Transferrin as a Metal Ion Mediator

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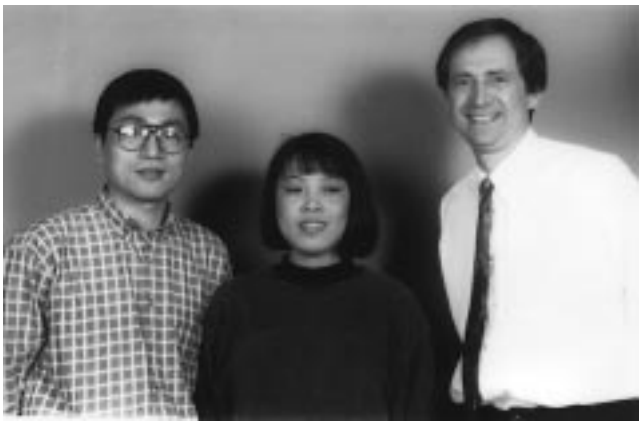

Hongzhe Sun (left) was born in Luoyang, the ancient capital of China, and received his B.Sc. and M.Sc. degrees form the Chinese Academy of Sciences in 1985 and 1990. He then became a research associate and lecturer at Nanjing University in China. In 1996 he obtained his Ph.D. from the University of London, working on the biological chemistry of bismuth drugs with Peter Sadler. After 2 years at the University of Edinburgh as a GlaxoWellcome Research Fellow, he took a position as an Assistant Professor in the Department of Chemistry at the University of Hong Kong in 1998. His current research interests are in the areas of biological inorganic chemistry and NMR spectroscopy.

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Peter Sadler (right) was born in Norwich, U.K., and obtained his B.A., M.A., and D.Phil. degrees in Chemistry at the University of Oxford from 1965 to 1971. His doctorate research on bioinorganic chemistry was under the direction of Professors Allen Hill and Bob Williams. After 2 years as a Medical Research Council Research Fellow at the University of Cambridge and National Institute for Medical Research, London, he joined the Department of Chemistry at Birkbeck College, University of London, where he was successively Lecturer, Reader in Biological Inorganic Chemistry, and Professor of Chemistry. In October 1996 he took up the Crum Brown Chair of Chemistry at the University of Edinburgh, where his research is centered on the design and mechanism of action of metal-based therapeutic agents. In 1993 he was the recipient of the Royal Society of Chemistry Award for Inorganic Biochemistry, and in 1999 he was elected to the Fellowship of the Royal Society of Edinburgh. He was Chairman of Action D8, The Chemistry of Metals in Medicine, of the European Community Coorporation in Science and Technology (COST) programme from 1996 to 1998 and has published over 270 papers.

I. Introduction

How can we try to ensure that a metal-based therapeutic or diagnostic agent reaches its target site? One way is to incorporate features which are recognized specifically by the target. We can seek to use natural recognition mechanisms. In the case of cells, the target may be an external membrane receptor, usually a protein, which has a high affinity for the metal complex. This is an attractive proposi-

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Table 1. Properties of the Transferrin Family

	serum transferrin (hTF)	ovotransferrin	lactoferrin (hLF)	melanotransferrin (p97)	ferric ion binding protein (FBP)
M_r (Da)	79 570	77 770	82 400	97 000	34 000
residues	679	686	703	719	309
lobes	2	2	2	2	1
glycans	2C	1C	1N, 1C	1N, 1C(?)	
disulfides	8N, 11C	6N, 9C	6N, 10C	7N, 7C	none (no Cys)
pI (differic)	5.6	5.8	8.7		ca. 9.4
source	serum	egg white	milk, tears, saliva, mucus	melanomas	gram-negative bacteria
ref	10,11	12,13	14	6	8,9

tion because natural recognition is likely to stimulate a course of events which includes transport through cell membranes, internalization, metal release, DNA communication, and other processes. This approach can form the basis of therapy if, for example, the metal site in the complex is tailored to affect the metal-release properties or the natural metal is replaced by an unnatural one. Therefore, knowledge of receptor-mediated metal uptake systems is likely to be very powerful for use in strategies for the design of pharmaceuticals.

A variety of specific metal uptake and transport pathways are now becoming understood at the molecular level. In the bacteria *Escherichia coli*, there is a system of membrane proteins (Fhu B, C, and D) which recognizes and transports iron hydroxamate siderophores, as well as heme and vitamin B_{12}.[1] *E. coli* also possesses FepA receptors which have a high affinity for ferric enterobactin.[2] In yeast, copper uptake is mediated by genes that play key roles in copper transport, which include Fre1- and Fre2-encoded Cu^{2+}/Fe^{3+} cell surface reductases and CTR1- and CTR3-encoded membrane-associated copper transport proteins. Copper can control gene expression at picomolar levels.[3] A final example is molybdenum, for which *E. coli* possesses a high-affinity periplasmic binding protein for molybdate (and tungstate).[4] Some of these systems may well have analogues in mammalian cells, although the best understood at present is the transferrin-based iron transport system.

