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Molecular structure, binding properties and dynamics of lactoferrin

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Abstract. Lactoferrin (Lf), a prominent protein in milk, many other secretory fluids and white blood cells, is a monomeric, 80-kDa glycoprotein, with a single polypeptide chain of about 690 amino acid residues. Amino acid sequence relationships place it in the wider transferrin family. Crystallographic analyses of human Lf, and of the Lfs from cow, horse, buffalo and camel, reveal a highly conserved three-dimensional structure, but with differences in detail between species. The molecule is folded into homologous N- and C-terminal lobes, each comprising two domains that enclose a conserved iron binding site. Iron binding and release is accompanied by domain movements that close or open the sites, and is influenced by cooperative interactions between the lobes. Patches of high positive charge on the surface contribute to other binding properties, but the attached glycan chains appear to have little impact on structure and function.

Key words. Lactoferrin; crystal structures; domains; conformational change; iron; cooperative interactions; surface charge; glycosylation.

Introduction

Lactoferrin (Lf) has been the subject of intensive structural and functional studies since it was first isolated, simultaneously in three separate laboratories, almost 50 years ago [1–3]. An 80-kDa glycoprotein, its most striking characteristic was its intense red colour when incubated in the presence of Fe^{3+} ions. This led to an early recognition that it was likely to be an analogous protein to the iron binding protein of serum, transferrin (Tf), leading to its description as lactotransferrin or lactoferrin. It is now apparent that Lf is not confined only to mammalian milks, but is also a component of many external secretions such as saliva, tears, semen and mucosal secretions [4], and is further an important constituent of the neutrophilic granules of leucocytes [5].

The most striking physicochemical feature of Lf is its very high affinity for iron. As for the related Tfs, two Fe^{3+} ions are bound, very tightly ($K \sim 10^{22}$ M) but reversibly, together with two synergistically bound CO_3^{2-} ions [6, 7]. This led to the early presumption that the biological role of Lf was in iron absorption in infant nutrition, or as a bacteriostatic protein because of its ability to deprive bacteria of iron essential for growth [8]. This has now been replaced, however, by recognition that it has a considerable variety of other functions, some dependent on its iron-binding properties, and others not. These functions, which are still being elucidated, are the subject of the accompanying reviews in this series.

The determination of the amino acid sequence of Lf in 1984 [9] confirmed that it belonged to the wider Tf family; the human Lf sequence was found to have ~60 % sequence identity with human serum Tf, and share the same internal duplication, whereby the N-terminal half of the protein had ~40 % sequence identity with the C-terminal half. The three-dimensional structure of human Lf, determined 3 years later [10, 11], showed that this internal sequence homology was expressed in a bilobal structure comprising N-terminal and C-terminal lobes, both with very similar folds and both possessing essentially identical iron binding sites. Subsequent crystal structures have

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revealed structural variations in the iron-free (apo) form, in Lfs of different species, complexes of different metal ions or anions, half-molecules and site-specific mutants. Between them, these offer a fairly complete picture of the molecular characteristics of Lf. Previous relevant reviews have focused on the Tf family as a whole [7], on the relationship between Lfs and Tfs [12, 13], on the structure of Lf itself [14] and on comparisons with other binding proteins [15]. Here we update the structural knowledge on Lf to set the framework for the discussion of function in the accompanying reviews.

What defines a Lf?

Lf appears to be found only in mammals, suggesting that its emergence had to do with infant nutrition and protection. In contrast, its close relative Tf can be found in species as distant as insects [7]. Presumably this means that Lf arose through duplication of the Tf gene at the time of emergence of mammals, with subsequent divergence in its amino acid sequence and functional properties. Current sequence databases contain amino acid sequences for the Lfs of nine species, human, pig, horse, cow, buffalo, sheep, goat, camel and mouse. Pairwise sequence identities range from a minimum of ~65 % to nearly 100 %. The main outliers in this group are human Lf and mouse Lf; these have 70–74 % and 65–70 % identity, respectively, with each of the others. On the other hand, cow, buffalo, goat and sheep Lfs share over 90 % sequence identity with each other and form an extremely closely related group.

