

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.

STUDIES ON LACTOFERRIN,
A METAL BINDING PROTEIN IN HUMAN MILK

A Thesis presented in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy
in Chemistry
at Massey University

Jeffrey Ernest Plowman

1979

"Its a bit late in the day to introduce the idea now, but almost any mammal's milk would be easier to modify than cow's milk. Pig's milk is actually nearest to human milk. Camel milk and mare's milk have a better balance for humans. Sheep's milk is OK and so is goat's. Reindeer milk would be a bit fat, dog's milk a bit thin. Now, otter's milk could be just right. Perhaps we should look into it."

M. Bateman ("Sunday Times" - London, 16 March 1975)

ABSTRACT

Lactoferrin (Lf), isolated from human colostrum, has been complexed with a variety of transition metal ions. In addition to binding two iron(III) or copper(II) ions it strongly and specifically binds two cobalt(III), chromium(III) and manganese(III) ions, in the presence of bicarbonate. Such strong, specific binding of copper(II) to lactoferrin will not occur in the absence of bicarbonate, instead only a weak interaction is observed. Lactoferrin has also been shown to weakly bind manganese(II), cobalt(II), nickel(II), zinc(II), cadmium(II), lead(II) and mercury(II), though manganese(II) will undergo aerial oxidation in the presence of the protein and bind as manganese(III). These metal ion protein complexes have been examined using the techniques of fluorescence, electronic, e.s.r. and Resonance Raman spectroscopy. The close similarity between the spectra of the complexes Fe_2Lf , Cu_2Lf , Co_2Lf , Mn_2Lf and Cr_2Lf with those of transferrin and ovotransferrin reinforce the postulate that the metal binding sites in all three proteins are similar. The sites in all three proteins are essentially rhombic in character and contain 2-3 tyrosyl residues and at least one histidyl residue. A distinct heterogeneity is evident from the e.s.r. spectrum of Cr_2Lf and metal ion replacement studies indicate that chromium in one site is more labile than the other. In addition to the specific sites lactoferrin appears to have a number of non-specific sites on the outside of the protein capable of weakly coordinating metal ions such as chromium(III) and copper(II). Differences evident in the non-specific sites available to copper when manganese(III) is bound to lactoferrin, to those available when iron(III), cobalt(III) and chromium(III) are bound, suggest that the stronger binding of manganese(III) results from it inducing a different conformational change in the protein to those other metals and one that favours the higher stability of its metal-protein complex.

A series of small molecular weight complexes of iron(III) with bi-, tri-, tetra- and hexadentate ligands, containing phenolate groups were prepared and examined by electronic,

e.s.r. and Mössbauer spectroscopy. Complexes of copper(II) with nitrogen bases and chloro- and bromo-substituted phenolates were prepared and examined by electronic and e.s.r. spectroscopy. Results from these studies would favour the involvement of three tyrosyl residues and two cis histidyl residues in the metal-binding sites of lactoferrin and from studies on the copper complexes it seems likely that one histidyl and one tyrosyl are axially coordinated to the metal. Evidence from a single crystal x-ray structure is presented which would favour the interaction of bicarbonate ion with iron(III) via a solvent (water) molecule.

ACKNOWLEDGEMENTS

I am most grateful to:

Dr E. W. Ainscough and Dr A. M. Brodie for their invaluable assistance, advice and encouragement through all stages of this project.

Dr W. C. Tennant, Dr D. G. McGavin and Dr A. R. Gainsford of Chemistry Division, D.S.I.R., Petone, for their assistance in running and analysing e.s.r. and Mössbauer spectra and for the use of their Mössbauer spectrometer.

Dr K. L. Brown of Chemistry Division, D.S.I.R., Petone, for the single crystal x-ray structural analysis of one of my compounds.

Professor N. F. Curtis of the Chemistry Department, Victoria University, Wellington, for the use of his Faraday Balance.

Professor A. D. Campbell of the Chemistry Department, Otago University, Dunedin, for microanalyses.

Professor T. M. Loehr of Oregon Graduate Centre, Beaverton, Oregon, for running and analysing spectra of some metal-lactoferrin solutions.

Dr A. W. Addison of the Chemistry Department, Drexel University, Philadelphia for supplying two of the ligands used in Chapter 2.

Dr S. V. Rumball, Dr E. N. Baker, Dr G. G. Midwinter, Mr A. Trow, Mr S. J. Bloor and others of the Chemistry/Biochemistry/Biophysics Department, Massey University, for their assistance and advice.

Mrs L. Reid for typing this thesis and Mrs J. Trow for preparation of some of the diagrams.

