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Metal and anion binding sites in lactoferrin and related proteins

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Abstract - The metal and anion binding sites of the protein lactoferrin (Lf) have been defined through crystallographic analyses of Fe_2Lf and Cu_2Lf. In both cases each metal ion is 6-coordinate, with four protein ligands (2 Tyr, 1 Asp, 1 His) and the synergistic CO_3^{2-} anion which binds as a bidentate ligand. The CO_3^{2-} fits into a pocket between the metal and the N-terminus of an α-helix. Binding of other metals and anions can be understood in terms of the protein structure and a suggested mechanism for binding and release. Differences between the two sites in Lf can also be explained. Striking similarities between Lf and a SO_4^{2-} binding protein suggest links between bacterial and mammalian binding proteins.

INTRODUCTION

Lactoferrin, together with serum transferrin and ovotransferrin, is a member of the family of iron-binding proteins, the transferrins (ref. 1), which play a key role in regulating iron levels in biological fluids. These proteins are monomeric glycoproteins, M_r 80,000, each with the ability to bind very tightly ($K \sim 10^{20}$) but reversibly two iron atoms, as Fe^{3+}. Other metals can be substituted for iron, in the two specific sites; those of similar size and charge such as Ga^{3+} and Al^{3+}, bind with affinities close to that of Fe^{3+}, but even much larger cations, such as lanthanides, can be accommodated. A striking feature of transferrin chemistry is the requirement that a suitable anion be bound with each metal ion. The relationship is synergistic in that neither cation nor anion is bound significantly in the absence of the other. CO_3^{2-} (or HCO_3^-) is the anion of highest affinity, but other anions with a carboxylate group can be substituted (ref. 2). We have addressed the structural basis for this unique anion-cation relationship, and for the great stability of the iron-protein complex, through crystallographic studies of human lactoferrin (Lf) (refs. 3, 4).

METAL AND ANION SITES IN LACTOFERRIN

The structure of human iron-lactoferrin, Fe_2Lf, initially determined at 3.2 Å resolution (ref. 3), has now been refined using crystallographic data to 2.2 Å. The present model, comprising 5319 protein atoms (691 amino acid residues) two Fe^{3+} ions, two CO_3^{2-} ions, and 320 water molecules, gives an R factor of 0.185. The bilobal molecule is shown below.

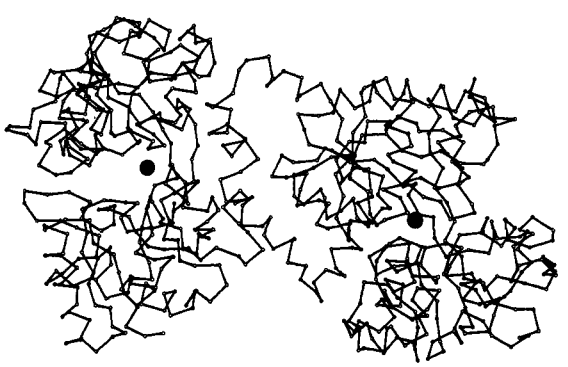

Fig. 1. C_α plot of human lactoferrin, showing its subdivision into 2 lobes, N-lobe (left) and C-lobe (right). The two iron atoms are shown as filled circles, bound in the cleft between the two structural domains of each lobe.

The transferrins have long been recognised as metal-phenolate complexes, and the binding of metals can be followed by UV difference spectroscopy, monitoring the ionisation of two tyrosine residues at each metal site (ref. 5). In lactoferrin, the protein provides four iron ligands at each site, *viz*. two phenolate oxygens, one carboxylate oxygen and one imidazole nitrogen, from 2 Tyr, 1 Asp and 1 His. A protrusion from the iron density, first

seen at lower resolution (ref. 3), has now been confirmed as the CO_3^{2-} anion, bound in bidentate mode to iron. This structure, 6-coordinate Fe(III), with a distorted octahedral geometry (Fig. 2), is the same at both iron sites. Independent crystallographic refinements of serum transferrin and a half-molecule fragment of transferrin (refs. 6, 7) confirm the same structure. There is no evidence for a water ligand; the reduction in coordination number seen by EXAFS (ref. 8) when ovotransferrin is freeze-dried must result from rearrangement of the iron ligands, e.g. movement of the CO_3^{2-}, following the loss of nearby H bonded (but non-coordinated) waters.

