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Lactoferrin and Iron: structural and dynamic aspects of binding and release

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Abstract

Lactoferrin (Lf) has long been recognized as a member of the transferrin family of proteins and an important regulator of the levels of free iron in the body fluids of mammals. Its ability to bind ferric iron with high affinity ($K_D \sim 10^{-20}$ M) and to retain it to low pH gives the protein bacteriostatic and antioxidant properties. This ability can be well understood in terms of its three dimensional (3D) structure. The molecule is folded into two homologous lobes (N- and C-lobes) with each lobe binding a single Fe^{3+} ion in a deep cleft between two domains. The iron sites are highly conserved, and highly favorable for iron binding. Iron binding and release are associated with large conformational changes in which the protein adopts either open or closed states. Comparison of available apolactoferrin structures suggests that iron binding is dependent on the dynamics of the open state. What triggers release of the tightly bound iron, however, and why lactoferrin retains iron to much lower pH than its serum homologue, transferrin, has been the subject of much speculation. Comparisons of structural and functional data on lactoferrins and transferrins now suggest that the key factor comes from cooperative interactions between the two lobes of the molecule, mediated by two α-helices.

Introduction

Lactoferrin has for many years teased and tantalized researchers seeking to understand its many activities and to define its *in vivo* role(s). Since its first purification from milk (Groves 1960, Johanson 1960, Montreuil *et al.* 1960), lactoferrin (Lf), also known as lactotransferrin, has been recognized as an iron binding protein and identified as a member of the transferrin (Tf) family of proteins. This led to the demonstration by Bullen *et al.* (1972) that Lf could protect against bacterial infection through its ability to deprive bacteria of iron necessary for growth, and consolidated the view that iron binding was at the heart of its biological activity.

Subsequent investigations have discovered other activities, however, some of them associated with iron binding but others not. Thus, Lf is an antioxidant because its iron binding ability inhibits the iron-catalyzed formation of free radicals (Baldwin *et al.* 1984). It also has the ability, however, to bind to various types of immune cells, and, by virtue of its high positive charge (pI $\sim$ 9), to bind anionic molecules such as DNA (Bennett & Davis 1982, He & Furmanski 1995), heparin (Van Berkel *et al.* 1997), and glycosaminoglycans (Mann *et al.* 1994). A bactericidal domain on the outside of the Lf molecule further gives it the ability to kill bacteria in an iron-independent manner (Bellamy *et al.* 1992), and Lf has also been shown to specifically inactivate several colonization factors from the pathogenic organism *Haemophilus influenzae* (Qiu *et al.* 1998), this time by proteolysis. Finally a recent report identifies Lf as an inhibitor of biofilm formation by pathogenic *Pseudomonas aeruginosa*, through iron sequestration (Singh *et al.* 2002). These discoveries, coupled with the presence of Lf, often in high concentrations, in mammalian milk, many other exocrine secretions and white blood cells has firmly established lactoferrin as one of the key protective factors in the defense mechanisms of mammals.

It is now over 15 years since the three-dimensional (3D) structure of human Lf was determined (Anderson *et al.* 1987, 1989), the first for any member of

the transferrin family. This has been followed by 3D structures for serum Tf (Bailey *et al.* 1988) and hen ovotransferrin (oTf) (Kurokawa *et al.* 1995), together with those of the Lfs from four different species, cow (Moore *et al.* 1998), buffalo (Karthikeyan *et al.* 1999), horse (Sharma *et al.* 1999a) and camel (Khan *et al.* 2001). All of these proteins have the same basic polypeptide fold, comprising two homologous globular lobes (N-lobe and C-lobe), each further divided into two domains (N1 and N2, C1 and C2). All Lfs, serum Tfs and oTfs have essentially identical iron binding sites, one per lobe, giving them the capacity to bind two Fe^{3+} ions, very tightly ($K \sim 10^{20}$ M) but reversibly (Aisen and Harris 1989, Baker 1994). Two CO_3^{2-} ions are also bound, concomitantly with the two Fe^{3+} ions, and the synergistic relationship between metal binding and anion binding is a unique feature, not seen in any other metal-binding proteins to date (Aisen & Harris 1989, Baker 1994).

Lf function must therefore be understood in terms of this common structure, shared with Tf and oTf. Here we focus on the factors that control iron binding and release. How does the protein structure enable iron to be bound with such high affinity yet also allow reversibility *via* a mechanism for release? What triggers release, and why is it that, despite virtually identical iron sites in the two proteins, Lf retains its iron to pH values as low as 3, whereas Tf iron is released at a pH of about 5.5 (Mazurier & Spik 1980, Baker 1994)?

