

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.

**Crystallographic and physicochemical studies on
anion-binding and deglycosylation of human lactoferrin**

A thesis presented in partial fulfilment of the requirements for the degree of
Master of Philosophy in Chemistry at Massey University

Heather Mary Baker
1995

ABSTRACT

Lactoferrin, a monomeric 80 kDa glycoprotein, is a major component of human milk and is found in many other exocrine secretions as well as in the neutrophilic granules of white blood cells. A member of the transferrin family of iron-binding proteins, lactoferrin has the ability to bind tightly but reversibly 2 Fe³⁺ ions with the concomitant binding of 2CO₃²⁻ ions. Crystal structure studies clearly demonstrate that the polypeptide chain is folded into two similar lobes, representing the N- and the C-terminal halves of the protein, and that each lobe contains one of the two very similar iron-binding sites. Transferrins also show considerable versatility in their binding properties, being able to bind many metal ions in place of Fe³⁺ and anions in place of CO₃²⁻. Differences between the two sites become more pronounced, however, with the substitution of non-native metals and anions, and the origins of this inequivalence have long been debated.

To investigate the means by which lactoferrin can accommodate anions larger than carbonate, the diferricdioxalato-lactoferrin (Fe₂(C₂O₄)₂Lf) complex was prepared and crystallised. The crystals, which were isomorphous with those of Fe₂(CO₃)₂Lf, were used to collect a complete 2.4 Å data set at the Photon Factory (Japan) synchrotron source. The structure was refined by restrained least squares methods to a final R factor of 0.196 for all 31758 reflections in the resolution range 8.0 to 2.4 Å. The polypeptide folding and domain closure were identical to those of the native Fe₂(CO₃)₂Lf. In contrast to the carbonate complex, however, in which the two binding sites appear almost identical, with the carbonate coordinating in a symmetrical bidentate mode to each iron, when oxalate is the anion, the coordination around the metal differs between the N- and the C-lobe. In the C-lobe, the oxalate has a symmetrical 1,2-bidentate coordination to the iron, but in the N-lobe this coordination is quite asymmetric (O_{1ox}-Fe = 1.87 Å, O_{2ox}-Fe = 2.55 Å). Analysis of the structure indicates that the stereochemistry of the oxalate coordination to the iron is influenced by the position of the anion-binding arginine. The position this arginine can adopt in each lobe is, in turn, influenced by residues more remote from the iron site and which differ between the N- and C-lobes.

All lactoferrins so far characterised are glycoproteins, but the importance of the glycan chains for structure and/or function has yet to be established. Enzymatic methods were used to deglycosylate human and bovine lactoferrins, and the native deglycosylated forms of the human protein were compared with respect to CD spectra, iron binding and release, stability to proteolysis and heat stability.

Deglycosylation was carried out at pH 6.0 on the iron-free form of lactoferrin, using an endoglycosidase preparation from *Flavobacterium meningosepticum*, comprising PNGase F and Endo F. Deglycosylation was rapid for human lactoferrin, being essentially complete within 12-24 hr. Only partial deglycosylation of bovine lactoferrin could be achieved under the same conditions, however, and this is attributed to the relative inaccessibility of at least one of the glycosylation sites.

The CD spectra of native and deglycosylated human lactoferrins were found to be essentially identical in the range 250-350 nm, implying the same three dimensional structures. Both also bind iron in identical fashion; 2 Fe³⁺ ions are bound and binding is complete within 1 minute. The release of iron as the pH was lowered from 8.0 to 2.0 also showed no significant difference, the pH at which 50% release had taken place being 3.2 and 3.0 respectively for native and deglycosylated proteins. Susceptibility to proteolytic digestion by bovine trypsin over a period of 24 hr showed similar fragmentation patterns and a similar time course for the reaction for both species. Iron binding ability as a function of temperature was used as a measure of heat stability; melting temperatures derived from these experiments were 64°C for native and 63°C for deglycosylated lactoferrin. Comparison of the three dimensional structures of glycosylated iron-lactoferrin with deglycosylated apo-lactoferrin are consistent with these results, showing only a small increase in flexibility near the glycosylation site, when the carbohydrate is removed.

