

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**The binding of small volatile molecules by
bovine β -lactoglobulin**

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Chemistry at Massey University

Yu-Ting Hsu

2008

Abstract

Bovine β -lactoglobulin (β -Lg) has been studied extensively but there is no clear identification of its biological function. Hydrophobic molecules have been observed binding into the hydrophobic calyx of β -Lg. By comparison with other members of lipocalin family, it is probable that β -Lg plays a role of transport of ligands, as ligands also bind into the central cavity of lipocalins. The structurally similar MUP is a pheromone-binding protein; therefore, it is possible that β -Lg may also fulfil a similar role. This study has begun to test this hypothesis by investigating the interactions between bovine β -Lg and several small volatile molecules (2-sec-4,5-dihydrothiazole, 3-methyl-2-butenal, 3-methyl-2-buten-1-ol and phenylacetic acid). The interactions between the volatile molecules and β -Lg were studied by both two-dimensional NMR spectroscopy and X-ray crystallographic methods. TOCSY spectra were recorded for β -Lg and the complex between β -Lg and the ligands. The observed chemical shifts in the H_N - H_α region are sensitive to the proximity of ligands, and hence chemical shift changes on ligand binding provide information on possible binding sites. It appears that several amino acids with hydrophobic sidechains are affected by interaction with volatile molecules at pH 2.0. The X-ray crystallographic study at pH 8.5 showed that the potential ligand, 2-sec-4,5-dihydrothiazole, may have decomposed into a linear 2-methyl-butanol. The refined structure ($R=0.281$, $R_{\text{free}}=0.354$ for reflections to 2.6 Å resolution) reveals that the potential ligand may bind to the central cavity in a manner similar to the binding of 12-bromodecanoic acid to β -Lg.

Acknowledgements

Thanks to:

My supervisors, Professor Geoff Jameson and Dr. Patrick Edwards for giving me the opportunity to perform this research and guiding me through this project, Dr. Gill Norris for giving assistance on circular dichroism experiments and Mr. Simon Oakley for advice on data X-ray data processing and structural refinement.

Abbreviations

BBP	Bilin-binding protein
δ	Chemical shift
CD	Circular dichroism
CCP4(i)	Collaborative Computational Project Numer 4 (interface)
COSY	Correlation spectroscopy
COOT	Crystallographic Object Orientated Toolkit
kD	KiloDalton
GUI	Graphical User Interface
HSQC	Heteronuclear multiple quantum coherence
Hz	Hertz
β -Lg	β -Lactoglobulin
α -LA	α -Lactalbumin
MUP	Mouse major urinary protein
3MB	3-Methyl-2-butenal
3MBOH	3-Methyl-2-buten-1-ol
mmol	Millimole
min(s)	Minute(s)
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser Enhancement
OBP	Odorant-binding protein
PAA	Phenylacetic acid
RBP	Retinol-binding protein
J	Scalar coupling constant

SDS	Sodium dodecyl sulfate
SBT	2-sec-Butyl-4,5-dihydrothiazole
TOSCY	Total correlation spectroscopy

Title page

Abstract iii

Acknowledgements iv

Abbreviations v

Table of contents vii

List of tables xii

List of figures xiii

Table of contents

Chapter 1 – Introduction

1.1 β -Lactoglobulin and the lipocalins	1
1.1.1 Structure and properties of β -Lg	3
1.1.2 Ligand binding to β -Lg	6
1.1.3 Mouse major urinary protein	
– possible implications for bovine β -Lg function	9
1.2 Selected volatile ligands for binding to β -Lg	12
1.2.1 Volatile compounds present in bovine milk	12
1.2.2 2-sec-Butyl-4,5-dihydrothiazole	13
1.2.3 3-Methyl-2-butenal	13
1.2.4 3-Methyl-2-buten-1-ol	14
1.3 Protein structure determination methods	15
1.3.1 Nuclear magnetic resonance spectroscopy	16
1.3.2 X-ray crystallography	20
1.4 Summary of goals	22

Chapter 2 – Experimental methods

2.1 Introduction	23
------------------	----

2.2 Synthesis of 2-sec-butyl-4,5-dihydrothiazole	24
2.3 Reduction of 3-methyl-2-butenal	29
2.4 Protein purification	31
2.5 Circular dichroism experiments	32
2.5.1 Results of circular dichroism studies	35
2.6 Cocrystallization of β -Lg and ligands	38
2.6.1 Protein crystallization	38
2.6.2 Vapor diffusion	38
2.6.3 Results	40
Chapter 3 – NMR studies of ligand binding to β-Lg	
3.1 Introduction	42
3.2 3-Methyl-2-butenal ligand-binding trial at pH 7.4	44
3.2.1 Results	44
3.3 3-Methyl-2-butenal ligand-binding experiment at pH 2.0	45
3.3.1 Results	46
3.4 3-Methyl-2-butenal titration experiment at pH 2 and at 700 MHz	50
3.4.1 Sample preparation	50
3.4.2 Results	50
3.5 Phenylacetic acid titration experiment	52