Given the close similarity of Lf and Tf it is relevant to ask how the two may be distinguished, and what properties define a Lf. Biological location is not definitive, since the milks of some species, such as rat, reportedly contain Tf rather than Lf [16]. The sequence relationships given above show that the Lfs form a highly conserved sequence family, but sequence identity between Lfs and Tfs is not much lower, at 60–65 % [7]. A more characteristic feature of Lfs is their highly basic character, with a pI typically greater than 9; this property should be predictable from sequence. Structurally, the feature that most readily distinguishes Lfs from Tfs is the peptide linker between the two lobes, which is helical in Lfs (see below), but irregular in Tfs, containing several proline residues. Functionally, most Lfs retain iron to much lower pH than Tfs (pH 3–4, compared with pH 5–6) [17], but camel Lf is an exception, losing iron from its N-lobe at pH 3–4, but from its C-lobe at pH 6–7 [18].

Overview of the three-dimensional structure

Crystal structures are now available for the Lfs of five species, human [11, 19], cow [20], buffalo [21], horse [22] and camel [18]. All share the same overall structural organization (fig. 1), which is shared also by Tf [23, 24]. The polypeptide is folded into two globular lobes, representing its N- and C-terminal halves (residues 1–333 and 345–691 in human Lf). These two lobes, referred to as the N-lobe and C-lobe, are linked by a short α-helix (residues 334–344 in human Lf); in Tfs, which otherwise share the same fold, this inter-lobe peptide is irregular and flexible. Non-covalent interactions, mostly hydrophobic, provide a cushion between the two lobes, with the C-terminal helix (residues 678–691 in human Lf) playing a large part.

Both lobes have the same fold, consistent with their sequence identity of ~40 %. In each lobe, two α/β domains, referred to as N1 and N2, or C1 and C2, enclose a deep cleft within which is the iron binding site. The α/β fold of each domain consists of a central, mostly parallel β-sheet, with α-helices packed against it. The helix N-termini face the interdomain cleft, making it somewhat positively charged, and one of the helices, H5 from the N2 (or C2) domain, serves as the binding site for the essential carbonate anion at the metal binding site. This overall fold for each lobe corresponds to a classic two-domain 'binding protein fold' that is shared by a large family of bacterial periplasmic proteins that transport ions and small molecules, and suggests a common evolutionary origin [25, 26].

Consistent with their similarity to the two-domain periplasmic binding proteins, half-molecules of Lf, comprising a single lobe, can be generated, either by proteolysis or by recombinant DNA methods. Proteolysis with

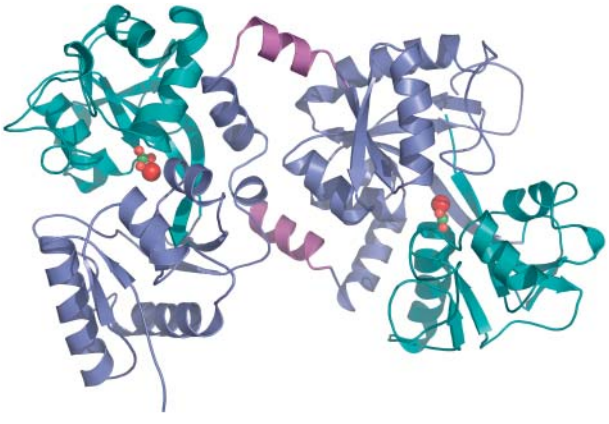

Figure 1. Ribbon diagram showing the characteristic polypeptide fold found for human Lf and all other members of the Tf family. The N-lobe is on the left, with its bound Fe^{3+} and CO_3^{2-} ions (shown as spherical atoms) in the cleft between the two domains, N1 (dark blue) and N2 (teal). Similarly, for the C-lobe (right) the C1 and C2 domains are coloured dark blue and teal, respectively with the Fe^{3+} and CO_3^{2-} ions in between. The two lobes are joined by a three-turn helix (magenta, top) and at the C-terminus a final helix (magenta, below) makes contact again between the lobes. This and other figures drawn with PyMol (W. L. DeLano; http://www.pymol.org).