Conclusions are that the *in vitro* physicochemical properties of lactoferrin are unaffected by the presence or absence of its glycan chains. *In vivo* studies may be necessary to establish the importance, if any, of glycosylation.

Acknowledgements

This thesis was made possible by the contributions and help of a number of people. My thanks are due in three quarters.

To my supervisors, Professors Andrew Brodie and Sylvia Rumball for their advice and encouragement throughout the course of this work.

To all members of the protein crystallography group (past and present), who contributed to the friendly and helpful working environment which made the course of this work so enjoyable.

To my family for their continuous support and encouragement from the beginning to the end of this project.

I am very grateful to Andrew and Sylvia who always made themselves available for discussion in spite of their busy schedules. Their expertise, thoughtful help, and time are very much appreciated.

While the members of the Chemistry and Biochemistry Department are gratefully acknowledged, special thanks go to all the protein crystallography group for their friendship, support, kindness and practical help. In particular I thank Dr Bryan Anderson for his help with the computing, Dr Gillian Norris for help in many aspects of the protein work and deglycosylation, Drs Clyde Smith and Musa Shongwe who shared a laboratory, and interest in the oxalate work, Richard Kingston who also shared a laboratory, many helpful discussions, books, and his computing skills and Jean King for help in preparation of this thesis.

I am especially grateful to my family, both parents and children, but above all I would like to express a particular debt of gratitude to Ted for first engendering a love of research, for all his encouragement and special support. Thanks Ted.

Table of Contents

	Page
Abstract	ii
Acknowledgements	iv
Table of Contents	v
List of Figures	viii
List of Tables	x
Abbreviations	xi
Chapter I An Introduction and Review of the Literature	1
I.1 Transferrin Family	1
Molecular Properties	2
Biological Roles of Lactoferrin	2
Physicochemical Properties	6
Protein Structure	7
Primary Structure	7
Fragment Studies	8
Crystallographic Studies	9
I.4 Metal and Anion Substitution	12
Metal Substitution	18
Anion Substitution	20
I.5 Differences Between the Two Sites	23
I.6 Questions to be Addressed by this Thesis	25
Chapter II Preparation of Diferricdioxalatolactoferrin	26
II.1 Experimental Procedures	26
Glassware and Dialysis Tubing	26
Solutions	26
Gel Electrophoresis	27
Purification of Apolactoferrin	28
Preparation of Diferricdioxalatolactoferrin	29
Preparation of Diferricdioxalatotransferrin	29
Preparation of Diferricdioxalatolactoferrin for EPR Spectroscopy	30
Crystallisation of Diferricdioxalatolactoferrin	30
II.2 Results	31
Purification of Apolactoferrin	31
Preparation of Diferricdioxalatolactoferrin	31
Electronic Spectra	32
Crystallisation	35

II.3 Discussion	35
Chapter III Crystal Structure of Diferricdioxalatolactoferrin	37
III.1 Methods	37
Stabilisation and Mounting of Crystals	37
Data Collection	37
Data Processing	38
III.2 Structure Determination	43
The Refinement Problem	43
Refinement Strategy	43
Structure Solution and Refinement	44
The Quality of the Final Model	48
III.3 Three-dimensional Structure of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	61
Secondary Structure	64
Comparison of Secondary Structure in the Two Lobes	65
Metal and Anion Sites	66
Solvent Structure	73
Chapter IV Deglycosylation of Lactoferrin and a Comparison of some Properties of the Native and Deglycosylated Forms	75
IV.1 Introduction	75
Functions of Glycoprotein Glycans	75
Glycosylation and Recombinant Proteins	77
Glycosylation of Transferrins	78
Methods of Deglycosylation	79
IV.2 Experimental	80
Preparation of Endo F and PNGase	80
Deglycosylation of Lactoferrin	82
Purification of Deglycosylated Lactoferrin	83
Tests for Carbohydrate	83
Iron Binding	84
Heat Treatment and Iron Binding	84
pH-mediated Iron Release	84
Circular Dichroism Spectra	85
Proteolysis Experiments	85
Structural Comparisons of Native and Deglycosylated Lactoferrins	86
IV.3 Results	87
Preparation of Endoglycosidase	87
Deglycosylation of Human Apolactoferrin	87