TABLE OF CONTENTS

	Page
Abstract	iii
Acknowledgements	v
Table of Contents	vi
List of Figures	vii
List of Tables	x
Introduction	1
Chapter 1 Lactoferrin	
1.1 Introduction	10
1.2 Experimental	10
1.3 Results and Discussion	19
1.4 Conclusion	58
Chapter 2 Small Molecule Complexes of Iron	
2.1 Introduction	60
2.2 Experimental	62
2.3 Results and Discussion	68
2.4 Conclusion	87
Chapter 3 Small Molecule Complexes of Copper and Manganese	
3.1 Introduction	90
3.2 Experimental	92
3.3 Results and Discussion	93
3.4 Conclusion	110
Appendix Publications arising from this Thesis	114
References	115

LIST OF FIGURES

Figure		Page
1	Proposed structure of the iron binding site of lactoferrin, transferrin and ovotransferrin.	7
1.1	CM-Sephadex C-50 chromatography of the supernatant prepared from human milk.	12
1.2(a)	SDS polyacrylamide gel electrophoresis two bands eluted from a CM-Sephadex C-50 column.	14
(b)	SDS polyacrylamide gel electrophoresis of lactoferrin.	
1.3	Polyacrylamide gel electrophoresis of lactoferrin at pH 2.9.	15
1.4	Uptake of iron complexes by citrate-treated apolactoferrin and apolactoferrin.	21
1.5	The influence on the uptake of 1:1 iron-citrate by apolactoferrin of other metal ions.	24
1.6	Visible absorption spectra of Fe_2Lf and Cu_2Lf , in phosphate buffer pH 7.6, 0.01M.	31
1.7	Visible absorption spectra of Co_2Lf and Mn_2Lf , in Tris buffer pH 7.4, 0.05M.	32
1.8	Visible absorption spectrum of Cr_2Lf , in Tris buffer pH 7.4, 0.05M.	33
1.9	Transition metal complexes of Mn_2Lf , Co_2Lf , Fe_2Lf , Cu_2Lf and Cr_2Lf .	34
1.10	E.S.R. spectra at -160°C of (a) Fe_2Lf in phosphate buffer, pH 7.6, 0.01M, and Fe_2Lf in phosphate buffer titrated to pH 2.0 with (b) citric acid and (c) phosphoric acid.	38
1.11	E.S.R. spectra at -160°C of a solution	39

	containing 1 mole of ferric perchlorate and 3 moles of citric acid at (a) pH 2.0, (b) pH 4.1 and (c) pH 4.7.	
1.12	E.S.R. spectra at -160°C in phosphate buffer 0.01M, pH 7.6 of (a) Cu_2Lf , (b) 2 molar equivalents of copper(II) added to Fe_2Lf and (c) bicarbonate-free copper-lactoferrin.	42
1.13	E.S.R. spectra at -160°C of (a) copper(II) in Tris buffer, pH 7.4, 0.05M and 2 molar equivalents of copper(II) added to (b) Mn_2Lf and (c) Cr_2Lf in Tris buffer.	43
1.14	E.S.R. spectra at -160°C of (a) and (b) Cr_2Lf in Tris buffer and (c) Cr_2Lf after the addition of one molar equivalent of iron(III).	46
1.15	Resonance Raman spectra of (a) Fe_2Lf , (b) Cu_2Lf , (c) Co_2Lf , (d) Mn_2Lf and (e) Cr_2Lf .	53
1.16	Resonance Raman excitation profiles of (a) Co_2Lf , (b) Cu_2Lf and (c) Fe_2Lf .	56
2.1	Nomenclature and structure of the ligands complexed with iron(III).	61
2.2	The molecular conformation of $[\text{Fe}(\text{IIIa})_2(\text{MeOH})_2]\text{NO}_3 \cdot \text{MeOH}$.	71
2.3	Visible absorption spectra of $[\text{Fe}(\text{IIIa})_3]1\frac{1}{2}\text{H}_2\text{O}$ and $[\text{Fe}(\text{IIIa})_2(\text{MeOH})_2]\text{NO}_3 \cdot 2\text{H}_2\text{O}$ in methanol, and diffuse reflectance spectrum of $[\text{Fe}(\text{IIIa})(\text{MeOH})_4]^{2+}$.	76
2.4	E.S.R. spectra at -160°C of (a) $[\text{Fe}(\text{IIIa})_3]1\frac{1}{2}\text{H}_2\text{O}$ and (b) $[\text{Fe}(\text{IIIa})_2(\text{MeOH})_2]\text{NO}_3 \cdot 2\text{H}_2\text{O}$ in Acetone, and (c) $[\text{Fe}(\text{IV})_3]1\frac{1}{2}\text{H}_2\text{O}$ in CH_2Cl_2 .	80
2.5	E.S.R. spectra at -160°C of (a)	81