trypsin gives 50- and 30-kDa fragments of human Lf [27] which are not true half-molecules (cleavage occurs within the N-lobe), but proteolysis with proteinase K cuts buffalo, cow and camel Lfs in the interlobe connecting peptide, giving authentic N- and C-terminal half-molecules [18, 28–30]. A recombinant N-lobe half-molecule of human Lf has also been generated [31]. These half-molecules are generally unchanged from their structures in the intact molecule, although some local changes occur where stabilizing interactions with the other lobe are lost [29, 30, 32]. They make very useful tools for testing the individual properties of the lobes, either by mutagenesis or with functional assays.

The two-lobe, four-domain structure provides the key to understanding the dynamic properties of Lf. As outlined below, metal binding and release are associated with large-scale rigid-body domain movements, in which the two domains of each lobe close over the bound metal or open to release it [33, 34]. The dynamic properties of the open (apo) form also provide the key to understanding metal binding. Not surprisingly, there are minor variations in the extent to which the domains close over a bound metal ion in the Lfs of different species, as a result of local sequence changes in the binding cleft, but the differences are so small as to be functionally not significant. On the other hand, the N- and C-lobes differ significantly in their relative orientations in the Lfs of different species (fig. 2), probably as a result of changed interactions in the hydrophobic 'cushion' between the lobes. For example, the lobe orientations in human Lf differ by 8.2° from those in horse Lf [22], 11.3° from cow Lf [20] and 14.8° from buffalo Lf [21]. The most likely consequence is that there may be differences in the extent or nature of cooperativity between the lobes, since this appears to depend on inter-lobe contacts.

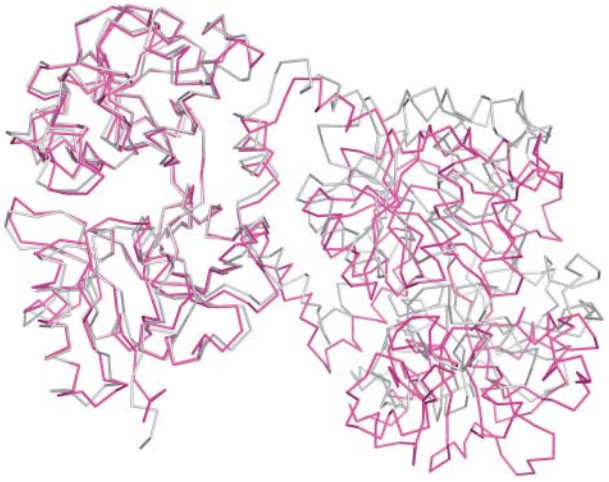

Figure 2. Altered lobe orientations in human (grey) and buffalo (pink) Lfs. When the N-lobes of the two proteins are superimposed, as shown, the C-lobes differ by ~15° in their relative orientations.