3.5.1 Results	52
3.6 Discussion and conclusions	56
Chapter 4 – The crystallographic studies	
4.1 Introduction	57
4.2 X-ray data collection system	59
4.3 Data collection	60
4.4 Data processing	62
4.4.1 MOSFLM	63
4.4.2 Scala/Truncate	64
4.4.3 Molecular replacement	64
4.4.4 Electron density map	67
4.5 Structure solution and refinement	68
4.6 Data processing of possible β -Lg-SBT complex at pH 8.5	70
4.7 Structural refinement of possible β -Lg-SBT complex at pH 8.5	71
4.8 Discussion and conclusions for X-ray structures	81
Chapter 5 – Experimental details	
5.1 Synthesis of 2-(trimethylsilyloxy)ethylamine	82
5.2 Synthesis of N-(2-trimethylsilyloxyethyl)-2-methylbutanamide	84

5.3 Synthesis of 2-sec-butyl-4,5-dihydrothiazole (SBT)	84
5.4 Reduction of 3-methyl-2-butenal	85
5.5 Protein purification	85
5.6 Circular dichroism studies of β -Lg	
and 2-sec-butyl-4,5-dihydrothiazole	86
5.7 Circular dichroism studies of β -Lg and 3-methyl-2-butenal	86
5.8 Ammonium sulfate screen for β -Lg and SBT	87
5.9 Ammonium sulfate screen for β -Lg and 3-methyl-2-buten-1-ol	88
Chapter 6 – Conclusions and perspectives	89
References	91

List of tables

Table 1.1: Amino acid substitutions for β -Lg A, B and C	5
Table 2.1: ^1H NMR chemical shifts for 3-methyl-2-butenal and 3-methyl-2-buten-1-ol	30
Table 3.1: Residues of β -Lg having significant changes in chemical shift in the 3MB binding experiment on the 500 MHz spectrometer	47
Table 4.1: Summary of data collection statistics for the β -Lg-SBT complex	70
Table 4.2: Data summary after molecular replacement	71
Table 4.3: Data summary following after rigid-body refinement	72
Table 4.4: Summary of R and R_{free} values for initial and 1 st round refinements	75
Table 4.5: Summary of R and R_{free} values for 2 nd to 4 th round refinements	76
Table 4.6: Summary of R and R_{free} values for refinements after all residues were added	76
Table 5.1: Literature and experimental values of ^1H NMR chemical shifts for 2-(trimethylsilyloxy)ethylamine	84

List of figures

Fig. 1.1:	Structure of plasma retinol-binding protein	2
Fig. 1.2:	Structure of major mouse urinary protein	2
Fig. 1.3:	Structure of β -Lg	2
Fig. 1.4:	Structure of β -Lg, showing location of the disulfide bridges	4
Fig. 1.5:	β -Lg with cholesterol complexed within the calyx	7
Fig. 1.6:	β -Lg with vitamin D ₂ complexed within the calyx	7
Fig. 1.7:	Top view of SBT bound in the central cavity of MUP1	10
Fig. 1.8:	Side view of β -Lg with 12-bromodecanoic acid bound in the central cavity	10
Fig. 1.9:	Butyric acid	12
Fig. 1.10:	Phenyacetic acid	12
Fig. 1.11:	Hexanoic acid	12
Fig. 1.12:	Furaldehyde	12
Fig. 1.13:	Structure of 2-sec-butyl-4,5-dihydrothiazole	13
Fig. 1.14:	Structure of 3-methyl-2-butenal (3MB)	14
Fig. 1.15:	3-Methyl-2-buten-1-ol.	14
Fig. 1.16:	TOCSY spectrum of β -Lg at pH 2.0 and 35 °C	18
Fig. 2.1:	Mechanism of Lawesson's reagent in equilibrium with the dithiophosphine ylide	24
Fig. 2.2:	Mechanism of thioxaphosphetane intermediate formation	25
Fig. 2.3:	Mechanism of formation of 2-sec-butyl-4,5-dihydrothiazole	26
Fig. 2.4:	¹ H NMR spectrum of 2-sec-butyl-4,5-dihydrothiazole	27
Fig. 2.5:	Literature ¹ H NMR spectrum of 2-sec-butyl-4,5-dihydrothiazole	27
Fig. 2.6:	Mass spectrum of 2-sec-butyl-4,5-dihydrothiazole	28