The serum transferrin system involves the specific recognition of Fe^{3+} (and not Fe^{2+}) along with an obligatory synergistic anion (usually carbonate). Only when transferrin is loaded with two Fe^{3+} ions does it bind strongly to its receptor, whereupon it is internalized by cells, the iron is released, and the protein is recirculated. There is potential for use in diagnosis and therapy because transferrin can bind strongly to a range of other metal ions apart from Fe^{3+}, and many heterometal−transferrin complexes are still recognized by the transferrin receptor. Moreover, metal uptake and release can be controlled both thermodynamically and kinetically. Transferrin mediation has significance wider than just for cells that communicate with blood serum because there are related proteins such as lactoferrin and ovotransferrin in other biological compartments. Moreover, not only do some virulent bacteria have transferrin receptors, but they also possess a transferrin-like molecule of their own.

In this article, we review the structures of the transferrin family of proteins, their anion- and metal binding properties, the mechanisms for metal release, receptor recognition, and their role in diagnostic and therapeutic approaches. It is clear that the chemistry of transferrin is important for understanding the role of metals in health, disease, therapy, and diagnosis.

II. Transferrin Family of Proteins

The transferrins are a class of iron binding proteins (Table 1) believed to originate with the evolutionary emergence of vertebrates or prevertebrates. It is typified by serum transferrin, the iron transport protein in blood. Serum transferrins appear to be present in all vertebrates, in crabs, and insects.[5] The concentration of human serum transferrin (hTF) is ca. 2.5 mg/mL (35 μM). The second member of the family, lactoferrin (LF), is widely present in a variety of secretory fluids such as milk, tears, bile, pancreatic juice, mucosal fluid, and white blood cells. Lactoferrin has a much higher isoelectric point (pI 8.7) than serum transferrin (pI 5.6) or ovotransferrin (pI 5.8) (Table 1). The third member of the family, ovotransferrin (OTF), is mainly present in egg white and the fourth, melanotransferrin (MTF), is a membrane-bound protein present at very low levels on the surface of normal cells.[6] This protein, originally called p97 from its apparent molecular mass (97 kDa), binds only one iron per molecule. Finally, the ferric ion binding protein (FBP, ca. 34 kDa) present in several Gram-negative bacteria.[7−9] is also considered as a member of the transferrin superfamily, although it is structurally homologous to only a single lobe of transferrin, binds to only one iron per molecule, and appears to have developed from a different origin.

The primary structures of more than 10 transferrins have been determined, and the sequences are available from various protein databases.[10−14] Levels of sequence identity between these proteins are very high, for example, 78% identity between rabbit and human serum transferrin. A similar level of identity (60−70%) for lactoferrins is also notable. There is even about a 60% sequence identity between serum transferrin and lactoferrin and ca. 40% identity between MTF and other transferrins. Of key significance to understanding the structure and function of transferrin is the 2-fold internal primary structure repeat. In each protein, the N-terminal lobe of the protein is homologous to its C-lobe, the level of identity being ca. 40% for human transferrin and ca. 30% in insect transferrin. This homology is also reflected in the three-dimensional structures of the proteins, and the iron binding sites (see below). Despite the structural similarities between the ferric ion binding proteins, most periplasmic transport

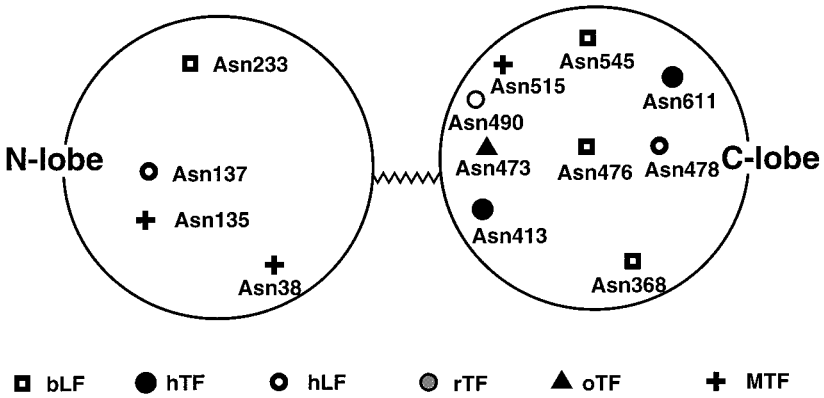


Figure 1. Distribution of glycan chains in various transferrins. All of them are attached to asparagine residues.

proteins (including *Haemophilus influenzae* FBP) share less than 20% sequence identity with one another and share less than 10% identity with the transferrins.

