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STRUCTURE AND REACTIVITY OF TRANSFERRINS

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I. Introduction

When ferric iron (preferably as ferric nitrilotriacetate) is added to egg white, with a little salt, and the mixture is shaken, a vivid red color develops. This simple experiment introduces the protein ovotransferrin (formerly known as conalbumin) and the most striking property of transferrins, their ability to rapidly and tightly bind iron.

The transferrins are a family of metal-binding proteins (Table I), which apparently owe their place in biology to the need to deal with the solution properties of iron. Under physiological conditions, the favored state of iron is Fe(III), but this is prone to rapid hydrolysis at concentrations greater than 10^{-17} M, ultimately leading to the precipitation of

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TABLE I

PROTEINS OF THE TRANSFERRIN FAMILY

Protein	Source	Function
Serum transferrin	Blood	Iron transport Protection (antibacterial, antioxidant) Growth factor?
Ovotransferrin (conalbumin)	Avian egg white	Protection (antibacterial, antioxidant)
Lactoferrin (lactotransferrin)	Milk, tears, saliva, and other secretions White blood cells	Protection (antibacterial, antioxidant) Iron absorption? Growth factor?
Melanotransferrin (p97)	Melanoma cell surface	Iron translocation?

insoluble ferric hydroxides (*1*). Bacteria have evolved iron chelation systems based on low molecular weight chelate compounds called siderophores (*2*); animals instead use proteins of the transferrin family for the solubilization, sequestration, and transport of ferric iron.

The transferrin family is typified by serum transferrin, the iron transport protein in blood from which the name is taken (literally "transport of iron"). Serum transferrins appear to be present in all vertebrates so far examined; they have been found in crabs and spiders (*3*) and appear likely to be widespread also in insects (*4*). A second member of the family, lactoferrin, is widespread in the secretory fluids of higher animals, including milk, tears, saliva, mucosal, and genital secretions (*5*), and in white blood cells (*6*); in species that possess both proteins, lactoferrin is distinguishable from serum transferrin by its higher isoelectric point (8–9 for lactoferrin compared with 5–6 for transferrin), its distinct sequence, and different bodily location and functional roles. The protein of avian egg white, referred to as ovotransferrin above, is actually a post-translationally modified form of the avian serum transferrin, differing only in its attached carbohydrate (*7*). Finally, the most recently recognized member of the transferrin family is a membrane-bound protein, which is present at low levels on the surface of normal cells, but becomes expressed at high levels on melanoma cells (*8*). This protein, at first called p97 from its apparent molecular weight (~97,000), is referred to here as melanotransferrin.

The transferrins are typically monomeric glycoproteins with a single polypeptide chain of 670–700 amino acids and a molecular weight of ~80,000. Their characteristic property is to bind, very tightly but reversibly, two Fe^{3+} ions together with two CO_3^{2-} ions. The relationship

between the metal ion and the anion, first recognized in the landmark paper of Schade and coworkers (*9*), is synergistic in the sense that neither is bound strongly in the absence of the other. The proteins are also characterized by an internal duplication in which their N-terminal and C-terminal halves are homologous (*10, 11*), with each carrying a single iron binding site. Within this general framework, there are variations. Melanotransferrin and the transferrin from the sphinx moth, *Manduca sexta,* each bind only one iron atom (*12, 13*) even though they are the same size as the other transferrins, and there have been reports of both smaller [*Pyura stolonifera,* M_r ~40,000 (*14*)] and larger [crab, *Cancer magister,* M_r ~150,000, (*15*)] transferrins; it is likely that more variations will be discovered.

There have been numerous reviews of transferrin chemistry. Earlier reviews by Aisen and Listowsky (*1*) and Chasteen (*16*) have been followed by extensive recent articles that have particularly emphasized the functional properties (*3*), the physical biochemistry (*17*), and the structures (*18*) of transferrins. Here we will concentrate on the bioinorganic chemistry of these intriguing proteins, with a particular emphasis on their binding properties, as shown by chemical and spectroscopic studies, but all set firmly in a structural perspective.

II. Biological Roles

The fundamental role of transferrins, shared by all (except, perhaps, melanotransferrin), is to control the levels of free iron in body fluids by binding, sequestering and transporting Fe^{3+} ions. This helps to maintain the availability of iron while preventing the deposition of insoluble ferric hydroxide aggregates. It also has two further effects. First, the proteins thus protect against the toxic effects of free iron that might otherwise catalyze the formation of the free radicals that damage cells (*19*). Second, they have bacteriostatic properties that probably are derived from the fact that *in vivo* the proteins are largely present in their apo- (iron-free) forms and are then able to bind iron so tightly that it is unavailable for bacterial growth. Breast-fed infants, for example, are protected from stomach upsets by the presence, in human milk, of lactoferrin, which inhibits bacterial growth. Similarly the antibacterial properties of egg white derive from the presence of ovotransferrin.

In addition to these general roles, certain of the transferrins have more specific functions. Serum transferrin has the role of transporting iron from sites of absorption to sites of storage and utilization. There

the iron-loaded transferrin binds to specific cell receptor molecules, is internalized, releases its iron inside the cell, and is then returned to the cell surface, from where it detaches and reenters circulation as the apoprotein (*20*). This process is shown diagrammatically in Fig. 1. Whether lactoferrin also has a role in iron absorption is not clear, but the presence of lactoferrin receptors in the gut and elsewhere (*21*, *22*), together with the high bioavailability of the iron in human milk, suggests that it could have. It must be stressed, however, that the iron release properties of lactoferrin are quite different from those of serum transferrin (see Section VI), and their respective receptors are also different.

Other metals may also be carried by transferrins, notably aluminium, implicated in Alzheimer's disease; Al^{3+} is bound by transferrins (*23*), suggesting a possible therapeutic use (*24*). All of the manganese in human milk is reportedly bound to lactoferrin (*25*), and it is highly likely that levels of other metal ions in biological fluids may be similarly controlled. The fact that the conformation of lactoferrin is the same whether Fe^{3+} or Cu^{2+} is bound (*26*; see also Section IV.B) suggests that transferrins carrying copper, and probably other metals, should bind equally well to receptors as those carrying iron. Moreover, the low degree of iron saturation of transferrins *in vivo* (~30% for serum transferrin, ~10% for lactoferrin) implies a potential capacity for binding other metal ions.

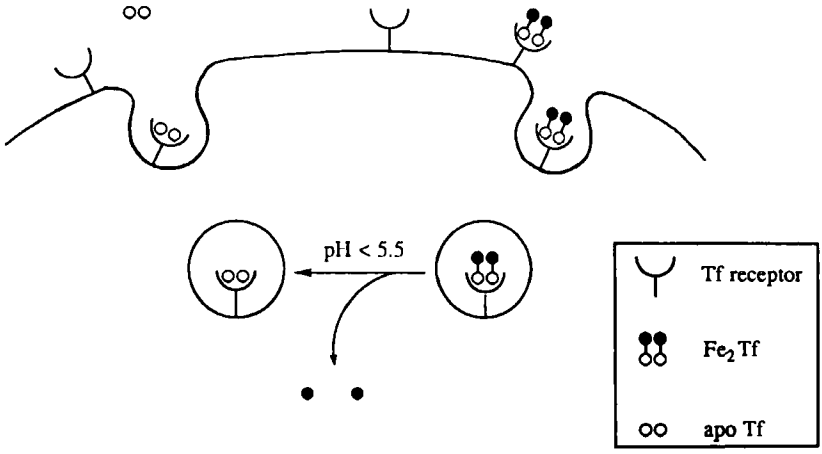


FIG. 1. Schematic representation of the cycle of iron delivery to cells by transferrin, showing the uptake of diferric transferrin by cell receptors, internalization, release of iron at the lower intracellular pH, and recycling and release of apotransferrin.

The above roles all depend on the ability of transferrins to bind metal ions reversibly. The other element in their biological functions is their ability to bind to cells. Both transferrin and lactoferrin have potent growth factor activity (*27, 28*), which depends on binding to cellular receptors (*28, 29*). Lactoferrin's ability to bind to a wide variety of cells (*30, 31*) has also brought suggestions of a role in modulating the immune and inflammatory responses (*31*). The bacteriostatic effects of lactoferrin and ovotransferrin, arising from iron deprivation, also appear to be supplemented by a bactericidal activity that results from direct contact with the bacteria (*32*). In the case of lactoferrin, a region of the molecular surface that probably attacks bacterial cell walls has been identified through isolation and characterization of a bactericidal peptide (*33*).

Finally, the one membrane-bound transferrin, melanotransferrin, although its function has not yet been established, may aid the rapid proliferation of tumor cells, perhaps through an ability to bind and translocate iron.

III. Transferrin Structure

A. Primary Structure

The large size of transferrins (670–700 residues), with the consequent difficulties of chemical sequencing, meant that it was not until 1982 that the first amino acid sequences, those of human serum transferrin (*10*) and chicken ovotransferrin (*34, 35*), were established. These were closely followed by that of human lactoferrin (*11*). The twofold internal repeat in each sequence (see below) was immediately apparent, and comparison of all three sequences then identified conserved tyrosines and histidines that were potential ligands for iron (*11*).

Since that time many more sequences have become available through the advent of recombinant DNA technology and the deduction of amino acid sequences from the base sequences of cloned DNA. At the present time, the primary structures (amino acid sequences) of 14 proteins of the transferrin family have been established. These include seven serum transferrins, from human (*10, 36*), pig (*37*), horse (*38*), rabbit (*39*), toad (*Xenopus laevis*) (*40*), sphinx moth (*M. sexta*) (*13*), and cockroach (*Blaberus discoidalis* (*4*); chicken (*34, 35*) and duck (*41*) ovotransferrins; four lactoferrins, from human (*11, 42*), mouse (*43*), pig (*44*) and cattle (*45, 46*); and the human tumor cell melanotransferrin (*47*). All of these sequences are available from sequence databases such as EMBL and SWISSPROT.

Levels of sequence identity between the proteins are generally high. For example, lactoferrins, which have so far been found only in mammals and are probably a more recent evolutionary development, have 60–70% sequence identity, as shown in Table II. A similar level of identity is found between the serum transferrins of higher animals and there is still 50–60% identity between these transferrins and lactoferrins. Thus these transferrins of the higher animals form a highly conserved family, likely to have very similar three-dimensional structures. The evolutionarily more distant insect transferrins and the membrane-associated melanotransferrin have diverged considerably further, but their level of identity with the higher transferrins (20–30% for the insect proteins and ~40% for melanotransferrin) is still sufficient to imply that they share the same three-dimensional structure.

Of fundamental significance to understanding transferrin structure and function is the two-fold internal amino acid sequence repeat. In each protein, the N-terminal half of the polypeptide is homologous with the C-terminal half, with the level of identity between the two halves ranging from 26–28% in the insect proteins to ~40% in higher transferrins and as high as 46% in melanotransferrin. This repeat is expressed

TABLE II

SEQUENCE IDENTITY (%) IN TRANSFERRIN FAMILY[a]

	Protein												
Protein	hTf	rTf	pTf	eTf	hLf	bLf	mLf	pLf	cOTf	XTf	cTf	MsTf	MTf
hTf		78	71	72	61	61	57	61	53	45	32	29	43
rTf	78		72	72	62	61	57	61	52	46	31	27	42
pTf	71	72		73	61	61	58	61	53	46	31	27	41
eTf	72	72	73		62	62	56	61	53	47	32	27	42
hLf	61	62	61	62		69	70	70	52	45	32	27	41
bLf	61	61	61	62	69		63	73	53	44	30	28	41
mLf	57	57	58	56	70	63		64	49	46	32	29	40
pLf	61	61	61	61	70	73	64		51	44	32	28	42
cOTf	53	52	53	53	52	53	49	51		46	31	25	40
XTf	45	46	46	47	45	44	46	44	46		33	23	38
cTf	32	31	31	32	32	30	32	32	31	33		45	30
MsTf	29	27	27	27	27	28	29	28	25	23	45		27
MTf	43	42	41	42	41	41	40	42	40	38	30	27	

[a] hTf, human transferrin; rTf, rabbit transferrin; pTf, porcine transferrin; eTf, equine transferrin; hLf, human lactoferrin; bLf, bovine lactoferrin; mLf, mouse lactoferrin; pLf, porcine lactoferrin; cOTf, chicken ovotransferrin; XTf, *Xenopus* transferrin; cTf, cockroach transferrin; MsTf, *M. sexta* transferrin; MTf, human melanotransferrin.

also in the three-dimensional structures of the proteins, which are bilobal, with a binding site in each lobe (Section III.B), and marks the transferrins as a classic example of proteins that have evolved via a gene duplication event. It is assumed that the gene for an ancestral 40-kDa protein with a single binding site has been doubled to give a bilobal, two-sited molecule of twice the size. A hypothetical scheme for transferrin evolution is given in Fig. 2.

A number of consequences flow from the structural duplication in transferrins. First, the degree of similarity between the two binding sites assumes considerable importance, both for biological function and for interpreting chemical and spectroscopic studies. Second, it means that stable half-molecules, each with a single binding site, can be prepared, either by limited proteolysis or by recombinant DNA methods.

Half-molecules offer the chance to examine the properties of single-sited molecules without the complications of seeing the "averaged" characteristics of the two sites. The most useful half-molecules are those prepared by recombinant DNA methods because the "cleavage" point is deliberately engineered. Two recombinant half-molecules have so far been prepared and characterized, the N-terminal half of human

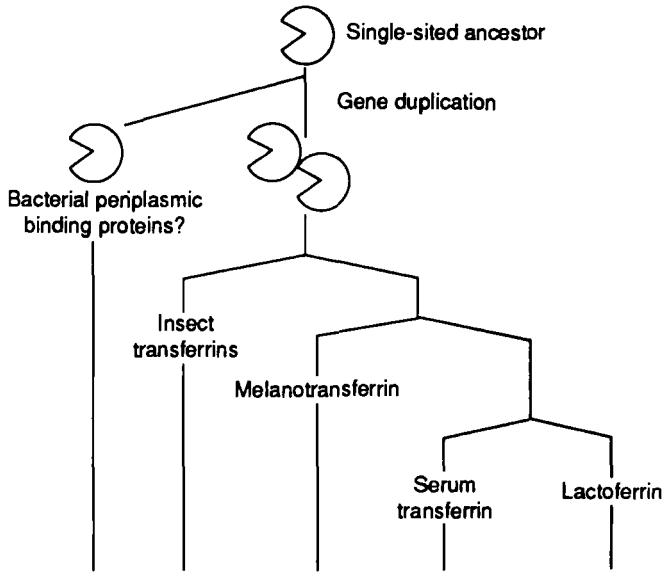


FIG. 2. Possible evolutionary development of the transferrin family, showing also the proposed relationship of bacterial periplasmic binding proteins (Section III.D).

serum transferrin, comprising residues 1–337 (*48*), and the N-terminal half of human lactoferrin, comprising residues 1–333 (*49*); both these half-molecules have as their C-terminal residues amino acids from within the connecting peptide that links the two lobes. No C-terminal half-molecule has yet been prepared by these methods, however.

Many proteolytic fragments of transferrins have been prepared over the years. They have played a significant part in analyzing the differences between the two sites, but they have the disadvantage that the cleavage sites are fortuitous in the sense that they depend on what enzyme is used and what sites happen to be offered by the protein structure; this is not usually predictable. The "best" half-molecule fragments are probably those prepared from chicken ovotransferrin, for which cleavage occurs in the connecting peptide to give stable N-terminal and C-terminal half-molecules (*50–52*). The use of immobilized subtilisin offers a particularly good preparative route (*52*). These half-molecules bind iron reversibly and also reassociate strongly (*53, 54*) to give a noncovalent complex capable of delivering iron to cells via the transferrin receptor (*55*). Half-molecule fragments of bovine serum transferrin with very similar, but not identical, spectroscopic properties have also been prepared (*56*); these and other fragments are well summarized in Ref. *17*.

The difficulties inherent in proteolytic fragments are illustrated by human lactoferrin, for which trypsin cleavage gives an N-terminal fragment of 30 kDa (residues 1–281) and a C-terminal fragment of 50 kDa (residues 282–691) (*57*). That is, cleavage occurs not in the connecting peptide but 50 amino acids earlier; the N-fragment thus lacks the last 50 amino acids of the N-lobe and its properties are likely to be different from those of the true half-molecules.

B. Three-Dimensional Structure

The first crystallographic studies on transferrins date back more than 20 years (*58*), and crystals of various transferrins have since been reported. These include the diferric forms of rabbit (*59*) and human (*60*) serum transferrins, hen (*61*) and duck (*62*) ovotransferrins, human (*63*) and bovine (*64*) lactoferrins, and the apo- (iron free) forms of human lactoferrin (*65*) and duck ovotransferrin (*62*). In spite of all this activity, the crystals in many cases have proved difficult to handle, and the X-ray analyses quite challenging. A low-resolution analysis of rabbit serum transferrin in 1979 demonstrated the bilobal nature of the molecule (*66*), but it was not until 1987, with the publication of the structure of human lactoferrin (*67*), that full details of a transferrin

structure emerged. The structure of rabbit serum transferrin soon followed (*68*), and it is likely that structure analyses of some of the other species will reach fruition over the next few years.

Complementing the structural studies of the intact transferrins, a number of fragments have also been crystallized, including proteolytic N-terminal half-molecules of rabbit serum transferrin (*69*) and chicken ovotransferrin (*70*), recombinant N-terminal half-molecules of human lactoferrin (*71*) and human serum transferrin (*72*), and a quarter-molecule fragment of duck ovotransferrin (*73*). All of these have now led to high-resolution structures (*74–77*).

The most detailed description of a complete transferrin molecule is that of human lactoferrin, at 2.8-Å resolution (*78*), and most of the data in the following sections come from this work and from refinement of the same structure at 2.1-Å resolution (*79*). As would be expected from the high level of sequence similarity, the three-dimensional structure of rabbit serum transferrin (*68*), although at lower resolution (3.3 Å), is completely consistent with that of lactoferrin; the differences are at the level of individual amino acid changes, together with some differences in lobe and domain orientations. These are discussed below (Section III.B.1).

Coordinates for human lactoferrin, in both diferric (*78*) and apo- (*80*) forms, for diferric rabbit serum transferrin (*68*), and for the three fragment structures, the proteolytic N-lobe of rabbit serum transferrin (*74*), the recombinant N-lobe of human lactoferrin (*75*) and the duck ovotransferrin quarter-molecule (*76*), all in their iron-bound forms, can be obtained from the Brookhaven Protein Data Bank (Brookhaven National Laboratory, Upton, New York).

1. General Organization

All transferrins characterized so far consist of a single polypeptide chain of 670–700 amino acid residues. The lactoferrin and serum transferrin structure analyses show that the folding (polypeptide chain conformation) is the same in both proteins and, given their sequence homology, can be assumed to hold for all proteins of the transferrin family.

The polypeptide chain is first of all folded into two globular lobes, representing the N-terminal and C-terminal halves of the molecule; these are referred to as the N-lobe and C-lobe, respectively. In human lactoferrin the N-lobe comprises residues 1–333 and the C-lobe, residues 345–691, whereas in rabbit serum transferrin the equivalent lobes comprise 1–328 and 342–676. Each lobe contains a single iron binding site, and each has essentially the same folding (described more fully in Section III.B.2). The two lobes do not have equivalent orientations

with respect to the molecule as a whole, however, being arranged "front-to-back" as in Fig. 3. This is one of the factors which may contribute to inequivalence in their functional properties.

The two lobes are joined by a short connecting peptide, which is the only covalent link between them. This peptide varies between different transferrins, both in length (7 to 14 residues—see Section III.C) and in conformation; in lactoferrin, it is 12 residues long and forms a three-turn helix, whereas in serum transferrin it is 14 residues long and has a much less regular structure.

