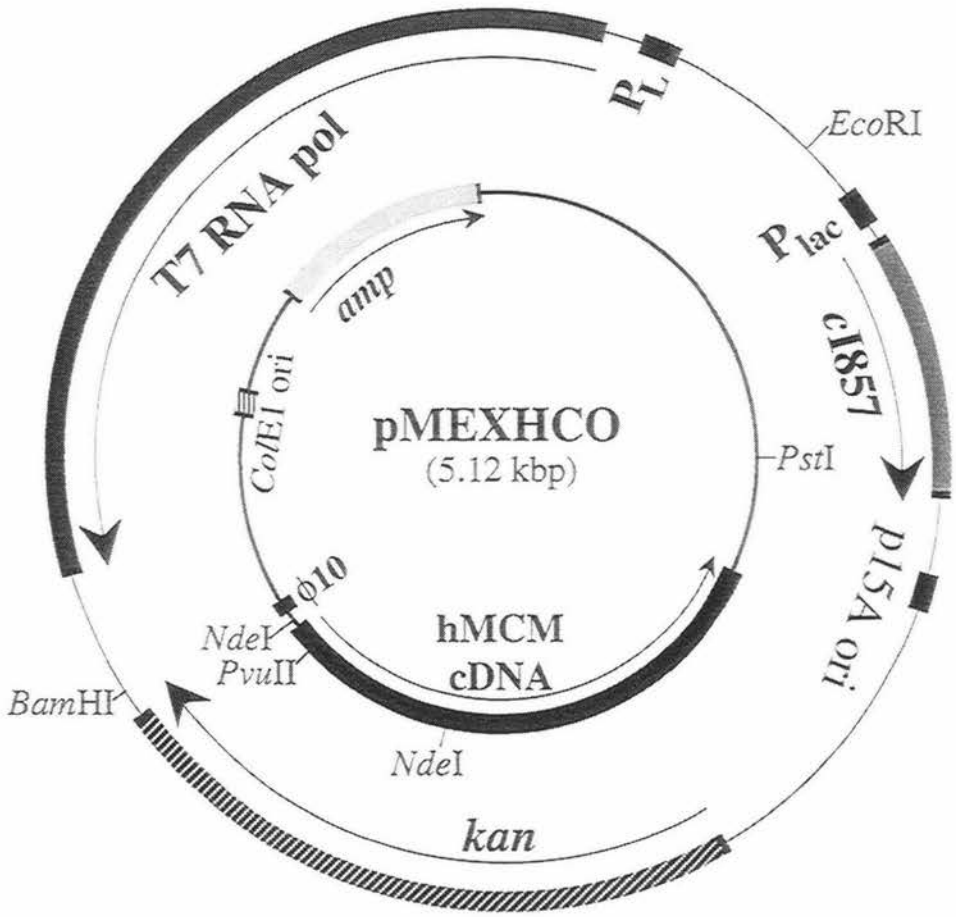
4.10 Future Work

hMCM purified from human liver had a specific acitivity of 14 units/mg (Fenton *et al.*, 1982). Using this figure approximate refolding efficiencies from this current study can be calculated; refolding by dialysis resulted in 1% refolding efficiency, refolding by rapid dilution resulted in 0.6% refolding efficiency and the large-scale refolding resulted in 0.3% refolding efficiency. Clearly a more efficient refolding method is required if large amounts of active hMCM are to be produced from inclusion bodies. Future work towards the production of active recombinant hMCM for structural studies might include, the use of a large-scale rapid dilution method with improved efficiency of refolding. The X-ray crystal structure of hMCM would complement the recently determined structure of the bacterial methylmalonyl-CoA mutase from *P. shermanii* (Mancia *et al.*, 1996). The high percent identity between the sequence of the α-subunit of *P. Shermanii* MCM and human mutase should facilitate the solution of the hMCM crystal structure using molecular replacement techniques (Thoma and Leadlay, 1996). Further work optimising the technique of refolding hMCM by size exclusion chromatography could also be well worthwhile.