Lactoferrin structure

Amino acid sequence

The amino acid sequence of human Lf (Metz-Boutigue *et al.* 1984) demonstrated the high degree of sequence identity with serum Tf ($\sim$60%), and a characteristic twofold internal sequence repeat indicative of an ancestral gene duplication; the N- and C-terminal halves have $\sim$40% sequence identity. The amino acid sequences for the Lfs of eight species (human, bovine, buffalo, camel, goat, horse, mouse, pig) are now in sequence databases and all share $\sim$70% sequence identity (human/mouse 70%, human/bovine 69%, mouse/bovine 63%), consistent with the strong conservation of their 3D structures.

Polypeptide folding

The canonical transferrin fold, found for all Lfs and Tfs, is shown in Figure 1, and has been described in detail (Baker 1994, Haridas *et al.* 1995). The polypeptide chain of about 690 amino acid residues is folded into two globular lobes, representing its N- and C-terminal halves (residues 1–333 and 345–691 in human Lf). The lobes are connected by a peptide of 10–15 residues (residues 334–344 in human Lf), which in Lfs forms a 3-turn α-helix, but in Tfs is irregular and flexible. There are non-covalent interactions, mostly hydrophobic, where the two lobes pack together.

Both lobes have the same fold, consistent with their sequence identity of $\sim$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. This 2-lobe, 4-domain structure provides the key to understanding the dynamic properties of Lf (see below). The fold of each lobe also corresponds to a classic 2-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 (Baker *et al.* 1987, Bruns *et al.* 1997).

Iron binding sites

The ligands for the bound Fe^{3+} ion are the same in both lobes, in all Lfs and in both serum Tf and oTf. These ligands comprise two tyrosine residues, one aspartic acid and one histidine (Tyr92, Tyr192, Asp60 and His253 in the Lf N-lobe), together with two oxygens from the synergistically bound anion (Figure 2). Both structural and spectroscopic data suggest that the latter is CO_3^{2-} rather than HCO_3^- (Baker 1994). The same set of ligands, although with the substitution of a phosphate ion for carbonate, is found in the iron binding site of the bacterial ferric binding protein from *H. influenzae*, even though the four protein ligands are contributed from different parts of the protein structure (Bruns *et al.* 1997). Mutagenesis of any of these ligands in Tf or Lf (e.g. Faber *et al.* 1996, Nicholson *et al.* 1997; Baker *et al.* 2003) substantially weakens iron binding.

The conclusion is that this set of ligands is chemically and geometrically ideal for high affinity, but reversible, iron binding (Baker 1994). The participation of a non-protein ligand, the CO_3^{2-} ion, in the iron coordination sphere, appears to be a special design feature. Loss of this endogenous ligand following protonation is a likely first step in the breakup of the iron site at low pH (MacGillivray *et al.* 1998) and is probably critical for efficient, controllable iron release in a