The CO_3^{2-} ion fits into a pocket between the iron atom and two positively charged protein groups, an arginine sidechain and a helix N-terminus (Fig. 2). Both of the latter are commonly recognised as anion-binding groups in proteins. The preference for CO_3^{2-} as the synergistic anion must result from its near-perfect fit into this pocket; the two coordinated oxygens also receive H bonds from protein NH groups, while the non-coordinated oxygen is involved in two H bonds (Fig. 4). All of the H bonds have close-to-optimal geometry. If anion binding precedes metal binding, the role of the anion may be to neutralise these positive groups in the binding cleft, and so create, with the carboxylate and phenolate oxygens, a highly anionic site, with a consequent high affinity for Fe^{3+}.

STRUCTURAL FACTORS IN BINDING AND RELEASE

The construction of each iron site (Fig. 3) suggests a mechanism for iron binding and release, and explains why a considerable variety of metal ions and anions can be accommodated. The iron lies buried at the back of a deep cleft between two protein structural domains. Behind it run two antiparallel strands of polypeptide chain, linking one domain to the other; flexing of these two 'backbone' strands would allow the cleft to open to permit iron release, or to close over a bound iron atom. A closed to open conformational transition is a feature of kinetic models for iron release (refs. 9, 10), and our own very recent crystallographic studies on iron-free lactoferrin (unpublished work) have directly demonstrated such an open conformation.

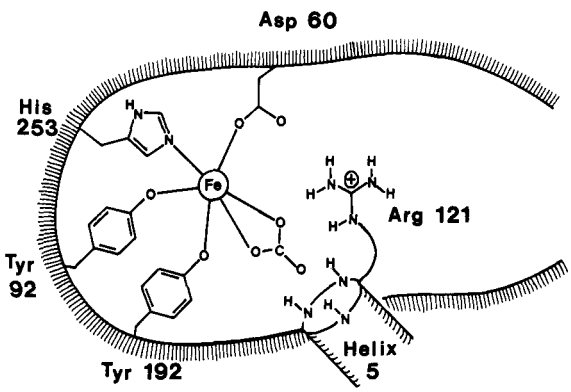

Fig. 2. Schematic diagram of iron and anion binding site in human lactoferrin (shown for N-lobe).

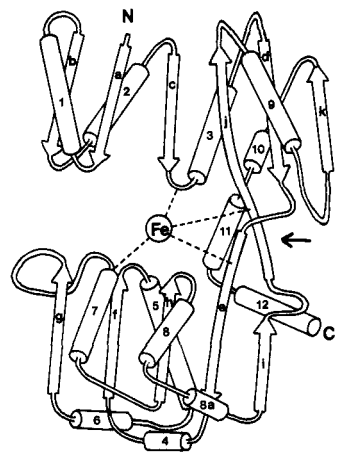

Fig. 3. Polypeptide chain folding in each lobe of Lf. (Helices cylinders, β-strands arrows.) Possible hinge point indicated ← .

In contrast to many metalloproteins, where two or more ligands are often closely spaced along the polypeptide chain (e.g. the copper binding loops of blue copper proteins), the ligands in lactoferrin are widely spaced, and belong to structurally distinct regions, - Asp 60 to domain 1, Tyr 192 to domain 2, and Tyr 92 and His 253 to the two backbone strands (Fig. 3). A model for binding, consistent with other binding proteins, is that iron binds first to one domain (probably domain 2, to which the CO_3^{2-} would already be bound), after which the other domain (1) closes over it to complete coordination. The carboxylate group of Asp 60, from domain 1, appears to play a key role. Domain closure brings two helix N-termini (of helix 3 in domain 1, and helix 5 in domain 2) close together. Their positive charges should repel, but the carboxylate group of Asp 60 intervenes, H bonding (through OD2) with both, while at the same time binding to iron through OD1.