The relative orientations of the two lobes also appear to vary from one transferrin to another. In human lactoferrin the C-lobe can be superimposed on to the N-lobe by a twofold screw axis rotation, a rotation of 180° followed by a translation of 25 Å along the rotation axis. In rabbit serum transferrin, however, the superposition requires a rotation of 167°, followed by a translation of 24.5 Å; i.e., there is a difference of ~13° between the lobe orientations in lactoferrin and transferrin (*81*). Whether this has any functional significance remains to be seen, but we may anticipate more variations of this type between

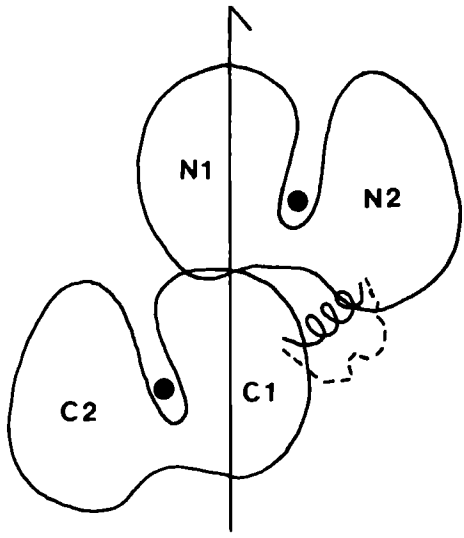


FIG. 3. Domain organization of transferrins. The N-terminal lobe (above) is divided into domains N1 and N2, and the C-terminal lobe (below), into domains C1 and C2. The two lobes are related by a screw axis, a rotation of ~180°, and a translation of ~25 Å. The two iron sites are identified with closed circles. The connecting peptide that joins the two lobes is helical in lactoferrin (solid line) and less regular in transferrin (dashed line).

different transferrins—in fact human and bovine lactoferrins have also been shown to differ in this respect (*82*).

Within each lobe there is a further subdivision into two separately folded domains. This is shown schematically in Fig. 3 and can be clearly seen in the ribbon diagram of Fig. 4. The domains in the N-lobe of lactoferrin are labeled N1 (residues 1–90 and 252–333) and N2 (residues 91–251), with the equivalent C-lobe domains being C1 (residues 345–433 and 596–691) and C2 (residues 434–595). This subdivision into domains also has a crucial functional significance as the cleft separating the two domains of each lobe houses the metal binding site, and the domain structure has major implications for mechanisms of binding and release; this is explored further in Sections III.B.2–III.B.4.

One feature of the domain structure is that the relative orientations of the two domains in each lobe vary slightly, despite the close structural correspondence. If the N2 and C2 domains of human lactoferrin are overlaid, the other domains do not quite match; the N1 domain must be rotated a further 6° to bring it into line with the C1 domain. The difference is that in lactoferrin the C-lobe domains are slightly more closed over their binding site than are the N-lobe domains. The

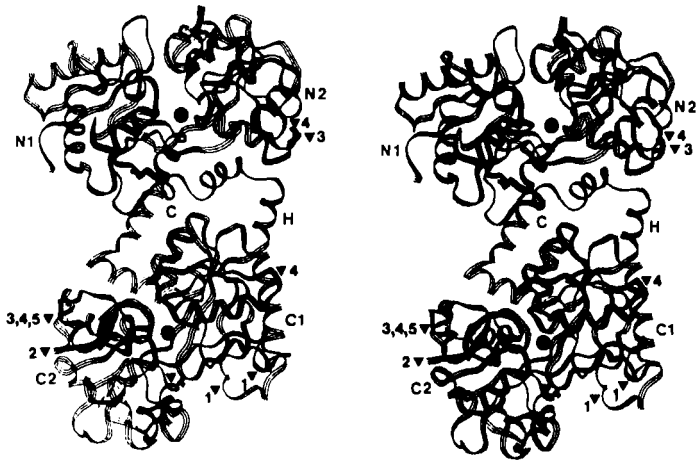


FIG. 4. Ribbon diagram of human diferric lactoferrin, showing the organization of the molecule, with the N-lobe above and C-lobe below. The four domains (N1, N2, C1, C2), the interlobe connecting peptide (H), and the C-terminal helix (C) are indicated. The glycosylation sites in various transferrins are shown by triangles and numbered (1, human transferrin; 2, rabbit transferrin; 3, human lactoferrin; 4, bovine lactoferrin; 5 chicken ovotransferrin). The interdomain "backbone" strands in each lobe can be seen behind the iron atoms. Adapted from Baker *et al.* (*82*), with permission.

two lobes of rabbit serum transferrin also appear to differ in this respect, but this time the N-lobe seems more closed. Similar small differences of domain closure are found in bacterial binding proteins (Section III.D).

A potentially confusing aspect of transferrin structure and function is that, prior to determination of the lactoferrin and transferrin structures, the N- and C-terminal halves of each molecule were often referred to as N- and C-domains. Given the clear subdivisions apparent in Fig. 4, however, the commonly accepted definition of protein domains (separately folded units; see Ref. *83*), and the functional importance of these units, it seems preferable to think of the transferrins as proteins comprising two lobes, but four domains.

A final point of general organization concerns the carbohydrate. All transferrins so far characterized, except apparently for one fish transferrin (*84*), are glycoproteins. There is, however, no pattern to the sites of attachment of the carbohydrate chains on different proteins—they appear almost randomly distributed over the protein surface (Fig. 4), strengthening the view that the carbohydrate plays no direct role in function. Rabbit serum transferrin, for example, has one carbohydrate chain, on its C-lobe (residue 490); human serum transferrin has two, both on the C-lobe (residues 416 and 611); human lactoferrin has two, one on each lobe (at residues 137 and 478); and bovine lactoferrin has four, one on the N-lobe (residue 233) and three on the C-lobe (residues 368, 476, and 545).

2. *Polypeptide Folding*

As noted above, the two lobes have very similar folding. This is only to be expected given their high (~40%) sequence identity. The differences, at the level of polypeptide folding, are confined primarily to small insertions and deletions in the loops that join secondary structure elements. These are almost all located on the molecular surface and do not disturb the basic structure—indeed 90% of the main chain atoms of the N-lobe of human lactoferrin can be superimposed on equivalent atoms in the C-lobe with a root-mean-square deviation of only ~1.2 Å. The agreement would be even closer were it not for the small difference in the closure of the two domains, described above.

The folding pattern for a typical transferrin lobe or half-molecule is shown schematically in Fig. 5. Each domain is based on a mixed β-sheet (i.e., a mixture of parallel and antiparallel β-strands) overlaid with α-helices, which pack against the two faces of the sheet. The first ~70 residues make a coherent unit of three parallel β-strands (a, b, c) and three helices (1, 2, 3), which form about half of the first domain (N1 or C1). A fourth parallel strand, made in two parts (d and e), then

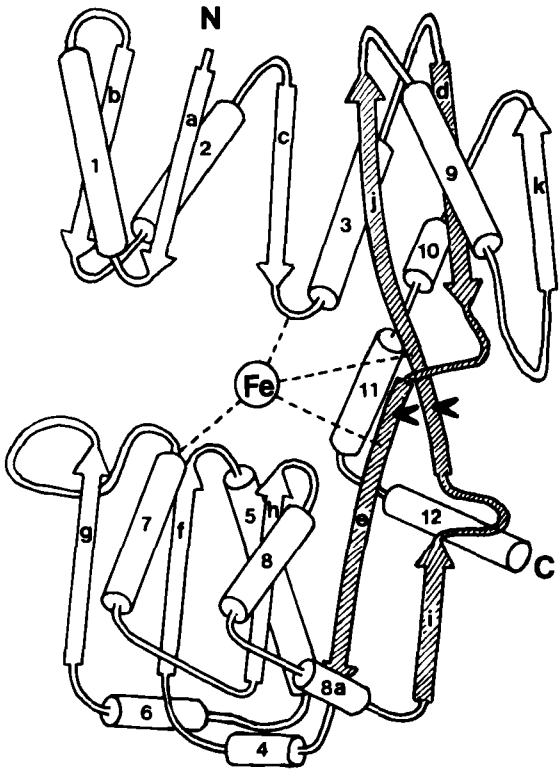


FIG. 5. Polypeptide folding pattern found in each lobe of human lactoferrin. Helices (cylinders) are numbered 1 to 12 and β-strands (arrows) are labeled "a" to "k" as in Anderson *et al.* (*78*). The interdomain backbone strands are shaded and the position of the hinge is indicated.

runs behind the iron site to begin the folding of the second domain, crossing over at about residue 90. The next ~160 residues form the whole of the second domain (N2 or C2), with five β-strands and a number of helices, before a second long extended strand (again in two parts, i and j) crosses back behind the iron site to complete the folding of the first domain. Finally, the chain crosses for a third time, finishing with a helix (*11*) packed against the second domain.

Three features of the folding pattern are of particular importance. First, the N-termini of many of the helices are directed toward the central binding cleft; the positive charge they carry should help attract anions into the binding cleft (see also Section V.A). Second, the two extended β-strands running behind the iron site and linking the two domains can be thought of as "backbone" strands (*85*). A hinge in these

two backbone strands is of crucial importance to the conformational change that occurs during binding and release (*80, 82, 86*). Third, the iron atom takes its protein ligands from four different parts of the structure, widely spaced along the polypeptide chain—one from domain 1, one from domain 2, and the other two from the two backbone strands. The importance of this latter feature for the design of the metal site is outlined below (Section III.B.4).

3. Disulfide Bonding

Both lobes contain disulfide bonds, the total number varying from 15 in chicken transferrin to 22 in human serum transferrin. Although they clearly contribute to the high stability of transferrins, to heat and denaturants, and hence to their relatively long lifetimes in circulation, it is not likely that they play any major role in *defining* the protein folding. Six disulfides [numbers 1–6 in the nomenclature of Williams (*87*)] are common to both lobes, in all transferrins, and can be regarded as the conserved "core" disulfides. Most are local in nature (Fig. 6), linking secondary structure elements, and none of them bridge between domains. Three more, numbers 7–9, are found in the C-lobes of nearly

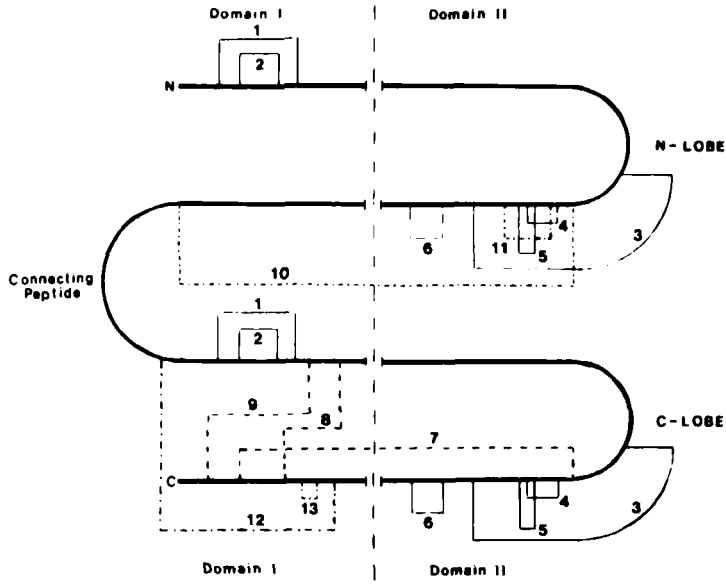


FIG. 6. Pattern of disulfide bridges commonly found in transferrins. Numbering corresponds to that introduced by Williams (*87*) and used in Table V. Figure taken from Bailey *et al.* (*68*), with permission.

all transferrins. These, in contrast, are long range (i.e., they join Cys residues that are far apart in the sequence) and probably make the C-lobe more rigid than the N-lobe. Number 7, which bridges between the C1 and C2 domains, may be of particular importance in modulating the iron binding and release properties of the C-lobe (*80, 85*; see also Sections III.B.5 and V.B). Others are much more variable in their occurrence (Section III. C)—some may influence the properties of different transferrins, but most of them probably just reflect natural variations in different species, with little functional effect.

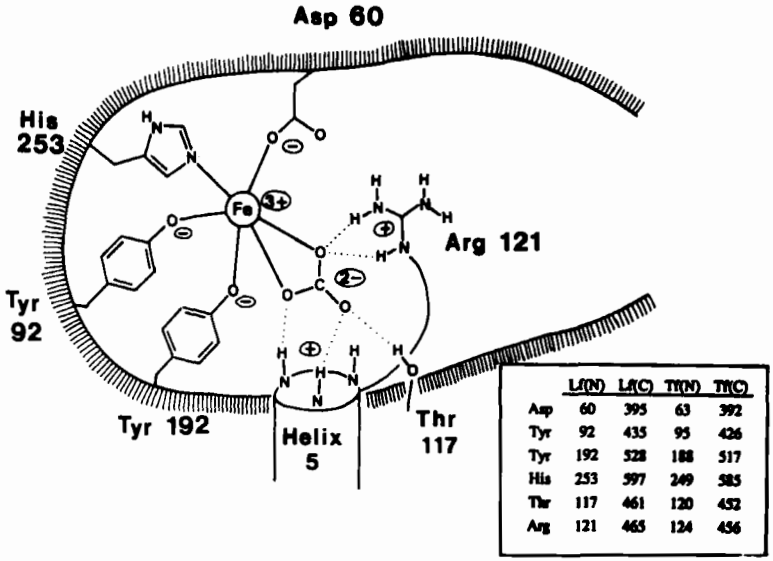
4. Metal and Anion Sites

Prior to first crystallographic analyses, many physicochemical studies were directed toward establishing the nature, number, and arrangement of ligands at the iron site. These have been reviewed extensively (*1, 3, 16, 17*). Four types of ligand were anticipated by these studies, the side chains of the amino acids tyrosine and histidine, the synergistic anion (normally bicarbonate), and water (or OH^- ion). The question was finally settled by X-ray crystallography, however, as there is a high degree of unanimity between the various structure analyses of diferric human lactoferrin (*78*) and rabbit transferrin (*68*) at resolutions of 2.1 and 3.3 Å respectively, and the iron-bound N-terminal half-molecules of human lactoferrin (*75*) and rabbit transferrin (*74*) at resolutions of 2.0 and 2.3 Å, respectively.

a. Iron Coordination. The archetypal transferrin iron binding site is shown schematically in Fig. 7, which is taken from the N-terminal lobe of human lactoferrin. The same organization, however, holds good for both the N-terminal and the C-terminal sites, in both lactoferrin and transferrin.

The iron coordination is distorted octahedral, with ligands provided by four amino acid sidechains from the protein, 2 Tyr, 1 His, and 1 Asp, together with the synergistically bound (bi)carbonate anion. Bond lengths are all around 2.0–2.2 Å (Fig. 8), consistent with EXAFS measurements, which indicate the coordination of six low-Z ligands (O or N) with bond distances of 1.9–2.1 Å (*88*). The bond angles show larger deviations from ideality, largely because of the small chelate "bite" angle of the (bi)carbonate anion, of ~65°.

The involvement of tyrosine and histidine ligands had been expected from spectroscopy; the electronic spectral band at ~465 nm, which is responsible for the orange–red color, is characteristic of iron–phenolate interaction (*89, 90*), and both EPR (*91, 92*) and NMR (*93*) studies indicated metal–histidine coordination. The presence of the Asp ligand

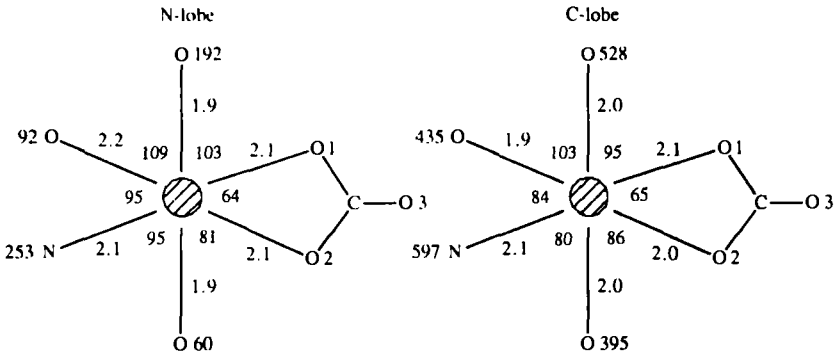


	Lf(N)	Lf(C)	Tf(N)	Tf(C)
Asp	60	395	63	392
Tyr	92	435	95	426
Tyr	192	528	188	517
His	253	597	249	585
Thr	117	461	120	452
Arg	121	465	124	456

FIG. 7. Schematic diagram of the characteristic transferrin metal and anion binding site. Numbering is as for the N-lobe of human lactoferrin, but the same arrangement of ligands is found in the C-lobe and in the N- and C-lobes of almost all transferrins (Table III). For reference, the residue numbers for human lactoferrin and human transferrin are shown in the inset.

was not anticipated, because its spectroscopic silence makes it difficult to detect, but the carboxylate group is an excellent ligand for Fe(III) and serves as such in many nonheme iron proteins (*94*). In the transferrins it appears to play a crucial role in the metal site, as it coordinates the metal through one carboxylate oxygen while hydrogen bonding between the two domains with its other oxygen (Fig. 9a).

With the anion occupying the two remaining coordination positions, there is no room for water in the immediate coordination sphere. The EXAFS (*95*) and NMR proton relaxation (*96*) measurements, which suggested a bound water, must be explained by the presence of several water molecules close to the metal, but not directly coordinated. The two most likely candidates are water molecules hydrogen bonded to the first Tyr ligand and to the His ligand, 3.8 and 6.5 Å, respectively, from the iron atom. These are present in both N- and C-sites in all the lactoferrin structures and in the half-molecule of rabbit transferrin. Another water molecule, attached to the active site Arg sidechain and 4.5 Å from the iron, has also been noted in transferrin (*74*). Although none of these water molecules are directly coordinated, they must contribute to the stability of the binding site, and changes in the water structure may be of importance for iron release.



O60-Fe-O92	88	O395-Fe-O435	83
O60-Fe-O192	161	O395-Fe-O528	170
O60-Fe-O1	84	O395-Fe-O1	92
O92-Fe-O1	92	O435-Fe-O1	95
O92-Fe-O2	155	O435-Fe-O2	157
O192-Fe-N253	84	O528-Fe-N597	93
O192-Fe-O2	86	O528-Fe-O2	91
N253-Fe-O1	167	N597-Fe-O1	172
N253-Fe-O2	106	N597-Fe-O2	115

FIG. 8. Bond lengths (Å) and angles (°) at the iron sites of human lactoferrin.

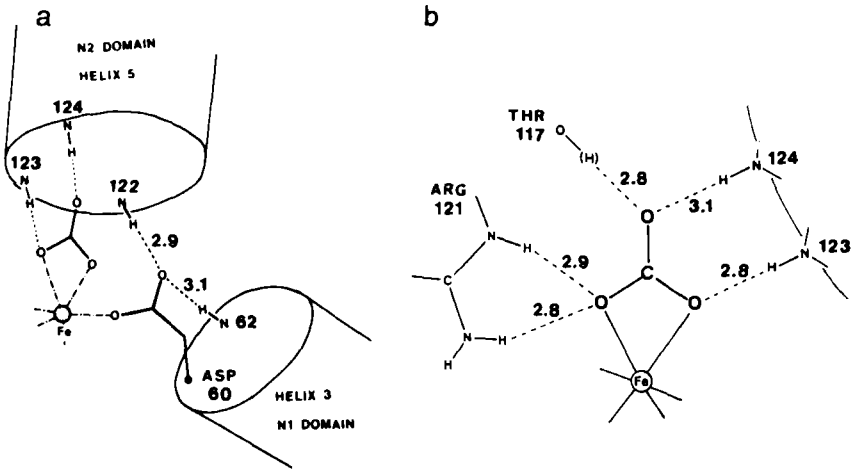


FIG. 9. Hydrogen bonding interactions in the transferrin binding site, with distances as in the N-lobe of human lactoferrin. (a) The Asp ligand, showing its position between two helix N-termini, and the important interdomain hydrogen bond to helix 5. (b) The carbonate ion, hydrogen bonded to the Arg sidechain and the helix 5 N-terminus.

b. Anion Binding. The anion occupies a pocket on domain 2 (N2 or C2). The pocket is formed by two positively charged groups, the side chain of an arginine residue and the N-terminus of an α-helix, number 5 in the nomenclature used for both transferrin (*68*) and lactoferrin (*78*). Note that helices have a partial positive charge, estimated at 0.5+ to 0.75+, because of their dipolar nature (*97*). The anion fits beautifully between protein and metal (Fig. 9b), in such a way that the full bonding potential of each oxygen is expressed, either in metal coordination or in hydrogen bonding. The hydrogen bonds are close to optimal in their geometry, with lengths ~2.8 Å, angles at the hydrogens close to 180°, and angles at the oxygens ~120°, emphasizing the near-perfect fit of the anion.