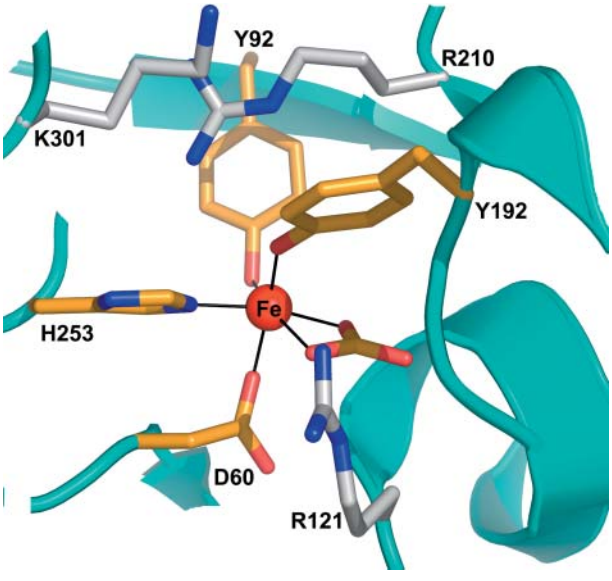

Figure 3. Canonical iron binding site, found in both lobes of all Lfs, shown here for the N-lobe of human Lf. The CO_3^{2-} ion, which binds in bidentate mode to the Fe^{3+} ion, is at the N-terminus of an α-helix, and interacts with also with an arginine residue (here Arg121). Two basic residues at the back of the iron site (here Arg210 and Lys301) are more commonly both lysine, in Lfs and Tfs, and are unique to the N-lobe binding sites.

Iron binding and the 'closed' holo-Lf structure

The defining structural feature of iron-bound (holo) Lf is that the two domains of each lobe enclose the bound Fe^{3+} ion, which is effectively sequestered away from the external environment. Four protein ligands, plus the synergistically bound CO_3^{2-} anion, are covalently bound to the metal ion, which thus crosslinks the two domains that enclose it [7, 11]. This explains the high stability of this structure and the difficulty of removing the bound metal without first destabilizing the protein structure, for example, at very low pH or perhaps by receptor action.

All Lfs and Tfs so far characterised have essentially identical metal and anion binding sites, which appear to be optimised for binding Fe^{3+} and CO_3^{2-}. This canonical binding site (fig. 3) has the same composition and geometry in both lobes of human, bovine, horse and buffalo Lfs, and in the equivalent binding sites in Tfs. It comprises four protein ligands (2 Tyr, 1 Asp, 1 His) that provide three negative charges to balance the 3+ charge of Fe^{3+}, together with a helix N-terminus and Arg sidechain whose positive charge balances the negative charge on the CO_3^{2-} anion. Mutagenesis experiments on the Asp, His and Tyr ligands [35–37] show that any change is strongly deleterious to iron binding, as is mutation of the Arg residue that helps bind the CO_3^{2-} anion [38]. That

this site has been selected by evolution is strongly suggested by the fact that the ferric binding protein in the periplasm of *Haemophilus influenzae* appears to have independently evolved an almost identical binding site within the same basic fold [26].

The conclusion is that this set of ligands, and their location in the cleft between two domains, is chemically and geometrically ideal for high-affinity, but reversible, iron binding [7]. The inclusion of a non-protein ligand, the CO_3^{2-} ion, in the iron coordination sphere, appears to be a special feature that gives pH control of iron release. Loss of this endogenous ligand following protonation is a likely first step in the breakup of the iron site at low pH [39] and is probably critical for efficient, controllable iron release in a way that would not be possible with a complete set of six protein ligands.

Binding of other metals and anions

Lf, like Tf, is traditionally thought of as an iron binding protein, with very high affinity for Fe^{3+} ions. Like Tf, however, it can also bind many other metal ions, albeit with lower affinity. These range from small, trivalent ions such as Ga^{3+} and Al^{3+}, through di- and trivalent transition metal ions such as VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+} and Zn^{2+}, to larger ions such as the trivalent lanthanides [7]. There is strong preference for higher oxidation states, as shown by the ability of Lf to oxidize Mn^{2+} to Mn^{3+} [40] and Ce^{3+} to Ce^{4+} [41]. Metal binding can be monitored by the large increase in ultraviolet (UV) absorption at 240–280 nm that results from ionization of the tyrosine ligands as they bind to the metal ion [7, 41]. This spectroscopic signature does not guarantee that the protein adopts the characteristic closed holo-Lf structure, but crystal structures of Lfs bound to Mn^{3+} [42], Sm^{3+} [43], Zn^{2+} [30] and Ce^{4+} [44] have all shown closed structures, suggesting that Lf could well have a physiological role in binding metal ions other than Fe^{3+}. It has been shown, for example, that all the manganese in milk is apparently bound to Lf [45], and physiological roles in the binding of vanadium [46] and zinc [47] have also been suggested. Given that Lf is usually found less than 10% saturated with iron, it must have the potential to bind trace quantities of many other metal ion species in vivo.