Fig. 2.7:	^1H NMR of 3-methyl-2-buten-1-ol in CDCl_3	30
Fig. 2.8:	^1H NMR spectrum of contaminated β -Lg A	31
Fig. 2.9:	UV-vis spectrum of 16 mg/mL SBT in phosphate buffer	34
Fig. 2.10:	UV-vis spectrum of 10 mg/mL 3MB in phosphate buffer	34
Fig. 2.11:	CD spectra of β -Lg solution and β -Lg-SBT solution	35
Fig. 2.12:	β -Lg and 3MB CD titration spectrum	36
Fig. 2.13:	Diagram of the hanging drop method	39
Fig. 2.14:	Pictures of β -Lg A and 3MBOH trigonal crystals at pH 7.7	40
Fig. 2.15:	Pictures of β -Lg A+B mixture and SBT crystals at pH 8.5	40
Fig. 2.16:	Pictures of β -Lg A+B mixture and PAA crystals at pH 6.1	41
Fig. 3.1:	Structure of β -Lg complexed with retinol	43
Fig. 3.2:	Amino acid side chains affected by complex formation with γ -decalactone (left) and β -ionone	43
Fig. 3.3:	NMR samples for 3MB binding trial	45
Fig.3.4:	Overlap spectrum of β -Lg reference solution (red) and β -Lg-3MB solution (blue) recorded on the 500 MHz spectrometer	46
Fig. 3.5:	Zoom in of residue Gly9 of overlapped spectra	48
Fig. 3.6:	Zoom in of residue Ser30 of overlapped spectra	48
Fig. 3.7:	Zoom in of residue Ile12 of overlapped spectra	48
Fig. 3.8:	Zoom in of residue Tyr102 of overlapped spectra	48
Fig. 3.9:	Zoom in of residue Ala132 of overlapped spectra	48
Fig. 3.10:	Residues that had significant chemical shift changes caused by addition of 3MB	49
Fig. 3.11:	Overlap of reference and titration (1 β -Lg:2 3MB) spectra	50
Fig. 3.12:	Overlap spectrum of reference and titration spectra for residue Tyr102	51

Fig. 3.13: Overlap of β -Lg reference and β -Lg-phenylacetic acid TOCSY spectra	53
Fig. 3.14: Overlap of reference and titration spectra for residue Tyr102	53
Fig. 3.15: Overlap of reference and titration spectra for residue Lys101	54
Fig. 3.16: Overlap of reference and titration spectra of residue Ala132	54
Fig. 3.17: Overlap of reference and titration spectra of residue Ala129	54
Fig. 3.18: Residues having significant chemical shift changes upon binding of phenylacetic acid	55
Fig. 4.1: Flow diagram for X-ray analysis of a macromolecule	57
Fig. 4.2: Protein X-ray diffraction system	59
Fig. 4.3: Friedel's Law	61
Fig. 4.4: Electron density map can be transformed from raw diffraction data	62
Fig. 4.5: Steps and programs used for X-ray diffraction data processing	63
Fig. 4.6: Crystallographic R -factor	68
Fig. 4.7: Programs used to generate $F_{calc}(\mathbf{h}), \varphi_{calc}(\mathbf{h})$ and to refine structural parameters	69
Fig. 4.8: The electron density maps of β -Lg after rigid-body refinement with Refmac5	73
Fig. 4.9: The electron density maps of β -Lg after restrained refinement with Refmac	74
Fig. 4.10: Electron density map contoured at 1.3σ with modeled SBT close to Met107 and Phe105	78
Fig.4.11: Electron density map contoured at 1.3σ with modeled	

<i>N</i> -ethyl-3-methyl-butanethioamide	
close to Met107 and Phe105	78
Fig.4.12: Side view of model of β -Lg and potential ligand	79
Fig. 4.13: Top view of model of β -Lg and potential ligand	79
Fig. 4.14: The side view of β -Lg composite omit map with potential ligand	80
Fig. 4.15: The top view of β -Lg composite omit map with potential ligand	80
Fig. 4.16: Overlap of electron density map, difference map and composite omit map	80
Fig. 5.1: ^1H NMR spectrum of 2-(trimethylsilyloxy)ethylamine	84
Fig. 5.2: A 6 x 4 crystal screen matrix	88