The form of the anion, whether carbonate or bicarbonate, has never been certain. Considerations of the number of protons that are released upon metal binding have suggested bicarbonate (*98*), but NMR spectroscopic studies (*99*) favor carbonate. The anion site revealed crystallographically strongly suggests carbonate because of the hydrogen bonding pattern (Fig. 9), the charge of at least 1.6+ on the protein and the bidentate iron coordination (*78*). Therefore the description as carbonate is probably preferable.

c. Design of the Metal Site. The design of the metal site is based upon two main requirements, that Fe^{3+} iron should be tightly bound and that binding should be reversible. The first requirement is met by the choice of ligands. The protein provides three anionic oxygen ligands in the form of one carboxylate oxygen and two phenolate oxygens (the Tyr ligands are deprotonated when the metal binds—see Section IV.A), as well as the single neutral imidazole nitrogen. Two further oxygens are provided by the carbonate ion. This arrangement should be highly favorable for Fe^{3+}, with its preference for anionic oxygen ligands, and this is reflected in the low redox potential of around −500 mV (*100*). The size of the metal binding site is such as to allow all metal—ligand bonds to be ~2.0 Å, as expected for Fe^{3+}, and the charge also complements a tripositive metal ion perfectly. The charge on the synergistic anion (2-, assuming carbonate) is approximately matched by the positive charge (~1.6+) of its binding pocket, leaving the 3-charge of the protein ligands to be matched by the 3+ charge of the metal ion. The exclusion of water from the coordination sphere also helps to prevent hydrolysis.

The requirement for reversibility of binding dictates the overall location and construction of the binding site. As noted before it lies in a deep cleft between two domains. The metal takes its ligands from four different parts of the protein structure, one (Asp) from domain 1, one

(the second Tyr) from domain 2, and two (His and the first Tyr) from the two polypeptide strands that cross over between the two domains at the back of the iron site (the backbone strands; Fig. 5). This means that when the domains are moved apart, to a more open configuration, through a hinge in the backbone strands, the metal site is necessarily pulled apart, leading to iron release. This is discussed further below (Section III.B.5).

The synergistic anion has a particular importance in helping to create the metal site. In the absence of a suitable anion, the positive charge of the Arg side chain and helix 5 N-terminus must inhibit metal ions from binding in the specific site. This presumably accounts for the very weak, nonspecific binding found for metal ions in the absence of suitable anions (*101*). Once the anion is in place, however, not only is this positive charge neutralized but the anion then offers two ligands for metal coordination. The intimate burial of the anion between metal and protein also suggests it could have a role in iron release—protonation of the carbonate ion, for example, could disrupt the hydrogen bonding pattern and help in the breakup of the binding site (see also Section V.B). Perhaps the presence of this one non-protein ligand, which can be displaced, is an essential part of ensuring reversibility of metal binding.

5. *Conformational Change*

Transferrins have long been known, from biophysical studies (*102, 103*), to undergo a substantial conformational change during iron binding and release. This takes the form that the protein becomes more compact when the metal is bound. Very important progress has recently been made in defining the nature of this conformational change and understanding its significance, primarily from crystallographic studies of apolactoferrin (*80*), but complemented by solution X-ray scattering measurements (*104–106*).

The apolactoferrin structure analysis was based on protein from which the carbohydrate had been removed enzymatically; this deglycosylated protein had identical properties of iron binding and release and identical spectroscopic parameters, but gave crystals of the apoprotein, which diffracted to high resolution, 2.0 Å (*65*). Two striking results came from the X-ray analysis (*80*). The N-lobe was shown to have undergone a very large conformational change, whereas the C-lobe remained essentially unchanged even though no metal was bound (Fig. 10).

The conformational change in the N-lobe involves an opening of the binding cleft through relative movement of the two domains; the N2 domain rotates 54° relative to N1, about an axis passing through the

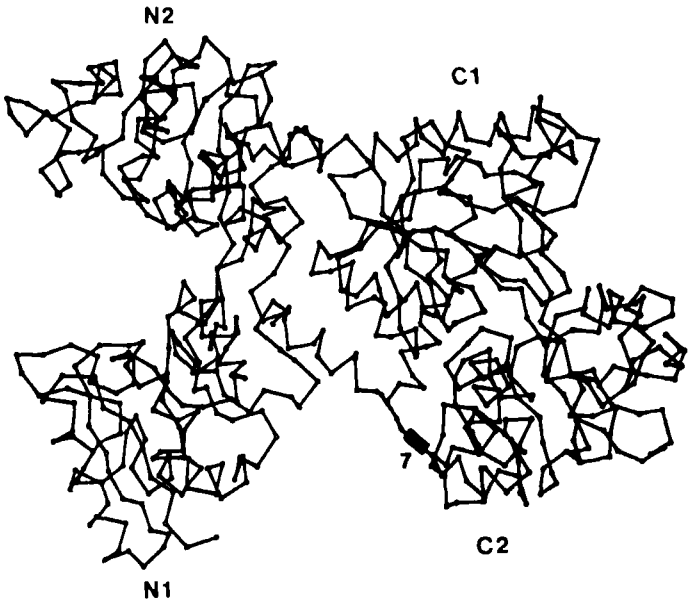


FIG. 10. Cα plot of the "one open, one closed" structure of human apolactoferrin (*80*). The open N-lobe is at left, the closed C-lobe, at right. The disulfide bridge 7 (residues 483–677), which may restrict the flexibility of the C-lobe and which has no counterpart in the N-lobe, is indicated at bottom right.

two backbone strands that run behind the iron site, connecting the two domains (Fig. 11). This is just as anticipated from the polypeptide folding (*85*). Analysis of the conformational change (*86*) shows that it is a rigid body movement in which neither domain shows any significant structural change and in which the hinge is localized to only a very small number of residues, 90–91 and 250–251. A further element is that a helix in the N2 domain (number 5) appears to pivot on another (number 11), which remains associated with the N1 domain (*80*), and there is a fine balance between the interfaces buried and exposed by this movement (*86*).

The 54° domain movement is one of the largest seen in any protein. It has the effect of opening the binding cleft wide, thus exposing a number of residues previously buried, including several basic side-chains. The position of the hinge neatly splits the two ligands provided by the backbone strands, Tyr 92 and His 253, so that in the "open" form Tyr 92 remains with domain N2 (and Tyr 192), whereas His remains with domain N1 (and the Asp ligand). This division of the ligands has important implications for mechanisms of binding and release (Section V).

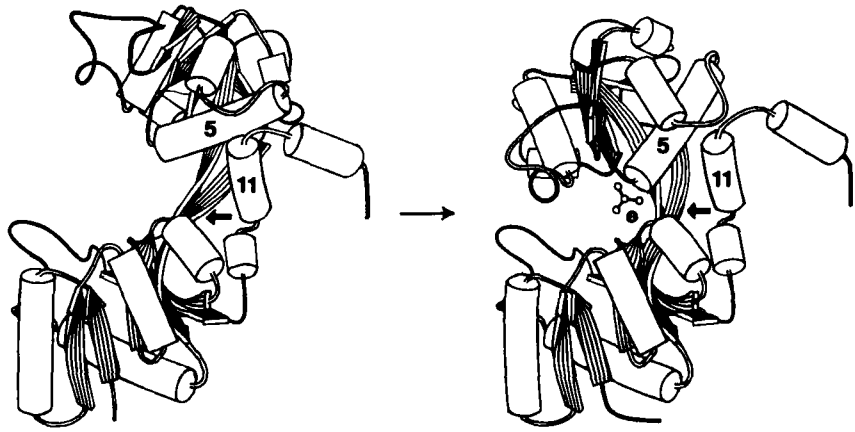


FIG. 11. Schematic diagrams of the "open" (left) and "closed" (right) forms, shown for the N-lobe of human lactoferrin. In the conformational change, helix 5 in domain N2 appears to pivot on helix 11. The hinge in the two backbone strands (at Thr 90 and Pro 251) is indicated with an arrow. Taken from Anderson *et al.* (*80*).

The discovery of a closed C-lobe in the apolactoferrin crystal structure was unexpected but has given some added insights into the likely dynamics of transferrins. It seemed certain the the C-lobe must be able to open in a similar way to the N-lobe, although it was known to bind iron more strongly (*107*) and release it more slowly (*108*), in transferrin at least, and it appeared to be conformationally less flexible (*108*). It was suggested that a disulfide bridge, number 7, which makes a link between the C1 and C2 domains and which has no counterpart in the N-lobe, might impose extra rigidity, although it would still be unlikely to totally prevent opening. Because no metal ion, anion, or other species that could be holding the two domains together was bound, it was concluded that the requirements of crystal packing had preferentially selected out one of several conformations existing in solution (*80*). The implications are of profound importance. Even in the absence of a bound metal ion or anion, both open and closed conformations must be accessible in solution, and there must be very little energy difference between them, given the weakness and small number of intermolecular contacts in the crystals.

Solution X-ray scattering measurements, which make use of the power of synchrotron radiation, have now added important elements to the picture. Closed and open structures give significantly different scattering profiles (Fig. 12), especially in the medium angle range (*104*). These studies have shown that the opening of the N-lobe of apolactoferrin seen in the crystal structure is consistent with the solu-

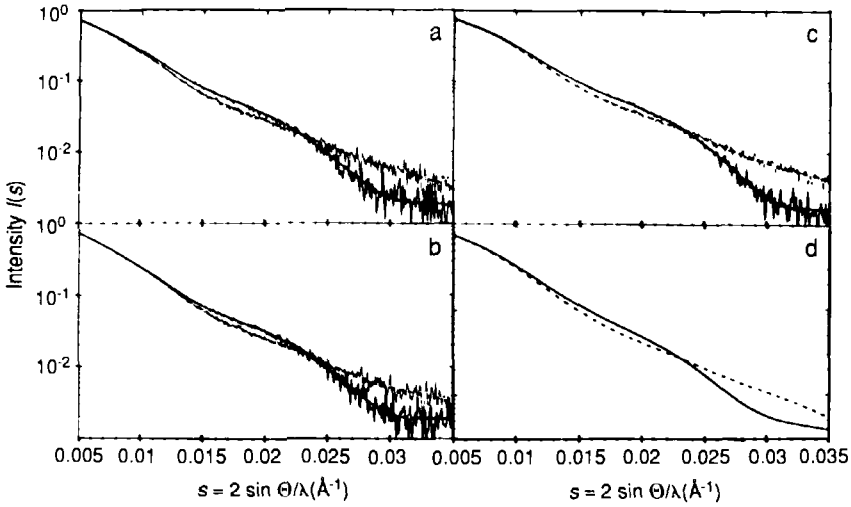


FIG. 12. Low-angle X-ray solution scattering profiles for the iron-free (--) and iron-loaded (——) forms of (a) human serum transferrin, (b) human lactoferrin, and (c) chicken ovotransferrin. In d, the calculated profiles for closed (—) and fully open (--) lactoferrin are shown. From Grossmann *et al.* (*104*), with permission.

tion scattering profile. They further show, however, that *both* lobes do open in solution, in contrast to the apolactoferrin crystal structure, with its closed C-lobe. There is, however, no real contradiction between the two types of study, which illustrate different aspects of the conformational variability. The solution scattering studies also show that the extent of closure can be affected by the particular metal ion bound (*105*; see also Section IV.B.5) and by mutations of ligands in the metal binding site (*106*).

The picture of conformational change has now been nicely completed by the analysis of a second crystal form of apolactoferrin in which *both* lobes were found to be open, just as in the solution studies (Fig. 13). Although the resolution is limited (3.5 Å), the opening of the N-lobe is again ~50°, whereas the opening of the C-lobe is somewhat less, at ~15° (*109*). This adds support to the earlier expectations (*80*) that the extra disulfide bridge might inhibit opening of the C-lobe and highlights the existence of structural and functional differences between the two lobes.

One final structure which merits comment is that of a monoferric form of human serum transferrin. In this structure, determined at 3.0 Å resolution (*110*) the N-lobe is metal-free and has an opening of ~50° relative to the iron-bound N-lobe of rabbit transferrin; the opening thus

FIG. 13. Stereo diagram of the fully open structure of human lactoferrin, determined crystallographically (*109*). The N-lobe is upper, the C-lobe, lower.

almost exactly matches that in apolactoferrin. The C-lobe, however, has iron bound, and has the expected closed configuration, essentially identical to that in rabbit transferrin.

6. *Minitransferrins: Half- and Quarter-Molecules*

The bilobal structure of transferrins means that half-molecules, representing either the N-terminal or C-terminal lobe, can be relatively easily prepared, either by limited proteolysis or by recombinant DNA methods (Section III.A). Relatively high-resolution crystal structures have been determined for three such half-molecules, the proteolytic N-lobes of rabbit transferrin (74) and chicken ovotransferrin (*77*) at 2.3 Å and the recombinant N-lobe of human lactoferrin at 2.0-Å resolution (*75*). These show that both the protein structure and the metal and anion binding sites are the same as in the intact parent structures. In fact comparison of the metal and anion sites of the lactoferrin and transferrin half-molecules with each other and with the N-lobe of lactoferrin shows very close correspondence; 92 atoms from the nine residues, plus metal and anion, making up the immediate binding site can be superimposed with an *rms* deviation of only 0.4 Å (*75*).

Solution scattering measurements on half-molecules show that they undergo very similar conformational changes, on iron binding or release, to those of the corresponding lobes of intact transferrins (*104*). This, together with the structural correspondence noted above, makes them valid as models of single-sited transferrins. The lactoferrin half-

molecule offers one note of warning, however. The structure analysis (*75*) showed that in the absence of contacts from the C-lobe, a helix, number 11, at the back of the iron site, had unraveled in the half-molecule to become an extended strand. This appears to be associated with altered properties of iron release (see also Section V.B).

Quarter-molecules (single domains) can also be prepared, although this is relatively straghtforward only for the N2 domain, which is made up of a single continuous piece of polypeptide. Both N1 and C1 domains comprise two noncontiguous sections of polypeptide, and preparation of a separate C2 domain would require that the disulfide bridge, number 7, linking it to C1, be broken first.

The crystal structure of the N2 domain (quarter-molecule) of duck ovotransferrin has recently been determined at 2.3-Å resolution (*76*). The anion site (which is formed entirely by residues of the N2 domain) is unchanged, as anticipated, and the iron atom is bound by the two carbonate oxygens and the phenolate oxygens of the two Tyr ligands (Fig. 14). This arrangement models a likely key intermediate in the uptake of iron by transferrins (see Section V.A). In the crystal structure the iron atom is actually six-coordinate, with the two remaining ligands coming from a piece of nonprotein density, which could be a glycine molecule resulting from the proteolytic preparation of the material. The folding within this single-domain fragment is the same as for the N2 domains of intact transferrins, again emphasizing that each domain is a stable entity and consistent with the idea that conformational change in transferrins occurs through rigid body movement of these domains.

C. Variations among Transferrins

The overall levels of sequence similarity between different transferrins (Section III.A) emphasize their family relationship and imply that they share a common three-dimensional structure. The variations in the iron-binding residues, and the residues which form the anion site, are listed in Table III. These show that the N-terminal site is highly conserved through all species. The Asp ligand and both Tyr are totally invariant, while the His ligand is invariant except in the two insect transferrins, where it appears to be changed to Gln; this latter substitution would result in the replacement of the imidazole nitrogen ligand by an amide oxygen, but should not otherwise alter the iron site significantly, as Gln and His have similar steric properties. In the N-terminal anion site, the Thr, which hydrogen bonds to one carbonate oxygen, is invariant, while the Arg residue is changed only to Lys, which like-

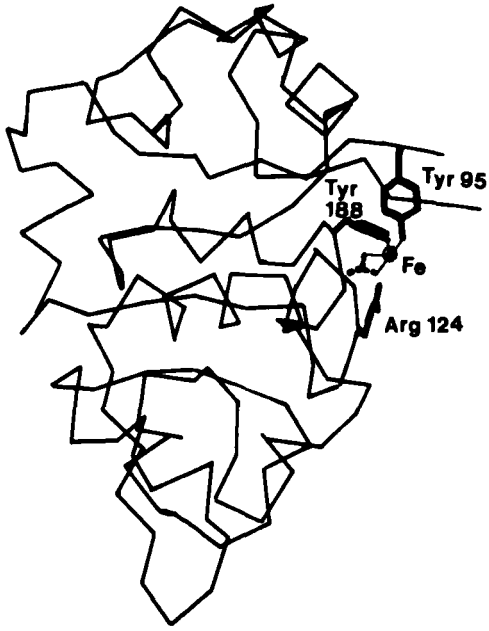


FIG. 14. The "quarter-molecule" 18-kDa duck ovotransferrin structure, showing the iron site at right, with coordination from the CO_3^{2-} ion and two Tyr residues. [The two remaining coordination positions are occupied by a non-protein ligand, possibly a glycine molecule (not shown)]. Adapted from Lindley *et al.* (*76*), with permission.

wise carries a positive charge. There do not seem to be any damaging changes at the helix N-terminus (residues 122–124)—thus all species seem likely to have near-identical N-terminal binding sites.

In the C-terminal site there is equally strong conservation in most species, but with two striking exceptions. In *M. sexta* transferrin both Tyr ligands are changed (to Asn and Asp), the His ligand is changed to Arg, which cannot serve as a ligand below pH ~11, and the anion-binding Arg is changed to Thr (which has no positive charge). In human melanotransferrin, the Asp ligand is changed to Ser and there are major changes in the anion site, with the hydrogen bond from Thr lost (change to Ala), the positive charge of the Arg lost (change to Ser), and the appearance of a Pro at the helix 5 N-terminus. Both these species, therefore, appear to have defective C-terminal sites, and iron titrations confirm that they each bind only one iron (*12, 13*), presumably in their "normal" N-terminal sites.

One other region adjacent to the iron site contains variations that may influence metal binding and release. In the N-lobe of human lacto-

TABLE III

SEQUENCE SIMILARITIES IN METAL AND ANION BINDING SITES[a]

	N-lobe residues[c]											
Protein[b]	60	92	192	253	117	121	122	123	124	210	216	301
Human Tf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Lys
Rabbit Tf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Lys
Pig Tf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Lys
Horse Tf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Lys
Human Lf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Arg	Glu	Lys
Bovine Lf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Lys
Mouse Lf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Arg	Glu	Lys
Pig Lf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Arg
Chicken Otf	Asp	Tyr	Tyr	His	Thr	Arg	Ser	Ala	Gly	Lys	Glu	Lys
Xenopus Tf	Asp	Tyr	Tyr	His	Thr	Lys	Thr	Ala	Gly	Lys	Glu	Gln
Cockroach Tf	Asp	Tyr	Tyr	Gln	Thr	Arg	Asn	Val	Gly	Lys	(Glu)	(Pro)
M. Sexta Tf	Asp	Tyr	Tyr	Gln	Thr	Arg	Asn	Val	Gly	Lys	(Pro)	(Pro)
Melano Tf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Val	Gly	Lys	Glu	Lys
	C-lobe residues[c]											
Protein[b]	395	435	528	597	461	465	466	467	468	546	552	644
Human Tf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Glu	Arg
Rabbit Tf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Gln	Arg
Pig Tf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Gln	Arg
Horse Tf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Gln	Arg
Human Lf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Gln	Asn
Bovine Lf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Glu	Asn
Mouse Lf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Gln	Asn
Pig Lf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Asp	Asn
Chicken Otf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Gln	Glu	Lys
Xenopus Tf	Asp	Tyr	Tyr	His	Thr	Arg	Thr	Ala	Gly	Lys	Glu	Lys
Cockroach Tf	Asp	Tyr	Tyr	His	Thr	Arg	Asn	Ala	Gly	Lys	Glu	Lys
M. Sexta Tf	Asp	Asn	Asp	Arg	Ser	Thr	Phe	Ser	Gly	Ser	His	Asn
Melano Tf	Ser	Tyr	Tyr	His	Ala	Ser	Pro	Ala	Gly	Arg	Asp	Lys

[a] Metal ligands, residues of the anion site (Thr and helix 5 N-terminus), and putative "trigger" residues.

[b] Tf, transferrin; Lf, lactoferrin; Otf, ovotransferrin. For references, see text, Section III.A.

[c] Human lactoferrin numbering. Residues in parentheses are those for which the alignment is uncertain.

ferrin two basic residues are located near the back of the iron site; Arg 210 hydrogen bonds to one Tyr ligand and nearby Lys 301 forms an interdomain salt bridge with Glu216. In transferrins both residues are Lys and there are structural differences in which Lys 206 (equivalent to Arg 210) does not directly hydrogen bond to the Tyr ligand, and the salt bridge is also not formed (see Section V.B). It has been suggested that protonation [a "pH trigger" (*77*)] or the binding of secondary, nonsynergistic anions [a "salt trigger" (*81*)] to these lysines could stimulate iron release and that these differences could then contribute to the characteristic differences between lactoferrins and transferrins (*77*,

81). These questions are discussed further in Section V.B. There are differences in the C-lobes as well but their significance is less clear—future mutagenesis studies may help.