Lf can also bind a variety of other anions in place of the physiological CO_3^{2-} ion. These are mostly carboxylate ions which are presumed to bind, like CO_3^{2-}, both to the helix 5 N-terminus and to the metal ion [7]. This has been demonstrated crystallographically for oxalate [48, 49], and should be possible for larger anions because of the large solvent-filled cavity that separates the two domains of each lobe. Indeed, this cavity suggests that Lfs could have an as-yet unrecognized functional role in binding anionic metabolites.

The 'open' apo-Lf structure

Biophysical studies show that the iron-free (apo) lactoferrin structure is much less compact than the holo form, and that a large conformational change accompanies metal binding and release [50]. The nature of this conformational change was first demonstrated by a crystal

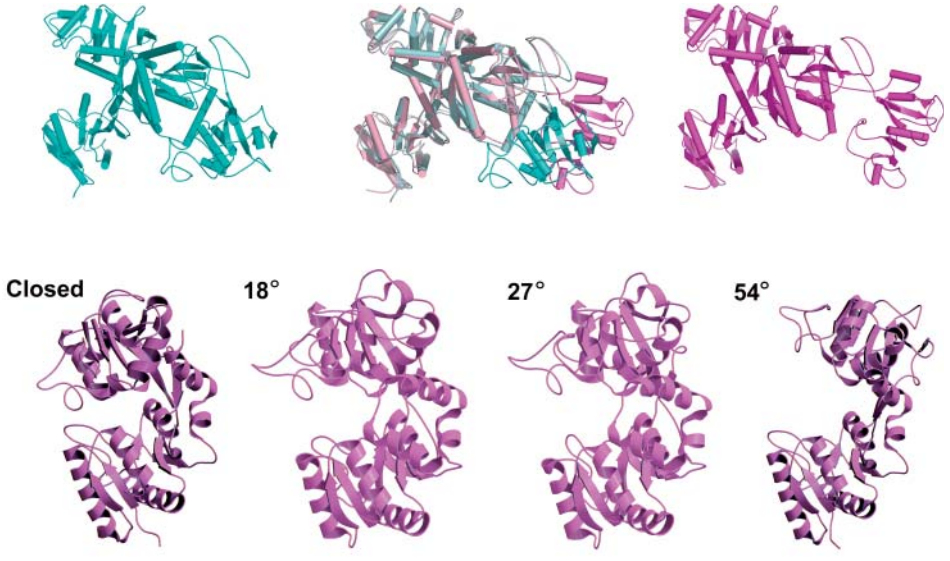

Figure 4. Variability in domain opening in apo-Lfs, seen in different crystal structures. At the bottom are shown the closed structure (as in the N-lobe of horse apoLf, and C-lobes of horse and human apoLfs); two partially open structures (seen for C-lobe structures of human apoLf); and the fully open structure (seen for the N-lobe of human apoLf, and both lobes of camel apoLf). Above left is the 'one-open, one-closed' human apoLf structure (N-lobe on left, C-lobe on right), shown in teal, and above right is the 'both lobes open' camel apoLf structure, in pink. In the middle, above, the two structures are superimposed on the basis of their N-lobes, showing the large difference in the orientations of their C2 domains.

structure analysis of human apo-lactoferrin (apoLf) [33], which showed that in this structure the N-lobe binding cleft was wide open, as a result of a 54° rigid-body rotation of one domain (N2) relative to the other (N1); a hinge in two polypeptide strands that run behind the iron site mediates this domain movement. Curiously, in this structure the C-lobe was still closed, even though no iron was bound, and this has proved to be an important clue to the dynamic behaviour of the apo-protein.

