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

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## 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 Fe<sup>3+</sup> ions with the concomitant binding of 2CO<sub>3</sub><sup>2-</sup> 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 Fe<sup>3+</sup> and anions in place of CO<sub>3</sub><sup>2-</sup>. 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 (Fe<sub>2</sub>(C<sub>2</sub>O<sub>4</sub>)<sub>2</sub>Lf) complex was prepared and crystallised. The crystals, which were isomorphous with those of Fe<sub>2</sub>(CO<sub>3</sub>)<sub>2</sub>Lf, 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 Fe<sub>2</sub>(CO<sub>3</sub>)<sub>2</sub>Lf. 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<sub>1ox</sub>-Fe = 1.87 Å, O<sub>2ox</sub>-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 Fe<sup>3+</sup> 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.

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## Abbreviations

|   |  |
|---|--|
| apoLf   | metal-free human lactoferrin   |
| apoTf   | metal-free human serum transferrin   |
| $\beta$ -me   | $\beta$ -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  |
| $\text{Cu}_2\text{Lf}$                                      | dicupriclactoferrin  |
| EDTA  | ethylenediaminetetraacetic acid  |
| Endo F  | endo $\beta$ -N-acetylglucosamidase F <sub>1</sub>                             |
| 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  |
| $\text{Fe}_2\text{Lf}$                                      | diferric human lactoferrin   |
| $\text{Fe}_2\text{Tf}$                                      | diferric transferrin   |
| FeLf <sub>N</sub>   | 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 <sup>4</sup> -(N-acetyl- $\beta$ -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  |