Two other variations merit comment. In the lactoferrins the connecting peptide is 12 residues long and contains no Pro residues (Table IV). It forms a regular α-helix in human lactoferrin (*67*) and probably all the other lactoferrins. In the mammalian transferrins it is longer (14 residues), however, contains Pro residues (and one or more Cys residues that form extra disulfide bonds), and has a very irregular conformation (*68*). These differences may be linked with the different lobe orientations found for the two proteins (Section III.B.1). In other members of the family the connecting peptide can be as short as 7 residues, as in the two insect transferrins (Table IV), or 9 residues, as in melanotransferrin. If differences in the connecting peptide are associated with different orientations and modes of association of the two lobes, then these may in turn be pointers to variations in functional properties.

Another variation that may affect the dynamic properties of different transferrins is in the number and distribution of disulfide bonds. Because conformational change is important for metal binding and release (Section V) the restraints imposed by these covalent bridges may be important. The likely distribution of disulfide bonds in different species is given in Table V. While these are deduced from sequence alignments and are thus tentative, several conclusions can be drawn. All species appear to have the same six conserved disulfide bonds in their N-lobe

TABLE IV

SEQUENCE VARIATIONS IN INTERLOBE CONNECTING PEPTIDE

Protein	Sequence[a]
	———α——— —β—
Human transferrin	Y E Y V T A I R N L R E G T **C** P E A P T D E **C** K **P** V K **W C**
Rabbit transferrin	Y E Y V T A V R N L R E G I **C** P D P L Q D E **C** K A V K **W C**
Pig transferrin	Y Q Y V T A L R N L R E E I S **P** D S S K N E **C** K K V R **W C**
Horse transferrin	Y E Y V T A I R N L R E D I R **P** E V **P** K D E **C** K K V K **W C**
Human lactoferrin	S G Y F T A I Q N L R K S ——E E E V A A R R A R V V **W C**
Mouse lactoferrin	F S Y T T S I Q N L N K K ——Q Q D V I A S K A R V T **W C**
Bovine lactoferrin	S R Y L T T L K N L R E T ——A E E V K A R Y T R V V **W C**
Pig lactoferrin	L P Y L T A I Q G L R E T ——A A E V E A R Q A K V V **W C**
Chicken ovotransferrin	F E Y Y S A I Q S M R K D ——Q L T P S **P** R E N R I Q **W C**
Xenopus transferrin	S R L F Q C I Q A L K E G V —K E D D S A A Q V K V R **W C**
Cockroach transferrin	K A N Y T D V I E R D ————————T G A **P** H R F V R F C
M. sexta transferrin	K A N Y T E V I E R G ————————N G A **P** E L V V R L C
Melanotransferrin	H E Y L H A M K G L L ———C ——D P N R L **P** P Y L R **W C**

[a] The one-letter code for amino acids is used (C, Cys; P, Pro; etc.). Alignment on either side of the connecting peptide is based on the lactoferrin and transferrin 3D structures. The last helix in the N-lobe and first β-strand in the C-lobe are indicated. Cys and Pro residues are in boldface.

TABLE V

DISULFIDE BRIDGES IN TRANSFERRINS

No.[a]	Residues[b]	hTf	rTf	pTf	eTf	hLf	bLf	mLf	pLf	cOtf	XTf	cTf	MsTf	MTf
							N-lobe							
1	9–45	+	+	+	+	+	+	+	+	+	+	+	+	+
2	19–36	+	+	+	+	+	+	+	+	+	+	+	+	+
3	115–198	+	+	+	+	+	+	+	+	+	+	+	+	+
4	157–173	+	+	+	+	+	+	+	+	+	+	+	+	+
5	170–181	+	+	+	+	+	+	+	+	+	?	+	+	+
6	231–245	+	+	+	+	+	+	+	+	+	+	+	+	+
10	134–336	+	+											+
11	160–183	+	+	+	+		+		+					
	26–283[c]										+			
	91–327[c]										+			
							C-lobe							
1	348–370	+	+	+	+	+	+	+	+	+	+	+	+	+
2	358–371	+	+	+	+	+	+	+	+	+	+	+	+	+
3	459–534	+	+	+	+	+	+	+	+	+	+	+	+	+
4	493–507	+	+	+	+	+	+	+	+	+	+	+	+	+
5	504–517	+	+	+	+	+	+	+	+	+	+	+		+
6	575–589	+	+	+	+	+	+	+	+	+	+	+	+	+
7	483–677	+	+	+	+	+	+	+	+	+	+	+	+	+
8	405–686	+	+	+	+	+	+	+	+	+	+	+		+
9	427–649	+	+	+	+	+	+	+	+	+	+			
12	342–608	+	+	+	+									
13	627–632	+	+	+	+	+	+	+	+					

[a] Numbering according to Williams (*87*).
[b] Residue numbering for human lactoferrin.
[c] Deduced from sequence and structure alignment.

(numbers 1–6), but several have one or two extra, one of which (number 10) may influence domain opening since it joins part of the N2 domain to the connecting peptide. *Xenopus* transferrin appears to be an outlier in its N-lobe disulfides. In the C-lobe, 9 disulfides are strongly conserved but interestingly the two species which do not bind iron in their C-lobe, *M. sexta* transferrin and melanotransferrin, are also the outliers with respect to the disulfide bonding, having fewer than other species.

D. SIMILARITIES WITH BACTERIAL BINDING PROTEINS

A remarkable feature of transferrin structure, discovered when the human lactoferrin structure was determined (*67, 85*), is the striking similarity with a group of bacterial binding proteins. These proteins, the bacterial periplasmic binding proteins, bind and transport certain small molecules, such as sugars, amino acids and oxyanions, through the periplasmic space before delivering them *via* specific receptors in the bacterial cell wall (*111*). They thus share with transferrins the

properties of strong but reversible binding and receptor-mediated release.

The structures of a number of the bacterial proteins have by now been determined (*111*). All are the same size (300–350 residues) as each lobe of transferrin. All are divided into two domains with the substrate bound in the interdomain cleft, just as for each transferrin half-molecule. In the bacterial proteins, as for transferrins, substrate binding is associated with a large-scale, hinge-bending, conformational change, likened to a "Venus flytrap" motion (*112*), in which the domains close over the bound substrate. For two of the proteins both closed (substrate-bound) and open (substrate-free) structures have been determined; for the maltodextrin binding protein the domain movement is about 35° (*113*), whereas for the lysine–ornithine–arginine binding protein it is 52° (*114*), almost exactly as in lactoferrin.

Most remarkably, one group of the bacterial binding proteins, which includes the two anion-binding proteins so far analyzed [specific for sulfate (*115*) and phosphate (*116*)], has even closer similarity. First, the polypeptide folding pattern in these proteins is almost identical to that in each lobe of lactoferrin (Fig. 15); the central β-sheet of each

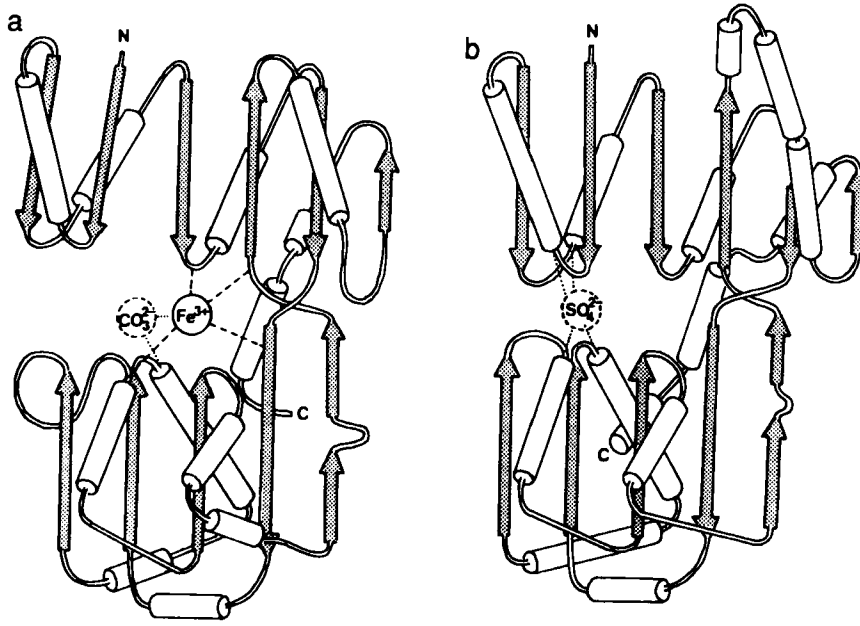


FIG. 15. Polypeptide folding patterns for (a) one-half of a transferrin molecule (the N-lobe of lactoferrin) and (b) the bacterial periplasmic sulfate-binding protein. Adapted from Baker *et al.* (*85*), with permission.

domain has the same topology; the backbone strands, where the hinge is located, have the same arrangement; and many of the helices match. Second, the anion binding site in each protein coincides with the site used to bind the carbonate ion in the transferrins; the largest single contributor to anion binding in each of the proteins is the N-terminus of the same helix (helix 5 in lactoferrin) together with a hydrogen bonding side chain (Arg in the transferrins, Ser in the bacterial proteins) attached at the N-terminus (Fig. 16). Of course there are also differences (Fig. 15), but the similarities in folding, function, and location of the anion binding site argue for a common evolutionary origin. In fact, what sequence similarity there is, is greater between lactoferrin and the sulfate-binding protein (15% identity when aligned by 3D structure) than between the sulfate- and phosphate-binding proteins (<10%).

Whether the two groups of proteins do share a common evolutionary origin is not clear, but they could have evolved from an ancestral anion-binding protein, with the metal-binding capacity of transferrin being added later, prior to the gene duplication that led to the present two-sited transferrins. A further connection may be provided by a recently discovered bacterial protein involved in iron transport (*117*)—perhaps this is the "missing link"? Irrespective of whether there is an evolutionary link, the structural and functional similarities are intriguing and revealing. Perhaps the transferrins could be regarded primarily as anion-binding proteins? The suggestion is not facetious, because the anion apparently binds first (*118–120*), although their physiological roles are clearly based on their metal-binding properties.

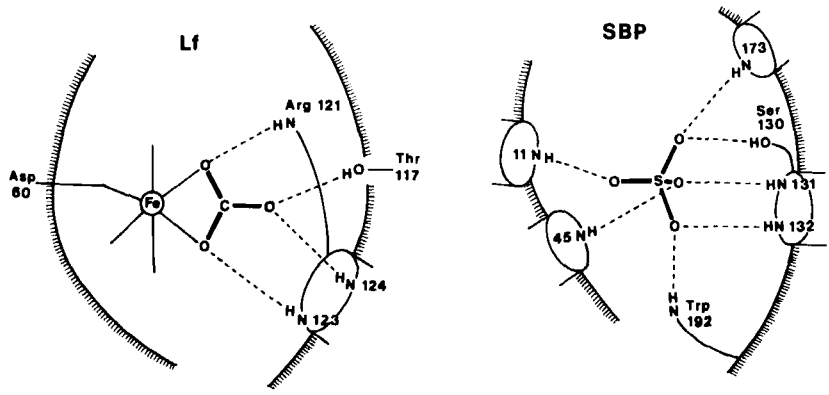


FIG. 16. Comparison of the anion binding sites in lactoferrin (left) and the bacterial sulfate-binding protein (right). From Baker *et al.* (*82*), with permission.

IV. Properties of the Metal and Anion Sites

A. Spectroscopic Monitors of Metal Binding

The orange–red color that develops when ferric iron is added to solutions of metal-free transferrins has traditionally provided the main indicator of iron binding in the two specific sites. The color is due to an intense charge-transfer absorption band at around 465 nm. The precise wavelength of maximum absorption varies slightly from one transferrin to another, in the range 460–470 nm, and it also represents an average of the maxima for the two sites, which differ slightly (Section IV.D). The similarity of the visible spectra to those of metal–phenolate complexes (*90*) suggested that they arise from tyrosine–metal interaction, and the charge transfer band is generally agreed to be derived from a ligand phenolate (π) → metal ($d\pi^*$) transition (*121*).

The rise in the 465-nm absorbance as Fe^{3+} is added to the apoprotein (generally as a ferric nitrilotriacetate or ferric citrate complex) can be used to monitor iron binding and forms the basis of iron titrations that demonstrate the presence of two specific sites per molecule (Fig. 17).

The ionization of tyrosine on metal binding ($PhOH \rightarrow PhO^- + H^+$) also causes a dramatic increase in absorbance in the UV region, both at 245 nm and ~290 nm, due to perturbations of the $\pi-\pi^*$ transitions of the aromatic ring. This effect has been widely exploited for metal titration studies, using UV difference spectroscopy (*122–125*). Typical metal titration difference spectra are shown in Fig. 18. These can also be used to determine the number of metal ions that are bound (Fig. 18) and to determine metal binding constants, at least for the weaker-binding metal ions (*126, 127*).

Fluorescence quenching measurements can also be used to monitor metal binding (*12, 128, 129*). The fluorescence spectra of transferrins, as for most proteins, are dominated by emission from tryptophan residues, but some of this is quenched if a cation, such as a transition metal ion or lanthanide ion, is bound nearby; one estimate is that energy can be transferred from tryptophan to Fe^{3+} or Cu^{2+} over distances up to ~20 Å (*128*). Metal binding is thus accompanied by a substantial decrease in transferrin fluorescence (e.g., Fig. 17). The decrease is nonlinear, however, with the binding of the first metal ion contributing a greater decrease than the second (*12, 128*). This is probably because some tryptophan residues lie between the two metal sites and their fluorescence is quenched no matter which metal site is occupied first; binding of the second metal ion would have little further effect on these residues. One study (*129*) suggested that iron bound at

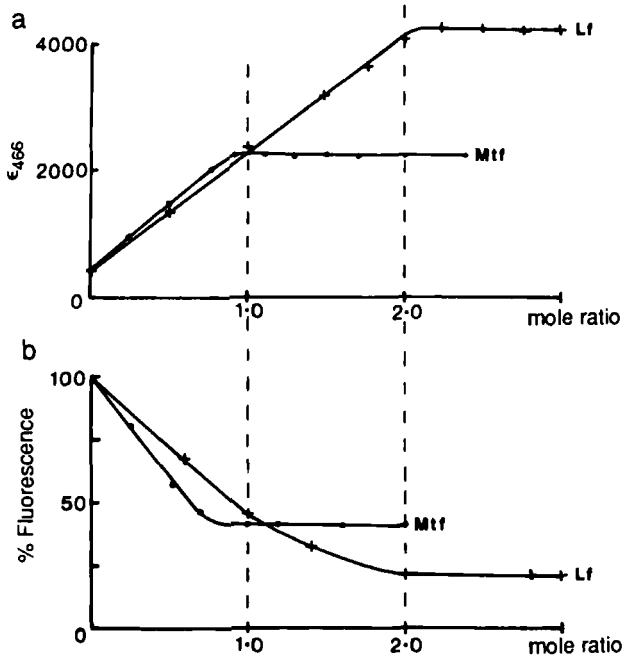


FIG. 17. Spectroscopic monitors of iron binding. In a, the rise in absorbance at 466 nm as Fe^{3+} is added is used to show that human melanotransferrin (Mtf) binds only one Fe^{3+} ion per molecule, compared with two for lactoferrin (Lf). In b, the decrease in fluorescence is used to demonstrate the same phenomenon. In each case, the mole ratio is moles Fe^{3+} added per mole of protein. From Baker *et al.* (*12*), with permission.

the C-terminal site produces more quenching than iron at the N-terminal site, but attempts to relate nonlinearity of fluorescence quenching to site binding preferences have tended to be inconclusive (*17*).

B. Metal Substitution and Spectroscopy

The transferrins are regarded primarily as iron-binding proteins. This is proper, because their affinity for Fe^{3+} is substantially greater than for any other metal ion and their physiological role is primarily concerned with the binding, sequestration, and transport of iron. They can, however, accommodate a wide variety of other metal ions, including most of the first row transition elements, several of the second and third row transition elements, group 13 metal ions, lanthanides, and actinides (Table VI). Most of these are unlikely to be physiologically significant (some exceptions are noted below), but many have been

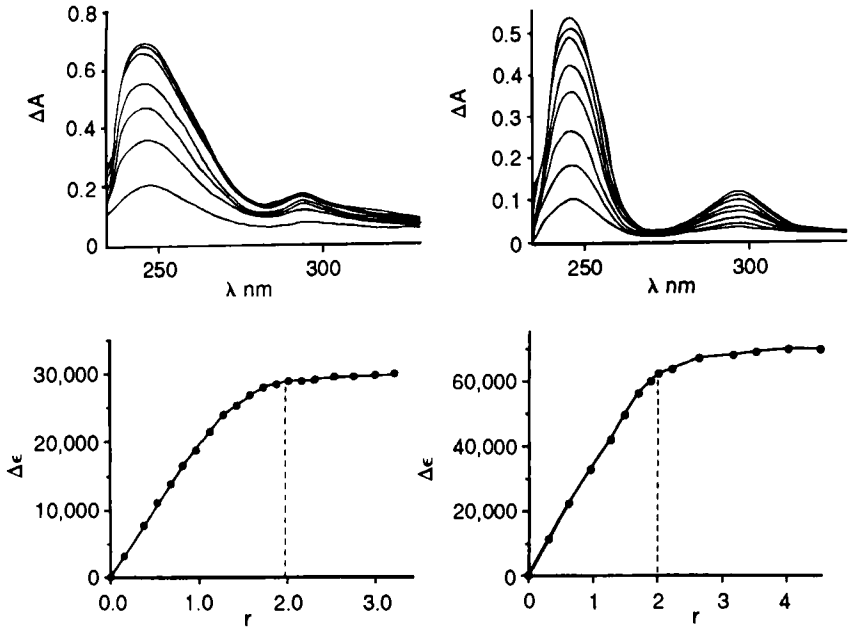


FIG. 18. Typical UV difference spectra, showing the increase in absorbance, ΔA, during the binding of (a) V^{3+} and (b) Yb^{3+} to human lactoferrin. The spectra are shown as a superimposed series of spectra, corresponding to successive increments of the added metal ion. Below each set of spectra is the corresponding titration curve, showing that in each case two metal ions are bound per molecule (r is the ratio of moles of the metal iron added per mole of protein).

TABLE VI

METAL IONS REPORTED TO BIND TO TRANSFERRINS

Transition elements			Group 13 elements	f-block elements	
Row 1	Row 2	Row 3		Lanthanides	Actinides
Sc^{3+} (130)	Ru^{3+} (141)	Hf^{4+} (142)	Al^{3+} (144)	La^{3+} (147)	Th^{4+} (151)
V^{3+}, VO^{2+}, VO_2^+ (131–133)	Cd^{2+} (140)	Pt^{2+} (143)	Ga^{3+} (126)	Ce^{3+}, Ce^{4+} (147)	Pu^{4+} (151)
Cr^{3+} (134, 135)			In^{3+} (145)	Pr^{3+} (148)	
Mn^{2+}, Mn^{3+} (134–136)			Tl^{3+} (146)	Nd^{3+} (148)	
Fe^{2+}, Fe^{3+} (137, 138)				Sm^{3+} (149)	
Co^{2+}, Co^{3+} (139, 140, 134–136)				Eu^{3+} (148)	
Ni^{2+} (137, 140)				Gd^{3+} (150)	
Cu^{2+} (89, 135, 136)				Tb^{3+} (148)	
Zn^{2+} (89, 127)				Ho^{3+} (148)	
				Er^{3+} (148)	

exploited for their spectroscopic properties in investigations of transferrin structure and function.

A feature of fundamental importance to understanding the metal-binding properties of transferrins is the strong preference for cations with a high positive charge. For iron this is shown by the binding constants for Fe^{2+} relative to Fe^{3+}, these being approximately 10^3 for Fe^{2+} (*137*) and 10^{20} for Fe^{3+} (*107, 138*). Likewise when manganese is added as Mn^{2+} it becomes oxidized to Mn^{3+} (*134, 135*), and all of the non-transition metal ions bound are tri- or tetravalent.

Binding of metals other than iron is generally inferred from spectroscopic observations, especially UV difference spectra that monitor binding to tyrosine ligands. It is, however, important to distinguish whether binding occurs at the two specific sites or at nonspecific sites that might be found at various locations on the molecular surface. To this end, Aisen (*152*) has proposed three criteria that might identify a metal ion as specifically bound, i.e.,

(i) no more than two metal ions bound per molecule,
(ii) binding to iron-saturated transferrin not observed, and
(iii) one (bi)carbonate, or other suitable anion, bound with each metal ion.

Most often it is the third criterion that has not been demonstrated to be met. The metal ions that have definitely been shown to bind specifically, by these criteria, are Cr^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+}, and Ga^{3+}, as well as Fe^{3+} (*152*), but it is highly probable that most of the species in Table VI do bind in the two specific sites.