Since then, the structure of a second crystal form of human apoLf has shown that the C-lobe does also adopt open forms, through the same kind of conformational change as was seen for the N-lobe; in this structure the C-lobe of one of the two apoLf molecules in the asymmetric unit is opened by 18°, and the other by 27° [13]. The crystal structures of the apo forms of other Lfs and Tfs show further diversity (fig. 4). The crystal structure of horse apoLf proved to have both lobes closed [51], whereas that of camel apoLf has both lobes wide open [18], and in the related apo-ovotransferrin, again both lobes are open [52].

We conclude from this variety of apoLf structures that in the absence of a bound metal ion to lock the two domains of each lobe together, the apo form is flexible. Although it probably exists mostly in the fully open form (i. e. with both lobes open), as implied by small-angle solution scattering [50], the small energy difference between open and closed forms enables it also to sample the closed state from time to time [7, 13]. This energy difference may be greater in the Lfs of some species than others – for example, camel Lf seems to favour the fully open apo structure more than other Lfs [18], and horse Lf somewhat less so [51]. Nevertheless, the crystal structures also reflect the fact that crystallization can select one out of several conformational states present in solution, so as to optimize crystal packing.

One important point, however, is that the conformational change(s) that differentiate the holo and apo forms of Lf are simple domain movements [33, 53]. The only residues whose solvent exposure changes are those that are inside the binding cleft of each lobe and a small number in the hinge region [52], and the vast majority of the molecular surface is unaffected – for example, the lactoferricin domain centred on helix 1 remains equally exposed in both forms.

Structural and dynamic aspects of iron binding and release

Metal ions are assumed to bind to the open form of apoLf, which allows unrestricted access to either free or complexed Fe^{3+} ions. Spectroscopic studies, and consideration of the three-dimensional structure, suggest that the CO_3^{2-} ion binds first [7]. This neutralizes the positive charge associated with the arginine residue (Arg121 in the Lf N-lobe) and the associated helix, and presents an incoming metal ion with four ligands (the two tyrosines and the two CO_3^{2-} oxygens) clustered on the inside surface of the N2 (or C2) domain. Direct evidence that this is the initial site of iron binding has come from the crystal structure of just such an intermediate, for camel Lf [54]. The completion of binding is believed to depend on dynamics. As the protein samples the closed state, through thermal fluctuations, two options exist. If it is iron-free it will simply open again, but if it is carrying a metal ion bound at the intermediate site on the N2 or C2 domain, it will be locked closed as binding to His and Asp ligands on the other domain occurs [13, 34].

Iron release depends on destabilization of the closed (holo) form. This could come from receptor binding, as is the case for Tf [55]. In the absence of receptor action, however, release is triggered by lowering the pH [17]. Protonation of the carbonate ion, and then the tyrosine and/or histidine ligands, as suggested by kinetic studies [56], should progressively weaken the iron coordination to the point where it no longer holds the two domains together; domain opening would then inevitably be followed by iron release. There remains the question of the characteristic difference between Lfs and Tfs, whereby Lfs release iron at pH 3–4, and Tfs at pH 5–6 [7, 17]. This difference may be functionally important, allowing Tf to release iron into cells, whereas Lf can scavenge iron under conditions in which it would be lost from Tf, allowing Lf to function in bacteriostasis, or modulation of inflammatory or other processes.