	[Fe(VIIa)]PF ₆ in Acetone and (b) [Fe(VIIb)]PF ₆ in MeOH.	
2.6	E.S.R. spectra at -160°C in MeOH of (a) a 1:1 solution of Fe(ClO ₄) ₃ and VIII, (b) a 1:1:1 solution of Fe(ClO ₄) ₃ , VIII and imidazole and (c) excess imidazole added to (b).	82
2.7	Room temperature Mössbauer spectra of (a) Na[Fe(Va)]4H ₂ O and (b) [Fe(II) ₃].	85
2.8	Room temperature Mössbauer spectra of (a) [Fe(IIIa) ₃]1½H ₂ O, (b) [Fe(IIIa) ₂ (MeOH) ₂]NO ₃ .2H ₂ O, (c) [Fe(IIIb) ₃]4H ₂ O and [Fe(IV ₃)]1½H ₂ O.	86
3.1	Nomenclature and structures of the ligands complexed with copper(II).	91
3.2	Visible absorption spectra of [Cu (tbp) ₂ (en)] and [Cu(tbp) ₂ (py) ₂] in ethanol.	99
3.3	E.S.R. spectra at -194°C of (a) [Cu(tbp) ₂ (py) ₂] in ethanol and (b) [Cu(cyclam)(pcp) ₂] in dmf.	106
3.4	E.S.R. spectra at -194°C of (a) [Cu(pcp) ₂ (collidine)] in acetone and (b) [Cu(pcp) ₂] in methanol.	107
3.5	Visible absorption spectrum of [Mn(SALENH ₄)(Acetato)](Acetone)1½H ₂ O in EtOH.	111
3.6	Proposed coordination sphere of iron(III) in the binding site of lactoferrin, transferrin and ovo- transferrin based on the evidence presented in this thesis.	113

LIST OF TABLES

Table		Page
1.	Concentration of lactoferrin in various mam- malian milks.	2
2.	Approximate concentration of lactoferrin in various secretions.	2
1.1	Times required to half saturate Apolacto- ferrin and Apotransferrin with Iron(III) and Copper(II).	22
1.2	Fluorescence Quenching of Apolactoferrin by Divalent and Trivalent Metal Ions.	26
1.3	Binding of Manganese(II) and Cobalt(II) by Apolactoferrin after the addition of Hydrogen Peroxide.	28
1.4	Binding of Manganese(II) and Cobalt(II) to Apolactoferrin in the absence of Hydrogen Peroxide.	28
1.5	Binding of two Iron(III) Equivalents by Metal-Lactoferrin Complexes.	29
1.6	Electronic Spectral Data for Human Lacto- ferrin Complexes.	30
1.7	Electronic Spectral Data for Cr(III) Complexes.	36
1.8	Characterisation of Iron(III) High Spin E.S.R. Spectra.	40
1.9	E.S.R. Spectra of Complexes of Iron(III) with Lactoferrin.	44
1.10	E.S.R. Spectra of Complexes of Chromium(III) with Lactoferrin and NTA.	45
1.11	E.S.R. Spectra of Complexes of Copper(II) with Lactoferrin and Copper(II) in Buffer Solutions.	48
1.12	E.S.R. Spectra of Copper(II) added to Fe_2Lf , Cr_2Lf and Mn_2Lf .	48
1.13	Bond Lengths of Manganese(III) Complexes.	51

1.14	Raman Spectral Data for Native and Metal-Substituted Human Lactoferrin.	54
2.1	Analytical Data of Small Molecular Weight Ferric Ion Complexes.	66
2.2	Conductivities and Magnetic Susceptibilities of Small Molecular Weight Ferric Ion Complexes.	67
2.3	Electronic Spectra of Small Molecular Weight Ferric Ion Complexes.	74
2.4	Electron Spin Resonance Spectra of Small Molecular Weight Ferric Ion Complexes.	79
2.5	Mössbauer Spectral Data of Small Molecular Weight Ferric Ion Complexes.	84
3.1	Colours and Analytical Data for the Copper Phenolate Complexes.	94
3.2	Conductivities and Magnetic Moments for the Copper Phenolate Complexes.	95
3.3	Diffuse Reflectance Spectra of the Copper Phenolate Complexes.	100
3.4	Electronic Solution Spectra of the Copper Phenolate Complexes.	101
3.5	Electron Spin Resonance Spectra of the Copper Phenolate Complexes.	108