Even where binding to the specific sites is indicated, however, this does not necessarily mean that all ligands are the same. UV difference and visible charge transfer spectra monitor binding to the tyrosine residues, which, together with the synergistic anion, belong to just one domain (domain 2). Such spectra may not show, therefore, whether the structure is closed (as with Fe^{3+}) or open and whether the Asp and His ligands are also coordinated or not. Crystallographic and biophysical studies (Section IV.B.5), or spectroscopic probes sensitive to the other ligands, are necessary to resolve these questions.

Binding constants have been determined for some metal ions, but their determination is complicated by the presence of two very similar sites and by the pH and (bi)carbonate-dependence of the values. Readers are referred to an excellent discussion by Aisen and Harris (*17*). For Fe^{3+}, at ambient HCO_3^- concentrations, the log K values are estimated as 19.4 and 20.7 for the two sites (*107*) and it is generally the case for most metal ions analyzed that the two sites differ by approximately one unit in their log K values (Table VII).

TABLE VII

SELECTED BINDING CONSTANTS FOR METALS BOUND TO TRANSFERRINS[a]

Metal ion	$[HCO_3^-]$	pH	log K_1	log K_2	Reference
Fe^{3+}	Ambient	7.4	20.7	19.4	*107*
Fe^{2+}	5 m*M*	7.4	3.2	2.5	*137*
Ga^{3+}	Ambient	7.4	18.1	17.1	*126*
Al^{3+}	5 m*M*	7.4	13.5	12.5	*153*
Zn^{2+}	Ambient	7.4	5.7	4.3	*127*
Ni^{2+}	5 m*M*	7.4	4.1	3.2	*137*
Nd^{3+}	Ambient	7.4	6.1	5.0	*149*
Sm^{3+}	Ambient	7.4	7.1	5.4	*149*
Gd^{3+}	Ambient	7.4	6.8	—	*150*

[a] Conditional equilibrium binding constants for the stated pH and $[HCO_3^-]$ (*17*).

1. *Metals of the First Transition Series*

The first row transition elements display a rich chemistry in their binding to transferrins. As noted above the trivalent oxidation states are highly favored, and although no binding constants have been reported, the very stable complexes formed with Cr^{3+}, Mn^{3+}, Co^{3+}, and Cu^{2+} have been extensively studied for transferrin (*134*), ovotransferrin (*136*), and lactoferrin (*135, 154*). Like Fe^{3+}, the elements Mn^{3+}, Co^{3+}, and Cu^{2+} give intensely colored complexes (brown, yellow, and yellow, respectively) because of the presence in the visible region of the phenolate (π) $\rightarrow$ metal ($d\pi^*$) LMCT band (*121*). Typical spectra are shown in Fig. 19. Cr^{3+} complexes, on the other hand, are pale blue–gray in color and their visible spectra contain only weak, spin-allowed *d–d* bands (*17*). Analyses by Patch and Carrano (*121*) suggest that the charge transfer band for Cr^{3+} is shifted to ~240 nm, where it would be obscured by the intense tyrosine $\pi-\pi^*$ transitions. Cr^{3+} gives rise to useful EPR spectra, which gave one of the first clear indications of differences between the two sites in transferrins (Section IV.D).

Vanadium can bind to transferrins in three different oxidation states. V(III) has been reported (*131*) as giving an air-stable complex with transferrin, probably as the simple cation V^{3+}; the corresponding lactoferrin complex of V(III) is, however, extremely air sensitive, being oxidized in minutes (*156*), and the transferrin result has been questioned (*133*). V(IV) forms a colorless complex with transferrins, shown by EPR spectroscopy to incorporate the vanadyl (VO^{2+}) ion (*155*). Displacement studies show that the VO^{2+} ions occupy the specific Fe^{3+} sites

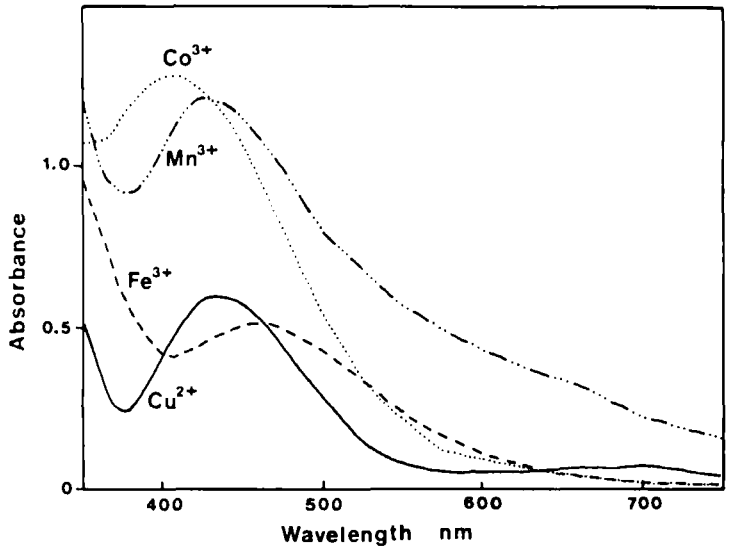


FIG. 19. The visible absorption spectra of various metal complexes of human lactoferrin; all spectra are for 1% protein solutions. Very similar spectra are obtained for transferrin and ovotransferrin. Adapted from Ainscough *et al.* (*135*), with permission.

and that the synergistic (bi)carbonate ion is required (*132*). Although oxidation to V(V) occurs rapidly in air, the V(IV) complex is stable under nitrogen and its d^1 electron configuration makes it another extremely useful EPR probe. In its highest oxidation state, V(V), vanadium probably binds to transferrins as the dioxovanadium(V) ion, VO_2^+, giving a colorless complex. An interesting feature here is that a synergistic anion is apparently not required (*133*), with the two *cis* oxygen atoms perhaps taking the place of two carbonate oxygens. The net charge of VO_2^+ is then the same as Fe^{3+}–CO_3^{2-}.

Most of the divalent transition metal ions bind only weakly to transferrins, the binding constants for Fe^{2+}, Ni^{2+}, and Zn^{2+} being approximately 10^3, 10^4, and 10^5, respectively (Table VII). Cu^{2+} may be an exception. Although no binding constants have been determined for any of its complexes with transferrins, and metal displacement studies indicate that it binds less strongly than Al^{3+} (*144*) and Mn^{3+} (*154*), it does form a very stable complex and has been used more than any other metal ion (apart from Fe^{3+}) for physicochemical studies on transferrins. With its d^9 electron configuration, Cu^{2+} has, like VO^{2+}, provided an excellent and much-used EPR probe of transferrin structure. It was the EPR spectra of Cu^{2+}–transferrin (*91*), –ovotransferrin (*157*), and –lactoferrin (*92*) complexes that provided the most compelling evidence

for a histidine ligand in the metal coordination sphere, prior to crystallographic analysis; the triplet superhyperfine splitting (Fig. 20) is characteristic of Cu(II) interaction with a single nitrogen ligand. Cu^{2+} is also the only metal ion, apart from Fe^{3+}, for which the details of its coordination geometry in a transferrin complex have been determined crystallographically; the structure of dicupric lactoferrin has recently been reported at 2.1-Å resolution (*26*) (see below). Cu^{2+}–transferrin complexes have also been used to examine the relationship between metal and anion binding (Section IV.C).

Because most of the first transition series elements are essential in biological systems there is considerable interest in the possibility that transferrins may be involved in their binding and translocation. This is certainly a distinct possibility, because neither transferrin nor lactoferrin is more than ~30% saturated with iron in body fluids. There is as yet little hard evidence, however. Transferrin has been reported to be the main carrier for manganese in blood (*158*), just as lactoferrin is in milk (*25*), and thermodynamic studies suggest that transferrin is also capable of competing with serum albumin for zinc under the

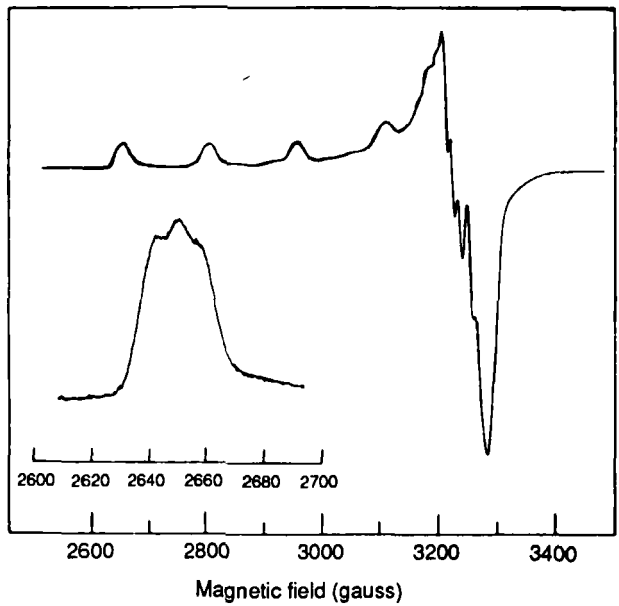


FIG. 20. The EPR spectrum of $^{65}Cu^{2+}$–transferrin–carbonate. The inset shows a low-field hyperfine line (at an eightfold increase in gain) that displays superhyperfine splitting resulting from the coordination of one nitrogen ligand in each site. Adapted from Zweier and Aisen (*91*), with permission.

conditions that prevail in circulation (*127*). Despite the biological importance of copper, however, surprisingly little is known of the likely role of transferrins in binding it *in vivo*.

2. *Group 13 Metals*

The group 13 metals are of particular interest because they have a characteristic 3+ charge, like Fe^{3+}, and because several of them are important medically. Aluminium is widely consumed in drinking water and foods and has been linked to various diseases, including dialysis encephalopathy and Alzheimer's disease (*24, 159*). Gallium is of pharmacological importance because $^{67}Ga^{3+}$ is an effective radiotracer, used as an imaging agent in diagnostic medicine (*160*), and two radioactive isotopes of indium, $^{111}In^{3+}$ and $^{113}In^{3+}$, have similar applications. Thallium, on the other hand is a potent poison. Transferrins are likely carriers of all of these species, and the stability and nature of the metal–transferrin complexes are important for several reasons: in determining where the metals are likely to accumulate and how the interaction is likely to be affected by competitive chelators.

The chemistry of the group 13 metals makes binding studies difficult, as careful control of pH and bicarbonate concentration is necessary to prevent formation of species such as $Al(OH)_4^-$ and $Ga(OH)_4^-$. Nevertheless, UV difference spectra have shown that Al^{3+}, Ga^{3+}, In^{3+}, and Tl^{3+} all form transferrin complexes with two metal ions per molecule (*126, 144–146*). $\Delta\varepsilon$ values imply the ionization of two tyrosines per bound metal ion, as for other specifically bound metals. NMR studies using ^{13}C-enriched bicarbonate show virtually identical spectra for Al^{3+} and Ga^{3+}, implying equivalent metal–anion environments (*99*).

Of the four metal ions, Ga^{3+} is most like Fe^{3+} in size, with an ionic radius of 0.62 Å compared with 0.65 Å for Fe^{3+} (*161*), and it is, accordingly, the most strongly bound. The binding constants for Ga^{3+}–transferrin are 10^{17} and 10^{18} for the two sites, almost comparable to those for Fe^{3+} and higher than those for any other metal ion for which data are available. Ga^{3+}–transferrin is recognized by transferrin receptors, providing the means by which Ga^{3+} enters tumor cells (*162*). Its diamagnetism and similar chemistry have also led Ga^{3+} to be used in place of Fe^{3+} in ^1H NMR studies of transferrin (*163*).

Al^{3+} (ionic radius, 0.54 Å) is smaller than Fe^{3+} but still forms a stable transferrin complex, with binding constants $10^{12.5}$ and $10.^{13.5}$; Al^{3+} is displaced by Fe^{3+} (*144*) but the Al_2Tf complex is stable enough that it has been shown to bind to cell surface receptors (*164*). Small-angle X-ray scattering experiments, however, indicate that the Al^{3+}–transferrin complex is conformationally distinct from the diferric

protein, implying perhaps an altered domain closure and altered receptor interactions (*105*).

In spite of its larger size, with an ionic radius of 0.80Å (*161*), In^{3+} appears to bind to transferrin with an affinity close to that of Fe^{3+} (*145*). In^{3+} displaces Cu^{2+} from copper-saturated ovotransferrin, and the In^{3+} even remains bound in the presence of an added twofold excess of Fe^{3+}. Indium–transferrin also migrates indistinguishably from iron–transferrin (*145*) and gives the same "closed" conformation, as judged by small-angle X-ray scattering (*105*).

Finally, thallium has been used as an NMR probe of transferrin through the binding of $^{205}Tl^{3+}$ (*146*). Although thallium is bound in both metal sites, they are distinguishable spectroscopically perhaps because the presence of a larger cation (radius of Tl^{3+}, 0.89 Å) accentuates the differences between the two sites (see also Section IV.D).

3. *Lanthanides*

The lanthanide ions resemble Fe^{3+} in their charge (3+), but are substantially larger, ranging in ionic radius from 0.86 Å for Lu^{3+} to 1.03 Å for La^{3+} (*161*). There have been a number of studies of lanthanide binding to transferrins, mostly aimed at using the metal ions as spectroscopic probes. Luk (*148*) used UV difference titrations to demonstrate binding of a series of lanthanide ions to human transferrin. The four smaller lanthanides used (Eu^{3+}, Tb^{3+}, Ho^{3+}, and Er^{3+} gave results indicative of binding at both sites, whereas two larger ions (Pr^{3+} and Nd^{3+}) bound only at one site; it was suggested that one of the specific transferrin sites was too small to accommodate the larger lanthanides. Harris (*149*) used UV difference titrations to determine binding constants for Sm^{3+} and Nd^{3+} (Table VII). He concluded that two metal ions were bound in each case and highlighted problems inherent in the bicarbonate dependence of specific metal binding. In the case of the lanthanides, this results in competition with the precipitation of insoluble lanthanide carbonates. Contradictory results have been obtained for Gd^{3+} binding to human transferrin. In a careful study, Zak and Aisen (*150*) found only one Gd^{3+} ion bound per molecule, showed this to be in the C-terminal site, and determined its binding constant (Table VII). In contrast, O'Hara and Koenig (*165*) obtained convincing evidence for two Gd^{3+} bound per molecule. The difficulties appear to arise from the relative weakness of lanthanide binding making it sensitive to competitive effects.

For lactoferrin, which binds metal ions more strongly than transferrin (*166*), all the lanthanide ions, from Yb^{3+} to La^{3+}, bind with two metal ions per molecule (*147*), with the log K_2 values ranging from 6.4

for Yb^{3+} to 4.8 for La^{3+}; binding at the second site thus becomes weaker as the ionic size increases. One striking result for lactoferrin is that Ce^{3+} initially forms a colorless Ce_2Lf complex, like the other lanthanides, but that on exposure to air the complex rapidly turns deep red. Ce^{3+} is oxidized to Ce^{4+} and a strong visible absorption band at 442 nm appears, with an extinction coefficient (ε = 4640 M^{-1} cm^{-1}) reminiscent of the transition metal ion LMCT bands (*147*). The smaller ionic radius of Ce^{4+} compared with that of Ce^{3+} (0.87 Å compared with 1.01 Å) may help to promote formation of this complex.

One other point of controversy concerns the $\Delta\varepsilon$ values obtained in UV difference titrations of lanthanide ions. These are significantly higher than the corresponding values for transition metal ions (7400–8700 M^{-1} cm^{-1} compared with 5000–6600 M^{-1} cm^{-1}), leading to debate about the number of tyrosines coordinated (*167*). Although it is possible that lanthanide binding could recruit a third Tyr ligand (*78*), careful comparisons of the $\Delta\varepsilon$ values with those of complexes with a small-molecule phenolate chelate analog suggests that the number is two per metal ion in all cases (*167*).

Lanthanide complexes of transferrin have been used for several purposes. Gd^{3+}–transferrin gives a characteristic EPR signal at g = 4.96, quite unlike the spectra for other Gd^{3+} complexes (*165*); Eu^{3+} has been used to probe differences between the two sites by Eu(III) excitation spectroscopy (*168*); and the luminescence of excited Tb^{3+} ions bound in one site of mixed-metal Tb^{3+}–Mn^{3+} and Tb^{3+}–Fe^{3+} transferrin complexes has been used to determine the intersite distance (*169*). The value obtained, 35.5 Å, compares well with the value of 42 Å later obtained from the lactoferrin crystal structure (*67*).

4. Actinides and Other Metal Ions

Very few metals of the second and third transition series have so far been shown to bind to transferrins. Ru(III) has been shown to bind to human transferrin, giving a deep red complex, when added as a chelate complex with nitrilotriacetate (*141*), and similar behavior has been found for Pt(II) when $PtCl_4^{2-}$ is added (*143*). Cadmium has been shown by UV difference spectroscopy to bind in both sites of ovotransferrin (*140*), and Cd_2OTf complexes have been used to investigate anion binding by ^{113}Cd and ^{13}C NMR spectroscopy using bicarbonate (*170*) and oxalate (*171*) as synergistic anion. Hafnium has been of particular interest because of its ability to model the binding of plutonium. Hf^{4+} has a similar size to Pu^{4+} (0.83 Å compared with 0.86 Å) and behaves similarly in its metabolism and interactions with biochemical ligands (*142*). ^{18}Hf also gives access to the spectroscopic method of perturbed

angular correlation of γ rays (*142*). The complex of Hf^{4+}–transferrin has hafnium bound at both sites (*172*), but appears not to have the same "closed" configuration as transferrin complexes of smaller cations (*105*).

The actinides plutonium, neptunium, protoactinium, and thorium (*151, 173*) bind to transferrin. The larger Th^{4+} ion (radius, 0.94 Å) still binds to both sites, although binding to the second site (probably the N-terminal site) is significantly weaker than that to the first and apparently involves only one Tyr ligand compared with two Tyr in the other (*151*). Although UV difference spectra for Pu^{4+} are equivocal (*174*), it seems likely that two Pu^{4+} are bound. The likely carrier properties of transferrin for Pu^{4+} makes the design of competitive chelators of some importance (*151*).

5. *Structural Aspects of Metal Substitution*

The metal ions that bind to transferrins are very diverse in size and in their coordination preferences, raising the question of what degree of induced fit there is in the metal sites. When the protein domains close over the bound metal ion (Section III.B.5), does the metal simply fit into a prepared site or are the details of the binding site moulded to some degree by the stereochemical preferences of the metal? This question is of great importance for correlating structure with spectroscopy.

The only metal ion other than Fe^{3+} for which crystallographic data are available is Cu^{2+}. In a high resolution study of Cu^{2+}-substituted lactoferrin, Smith *et al.* (*26*) showed that although the overall structure of the molecule is not changed at all, compared with the diferric protein, the metal sites are subtly different. Small movements in the metal ion position (1.0 Å in the N-terminal site, 0.4 Å in the C-terminal site) bring about a change in the metal coordination geometry and increase the nonequivalence of the two sites. In the N-site the copper is five coordinate, square pyramidal, with a long (2.8 Å) apical bond to one Tyr ligand (Tyr 92) and a monodentate (bi)carbonate ion (Fig. 21); the monodentate coordination of the latter results primarily from the copper movement but is also facilitated by a slight (20°) rotation of the anion. In the C-site, however, the metal ion remains six-coordinate but more distorted from regular octahedral geometry than is the case for Fe^{3+}. Corroboration for these crystallographic observations comes from an EXAFS study of Cu^{2+} binding to ovotransferrin, which is indicative of one five-coordinate site and one six-coordinate (*175*).