Transferrins are single-chain glycoproteins containing ca. 700 amino acids with molecular masses of approximately 80 kDa (human serum transferrin (hTF), 679 amino acids 79 750 Da; chicken ovotransferrin (oTF), 686 amino acids, 78 000 Da). The functions of transferrins include iron transport (in blood) and antimicrobial activity (ovotransferrin and lactoferrin). Recognition of transferrin by cells is via receptor-mediated endocytosis. Only diferric transferrin (two iron sites occupied) and not the apo form binds strongly to the receptor protein on the surface of the cells and is taken up. Inside the cell, diferric transferrin is held in membrane-bound vesicles (endosomes) where the pH is lowered from the extracellular value of 7.4 to about 5.5 and Fe^{3+} is released. Apo-transferrin remains bound to its receptor due to its high affinity for the receptor at acidic pH and is recycled back to the surface of the cell. At extracellular physiological pH, apo-transferrin dissociates from its receptor due to its low affinity at pH 7.4, released into the circulation, and reutilized (see below).[12,13,15,16]

The half-life of transferrin in human circulation is 7.6 days, and the lifetime of transferrin-bound Fe is 1.7 h.[17] During its lifetime, a molecule of transferrin undergoes ca. 100 cycles of iron binding. A rabbit reticulocyte exhibits steady-state binding of about 124 000 transferrin molecules and takes up 90 000 Fe atoms per minute, and the transfer of Fe from protein to cell takes less than 3 min.[17]

All transferrins characterized so far are glycosylated, except those from fishes.[15] All of the identified glycan chains are attached to asparagine (Asn) residues of the proteins, and they appear almost randomly distributed over the protein surface (Figure 1). These glycans are specific for each transferrin and for a given transferrin are specific to the species. The role of the carbohydrate residues is still unclear. They appear to play no direct role in transferrin binding to cell receptors.[19] Deglycosylation of hTF (including the use of recombinant hTF, which is not glycosylated) has no effect on iron binding, recognition by reticulocytes or HeLa cells, or on iron transport into cells compared to isolated hTF.[19,20] However, recent studies have suggested that glycans may play a role in maintaining the protein in a biologically active conformation. For example, the glycans in rabbit serum transferrin form a bridge between the two lobes of the protein. In diferric bovine lactoferrin, the glycan chain attached to Asn545 is sandwiched between two domains of the C-lobe near the back of the iron site and may restrict domain movements or modulate iron release from the C-lobe. In hTF and hLF, the glycan chains are in external positions and may provide recognition signals.[21]

III. Structures

A. Protein Conformation

The three-dimensional structures of monoferric human serum transferrin (Fe_C−hTF with Fe^{3+} in the C-lobe),[22] recombinant apo- and monoferric-N-lobe human transferrin,[23,24] apo- and diferric- human lactoferrin (hLF),[25−29] diferric bovine lactoferrin,[30] diferric hen ovotransferrin (hen oTF),[31] apo- and diferric duck ovotransferrin,[32,33] and diferric rabbit serum transferrin (rTF)[34] have been determined by X-ray crystallography (Table 2). The X-ray crystal structures show that the polypeptide folding is very similar for all proteins of the transferrin family. This is attributed to their high (∼40%) sequence identity. The polypeptide chain is first folded into two globular lobes, representing the N-terminal (first ca. 330 residues) and C-terminal (last ca. 330 residues) halves of the molecule, referred to as the N-lobe and C-lobe, respectively. Thus, transferrins are bilobal, and the two structurally similar lobes (N-lobe and C-lobe) each of 40 kDa are joined by a short peptide chain (Figure 2), which is a random coil in human serum transferrin but a three-turn helix in lactoferrin. Each lobe is further divided into two domains of similar size which have alternating α-helical and β-sheet segments (lactoferrin N-lobe: domain I residues 1−90 and 252−333, domain II residues 91−251), a common feature for binding proteins of the "Venus fly-trap" family.[35] These domains have a crucial functional significance since the cleft separating the two domains of each lobe houses the metal binding site. Each domain consists of a mixed β-sheet overlaid with α-helices. This folding pattern is particularly important since the N-terminii of many helices are directed toward the central binding cleft and the partial positive charges they carry may help