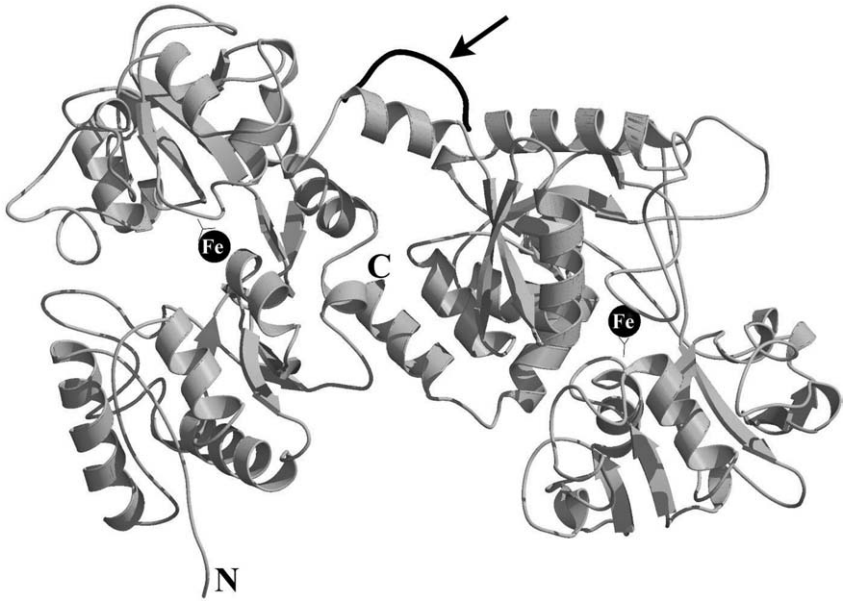

Fig. 1. Ribbon diagram showing the canonical transferrin fold. The N-lobe is on the left and the C-lobe on the right. The structure shown is for the holo form, with the two domains of each lobe closed over the bound iron atom (labelled black sphere). The connecting peptide that joins the two lobes (top, center) is helical in lactoferrins but irregular and flexible in transferrins (shown in black, arrowed). The N-terminus is labelled (N), as is the C-terminus (C) where the C-terminal helix abuts the N-lobe and may mediate cooperativity.

way that would not be possible with a complete set of six protein ligands.

Conformational change

Biophysical studies (Kilar & Simon 1980, Grossmann *et al.* 1992) demonstrated that a large conformational change accompanied binding and release, with the iron-bound form being much more compact. The nature of this conformational change (Figure 3) was first demonstrated by the crystal structure of human apolactoferrin (apoLf), determined in 1990 (Anderson *et al.* 1990). In this structure the N-lobe is in an open state, as a result of a 54 deg. rigid-body rotation of one domain (N2) relative to the other (N1). This movement is made possible by a hinge in two polypeptide strands that run behind the iron site, connecting the two domains. 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 behavior of the apo-protein. Since then, the structure of a second crystal form of apoLf has shown that the C-lobe can 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 deg., and the other by 27 deg. (Baker *et al.* 1997, Baker *et al.* 2002). Other crystal structures show further diversity. The structure of horse apoLf has both lobes closed (Sharma *et al.* 1999b), the structure of camel apoLf has both lobes open (Khan *et al.* 2001a), and in the related apoovotransferrin, again both lobes are open (Kurokawa *et al.* 1999).

There is no evidence to indicate that different Lfs and Tfs operate in different ways, and solution X-ray scattering studies indicate that human Lf, Tf and oTf all behave the same. Our conclusion is that in the iron-free state, the structures of the open and closed forms are very similar in energy, and while the apo proteins may be predominantly in the open state (as shown by the solution scattering studies) thermal fluctuations allow them to sample the closed state from time to time. Crystallization processes may then select whichever conformer best supports crystal packing, giving structures with both lobes open, both closed, and one open, one closed.

Iron binding and release

The functional challenge facing proteins of the transferrin family is that of combining high affinity iron binding with a suitable mechanism for release of this bound iron. The binding affinity is high enough ($K_D \sim$