The degree to which a given metal can stabilize the "closed" configuration seen for the diferric proteins (*67, 68*) has been addressed by small angle X-ray scattering studies (*105*). These show that Cu_2OTf is

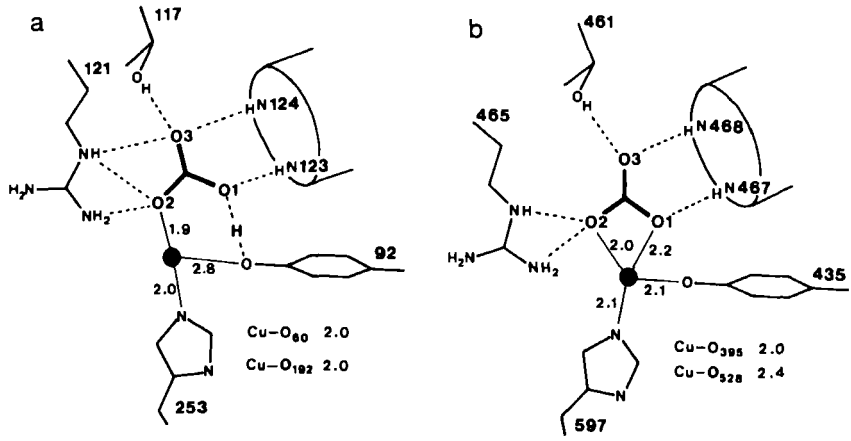


FIG. 21. Metal and anion binding in the crystal structure of Cu^{2+}-substituted human lactoferrin. (a) The five-coordinate N-lobe copper site, in which either the anion or Tyr 92 may be protonated, and (b) the six-coordinate C-lobe copper site. In each case the axial ligands, Asp 60 and Tyr 192 in the N-lobe and Asp 395 and Tyr 528 in the C-lobe, are present but not shown. Adapted from Shongwe *et al.* (*192*), with permission.

conformationally indistinguishable from the diferric protein, in accord with the crystallographic results for lactoferrin. The binding of In^{3+} also gave the same closure of the domains over the metal ions, as did Al^{3+}, although with some difference in magnitude in the latter case. In contrast, Hf^{4+} did not give the same domain closure, apparently remaining open as for the apoprotein, even though its ionic radius is only slightly greater than that of In^{3+} (0.83 Å compared with 0.80 Å). This may not represent an absolute size cutoff, however, for the closed structure. The crystallographic results for Cu_2Lf (*26*) show there is some room for adjustment in the metal sites, and whether a particular metal is able to stabilize the closed ferric-like structure must depend on a subtle interplay between size, geometrical preferences, and binding strength. It seems likely that most of the smaller metal ions, especially transition metal ions such as Cr^{3+}, Mn^{3+}, and Co^{3+}, will favor the closed state, whereas most of the larger ions may not. The distinction is critical for the metabolic fate of transferrin-bound metals, because presumably only the closed structure is recognized by transferrin receptors (*105*).

The concept that transferrin complexes of larger metal ions may not exhibit the same closed structure as that of Fe^{3+} is not incompatible with spectroscopic studies. UV difference spectra reflect tyrosine coordination, and it is known that the "open", metal-free structure has both tyrosines close together, adjacent to the (bi)carbonate site on domain 2 (*80*), but far (8 to 9 Å) away from the remaining Asp and His ligands

on domain 1. Thus a lanthanide could bind to both Tyr and the anion, in the open configuration, and water molecules or other species could bind to the other coordination sites instead of the Asp and His ligands. This should not greatly affect the UV difference spectra, although it could perhaps explain the differences in $\Delta\varepsilon$ values. Ultimately crystallographic studies of other metal-substituted transferrins are needed to define the coordination of some of the larger metal ions.

C. Anion Binding

Ever since the pioneering studies of Schade and coworkers (*9*) and Warner and Weber (*176*) the interdependence of metal and anion binding has been recognized as a hallmark of transferrin chemistry. The relationship is synergistic in the sense that neither is bound strongly in the absence of the other; the log K value for the binding of bicarbonate to apotransferrin, measured from UV difference spectroscopy, is only 2.73 (*177*), whereas Fe^{3+} binds only very weakly and nonspecifically in the absence of a suitable anion (*101*). Together, however, they give the strong specific binding characteristic of transferrins. Anions that are able to promote such binding are referred to as synergistic anions (*178*) to distinguish them from other, secondary anions that bind more weakly, at different sites, and have a modulatory effect on the specific sites (Section IV.C.3).

1. Characteristics of Synergistic Anions

In physiological media, carbonate, derived from dissolved carbon dioxide, fills the role of the synergistic anion. (Note that the form of this anion is variously given as carbonate or bicarbonate, but that a description as carbonate is now probably preferable, for the reasons given in Section III.B.4.) Some other anions can, however, substitute under carbonate-free conditions. A milestone toward understanding their characteristics was a study of some 30 anions, both organic and inorganic, using four different synthetic routes to the Fe^{3+}–anion–transferrin complexes, and monitoring binding by the appearance of the Fe^{3+} charge transfer band (*178*). This study, by Schlabach and Bates, showed that many organic anions could substitute for carbonate, but that none of the inorganic anions, including sulfate, nitrate, and phosphate could do so. The latter observations confirmed earlier conclusions by Aisen *et al.* (*179*).

A selection of synergistic anions is shown in Fig. 22. The common features of all such anions are the presence of a carboxylate group and, one or two carbon atoms removed, a second (proximal) electron donor

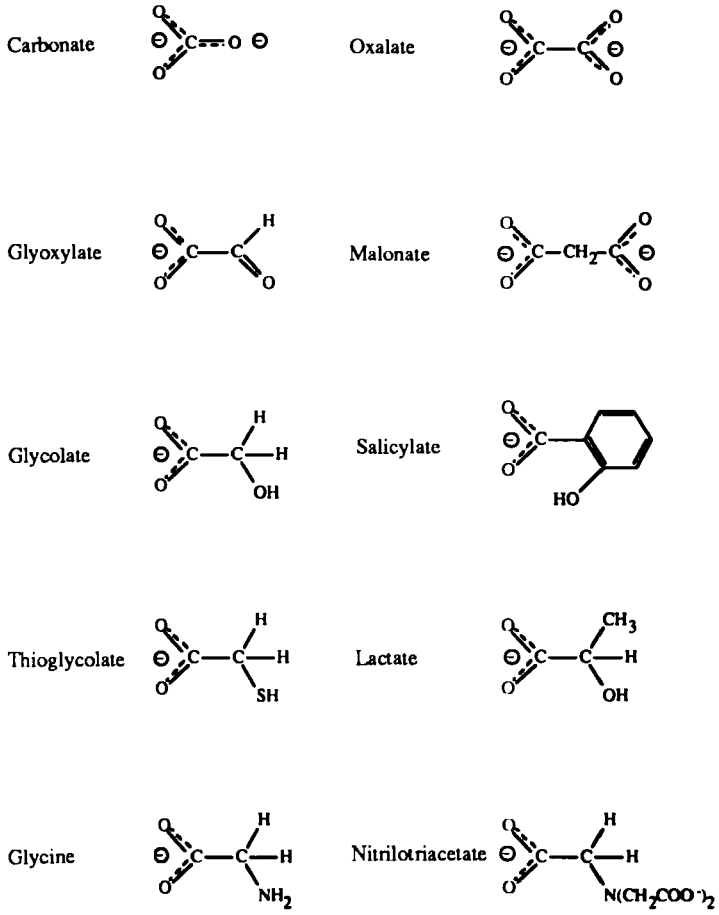


FIG. 22. A selection of synergistic anions bound by transferrins. From Schlabach and Bates (*178*).

group that has the potential to act as a metal ligand. This second group may, for example, be a second carboxylate (oxalate, malonate), hydroxyl (glycolate), thiol (thioglycolate), amino (glycine), carbonyl (glyoxylate), etc. Some surprisingly large anions, such as the dye xylenol orange (*180*), can fill this role, but there are some steric restrictions; e.g., lactate can act as a synergistic anion but methyl-lactate cannot (*178*). Much attention has been paid to citrate because it is used in chelate competition experiments but it appears it is not a synergistic anion for transferrins.

2. *Metal–Anion Interactions*

Prior to the crystallographic demonstration that carbonate binds directly to the iron atom, in bidentate mode, in diferric lactoferrin (*78*), much debate surrounded the nature of the metal–anion–protein interactions and the functional role of the synergistic anion. Experiments directed at elucidating these questions, together with the crystallographic results, are now beginning to give a much clearer picture.

Both electronic absorption spectra and EPR spectra are sensitive to the particular anion used in Fe^{3+}–anion–transferrin complexes. The characteristic charge transfer band in the visible spectrum varies considerably in intensity and λ_{max} as the proximal electron donor group on the anion is changed (Fig. 23), implying direct metal–anion bonding, and the EPR spectra of the carbonate and oxalate complexes of diferric transferrins are extremely different (Fig. 24). Many other spectroscopic studies have also suggested direct bonding of the anion to the metal, including electron-spin-echo studies of Fe^{3+}–, Cu^{2+}–, and VO^{2+}–transferrins with either carbonate or oxalate as synergistic anion (*181–184*),

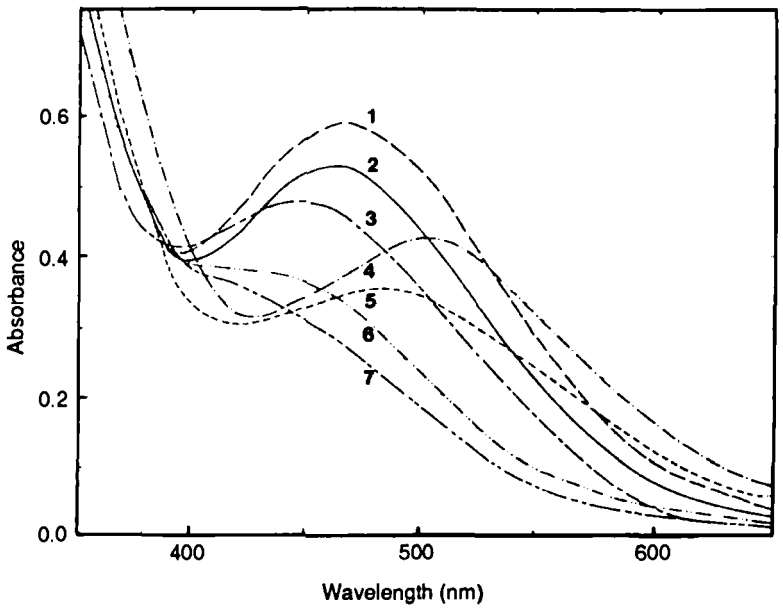


FIG. 23. Visible absorption spectra for diferric transferrin complexes utilizing various synergistic anions, showing the variation in λ_{max} for the charge transfer band. Anions are 1, nitrilotriacetate; 2, carbonate; 3, salicylate; 4, thioglycolate; 5, glycine; 6, glyoxylate; and 7, glycolate. From Schlabach and Bates (*178*), with permission.

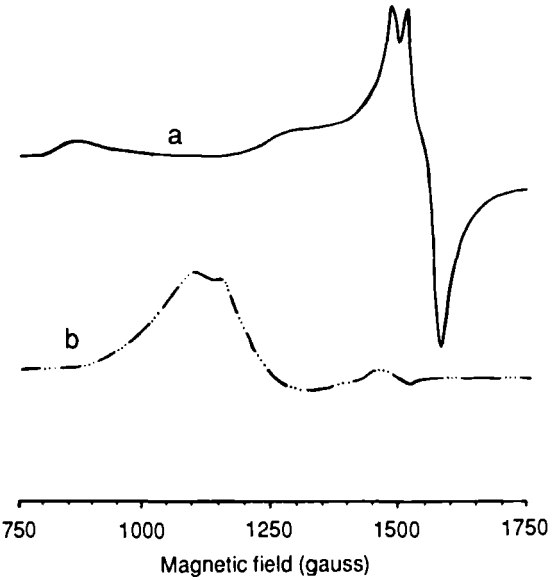


FIG. 24. EPR spectra for diferric lactoferrin with (a) carbonate and (b) oxalate as synergistic anion. Adapted from Shongwe *et al.* (*192*), with permission.

EPR studies of VO^{2+}–transferrins (*185*), ^{13}C NMR studies of Fe^{3+}–, Al^{3+}–, Ga^{3+}–, and Zn^{2+}–transferrins (*99, 186, 187*), and resonance Raman and EXAFS studies of anion binding to Fe^{3+}–ovotransferrin (*188*).

A focal point in the interpretation of anion-binding studies was the far-sighted "interlocking sites" model of Schlabach and Bates (*178*). In this model (Fig. 25) the anion was proposed to bind to the metal ion through the proximal electron donor group L and to a positively charged region of the protein through its carboxylate group. In this way it bridged between metal ion and protein. Support for the involvement of a cationic group, probably lysine or arginine, was later found from chemical modification (*189*) and NMR (*187*) studies.

The crystallographic results for lactoferrin (*75, 78*), transferrin (*68, 74*), and ovotransferrin (*77*) confirm that the carbonate ion does bridge between metal ion and protein, binding directly, in bidentate mode, to the metal and hydrogen bonding to a positively charged region of the protein. The latter comprises an arginine sidechain and a helix N-terminus (Section III.B.4). The role of the anion is then to

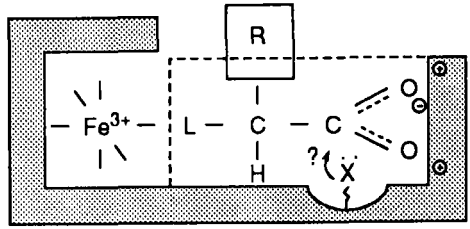


FIG. 25. A schematic representation of the "interlocking sites" model for the binding of anions to transferrins. From Schlabach and Bates (*178*), with permission.

(i) neutralize the positive charge on the protein that might otherwise inhibit the binding of a cation nearby,

(ii) partially prepare the site for metal binding by providing two potential ligands, and

(iii) occupy two coordination sites on the metal ion, leaving no space for the attack of water.

In addition, protonation of the anion may play a role in the breakup of the metal site, leading to metal ion release (Section V.B)

Extension of the carbonate binding mode to other anions does not follow automatically. Resonance Raman and EXAFS studies on the synergistic anions thioglycolate and 2,3-dihydroxybenzoate (*188*) confirmed that the proximal electron donor group L was bound to the metal, as in the interlocking sites model, but gave no indication whether the carboxylate group might also be coordinated. ^{13}C NMR spectra showed that the two carbon atoms of oxalate displayed different chemical shifts indicative of different environments; either only one carboxylate was coordinated or both were coordinated but had different protein environments (*99*). On the other hand, both modeling (*190*) and electron-spin-echo (*183, 191*) studies favored 1,2-bidentate coordination of oxalate, through both carboxylates. The latter study included a detailed analysis of 18 anions and showed convincingly that binding should occur through both carboxylate and proximal (L) groups (*191*).

Confirmation has come through crystallographic studies of oxalate binding to lactoferrin. The crystal structure of a hybrid complex of copper–lactoferrin, at 2.2-Å resolution, has carbonate in the N-terminal site and oxalate in the C-terminal site (*192*). The oxalate ion is bound to the metal ion (Cu^{2+} in this case) in 1,2-bidentate mode, as anticipated (*190, 191*), i.e., through both carboxylates (Fig. 26a). One

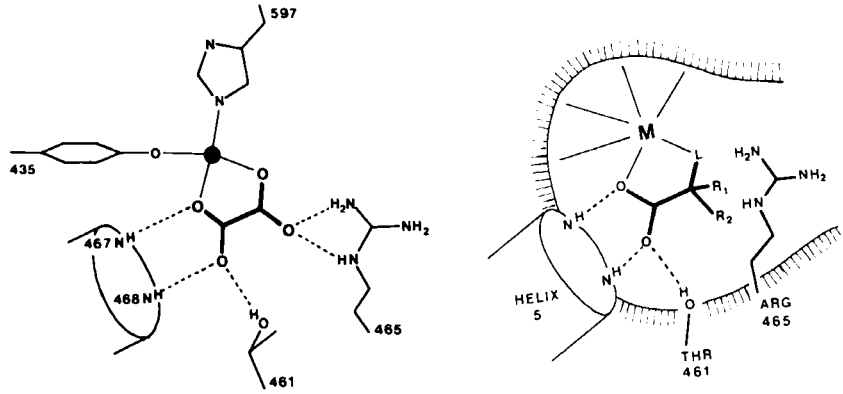


FIG. 26. Binding modes for anions other than carbonate. In (a) the mode of binding of oxalate to human lactoferrin, as determined crystallographically (*192, 193*), is shown. In b is a generalized model for synergistic anion binding to transferrins, based on EPR studies (*191*) and the crystallographic results for oxalate. From Shongwe *et al.* (*192*), with permission.

carboxylate coordinates the metal and is hydrogen bonded (like carbonate) to the helix 5 N-terminus and Thr 461; the other carboxylate also coordinates the metal and is hydrogen bonded to Arg 465. The same arrangement is found in a second crystal structure, that of diferric-dioxalato-lactoferrin at 2.3-Å resolution (*193*), this time with oxalate in both lobes and Fe^{3+} as the metal ion. The oxalate binding mode is thus independent of the metal ion. In the diferric case, however, oxalate binding in the C-lobe is symmetric, with two Fe–O bonds of 2.0 Å, but in the N-lobe it is asymmetric with bonds of 2.0 and 2.6 Å, emphasizing the slight differences between the sites, especially when "nonnative" metals or anions are bound (Section IV.D).

The question of how relatively large anions can be accommodated is answered in part by the lactoferrin structures. One oxalate carboxyl group occupies the carbonate site. To accommodate the other carboxyl group the Arg sidechain must be displaced 2–3 Å away from the metal, but this is possible because the interdomain cleft contains a large solvent-filled cavity next to the binding site and this allows room for movement (*78, 82*). There is thus some inner flexibility in the binding cleft. A very important caveat, however, is that anions have been defined as synergistic if the characteristic charge transfer spectrum is obtained when iron is bound. This monitors tyrosine binding but does not prove that the fully closed structure is adopted, because both Tyr and the anion are associated with domain 2 only. Thus it is conceivable

that some very large anions, such as xylenol orange (*180*), may not lead to the same closed protein structure as carbonate and oxalate.

Both the EPR (*191*) and crystallographic (*192, 193*) analyses lead to an elaboration of the Schlabach–Bates model, shown in Fig. 26b. The carboxylate group of synergistic anions is bound to the helix N-terminus and the Thr sidechain and can simultaneously coordinate the metal through one oxygen. The electron donor group L occupies a second coordination position (giving bidentate binding) and the Arg sidechain is pushed aside.

The failure of phosphate and sulfate to act as synergistic anions must be due to their tetrahedral structure. Both anions bind to the apoprotein, more strongly than carbonate in fact (*177*), but are presumably unable to provide the right geometry for the specific metal–anion–protein complex. Nitrate, although isostructural with carbonate, cannot act as a synergistic anion, probably because of its lesser charge (1-, compared with 2- for CO_3^{2-}), which would still leave a net positive charge at the anion site, and because of weaker hydrogen bonding ability, arising from the lesser polarity of N–O bonds compared with C–O bonds. Nitrate does not appear to bind to the apoprotein at all (*177*).

Carbonate appears to have a higher affinity than any other anion, probably because of its perfect fit between metal ion and protein, and the need for other anions to displace the arginine sidechain. The affinity is, however, metal dependent, emphasizing the synergistic relation between metal and anion binding. With Cu^{2+} as the metal ion, carbonate can be displaced from the $Cu_2(CO_3)_2Lf$ complex by an added large excess of oxalate; no displacement occurs, however, when Fe^{3+} is the metal ion, even with a very large (200-fold) excess of oxalate (*192*). Further, competition experiments, using equal concentrations of oxalate and carbonate with lactoferrin, show that under these conditions Fe^{3+} binds carbonate preferentially over oxalate in both sites, whereas Cu^{2+} prefers oxalate to carbonate in its C-terminal site, but carbonate to oxalate in its N-terminal site. The differences have been attributed to the different copper geometries supported in the two sites (*192*).

3. *Nonsynergistic Anions*

Nonsynergistic anions, bound at secondary sites, have attracted attention because of their possible role in metal ion release (Section V.B). The ability of nonsynergistic anions to perturb the EPR spectra of transferrins was first shown by Price and Gibson (*194*), in a study of the known chaotropic ion ClO_4^-. This effect was subsequently exploited by Folajtar and Chasteen (*195*), using EPR difference spectra to study

the binding of a series of such anions to human transferrin; their binding followed the trend $SCN^- > ClO_4^- > HP_2O_7^{3-} > ATP^{3-} > Cl^-$, whereas other anions ($HPO_4^{2-}$, AMP^{2-}, SO_4^{2-}, F^-, BF_4^-, HCO_3^-) had negligible effects. The order $SCN^- > ClO_4^- > Cl^-$ follows the lyotropic series for the binding of anions to positively charged sites on proteins (*196*) but the binding appears to be relatively specific, rather than simply chaotropic, at least for a given transferrin. Two Cl^- ions are bound to each lobe of human transferrin, giving four in all. Moreover they are bound in pairwise fashion, with strong positive cooperativity.

Two possible sites for the binding of nonsynergistic anions to transferrins may be identified from the crystal structures (Fig. 27), although there could be others given the large number of positively charged residues (lysine and arginine) on such large proteins. The first is the "essential" arginine at the synergistic anion site of each lobe. Binding to the other side of this arginine could perturb the interactions it makes with the synergistic anion and thus indirectly perturb the metal site. The second may involve the positively charged side chains behind the iron site, near the hinge region. In the N-lobes of serum transferrin and ovotransferrin, a pair of lysines are hydrogen bonded (*77, 81*), and disruption of this unusual arrangement by anion binding could perturb the iron site (*81*). If this is the secondary anion site, however, it must be different between different transferrins and between N- and C-lobes, because of the sequence differences that exist (Section III.C).