Another possible means of producing soluble active recombinant hMCM *in vivo* is to change the expression system. For example, an *E. coli* secretion vector could be used to export hMCM into the periplasmic space of the cell (e.g. Van Heeke *et al.*, 1993). Other possible expression systems to try are expression in tissue culture

cells (Andrews *et al.*, 1993) or in a yeast expression system (e.g. the methylotrophic yeast *Pichia pastoris*, Zhu *et al.*, 1997). One advantage of both yeast and *E. coli* expression systems is that these organisms have no intrinsic MCM activity that would interfere with the purification of the expressed protein. *E. coli* does have a mutase-like gene (Roy and Leadlay, 1992), but this gene is silent in *E. coli* under normal conditions. Finally, it may be possible to obtain sufficient native MCM from an abundant mammalian source, such as sheep liver mitochondria, to pursue structural studies on MCM.

APPENDIX 1



pGP1-2

(7.20 kbp)

Figure 1: Plasmids for hMCM Expression in SRP84/pGp1-2/pMEXHCO.

The gene for T7 RNA polymerase gene on pGP1-2 (Tabor and Richardson, 1995) is transcribed when a brief 42 °C heat shock denatures the temperature sensitive repressor protein cI857 that blocks the PL promoter. T7 RNA polymerase recognisesthe strong ϕ10 T7 promoter in pMEXHCO and transcribes the hMCM cDNA. pMEXHCO was prepared by M.L.Patchett (unpublished).