Confirmation of Deglycosylation	89
Circular Dichroism	91
Iron-binding Properties	92
Protein Stability	94
Structural Comparisons of Deglycosylated and Native Lactoferrins	96
Chapter V Discussion	100
V.1 Anion Binding by Lactoferrin	100
Anion Binding and the Schlabach Bates Model	100
Oxalate Binding to Transferrins	100
Metal-anion Relationships	102
Extension to Other Anions	105
Inequivalence of the Sites	108
Extension to Other Transferrins	110
Anion Binding and Domain Closure	112
V.2 The Role of Glycosylation of Lactoferrin	113
Appendix I	120
References	127

List of Figures

Figure		Page
I.1	The visible absorption spectra of Fe_2Tf complexes with various anions	7
I.2	Ribbon diagram of structure of Fe_2Lf	10
I.3	Folding patterns N- and C-lobes of diferric lactoferrin	11
I.4	Schematic diagram of iron and anion binding sites of lactoferrin	12
I.5	Hydrogen-bonding environment of the carbonate anion in lactoferrin	14
I.6	Folding patterns of diferric lactoferrin (N-lobe) and the sulphate binding protein	15
I.7	Anion-binding sites of lactoferrin and bacterial sulphate-binding protein	16
I.8	N-lobe of lactoferrin, showing the 'open' form of apolactoferrin and the 'closed' form of diferric lactoferrin	17
I.9	Four possible conformations of a single lobe of lactoferrin	18
I.10	'Interlocking sites' model of anion binding to transferrins	20
I.11	Generalised model for synergistic anion binding to transferrins	22
II.1	Elution profile of apolactoferrin from a Sephadex CM-50 column	31
II.2	Fe^{3+} and oxalate titrations with apolactoferrin	32
II.3a	Electronic visible spectra of oxalate and carbonate complexes of diferric lactoferrin	33
II.3b	Solutions of oxalate and carbonate complexes of diferric lactoferrin	33
II.4	EPR spectra of carbonate, mixed, and oxalate complexes of diferric lactoferrin	34
II.5	Crystal of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	35
II.6	Reaction scheme for preparation and interconversion of carbonate and oxalate complexes of diferric lactoferrin	36
III.1	The percentage of unique reflections in each resolution shell in the final data set for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	42
III.2	$\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$ - $\text{Fe}_2(\text{CO}_3)_2\text{Lf}$ difference electron density maps	45
III.3	Luzzati plot of resolution against the R factor for the final model of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	50
III.4	Ramachandran plot of the ϕ and ψ angles for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	52
III.5	PROCHECK assessments for the correctness of the mainchain parameters of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$ model	53
III.6	PROCHECK assessments for the correctness of sidechain parameters of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$ model	54
III.7	Plot of Chi-1 vs Chi-2 for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	55

Figure	Page	
III.8	Real space correlation coefficient against residue number, and temperature factor against residue number for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	57
III.9	Examples of well defined electron density	58
III.10	Examples of the most poorly defined electron density	59
III.11	Schematic representation of the secondary structure elements for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	62
III.12	Stereo $\text{C}\alpha$ plot of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	62
III.13	Richardson-type representations of N- and C-lobes of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	63
III.14	Views of $2F_0-F_c$ electron density in the oxalate-binding site of N-lobe and C-lobe of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	70
III.15	Stereo views of the iron-binding site of the N-lobe and C-lobe of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	71
III.16	Superpositions of the oxalate ions from the N- and C-lobes of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	72
III.17	Superpositions of the Arginine 121 on Arginine 465 of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	73
IV.1	Primary structure of human lactoferrin glycans	79
IV.2	SDS gel assay for endoglycosidase activity	82
IV.3	Elution profile for gel filtration purification of endoglycosidase	87
IV.4	SDS gel monitoring deglycosylation of human apolactoferrin	88
IV.5	Carbohydrate analysis of native and deglycosylated lactoferrin	90
IV.6	CD spectra of native and deglycosylated apolactoferrin	91
IV.7	Iron binding by native and deglycosylated apolactoferrin	92
IV.8	pH mediated iron release from native and deglycosylated lactoferrin	93
IV.9	Effect of trypsin digestion on native and deglycosylated lactoferrin	94
IV.10	Thermal stability of native and deglycosylated lactoferrin	96
IV.11	Plots of B factor versus residue number for Fe_2Lf , $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$, ApoLf (deglycos), FeLf_N (deglycos)	98
V.1	Oxalate coordination in small molecule structures	101
V.2	Energy levels for $\text{Fe}_2(\text{CO}_3)_2\text{Lf}$, $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$, $\text{Cu}(\text{CO}_3)_2\text{Lf}$ and $\text{Cu}(\text{C}_2\text{O}_4)_2\text{Lf}$	104
V.3	Generalised model for anion binding to transferrins	105
V.4	Structure of bovine lactoferrin around the glycosylation site Asn 545	114
V.5	Glycosylation sites of the lactoferrin family	116
V.6	Glycosylation sites of the transferrin family	117