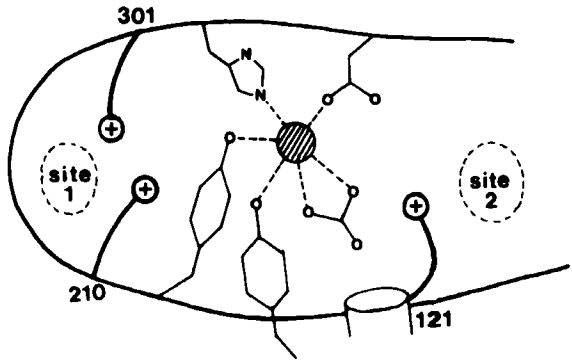


FIG. 27. Possible sites for the binding of secondary, nonsynergistic anions, which may perturb the iron site and modulate release. Residue 121 is the "essential" arginine. Residues 210 and 301 are located behind the iron site, near the Tyr ligands and the hinge region. Numbering is as for the N-lobe of lactoferrin. The identities of these residues, which vary in the two lobes and between different transferrins, can be seen in Table III.

An effect that is almost certainly related is that of salts on the relative stabilities of the two metal binding sites and on the kinetics of metal ion release. Increasing concentrations of salts, such as NaF, NaCl, NaBr, NaI, $NaNO_3$, Na_2SO_4, and $NaClO_4$, increase the stability of the N-terminal site relative to the C-terminal site of human transferrin (*197*). In the presence of an accepting chelator, salts also accelerate iron release from the C-site (*197, 198*), thus reversing the normal order (N-site faster than C-site). This is discussed further in Section V.B. Caution should be exercised, however, in extrapolating results from one transferrin to another, however, because sequence differences are likely to alter these weak, secondary binding sites.

4. Conformational Differences Associated with Anion Binding

Spectroscopic studies have consistently demonstrated the existence of multiple conformational states for the metal sites in transferrins, especially when using metal ions other than Fe^{3+} and anions other than CO_3^{2-}. The differences are not necessarily related to intrinsic geometrical differences between the two sites in each molecule, but also reflect changes dependent on pH, the nature of the synergistic anion, or salt effects.

For Co^{2+}-substituted ovotransferrin, for example, not only are the N- and C-sites distinguishable by CD spectroscopy, when oxalate is the anion, but ^1H NMR spectra reveal the existence of conformers (*139*). For VO^{2+}-substituted transferrin, the EPR spectra were examined using 16 different anions (*185*), and the resultant spectra could be grouped into two classes, A and B, which were anion dependent. Anions with one carboxylate and a nonionized electron donor group L gave only class B spectra, whereas dicarboxylate anions gave both class A and B spectra. For the latter anions, transition between class A and class B spectra was associated with ionization of a protein group of pK ~10.0. A pH-dependent change is also seen in the EPR spectrum of Cu^{2+}-substituted ovotransferrin, with carbonate as the associated anion, this time associated with ionization of a group of pK ~9.5 (*157*). EPR spectra of monoferric transferrins have also shown that each site (N or C) exhibits two types of spectrum and that the equilibrium between the two is affected by added NaCl (*191*); this equilibrium is presumably the cause of the salt-induced EPR perturbations noted by Folajtar and Chasteen (*195*).

Some tentative conclusions about the nature of these conformational differences may be drawn from the crystallographic studies of Cu^{2+} and oxalate-substituted lactoferrins (*26, 192, 193*). Anions which gave class A spectra with VO^{2+}-substituted transferrins are those that can

interact with the "essential" arginine as in Fig. 26, i.e., dicarboxylate anions. It may therefore be the ionization of this arginine that determines the conformer, perhaps by determining the degree of asymmetry in the bidentate anion binding. In the Cu^{2+} complexes, the geometry in the N-lobe of copper–lactoferrin suggests that either the anion or Tyr 92 is protonated (Fig. 21), and it may be the loss of this proton at higher pH that causes the change to a bidentate carbonate, as seen in the C-lobe, and gives the EPR change seen for Cu^{2+}-ovotransferrin (*157*).

In general, given that symmetric bidentate, asymmetric bidentate, and even monodentate anion configurations are seen in the various lactoferrin structures, it seems likely that it is changes of this nature that are detected spectroscopically.

D. Differences between the Two Sites

Proteins of the transferrin family share a common evolutionary history, which has resulted in the presence of two homologous halves to each molecule (Sections III.A and III.B.1). Except for the two outliers, melanotransferrin and *M. sexta* transferrin, each also has two metal binding sites. This raises a number of questions, which have been the subject of much debate over the years (*1, 3, 16, 17*). Why are there two sites? How similar are they, and do the differences between them have any functional or physiological significance? Is there any cooperativity between them?

1. Structural Comparison

The four metal-binding amino acid residues (2 Tyr, 1 Asp, 1 His) are present in both N- and C-sites of all transferrins so far sequenced, apart from melanotransferrin and the insect proteins (Table III). The same is true of the anion-binding Arg and Thr residues, and the residues at the N-terminus of the anion-binding helix are also strongly conserved. Superposition of the 81 common atoms of these residues, plus metal and anion, shows that their *rms* deviation in the N- and C-sites of diferric human lactoferrin is only 0.3 Å. This close structural similarity is reflected in their spectroscopic properties. Where these have been compared, with the "physiological" Fe^{3+} and CO_3^{2-} ions bound, they are so similar as to be virtually identical (*107, 56, 199*). Nevertheless, there are a number of factors that can potentially lead to inequivalence in properties:

(i) Outside the immediate binding site there are sequence differences between the two lobes, e.g., in the basic residues behind the metal site,

in the residues that line the binding cleft beyond the "essential" arginine (Fig. 7), and in the pattern of disulfides.

(ii) The "front-to-back" packing of the two lobes (Fig. 3) means that the two binding clefts have different environments with respect to the molecule as a whole (the N-terminal cleft is more exposed and accessible).

(iii) The fact that each binding site is created by closure of two proteins domains over metal and anion and that there is considerable "empty space" in the interdomain cleft (i.e., filled only by solvent) gives potential for 3D structural differences, especially when different metal ions and anions are bound.

2. Differences in Properties

Where chemical or physical differences can be detected between the two sites, there remains the problem of distinguishing which site is which. For serum transferrin this is helped immensely by the ability to prepare monoferric forms, loaded in either the N- or C-site (*198, 200*), and to be able to separate them by electrophoresis in 6 *M* urea, the Makey–Seal method (*201*). This enabled the so-called A and B sites, differentiated in earlier studies, to be identified with the C- and N-terminal sites, respectively (*202*). Comparisons of the diferric proteins with N- and C-loaded monoferric transferrins or (more recently) recombinant half-molecules have by now revealed a number of inequivalences.

Both kinetic and thermodynamic effects differentiate the two lobes of transferrins. Aisen *et al.* (*107*) have shown that C-terminal site of transferrin binds iron more strongly than the N-terminal site, with their effective binding constants differing by a factor of about 20. The C-terminal site also appears to be the more strongly binding site for other metal ions, for example, in lanthanide binding (*149, 150*). Iron release also differs, with the rate of iron release being faster for the N-terminal site (*108*). These two effects, tighter binding and slower release from the C-lobe, may be linked to its lesser flexibility (*85*), as seen in thermodynamic measurements (*108*) and inferred from the "one open/one-closed" apolactoferrin structure (*80, 82*) (see Section III.B.5). The reduced flexibility of the C-lobe may arise from the presence of an extra disulfide bridge [483–677, lactoferrin numbering, or number 7 in the nomenclature of Williams (*87*)]. This disulfide, which has no equivalent in the N-lobe of any transferrin, adds an extra constraint between the two domains of the C-lobe (Fig. 10). Predictions (*80*) that it would inhibit opening of the C-lobe have been born out by the lesser opening of this lobe seen in the fully open apolactoferrin structure (*109*).

The two sites also differ in their pH stability towards iron release. Experiments on serum transferrin showed that one site loses iron at a pH near 6.0, and the other at a pH nearer 5.0 (*203, 204*), giving a distinctly biphasic pH-induced release profile (Fig. 28). The acid-stable A site was later shown to be the C-terminal site (*202*). It is this differential response to pH, together with kinetic effects (below), that enables N-terminal and C-terminal monoferric transferrins to be prepared (*200*). Although the N-terminal site is more labile, both kinetically and to acid, the reasons are not necessarily the same; the acid stability may depend on the protonation of specific residues (Section V.B) and is likely to differ somewhat from one transferrin to another in response to sequence changes. The biphasic acid-induced release of iron seen for transferrin is not shared by lactoferrin. Although biphasic release from lactoferrin, in the presence at EDTA, has been reported (*205*), under most conditions both sites release iron essentially together at a pH(2.5–4.0) several units lower than that for transferrin (Fig. 28).

The two sites (in transferrin, at least) also show differences in iron loading behaviour. *In vitro,* when Fe^{3+} is added as a chelate complex, there are differences in which site is preferentially loaded, depending on the nature of the chelate ligand; these differences are apparently kinetically determined and differ from one transferrin to another (*17*).

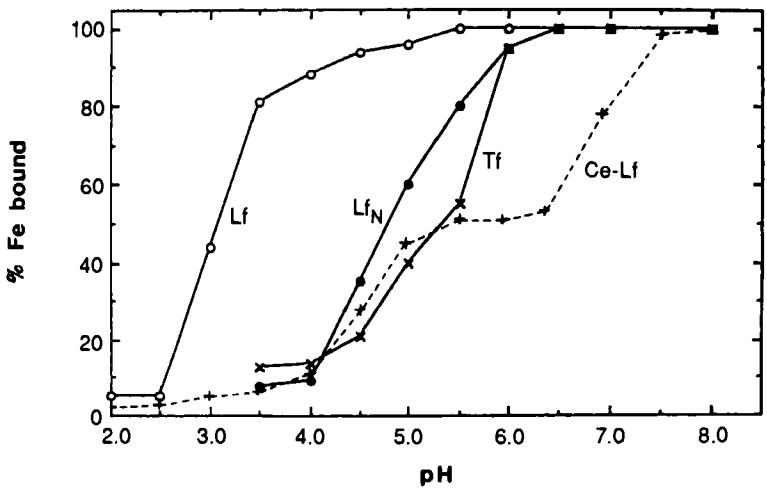


FIG. 28. The pH dependence of iron release from human serum transferrin (Tf), human lactoferrin (Lf), and the recombinant N-terminal half-molecule of human lactoferrin (Lf_N). Also shown is a plot (dashed line) for the release of cerium from Ce^{4+}-substituted lactoferrin, demonstrating the increased difference between the two sites for metal ions other than Fe^{3+}.

In vivo, a study of fresh serum showed that the N-site of human transferrin was more highly occupied, the average distribution in 22 samples being 39% apo-Tf, 23% Fe_N-Tf, 11% Fe_C-Tf, and 27% Fe_2-Tf (*206*).

3. *Metal and Anion Substitution*

Differences between the two sites become more pronounced for metal ions other than Fe^{3+} and anions other than CO_3^{2-}. The differences are most pronounced for larger metal ions such as lanthanides. For transferrin some of the larger lanthanides appear to bind in only one of the two sites (Section IV.B.3), and for lactoferrin, although binding occurs in both sites, the second metal ion binds much more weakly, as shown by the curvature of the UV difference titration graph (Fig. 18); the biphasic release of Ce^{4+} from lactoferrin contrasts with that of Fe^{3+} (Fig. 28). Even metal ions of the first transition series, of similar size to Fe^{3+}, enhance the differences between the two sites. When Cr^{3+} is bound to either transferrin (*134*) or lactoferrin (*154*), different EPR signals are seen for the two sites, and one Cr^{3+} ion is much more readily displaced by Fe^{3+} than the other. Likewise, the EPR spectra of VO^{2+}-substituted transferrin indicate different metal configurations in the two sites (*207*), as do ^{13}C NMR studies of Co^{2+}-substituted ovotransferrin (*139*). In these cases one metal ion is also released much more readily than the other as the pH is lowered.

The crystal structure of copper–lactoferrin (*26*) shows the kind of differences that may occur. In one site the coordination geometry is six-coordinate, distorted octahedral, whereas in the other it is five-coordinate, square pyramidal. One could suggest that the sites are optimized for the binding of Fe^{3+} and CO_3^{2-} and that there is an element of misfitting when a different metal ion, with different size, stereochemical requirements, or both, is bound. The sites can adjust, with small movements, but these are different in the two sites.

Distinct differences are also seen when anions other than CO_3^{2-} are used. The crystal structure of oxalate-substituted diferric lactoferrin shows differences in the anion binding in the two sites; in the C-site the oxalate is symmetric bidentate, whereas in the N-site it is asymmetric (*193*). When Cu^{2+} is the metal ion the oxalate binding differences become even more pronounced. Copper–transferrin binds oxalate only in its N-terminal site (*91*). Copper–lactoferrin and copper–ovotransferrin each bind two oxalate ions but binding occurs preferentially in the C-lobe (*157, 192*). These different affinities mean that hybrid complexes can be prepared with oxalate in one site and carbonate in the other (*92, 157, 192*). The use of oxalate as synergistic anion gives rise to spectroscopically distinct sites for other metal ions also (*171*).

The X-ray structural studies on lactoferrin show that it is not simply a question of how much room there is for a larger anion or cation in a given site. The N-terminal site in lactoferrin apparently has more room than the C-terminal site, yet it is the C-terminal site that is preferentially occupied by oxalate (*192*); perhaps the explanation is that the more favorable square pyramidal copper geometry in the N-site (with monodentate anion) makes it less amenable to substitution of oxalate. In general, the two sites have enough flexibility that the precise structure adopted depends on the particular metal and anion, emphasizing the synergistic relationship between the two and making the result of a given substitution rather hard to predict.

4. Functional Aspects

The small but significant difference in iron release from the two sites of transferrin led Fletcher and Huehns (*208*) to suggest that they might have different biological functions. It was suggested that one site might be involved in iron transport and release and the other, more as a storage site (for antibacterial or iron defense purposes). Although the idea has remained controversial (e.g., see discussion in Ref. *3*), several recent observations have led to renewed interest. The apolactoferrin structure (*80*) suggested a distinct difference in flexibility between the two lobes, which should also apply to transferrin. Studies of transferrin–receptor interactions (*209*) have also shown that the receptor specifically acts on the C-lobe, prying it open to release the iron, whereas the N-lobe loses iron by the long-proposed acid-mediated release (*20*). This neatly explains the observation that circulating transferrin has more iron in its N-lobe, whereas the less facile C-lobe release would have been expected to lead to a buildup in the C-site.

These observations come together with the evolutionary comparisons, which show that the N-lobe is highly conserved, through all species, whereas the C-lobe has become diversified. In serum transferrins, ovotransferrins, and lactoferrins it releases iron less readily than the N-lobe, because of its lesser flexibility, whereas in melanotransferrin and hornworm transferrin it no longer binds iron at all. Perhaps C-site binding has only remained where a receptor mechanism exists to extract iron from this site?

Finally the question of whether there is any cooperativity between the sites remains to be addressed. Although evidence of cooperativity from solution studies has mostly been equivocal (*3*), there are certainly structural interactions between the two lobes, involving helices from each (*78, 85*), and studies of a half-molecule fragment of lactoferrin have shown that separating the N-lobe from the C-lobe gives it altered

properties of iron release (*49*). Thermodynamic studies have shown that binding at one site is signaled to the other, presumably through changes in interlobe interactions (*210*) and it seems likely that the binding properties of each lobe are modified by the presence of the other.

V. Mechanisms of Binding and Release

The most striking feature of transferrin chemistry is that iron is bound with extraordinary avidity, yet it can be released without any denaturation and the protein can be recycled through many cycles of uptake and release. The mechanisms by which this is done are of fundamental importance to understanding biological transport processes.

A. Uptake of Iron

1. Mechanism of Binding

In vivo uptake of iron by transferrins usually involves its addition as a ferric–chelate complex, to prevent hydrolytic attack on the ferric ion (*211*). Complexes such as ferric citrate and ferric nitrilotriacetate are commonly used. Under these conditions, kinetic schemes for the uptake of iron by transferrins have identified five steps in the formation of the specific metal–anion–transferrin ternary complex (*120*). These may be summarized as follows.

1. Binding of the (bi)carbonate anion to apotransferrin.
2. Detachment of one or more ligands from the added metal chelate.
3. Formation of a quaternary transferrin–anion–metal–chelate complex.
4. Loss of the chelate ligands(s).
5. Conformational change to the final specific transferrin complex.

Evidence that the anion binds first comes from kinetic data (*119*) and from spectroscopic results, in which both ^{1}H NMR (*118*) and UV difference (*177*) spectra indicate that the anion binds to the apoprotein. Strong support comes from the 3D structural data; the positive charge at the anion site should deter metal binding until it is neutralized by a suitable anion (*78, 85*). Suggestions that nitrilotriacetate transiently occupies the anion site when iron is added as a Fe^{3+}–NTA complex (*212*) may imply that the early steps can vary depending on the form of the added iron, but the key point probably remains that the anion site must be occupied as a first step. Spectroscopic evidence for the

quaternary complex envisaged in Step 3 has been obtained from studies using ferric-acetohydroxamate in iron uptake experiments (*120*).

The above mechanism is totally consistent with the crystallographic results from the various forms of lactoferrin and transferrin (Section III.B). These lead to a structural model of binding shown pictorially in Fig. 29. In the first step the synergistic anion (usually carbonate) is bound in the specific site on domain 2 of each lobe. Binding may be preceded by electrostatic attraction from the exposed helix N-termini and several basic sidechains in the open interdomain cleft.

With the anion bound, four of the six iron ligands are in place on

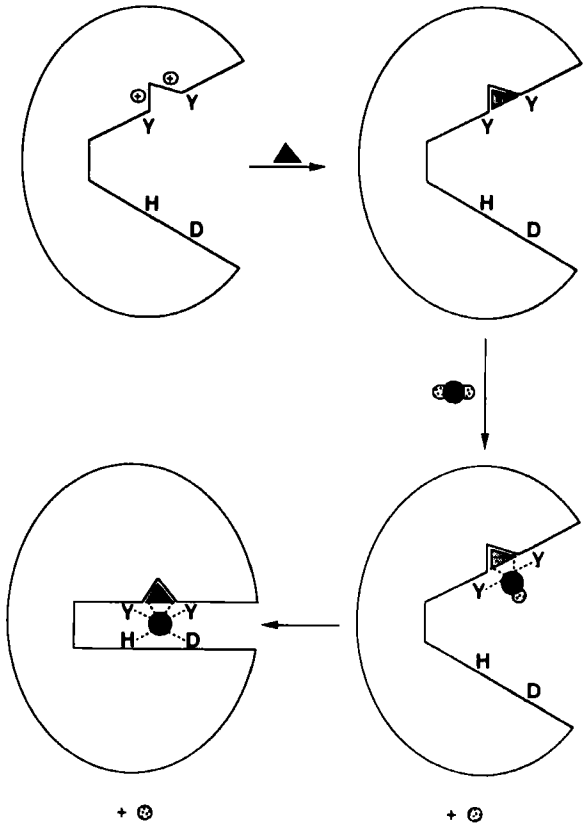


FIG. 29. A structural model of the steps involved in the *in vitro* uptake of iron by transferrins, shown for one lobe. (●) Iron; (▲) carbonate; Y, Tyr ligands; H, His ligand; D, Asp ligand; (○) chelate ligands. The positive charge at the anion site is due to the helix N-terminus and the Arg side chain. (Note that this is for the case in which Fe^{3+} is added as a chelate complex.)

domain 2 (two carbonate oxygens and two Tyr sidechains); the metal then binds to these groups, possibly with some of its chelating ligands still attached, to give the quaternary complex. A model for such an intermediate is provided by the 18-kDa domain 2 fragment of duck ovotransferrin, whose crystal structure has been determined by Lindley *et al.* (*76*). In this structure the iron atom is bound to the bidentate carbonate ion and both Tyr residues, with the remaining two coordination sites occupied by a non-protein ligand, possibly a glycine molecule (Fig. 14).