Closure of the two domains over the metal (rather than metal binding in a prepared site of defined size and geometry) explains the accommodation of a variety of metal ions, from Al^{3+} (ionic radius 0.51 Å), through trivalent transition metal ions such as Cr^{3+}, Mn^{3+}, Fe^{3+},

Co^{3+} (radii 0.63-0.66 Å), divalent ions such as Cu^{2+} and Zn^{2+} (radii 0.72-0.74 Å) to trivalent lanthanides (radii 0.85-1.04 Å). Binding of the larger cations would be at the expense of a lesser closure of the domains. Moreover only the Asp ligand is constrained (by H bonding to the protein) and the others could adjust to the coordination of different metals. The preference for Fe^{3+} (and CO_3^{2-}) is explained by the optimal protein-anion and protein-protein (involving the Asp ligand) H bonds that result. Larger cations would certainly disrupt this structure, leading to lower stability.

The substitution of other anions for CO_3^{2-}, as synergistic anion, can now also be understood in terms of the lactoferrin structure. All synergistic anions appear to have in common a carboxylate group and another potential electron donor ligand L on the adjacent carbon (ref. 2). Model-building into the lactoferrin structure suggests that these anions could bind as shown for oxalate in Fig. 4. For simultaneous binding of the carboxyl group to the helix 5 N-terminus (residues 123-124) and of L to the metal, these other anions cannot bind exactly as CO_3^{2-} does, and the arginine sidechain must be partially displaced. This is possible because there is a large cavity beyond the arginine (Fig. 2), filled only with water; indeed the size of this cavity clearly accounts for the binding of some quite bulky anions. The failure of small ions such as SO_4^{2-} and NO_3^-, to substitute for CO_3^{2-} must depend on their different steric and H bonding characteristics.

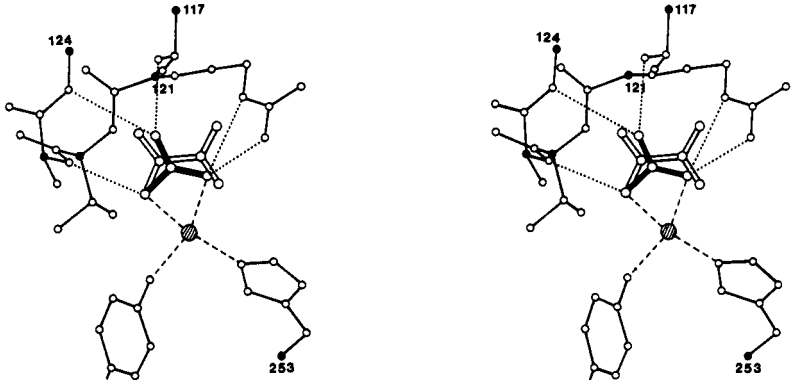

Fig. 4. Anion binding in Lf. The CO_3^{2-} (filled bonds) is H bonded to Thr 117, Arg 121 and the NH groups of 123 and 124. The same structure is found in serum transferrin (ref. 7). A modelled oxalate (open bonds) is also shown.

COPPER-LACTOFERRIN

To obtain structural data on the binding of metals other than iron we have prepared and crystallised copper-substituted lactoferrin, Cu_2Lf. The crystals are isomorphous with those of Fe_2Lf (cell dimensions a = 155.9, b = 96.9, c = 55.95 Å, space group $P2_12_12_1$ for Cu_2Lf, compared with a = 156.2, b = 97.3, c = 55.85 Å, space group $P2_12_12_1$ for Fe_2Lf). Crystallographic refinement at 2.7 Å resolution (current R factor 0.22) shows that the two copper atoms occupy the same sites as iron, with only minor differences in coordination geometry. There are indications that the orientation of the CO_3^{2-} anion in the N-lobe is slightly different from that in Fe_2Lf, being less bidentate, and that one Tyr ligand (Tyr 92) makes a slightly longer bond, giving a geometry changed somewhat towards distorted tetragonal. Although these observations must be tested by refinement at higher resolution, they are consistent with spectroscopic data (ref. 11).