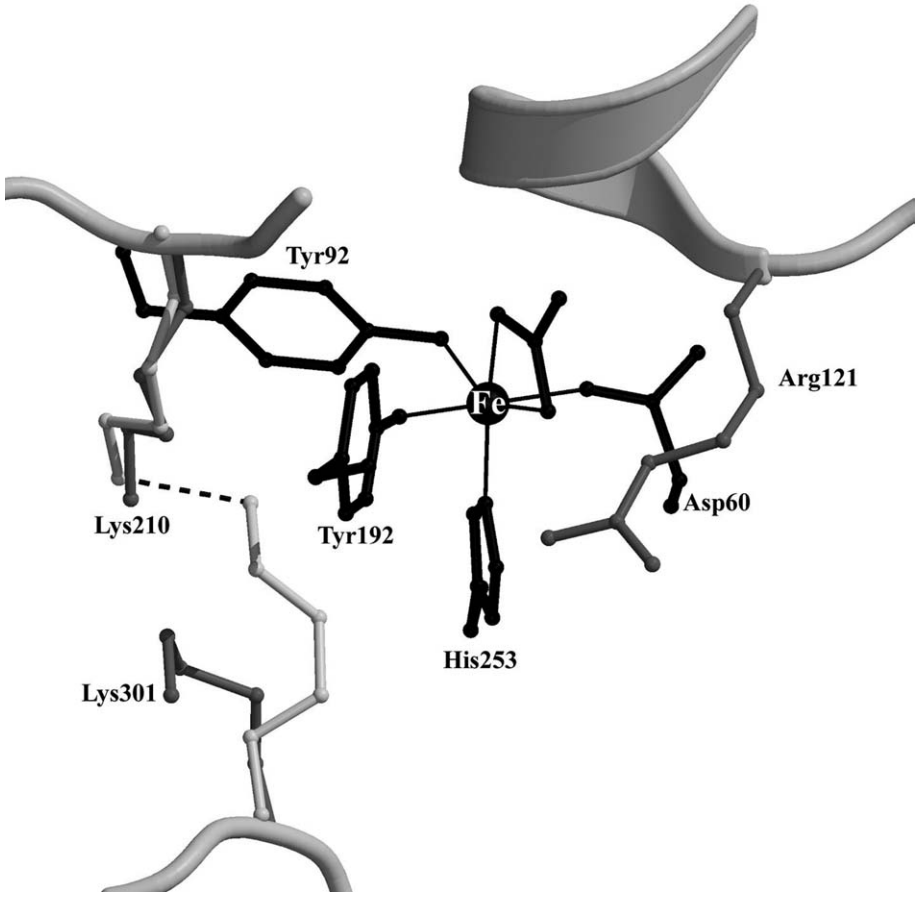

Fig. 2. The canonical iron binding site found in lactoferrin and transferrin. Residue numbering is as for the N-lobe of bovine lactoferrin. Two lysine residues form a dilysine pair, linked by a hydrogen bond (dotted line), in transferrin and may modulate iron release; in bovine lactoferrin and many other lactoferrins, however, two equivalent lysines are present (Lys210 and Lys301) but do not interact as Lys301 is turned away.

10^{-20} M) that in the presence of Lf or Tf the concentration of free iron in body fluids cannot exceed 10^{-18} M. This prevents the precipitation of iron as insoluble hydroxides and gives bacteriostatic and antioxidant protection (Baldwin *et al.* 1984). At the same time, Tf must be able to release this tightly-bound iron, in its role of iron delivery to cells (Octave *et al.* 1983) and the characterization of intestinal Lf receptors (Suzuki *et al.* 2001) now suggests that the same may apply to this protein. How is iron taken up so efficiently, how does release occur, and how is it triggered?

Importance of dynamics in iron binding

We assume that binding occurs to the open form of the apo-protein, which allows unrestricted access to either free or complexed Fe^{3+} ions. Both spectroscopic studies, and consideration of the 3D structure suggest that the CO_3^{2-} ion binds first. This neutralizes the positive charge associated with the arginine residue (Arg121 in the Lf N-lobe) and the associated helix, and presents incoming iron with four ligands (Tyr192, Tyr92 and the two CO_3^{2-} oxygens) clustered together on the inside surface of the N2 domain. This is the presumed site of initial iron binding (Baker 1994, Baker *et al.* 2002). Indeed, the crystal structure of just such an intermediate, for camel Lf, has recently been determined (Khan *et al.* 2001b). The question then arises: how do the other two ligands, Asp60 and His253, which are 9–10 A away on the other (N1) domain 'see' the iron atom and complete its coordination? We suggest that the dynamics of the open form are crucial, and that as it 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 iron bound at its intermediate site on the N2 domain, it will be locked closed as the iron atom encounters the other two ligands.