Research on Tf has focused on a pair of lysine residues, Lys206 and Lys296, which are hydrogen bonded together in the N-lobe of Tf and are proposed to act as a pH-sensitive trigger [57]. Protonation at low pH is then suggested to cause repulsion that triggers domain opening. The situation is more complex than this, however, since all Lfs except human and mouse also have two lysines that are sequentially and spatially equivalent, yet these proteins behave as typical Lfs in terms of iron release [20]. Mutagenesis studies are also equivocal as to the role of these two basic residues [58–60]. An important contribution to this debate comes from studies of N-lobe half-molecules of Lf and Tf, which indicate similar iron-release pH profiles for these species [31]. This suggests that interactions between the two lobes of Lf play a key role in ensuring iron retention to low pH.

Further evidence for the importance of interactions between the two lobes of Lf came from a study of mutants in which iron binding by either the N-lobe or C-lobe site was specifically eliminated by mutagenesis [37]. When the N-lobe site was knocked out, the C-lobe site behaved as normal. However, when the C-lobe site was knocked out, the N-lobe site still bound iron, but more weakly than in native Lf, and released iron at pH 6, as for the N-

lobe of Tf. The conclusion is that the specific difference between Lf and Tf is a function of different binding in their N-lobes, and that the enhanced iron retention of the Lf N-lobe is a result of cooperative interactions with the C-lobe [37] that do not occur for Tf. Consideration of the available Lf structures suggests that the C-terminal helix, which contacts the N-lobe close to the hinge, plays a key role, as does the helical linker peptide between the two lobes of Lf [61]. The latter is a Lf-specific feature which compares with the flexible, disordered linker of Tfs, and it is proposed that the rigidity of the helical linker in Lfs allows a stronger interaction between the two lobes that stabilizes iron binding in the N-lobe, delaying the onset of iron release to low pH [34].

Surface properties

Many of the proven or proposed functions of Lf, apart from its iron binding activity, depend on its surface properties. All Lfs so far characterized have high isoelectric points (pI about 9), and this strongly cationic nature must be a major factor in its ability to bind, perhaps rather indiscriminately, to many different cell types and to many anionic molecules. From the known crystal structures of the five Lfs so far analysed it is clear that the distribution of positive charge on the protein surface is highly uneven (fig. 5). There are three notable concentrations of positive charge, (i) at the N-terminus (residues 1–7), (ii) along the outside of the first helix (residues 13–30), and (iii) in the inter-lobe region, close to the connecting helix.

The first helix comprises the major part of lactoferricin, the bactericidal domain identified by Bellamy et al. [62]. In the intact proteins, the antibacterial properties of this region probably result from disruption of cell membranes by the basic residues (Lys and Arg) arrayed along the outside of this helix, which is highly solvent-exposed on the surface of the N-lobe (fig. 1). All Lfs have five or six basic residues along this helix, and the crystal structures show that these project freely into solution in a manner that is unaffected by the iron status of the Lf molecule. Thus this antibacterial domain should be a feature of all Lfs.

The N-terminal residues 1–5 of the polypeptide extend out from the protein surface, and in all Lf crystal structures are highly flexible. The strong concentration of positive charge here and at the adjacent C-terminal end of the first helix (residues 27–30) forms the proposed binding site for DNA, LPS, heparin and glycosaminoglycans [63, 64]. What is also apparent, however, is that the four Arg residues at the human Lf N-terminus are highly atypical of lactoferrins generally, suggesting that some of these binding properties could be species-specific [14]; so far no equivalent functional studies have been performed on other Lfs .