DIFFERENCES BETWEEN BINDING SITES

The two binding sites in lactoferrin are identical with respect to both the iron ligands and the groups that define the anion sites. The same appears true of other transferrins, as shown by structure and sequence comparisons (refs. 4, 6); Asp 60, Tyr 92, Tyr 192, His 253, Thr 117, and Arg 121 are invariant, and residues 122-125 at the helix N-terminus are highly conserved. Only in the C-lobe of melanotransferrin are there major changes (Asp → Ser, Thr → Ala, Arg → Ser), suggesting it may not bind iron. Nevertheless there are small but distinct differences, both between different transferrins and between the N- and C-terminal sites in any given molecule (ref. 1). Possible explanations for the stronger binding and slower release by the C-terminal site have been given previously (refs. 3, 4). Refinement of the Lf structure now shows that the C-lobe is slightly more closed over the iron than the N-lobe, resulting from a relative domain rotation of ~5°. At the lips of the cleft the two domains are ~2 Å closer together in the C-lobe than in the N-

lobe and while the differences are much less at the iron site (~0.2-0.3 Å) they do appear to produce a slightly tighter fit of metal and anion in the C-lobe. This may also explain why some larger cations can apparently only be accommodated in one site (ref. 12), and why copper-substituted transferrins can bind oxalate as the synergistic anion only in the N-lobe site (ref. 11).

COMPARISON WITH OTHER BINDING PROTEINS

The striking similarity between lactoferrin and one member of a group of bacterial binding proteins, the sulphate-binding protein (SBP) from Salmonella typhimurium (ref. 13), has been noted before (refs. 3, 4). The similarity includes size, domain structure, polypeptide chain folding and the importance of conformational change in binding and release. Now that the anion site in Lf has been established there are also seen to be similarities in the binding of the CO_3^{2-} and SO_4^{2-} anions (Fig. 5). In both Lf and SBP the anion is primarily associated with the N-terminus of a particular helix (5) in domain 2, through H bonds from two NH groups and a sidechain, to three anion oxygens. In Lf the anion is then linked to domain 1 via the iron atom and its Asp ligand and in SBP via two more hydrogen bonds from helix N-termini. This suggests that the initial event in binding is the binding of the anion to domain 2. For Lf this would be followed by iron binding and the closure of domain 1 over the site; for SBP simply by the closure of domain 1.

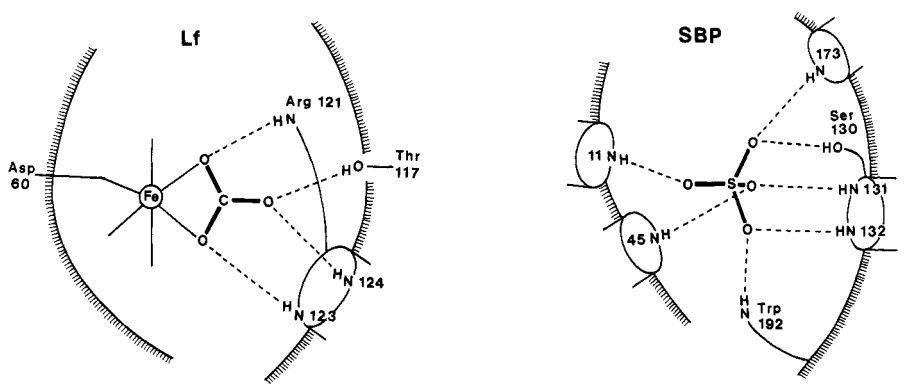

Fig. 5. Anion binding by lactoferrin (left) and sulphate binding protein (right).

These similarities between lactoferrin and SBP are such as to suggest that the two families of binding proteins may have diverged from a common ion-binding precursor molecule (with a subsequent doubling of transferrin, through gene duplication, giving the present two-sited molecule). Perhaps the transferrins can be regarded as anion binding proteins which have evolved to have an additional metal-binding function?

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