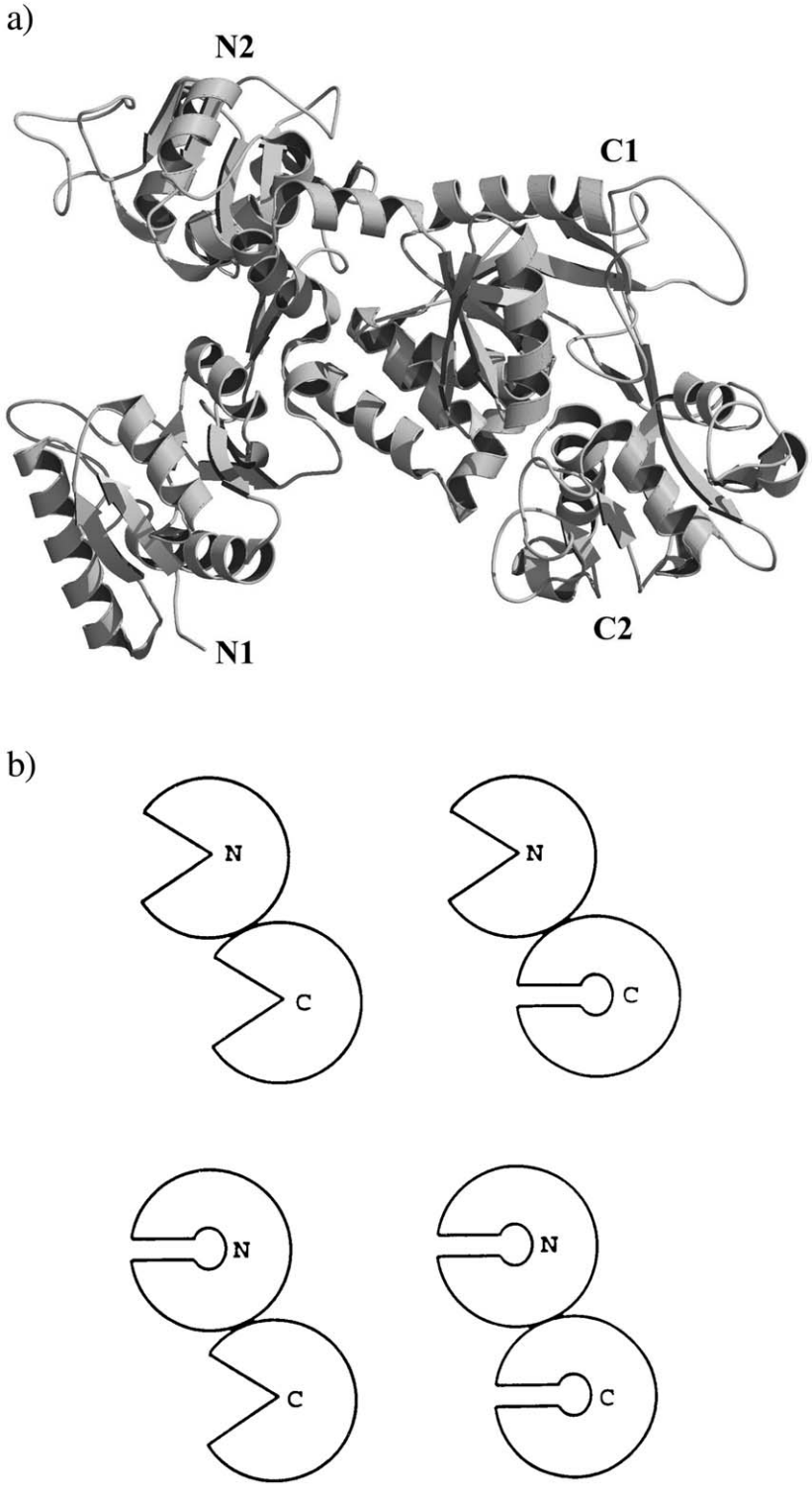

Fig. 3. Conformational change in lactoferrin. In (a) the open N-lobe of human apolactoferrin can be contrasted with the closed form shown in Figure 1. In the apolactoferrin structure determined by Anderson *et al.* (1990) shown here, the C-lobe was closed but iron-free. In (b) the possible structures that could exist for the apo form in solution are shown schematically, including structures with both lobes open, one open one closed, and both closed.

Control of iron release

Both Lf and serum Tf share the property that their bound iron is spontaneously released *in vitro* as the pH is lowered. For Tf, this loss of iron at low pH is considered to be important for iron delivery to cells (Octave *et al.* 1983); the iron-carrying Tf molecule binds to a receptor, is internalized by receptor-mediated endocytosis, releases its iron and is recycled to the cell surface. Although there are indications that the receptor plays an active part in this process, the ability of Tf to begin releasing iron at the endosomal pH of about 5.5 is also a critical factor.

We now consider two questions: (i) how does pH trigger the spontaneous release of iron, and (ii) why does Lf retain iron to much lower pH than Tf (3.0, compared with 5.5 for Tf), even though both iron binding sites appear the same? Kinetic studies of iron release show that several protonation events occur (El Hage Chahine & Pakdaman 1995), and both this work and crystallographic studies of Tf (MacGillivray *et al.* 1998) suggest that protonation of the carbonate ion occurs first, with a likely subsequent step being the protonation of the His ligand (Jeffrey *et al.* 1998). This weakens iron binding as ligands dissociate, and the domains move apart in completion of iron release.

This does not explain why Lf and Tf differ, however, as both share identical iron ligands. An observation from comparisons of the structures of Lf and Tf suggested that two lysine residues, Lys206 and Lys296, that formed an unusual hydrogen bonded pair in Tf might hold the key (Baker & Lindley 1992). Observation of a similar dilysine pair in oTf prompted the suggestion that these might provide a trigger for iron release (Dewan *et al.* 1993), since protonation would cause repulsion between the two (now positively-charged) lysines and cause domain opening. It was noted that human Lf did not have such a dilysine pair, as the equivalent residues were Arg210 and Lys301 and they did not interact; Lys 301 took on an alternative conformation from Lys296 in Tf. It has since been shown that the dilysine pair does indeed play a role in iron release, since mutation of either lysine residue in the Tf N-lobe drastically slows release, by a factor of 10^4, and iron is retained to lower pH (4.5, compared with 5.5 for the wild type) (Nurizzo *et al.* 2001).