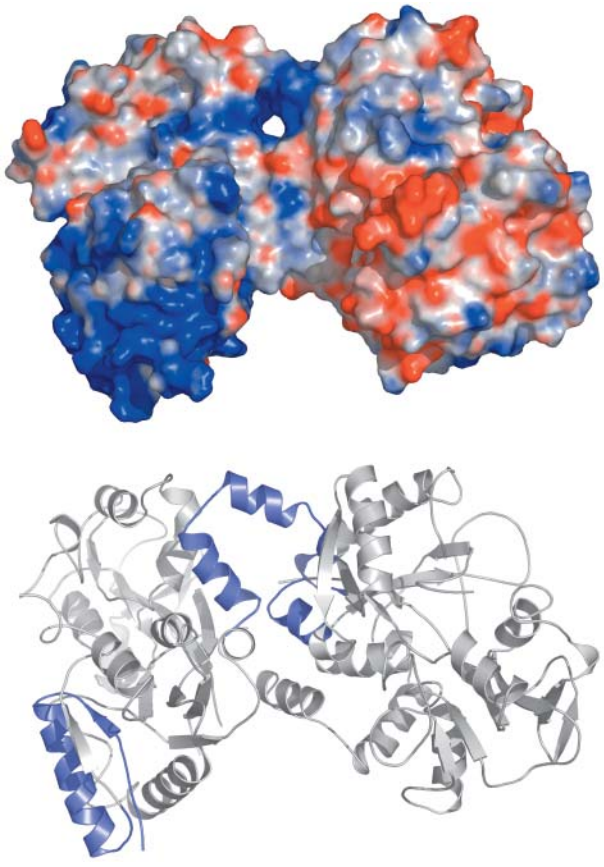

Figure 5. Space-filling representation of the iron-bound form of human Lf, showing the distribution of surface charge (blue, positive; red, negative). For reference, a ribbon diagram in the same orientation is shown below. The most striking areas of positive charge correspond with the bactericidal Lf domain of the N1 domain (lower left, with the N-terminus and Lf domain depicted in blue in the ribbon diagram) and the region around the connecting helix (upper middle).

Glycosylation

All Lfs appear to be glycosylated, although the number and location of potential glycosylation sites, and the sites actually used, vary from protein to protein. Thus, mouse Lf has only one potential N-glycosylation site (Asn476), human Lf three (Asn137, Asn478 and Asn623) and cow, goat and sheep Lf five (233, 281, 368, 476 and 545). From the available data, the number of sites actually used appears to be smaller, however, with two sites glycosylated in human Lf (Asn137 and Asn478) [19] and four in cow Lf (Asn233, Asn368, Asn476 and Asn545) [20].

Neither the nature nor the location of the bound carbohydrate appears to affect the polypeptide folding. All Lfs have the same fold, irrespective of the sites of glycosylation, and a comparison of native human milk Lf and recombinant human Lf expressed in *Aspergillus awamori* showed identical protein structures despite different glycan structures

[65]. Most of the glycosylation sites are highly exposed, on the protein surface, but there are two for which this is not the case, and which are likely to affect protein dynamics and/or function. Asn545, in cow, buffalo, sheep and goat Lfs, lies in a surface cleft between the two domains of the C-lobe; by providing additional interactions with both domains, it may help to stabilize the closed state [20, 30]. In contrast, Asn518 in camel Lf is located in the iron-binding cleft of the C-lobe, and glycosylation at this site must inhibit domain closure and is probably a major factor in the weakened iron binding and facile release by the camel Lf C-lobe [18]. Crystal structures show that the carbohydrate chains are generally flexible and disordered, and only for the Asn545 site in cow Lf can a significant number of carbohydrate residues be modelled [20, 30]. A comparison of native and deglycosylated human Lf [66] shows that its binding properties, both for iron and for other molecules, are unaffected, but the loss of carbohydrate increases its sensitivity to proteolysis.

Conclusions

The detailed molecular and atomic structure of Lf is now well known. Although crystal structures essentially give 'snapshots' of the structure, enough are now available, of both apo (metal-free) and holo (metal-bound) states, to give a good understanding of conformational dynamics. Metal binding and release are facilitated by the flexibility of the apo form, and strong retention by the relative rigidity of the holo form. The next major challenge is to understand the structural basis of receptor binding and of some of the more recently discovered functions of this fascinating molecule. Subtle structural differences between the Lfs of different species also point to the possibility that some activities of Lf could be species-specific, and the need therefore to extend functional studies across the full range of species.

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