APPENDIX 2

Edited SWISS-PROT database entry for hMCM.

```
ID   MUTA_HUMAN      STANDARD;       PRT;   750 AA.
DT   01-FEB-1996 (REL. 33, LAST ANNOTATION UPDATE)
DE   METHYLMALONYL-COA MUTASE PRECURSOR (EC 5.4.99.2) (MCM).
GN   MUT.
OS   HOMO SAPIENS (HUMAN).
RP   SEQUENCE FROM N.A., AND PARTIAL SEQUENCE.
RC   TISSUE=LIVER;
RA   JANSEN R., KALOUSEK F., FENTON W.A., ROSENBERG L.E., LEDLEY F.D.;
RL   GENOMICS 4:198-205(1989).
RA   NHAM S.U., WILKEMEYER M.F., LEDLEY F.D.;
RL   GENOMICS 8:710-716(1990).
RA   RAFF M.L., CRANE A.M., JANSEN R., LEDLEY F.D., ROSENBLATT D.S.;
RL   J. CLIN. INVEST. 87:203-207(1991).
RA   JANSEN R., LEDLEY F.D.;
RL   AM. J. HUM. GENET. 47:808-814(1990).
RA   CRANE A.M., JANSEN R., ANDREWS E.R., LEDLEY F.D.;
RL   J. CLIN. INVEST. 89:385-391(1992).
RA   CRANE A.M., MARTIN L.S., VALLE D., LEDLEY F.D.;
RL   HUM. GENET. 89:259-264(1992).
CC   -!- FUNCTION: INVOLVED, IN MAN, IN THE DEGRADATION OF SEVERAL AMINO
CC       ACIDS, ODD-CHAIN FATTY ACIDS AND CHOLESTEROL VIA PROPIONYL-COA TO
CC       THE TRICARBOXYLIC ACID CYCLE. MCM HAS DIFFERENT FUNCTIONS IN
CC       OTHER SPECIES.
CC   -!- CATALYTIC ACTIVITY: (R)-2-METHYL-3-OXOPROPANOYL-COA =
CC       SUCCINYL-COA.
CC   -!- SUBUNIT: HOMODIMER.
CC   -!- COFACTOR: ADENOSYLCOBALAMIN.
CC   -!- SUBCELLULAR LOCATION: MITOCHONDRIAL MATRIX.
CC   -!- DISEASE: DEFECTS IN MUT ARE THE CAUSE OF AN OFTEN FATAL DISORDER
CC       OF ORGANIC ACID METABOLISM TERMED METHYLMALONIC ACIDEMIA. TWO
CC       FORMS OF THE DISEASE ARE DISTINGUISHED BY THE PRESENCE (MUT-) OR
CC       ABSENCE (MUT0) OF RESIDUAL ENZYME ACTIVITY.
KW   MITOCHONDRION; TRANSIT PEPTIDE; ISOMERASE; VITAMIN B12; COBALT;
KW   DISEASE MUTATION; POLYMORPHISM.
FT   TRANSIT      1     32      MITOCHONDRION.
FT   CHAIN       33    750      METHYLMALONYL-COA MUTASE.
FT   VARIANT     93     93      R -> H (IN METHYLMALONICACIDURIA; MUT0).
FT   VARIANT    105    105      W -> R (IN METHYLMALONICACIDURIA).
FT   VARIANT    377    377      A -> E (IN METHYLMALONICACIDURIA; MUT0).
FT   VARIANT    532    532      H -> R.
FT   VARIANT    671    671      V -> I.
FT   VARIANT    717    717      G -> V (IN METHYLMALONICACIDURIA; MUT-;
FT                              INTERFERS WITH THE BINDING OF THE
FT                              COFACTOR TO THE APOENZYME).
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SQ SEQUENCE 750 AA; 83101 MW; 909261B7 CRC32;

MUTA_HUMAN Length: 750 February 2, 1998 15:16 Type: P Check: 9041
..

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   1 MLRAKNQLFL LSPHYLRQVK ESSGSRLIQQ RLLHQQQPLH PEWAALAKKQ
  51 LKGKNPEDLI WHTPEGISIK PLYSKRDIMD LPEELPGVKP FTRGPYPIMY
 101 TFRPWTIRQY AGFSTVEESN KFYKDNIKAG QQGLSVAFDL ATHRGYDSDN
 151 PRVRGDVGMA GVAIDTVEDT KILFDGIPLE KMSVSMTMNG AVIPVLANFI
 201 VTGEEQGVPK EKLTGTIQND ILKEFMVRNT YIFPPEPSMK IIADIFEYTA
 251 KHMPKFNSIS ISGYHMQEAG ADAILELAYT LADGLEYSRT GLQAGLTIDE
 301 FAPRLSFFWG IGMNFYMEIA KMRAGRRLWA HLIEKMFQPK NSKSLLLRAH
 351 CQTSGWSLTE QDPYNNIVRT AIEAMAAVFG GTQSLHTNSF DEALGLPTVK
 401 SARIARNTQI IIQEESGIPK VADPWGGSYM MECLTNDVYD AALKLINEIE
 451 EMGGMAKAVA EGIPKLRIEE CAARRQARID SGSEVIVGVN KYQLEKEDAV
 501 EVLAIDNTSV RNRQIEKLKK IKSSRDQALA EHCLAALTEC AASGDGNILA
 551 LAVDASRARC TVGEITDALK KVFGEHKAND RMVSGAYRQE FGESKEITSA
 601 IKRVHKFMER EGRRPRLLVA KMGQDGHDRG AKVIATGFAD LGFDVDIGPL
 651 FQTPREVAQQ AVDADVHAVG VSTLAAGHKT LVPELIKELN SLGRPDILVM
 701 CGGVIPPQDY EFLFEVGVSN VFGPGTRIPK AAVQVLDDIE KCLEKKQQSV
```

The mitochondrial leader sequence of the hMCM preprotein is underlined.

The hMCM cDNA in pMEXHCO codes for a protein with the predicted N-terminal sequence MLHQQQ. This sequence was confirmed by N-terminal sequencing of the hMCM inclusion bodies; the N-terminal methionine was not cleaved (M. Patchett, pers comm.).

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