Recent results show, however, that the dilysine pair in Tf cannot fully explain the characteristic difference between Lfs and Tfs. First, Lf retains iron to much lower pH, approximately 3.0, before release begins,

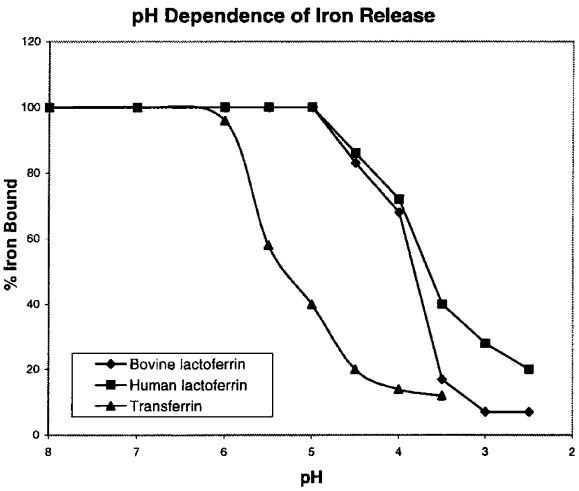

Fig. 4. pH dependence of iron release shown for bovine lactoferrin, human lactoferrin and human serum transferrin, determined using the method described in Day *et al.* (1992).

a difference of 2.5 pH units. Second, bovine, buffalo, horse and camel Lfs all have two lysine residues, Lys210 and Lys301, in positions equivalent to Lys206 and Lys296 in Tf, but these do not form a dilysine pair, and the proteins behave as typical lactoferrins. As shown in Figure 4, bovine Lf, with its two lysines, releases iron at only marginally higher pH than human Lf. Third, mutation of Arg210 to Lys in human Lf makes it only slightly more pH sensitive, by 0.7 pH units (Peterson *et al.* 2000).

The key difference between Lf and Tf appears to be a cooperative interaction between the two lobes in Lf that does not occur for Tf. Iron release from the isolated N-lobe half-molecule of Lf begins at pH 5 (Day *et al.* 1992), similar to transferrin (pH 5.5) and well above the pH at which iron release begins for intact human Lf (pH 3.0). This implies that in the absence of the C-lobe iron binding is substantially destabilized. Furthermore, mutagenic studies of intact human Lf (Ward *et al.* 1996) have shown that when iron binding in the N-lobe is disabled, iron binding in the C-lobe is unaffected, but when binding in the C-lobe is disabled, iron binding in the N-lobe is destabilized, occurring at pH $\sim$5.0. The conclusion is that there are cooperative interactions between the two lobes of Lf through which iron binding in the C-lobe substantially stabilizes binding in the N-lobe. This contrasts with Tf, for which the isolated N-lobe has an iron release pH profile that is virtually identical with that of the N-lobe in the intact protein. What mediates this interaction? Consideration of the Lf structure suggests that the C-

terminal helix, which contacts the N-lobe close to the hinge, plays a role (Jameson *et al.* 1998). Why does the same phenomenon not occur in Tf? Here we propose that it is the peptide that links the two lobes that is important. This linker peptide constitutes a characteristic difference between Lfs and Tfs; in all Lfs so far characterized it forms an α-helix, whereas in all Tfs, and in oTf, it has a flexible, extended and irregular structure. We suggest 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.

Conclusions

Lactoferrin and transferrin form a team that control iron in body fluids. They share highly conserved sequences, structures and iron binding sites, and a mechanism for iron binding and release that involves a large-scale conformational change between open and closed forms. Dynamic fluctuations of the open apoform play an important role in iron binding and in the transition to the metal-bound closed form. In turn, destabilization of iron binding at low pH stimulates iron release and transition to the open form. Although similar in many respects, however, Lf and Tf have become differentiated for different roles. Tf releases iron at a pH of ~ 5.5, consistent with its role in iron uptake by cells. Lf, however, which is found in many exocrine secretions and white blood cells, retains iron to much lower pH, ~ 3.0, giving it potent protective properties. This stabilization of iron binding at acid pH appears to stem primarily from cooperative interactions between the two lobes.

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