The final step in binding involves the closure of the two domains over the metal ion. Any remaining chelate groups are expelled as the metal ion binds to the Asp and His ligands to complete its coordination. The closed configuration is locked together by the Asp ligand, which plays a critical role in the metal-bound structure (*78*). Not only does the Asp carboxylate bind to the metal ion, but also it is involved in a strong hydrogen bonding interaction between the two domains. Its nonligated carboxylate oxygen atom receives a hydrogen bond from the NH group of residue 122 (466 in the C-lobe) in domain 2 as well as from the NH of residue 62 (397) in domain 1. The strength of these interactions is probably enhanced by the fact that both NH groups are at positively charged helix N-termini, helix 3 and helix 5 (Fig. 9a), and their importance is emphasized by the fact that when the Asp ligand is mutated to Ser which has only a single hydroxyl group, no stable closed structure appears to be formed (*106*).

2. *Importance of Dynamics*

Protein dynamics clearly plays a crucial role in metal binding and release. With respect to metal binding, the open structure described in Section III.B.5 should not be taken to imply that in the absence of a bound metal ion the cleft is always open. In fact the closed but metal-free C-lobe seen for one form of apolactoferrin (*80*) suggests that in the absence of a metal ion very little energy separates the open and closed states, and in fact there may be a dynamic equilibrium between them (*82*); at the very least the closed configuration may now and again be sampled. Similar conclusions have been drawn for bacterial binding proteins for which closed but ligand-free structures have also been observed (*213*).

The importance of this model is that in the intermediate in which the metal ion is bound to domain 2 (step 3 in Fig. 29) it would be some 8 to 9 Å away from the Asp and His ligands on domain 1, as judged by the extent of opening of the apolactoferrin (*80*) and C-terminal monoferric transferrin (*110*) structures. How then does it "find" these

two ligands to complete its coordination? Only if the dynamics of an equilibrium allows it to explore the closed structure, at which time the ligands will be able to lock on to the metal. Such dynamics are common in proteins, especially where the movement of domains (*214*) or flexible "lids" or "flaps" (*215*) are concerned.

Finally, the Asp ligand has been described as a "trigger" associated with domain closure (*106*). However, at a distance of 9 Å from a metal ion bound to domain 2 it is hard to see how it could induce closure. Rather, it should be seen as a lock that holds the closed structure in place once the protein dynamics have brought the domains close together.

B. Release of Iron

Iron release can be stimulated by a number of factors that can operate individually or together. *In vitro,* these include reduction of Fe^{3+} to the much more weakly bound Fe^{2+}, the use of competitive chelators, and the acid liability of the two sites, which results in iron release at low pH. *In vivo,* receptor interactions are of fundamental importance. Added to these are the modulatory effects of ionic strength (*216*) and nonsynergistic anions such as Cl^- and ClO_4^- (*198*).

1. Kinetics

Most kinetic studies of iron release have focused on pathways involving the use of chelate ligands such as EDTA (*217*), pyrophosphate (*218–220*), phosphonates (*220, 221*), catecholates (*108, 216*), hydroxamates (*120*), and nitrilotriacetate (*221*). In many cases, simple saturation kinetics are observed, and interpreted in terms of the formation of a quaternary complex, ligand–Fe–transferrin–CO_3^{2-} (*120, 122*). The failure to observe this complex spectroscopically [in contrast to iron uptake studies (*120*)] has been explained in terms of a rate-limiting conformational change, giving a basic three-step mechanism, which is essentially the reverse of that given for iron uptake (Section V.A.1).

1. A rate-limiting conformational change of the diferric protein from closed to open configuration.
2. Rapid attack of the chelator to give a quaternary complex.
3. Rapid decay of the quaternary complex to products.

The kinetics are, however, considerably more complex. Both pH and salt affect the two sites differently (Section IV.D.2), so that iron release kinetics are very different at pH 6 compared with those at pH 7.4, for example. The kinetics are also dependent on the particular chelator

used; this is not surprising because most of the ligands used are anionic, many of them able to bind quite strongly to transferrins even in the absence of iron (*177*).

There is now accumulating evidence of more than one pathway for release. Harris and coworkers (*221, 223, 224*) identified two parallel pathways, operative at either site, one being the saturation pathway envisaged above, the other being first order with respect to chelate concentration. Their relative importance can depend both on the nature of the chelator and on concentrations. Using pyrophosphate, iron removal from the N-site follows only the saturation pathway, whereas for the C-site both pathways operate (*223*); using nitrilotris(methylenephosphonate) (NTP) the saturation pathway is favored, but using nitrilotriacetate (NTA) the first-order pathway operates at both sites (*221*). The first-order pathway may be associated with interactions involving the chelate anion; it could either substitute for carbonate in the specific site (*223*) or bind to some other allosteric effector site, such as that occupied by Cl^- or ClO_4^- (*219*).

Much attention has been paid to the significance of salts (*217*) and nonsynergistic anions (*195, 198*) in promoting or modulating iron release, especially given the important observation that iron release extrapolates to zero as the ionic strength of the medium nears zero (*216*). A variety of anions accelerate the first-order pathway (*220*), with ClO_4^- being the most active species; this has been attributed to the presence of cationic groups near the metal binding site, which form a specific effector or allosteric anion-binding site (*219, 220*). The complex kinetics may result from competition between chelate anions and effector anions for such sites. Egan *et al.* (*225*) have further analyzed the release of iron from C-terminal monoferric transferrin to pyrophosphate in terms of the existence of a kinetically significant anion binding (KISAB) site. Here it is envisaged that two pathways operate, with either pyrophosphate or an added anion occupying this site. Comparisons of release from free and receptor-complexed transferrin also show that the release-promoting effects of the receptor and of the anion (in this case Cl^-) are independent of each other (*226*).

Many studies have noted weak cooperativity between the sites during iron release (*3*). One recent analysis used mixed-metal transferrins, with kinetically inert Co^{3+} in one site and Fe^{3+} in the other (*221, 224*). With pyrophosphate, release of iron from the C-site was accelerated by the presence of a metal in the N-site, but no corresponding effect was seen for iron release from the N-site. The cooperative effects were also weaker and somewhat different for different chelators (*221*).

Finally it is important to realize that most studies of iron release

have focused on human serum transferrin. Many of the finer details may be dependent on the interactions of chelators, salts, etc., with residues that are within the binding cleft but outside the immediate iron site; these residues tend to vary from one species to another and from one transferrin to another, and it is likely that kinetic details will also.

2. *Structural Aspects of Iron Release*

The conformational change from closed to open configuration is a key feature of models for iron release. The nature of this change can now be inferred from crystallographic and solution studies (Section III.B.5). What is less clear is how the conformational change is triggered. *In vivo,* receptor binding is clearly involved, but pH and salt effects can also play a part. *In vitro,* reduction of the pH is in itself sufficient. In attempting to understand iron release in structural terms one must look for potential protonation and anion binding sites and seek more knowledge of the interactions made with transferrin receptors.

The effect of pH differs for the two sites of transferrin and differs between transferrin and lactoferrin. When titrated with acid, in the absence of chelators, serum transferrin loses iron over the pH range 6.0 to 4.0; release is biphasic (Fig. 28), with iron lost from the more acid-labile N-lobe site first (Section IV.D.1). Lactoferrin, on the other hand, is distinctly more stable in acid, with release occurring 2 pH units lower, over the pH range 4.0 to 2.5, and the two sites losing iron essentially together.

Several explanations for the effect of pH on iron removal have been put forward. Protonation of the carbonate ion could cause repulsion between it and the anion-binding Arg residue (121 in the N-lobe, 465 in the C-lobe), or promote a change from bidentate to monodentate coordination, as seen in the N-lobe of copper–lactoferrin (*26*). Either effect could then be the first step in the breakup of the Fe^{3+}–transferrin complex. An alternative site where protonation could stimulate iron release is at the back of the iron site, in the hinge region. It is here that distinct differences between lactoferrin and transferrin, involving ionizable residues, are found (Fig. 30). In the N-lobes of both rabbit transferrin (*81*) and chicken ovotransferrin (*77*), a pair of lysine residues (206 and 296, transferrin numbering) are in hydrogen bonded contact, implying that one is in its neutral form; protonation of this lysine would break this interdomain interaction and could destabilize the closed structure. The pair of lysines has been referred to as a "dilysine trigger" (*77*). In the C-lobe of transferrin a different combination of charged residues is found (a salt bridge Lys $\cdots$ Asp $\cdots$ Arg), which

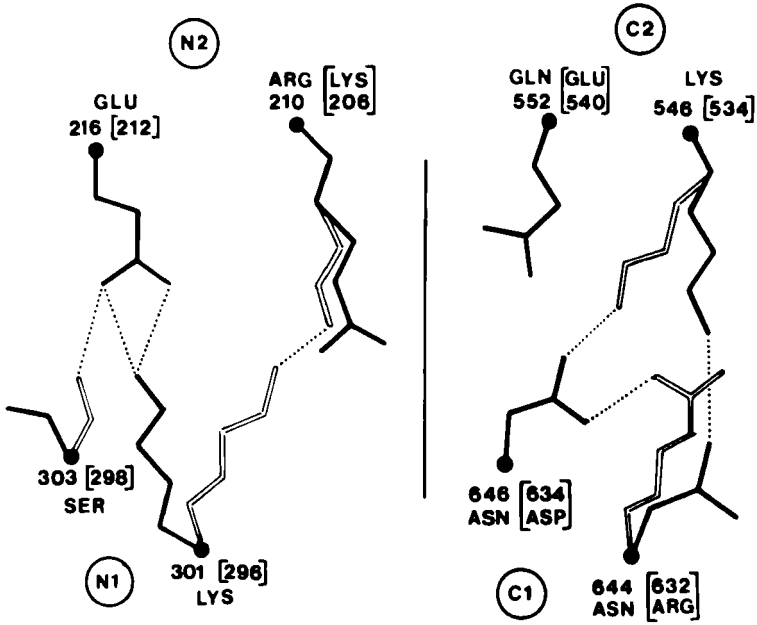


FIG. 30. Residues at the back of the iron site, near the hinge region, that may be implicated in the stimulation or modulation of iron release. The interactions present in human lactoferrin and rabbit transferrin are compared. Where the conformations are different, lactoferrin residues are shown with solid bonds, transferrin, with open bonds. Where the residues differ in identity or number, those for transferrin are in parentheses.

would be less easily protonated. In the N-lobe of human lactoferrin the interactions are different again; one Lys is changed to Arg (Table III) and a conformational difference leads to a Glu · · · Lys ion pair in place of the Lys · · · Lys pair. This could certainly account for the greater acid stability of the lactoferrin N-lobe site. (The situation may not be so simple, however, because the sequences of both bovine and porcine lactoferrins have both lysines.) Crystallographic and mutagenesis studies will be required to disentangle these effects.

Studies of half-molecule fragments also suggest that the region at the back of the iron site, near the hinge in the interdomain connecting strands, could be the site where protonation stimulates release. The recombinant N-terminal half-molecule of human lactoferrin releases iron over the pH range 6.0 to 4.0 (Fig. 28), approximately 2 pH units higher than that of intact lactoferrin, but very similar to transferrin (*49, 205*). The crystal structure shows that this decreased acid stability is associated with the loss of stabilizing contacts normally made by the C-lobe, leading to unwinding of a helix at the back of the iron site and

increased solvent exposure of both this region and the hinge (*75*). A proteolytic fragment of lactoferrin that lacks even more of the structure in this region is correspondingly more acid labile (*227*).

Potential anion-binding sites that could stimulate iron release have been discussed in Section IV.C.3 and are shown in Fig. 27. Binding to sites near the hinge, such as the Lys · · · Lys pair in the N-lobe of serum transferrin, has the potential either to promote domain opening, by disrupting interdomain interactions or the nearby hinge, or to perturb the iron site via the iron ligands; the Lys · · · Lys pair in transferrin and Arg 210 in lactoferrin are hydrogen bonded to the Tyr ligands (75, 77, 78). Binding to the "essential" Arg residue could disrupt the metal–synergistic anion interaction, leading to a change to monodentate coordination or complete displacement of the synergistic anion.

Defined structural pathways for iron release may exist. If some chelate anions bind to cationic groups on the protein first, as suggested (*221*), the metal could be passed to this site en route to the outside. If binding was to the "essential" arginine, which also helps hold the synergistic anion, the metal might just transfer from one to the other. An intriguing observation concerns interactions with cyanide. The iron in transferrin is usually high-spin Fe^{3+}, but it can be converted to low spin in the presence of CN^- (*228*); flash-freezing then traps an intermediate in which CN^- is exchanged for some of the normal transferrin ligands in the C-lobe. X-ray absorption studies of this intermediate indicate that it involves at least two Tyr ligands, but that at least one of them may be different from those normally ligated (*81*). This could implicate a Tyr-mediated pathway.

Studies of the transferrin receptor indicate that it acts preferentially on the less flexible C-lobe, to stimulate release from this site (*209*), and that the receptor and anion-binding effects are independent (*226*). The interdomain strands that contain the C-lobe hinge are highly exposed to the external environment and are rich in charged residues; they could be involved in binding the receptor, simple anions (such as Cl^-), or both. The problem at present is that so many potential sites exist. What is required is detailed knowledge of transferrin–receptor interactions, either through the crystallographic studies that have already been initiated (*229*) or by mutagenesis.

VI. Recombinant DNA Studies

The past six years have been an explosion of new results from X-ray crystallographic studies, which have added a new dimension to our

understanding of transferrin chemistry. The next few years should increasingly see another powerful approach, that of recombinant DNA studies, brought to bear. These techniques allow the production of mutant transferrins, in which single, selected amino acids are changed. Alternatively, chimeric transferrins in which part of one molecule is substituted into another can be constructed. Mutagenesis can be carried out either on the whole transferrin molecules or on half-molecules, because half-molecules of defined length can be made.

The first successful expression of any transferrin was reported in 1990 when Funk *et al.* (*48*) isolated the cDNA for human serum transferrin and introduced a stop signal following the codon for Asp 337; the resulting DNA construct thus coded for the N-terminal half-molecule. This recombinant human transferrin N-terminal half-molecule, hTf/2N, was expressed in a cell culture system incorporating baby hamster kidney cells, after unsuccessful attempts at expression in *Escherichia coli*. Similar methods have also been used to obtain expression of full-length human serum transferrin (*230*) and both the N-terminal half-molecule (*49*) and the whole molecule (*231*) of human lactoferrin. The use of an animal cell culture expression system has advantages in ensuring correct folding of the recombinant proteins and allowing glycosylation to occur, but is more laborious and gives lower levels of expression than are usually possible with *E. coli*. All the same, expression levels of 20–40 mg per liter of culture medium have been reported (*48, 49*), ample quantities for characterization. (The recombinant proteins are secreted into the culture medium.) A recent report of the expression of human serum transferrin in *E. coli* (*232*) suggests that higher levels may be attainable although little characterization of the protein obtained has yet been carried out.

The recombinant whole molecules are both expressed in glycosylated form, although the glycosylation patterns differ from the proteins isolated from natural sources. The recombinant human transferrin binds to receptors both in its glycosylated form and as a nonglycosylated mutant, showing that the carbohydrate is not required for receptor binding (*230*). Recombinant human lactoferrin shows identical spectroscopic properties and shows an identical profile of pH-dependent iron release when compared with human milk lactoferrin (*231*).

The half-molecules differ somewhat, although this is not unexpected, because the properties of the whole molecules are an amalgam of those of their two slightly different sites. Moreover, the lactoferrin half-molecule shows that its iron release properties are changed as a result of the loss of interactions from the other lobe (Ref. *49*; see also Section V.B.2 and Fig. 28). The visible λ_{max} for the transferrin half-molecule

is increased from 465 nm (native transferrin) to 473 nm (*233*), whereas that for the lactoferrin half-molecule is reduced from 465 to 454 nm. The reasons are not clear, but presumably have to do with the fine detail of the two metal sites, at a level that probably even high resolution X-ray analyses may not explain.

A number of site-specific mutants have already been prepared, both of transferrin (*233*) and of lactoferrin (*234*). In all cases the mutations have been made in transferrin or lactoferrin *half-molecules* and the targets have been amino acids in and around the metal binding sites. Characterization has been limited so far to their visible spectra and some measures of iron binding properties; for lactoferrin the pH dependence of iron release has been determined (*234*), whereas for transferrin the strength of iron binding has been inferred from the migration of the mutant proteins on urea gels (*233*). In neither case have binding constants yet been determined.

Already some intriguing observations have resulted, however, giving a glimpse of what may be to come with fuller characterization. Most mutations alter the value of λ_{max} (Table VIII). This is true even of residues outside the immediate binding site, such as Lys 206 and His 207 in transferrin (*233*), showing that even changes some distance away can perturb the metal site. Lys 206 is one of the two lysines that are hydrogen bonded together behind the iron site and that are potential sites for protonation and salt effects (Section V.B.2). Interestingly, mutations of Lys 206 to Gln and of His 207 to Glu, both mutations that reduce the positive charge, appear to increase the strength of iron binding.

Mutation of the Asp ligand to Ser, as in the C-lobe of melanotransfer-

TABLE VIII

PROPERTIES OF RECOMBINANT TRANSFERRINS[a]

Transferrins[b]	λ_{max}	Iron binding	Lactoferrins	λ_{max}	Iron release (pH)
N-lobe (Tf_N)	473	Strong	N-lobe (Lf_N)	454	5.5–4.0
Asp 63 Ser–Tf_N	420	Weak	Asp 60 Ser–Lf_N	434	7.0–5.0
Asp 63 Cys–Tf_N	440	Weak	Arg 121 Ser–Lf_N	454	5.5–4.0
Gly 65 Arg–Tf_N	468	Weak	Arg 121 Asp–Lf_N	472	>7.0
Lys 206 Gln–Tf_N	460	Strong	Asp 60 Ser, Arg 121 Ser } Lf_N	472	>7.0
His 207 Glu–Tf_N	484	Strong			

[a] Notation for mutants: Asp 63 Ser means that Asp 63 is mutated to Ser.

[b] Asp 63 in transferrin corresponds to Asp 60 in lactoferrin, and similarly Gly 65, to Gly 62; Arg 124, to Arg 121; Lys 206, to Arg 210; and His 207, to Glu 211.

rin, does not abolish iron binding, but reduces λ_{max} considerably (the protein is yellow) and weakens binding considerably. The transferrin mutant (Asp 63 Ser) loses iron on urea gels, traveling as the apoprotein (*233*), and low-angle X-ray solution scattering studies (*106*) suggest that it does not form the usual closed structure when iron is bound. The lactoferrin mutant (Asp 60 Ser) loses iron below pH 7, considerably more readily than the wild-type protein. Mutations of Asp 63 to Cys in transferrin, and of the nearby Gly 65 to Arg, have similar effects, again possibly inhibiting domain closure.

Mutation of the anion-binding Arg 121 to Ser in lactoferrin does not alter λ_{max} or significantly perturb the pH dependence of iron release, implying that the Arg residue is not essential for anion binding and arguing against a repulsion between it and a protonated anion as an important factor in iron release. Intriguingly, the double mutation Asp 60 to Ser and Arg 121 to Ser in the lactoferrin half-molecule (matching mutations in the C-lobe of melanotransferrin) weakens but does not abolish iron binding.

VII. Concluding Remarks

Historically, our understanding of transferrin chemistry has depended to a large extent on spectroscopic and other physicochemical approaches. Crystallographic studies over the past few years have added a new structural dimension. The iron ligands have been established definitively and the open and closed forms of the protein have been defined. Other groups in proximity to the metal and anion sites, with possible modulatory roles, can also be identified. It is thus an opportune time to reexamine the complementary role of spectroscopy. For example, UV difference spectra principally monitor the binding of the Tyr ligands to a metal ion, and the same is true, to a first approximation, of the visible charge transfer spectra. Because the Tyr ligands are associated with only one domain (domain 2), these techniques indicate only binding to this domain and do not show whether an open or a closed structure is adopted for a particular metal ion (or anion). Binding to the ligands of the other domain may be indicated by techniques sensitive to the His ligand, e.g., NMR. Thus combinations of approaches, including techniques such as low-angle solution scattering, and the ultimate power of X-ray crystallography can be used to address important questions. Does binding a particular metal ion or anion result in an open or closed structure? If so, what are the implications for the transport of such species?

A second major challenge and opportunity for bioinorganic chemists is to use the power offered by recombinant DNA technology. To be able to compare spectroscopic and other results from mutants in which single amino acids have been changed gives a unique possibility to really understand such complex systems. This will also require complementary X-ray structure analyses in order to disentangle the effects of structure and chemistry. (Interpretations of mutagenesis experiments are seldom straightforward.)

At the functional and physiological levels, perhaps the greatest need is a better understanding of transferrin–receptor interactions. Mutagenesis experiments again will contribute to this, but the crystal structure of a transferrin–receptor complex would be the ultimate prize. Although this chapter has concentrated on structure, as well as metal and anion binding properties, in the end it is because of their physiological roles, actual or possible, that we study transferrins and find such fascination in their chemistry.

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