List of Tables

Table		Page
I.1	The occurrence of lactoferrin in various bodily secretions	3
I.2	Residues involved in metal and anion binding in the transferrins	13
II.1	Absorption maxima and extinction coefficients of transferrins	34
III.1	Image plate data collection statistics	38
III.2	Data collection and processing statistics for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	39
III.3	Merging of intensity data	41
III.4	Statistics on the final data set for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	42
III.5	Comparison of unit cell dimensions of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$ and $\text{Fe}_2(\text{CO}_3)_2\text{Lf}$	43
III.6	Course of refinement of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	47
III.7	Refinement statistics for $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	49
III.8	Poorly defined regions of the structure	60
III.9	Residue assignments for the secondary structure of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	64
III.10	Bond lengths and angles at the iron sites of $\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	67
III.11	Hydrogen bonding interactions around the oxalate ions	69
IV.1	Functions of glycoprotein glycans	76
IV.2	<i>RMS</i> deviation in $\text{C}\alpha$ positions of N2 domains of glycosylated and deglycosylated lactoferrins	97
IV.3	Comparison of normalised B values of glycosylated and deglycosylated lactoferrin for residues 134-144	99
V.1	Comparison of hydrogen-bonding potential of synergistic anions, and stability of anion-transferrin complex	107
V.2	Residues that may modulate anion binding in human lactoferrin	109
V.3	Residues that may modulate anion binding in transferrins	110
V.4	Potential glycosylation sites of lactoferrins	116

Abbreviations

apoLf	metal-free human lactoferrin
apoTf	metal-free human serum transferrin
β -me	β -mercaptoethanol
bLf	bovine lactoferrin
CD	circular dichroism
cOTf	chicken ovotransferrin
$\text{Cu}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	dicupricdioxalatolactoferrin
$\text{Cu}_2(\text{CO}_3)(\text{C}_2\text{O}_4)_2\text{Lf}$	dicupric-(carbonato-oxalato)lactoferrin
Cu_2Lf	dicupriclactoferrin
EDTA	ethylenediaminetetraacetic acid
Endo F	endo β -N-acetylglucosamidase F ₁
EPR	electron paramagnetic resonance
ESEEM	electron spin echo envelope modulation
EXAFS	extended-X-ray absorption-fine-structure
$\text{Fe}_2(\text{C}_2\text{O}_4)_2\text{Lf}$	diferricdioxalatolactoferrin
$\text{Fe}_2(\text{CO}_3)(\text{C}_2\text{O}_4)_2\text{Lf}$	diferric-(carbonato-oxalato)lactoferrin
Fe_2Lf	diferric human lactoferrin
Fe_2Tf	diferric transferrin
FeLf _N	recombinant human N lobe lactoferrin, with iron
hTf	human transferrin
Lf	lactoferrin
mLf	mouse lactoferrin
MPD	2-methyl-2,4 pentanediol
msTf	Tobacco Hornworm transferrin
MTf	melanotransferrin
nmr	nuclear magnetic resonance
NTA	nitrilotriacetic acid
OTf	ovotransferrin
ovoTf	ovotransferrin
PAGE	polyacrylamide gel electrophoresis
PNGase	peptide-N ⁴ -(N-acetyl- β -D-glucosaminy)asparagine amidase F
rTf	rabbit transferrin
SDS	sodium dodecylsulphate
Tf	serum transferrin
UV/vis	ultraviolet and visible electronic absorption spectroscopy
XTf	Xenopus transferrin