Table 2. X-ray Crystal Structures of Transferrins

transferrin	residues	metal binding ligands[a]	geometry	resolution (Å)	pH	comments	refs
Fe_c−hTF	679	C-lobe: Y426, Y517, H585, D392, and bidentate carbonate	distorted octahedral	2.6	5.75	N-lobe open (apo), C-lobe closed (Fe^{3+})	22
Fe−hTF/2N	337 (N-lobe)	Y95, Y188, H249, D63, bidentate carbonate	distorted octahedral	1.6	5.75	Arg124 and carbonate disorder	24
Fe−hTF/2N	337 (N-lobe)	Y95, Y188, H249, D63, and bidentate carbonate	distorted octahedral	1.8	6.1	cleft closed to same extent as in the N-lobe of human lactoferrin	24
Apo−hTF/2N	337 (N-lobe)			2.2, 3.2	5.3	two crystal forms	23
Fe_2-rTF	676	N-lobe: Y95, Y188, H249, D63, bidentate carbonate C-lobe: Y426, Y517, H585, D392, bidentate carbonate	distorted octahedral	3.3	6.0	carbohydrate moiety is not defined in the structure	34
Fe_2−hLF	692	N-lobe: Y92, Y192, H253, D60, bidentate carbonate C-lobe: Y435, Y528, H597, D395, bidentate carbonate	octahedral	2.2	7.8	carbohydrate partially defined	27−29
Apo−hLF	692			2.8	8.2	N-lobe open and C-lobe closed	25
Apo−hLF	692			2.0	7.8	N-lobe open and C-lobe closed Cl^- bound to both lobes	26
Cu_2-ox−hLF	692	N-lobe: Y92, Y192, H253, D60, and monodentate oxalate C-lobe: Y435, Y528, H597, D395 and bidentate oxalate	square pyramidal C-lobe: octahedral	2.0	7.8	oxalate as anion	45,46
Fe−hLF/2N	333	Y92, Y192, H253, D60, bidentate carbonate	octahedral	2.0	8.0		41
D60S Fe−hLF/2N	333	Y92, Y192, H253, bidentate carbonate, H_2O	octahedral	2.05	8.0	mutant, cleft is still closed	57
Fe_2-bLF	689	N-lobe: D60, Y92, Y192, H253, bidentate carbonate	octahedral	2.8	7.7	three carbohydrate chains in the C-lobe (Asn368, Asn476 and Asn545) were clearly defined	30
Fe_2-oTF (chicken)	686	N-lobe: D60, Y92, Y191, H250, bidentate carbonate C-lobe: D395, Y431, Y524, H592, bidentate carbonate	distorted oictahedral	2.4	5.9	carbohydrate chain (attached to Asn473) is not defined dilysine trigger: Lys−Lys (2.9 Å) in the N-lobe and Lys638−Gln541 (3.0 Å) in the C-lobe	31
Fe_2-oTF (duck)	686	N-lobe: D60, Y92, Y191, H250, bidentate carbonate C-lobe: D395, Y431, Y524, H592, bidentate carbonate	distorted octahedral	2.35	5.8	both lobes in the closed form carbohydrate chains not defined dilysine trigger found only in the N-lobe (Lys209−Lys301, 2.62 Å)	33
Apo-oTF (duck)	686			4		both lobes in the open form distance between NZ atoms of Lys209−Lys301 increased to 8.14 Å	32
Fe-oTF/2N	332	D60, Y92, Y191, H250, bidentate carbonate	octahedral	2.3	5.9	no attached carbohydrate pH-sensitive dilysine trigger between Lys209 and Lys301 (side-chain NZ atoms 2.3 Å apart)	55
Fe-oTF/4N2	249	Y95, Y188, bidentate carbonate, and possible glycine or two H_2O	octahedral	2.3	7.8		147
FBP[a]	309	H9, E57, Y195, Y196, H_2O, monodentate phosphate	octahedral	1.6	6.6	solvent-exposed Fe^{3+} site phosphate site similar to that in phosphate binding protein	5

[a] Numbering of residues: Y92 (435) in hLF is equivalent to Y95 (426) in hTF or oTF.

to attract anions into the binding cleft. Two extended β-strands run behind the iron site and link the two domains and can be thought of as backbone strands crucial to the conformational change during binding of metal ions.

B. Metal Binding Sites

Before the crystal structure of transferrin was solved, electron paramagnetic resonance (EPR) spectroscopy was widely used to study Fe^{3+}, Cu^{2+}, Cr^{3+},

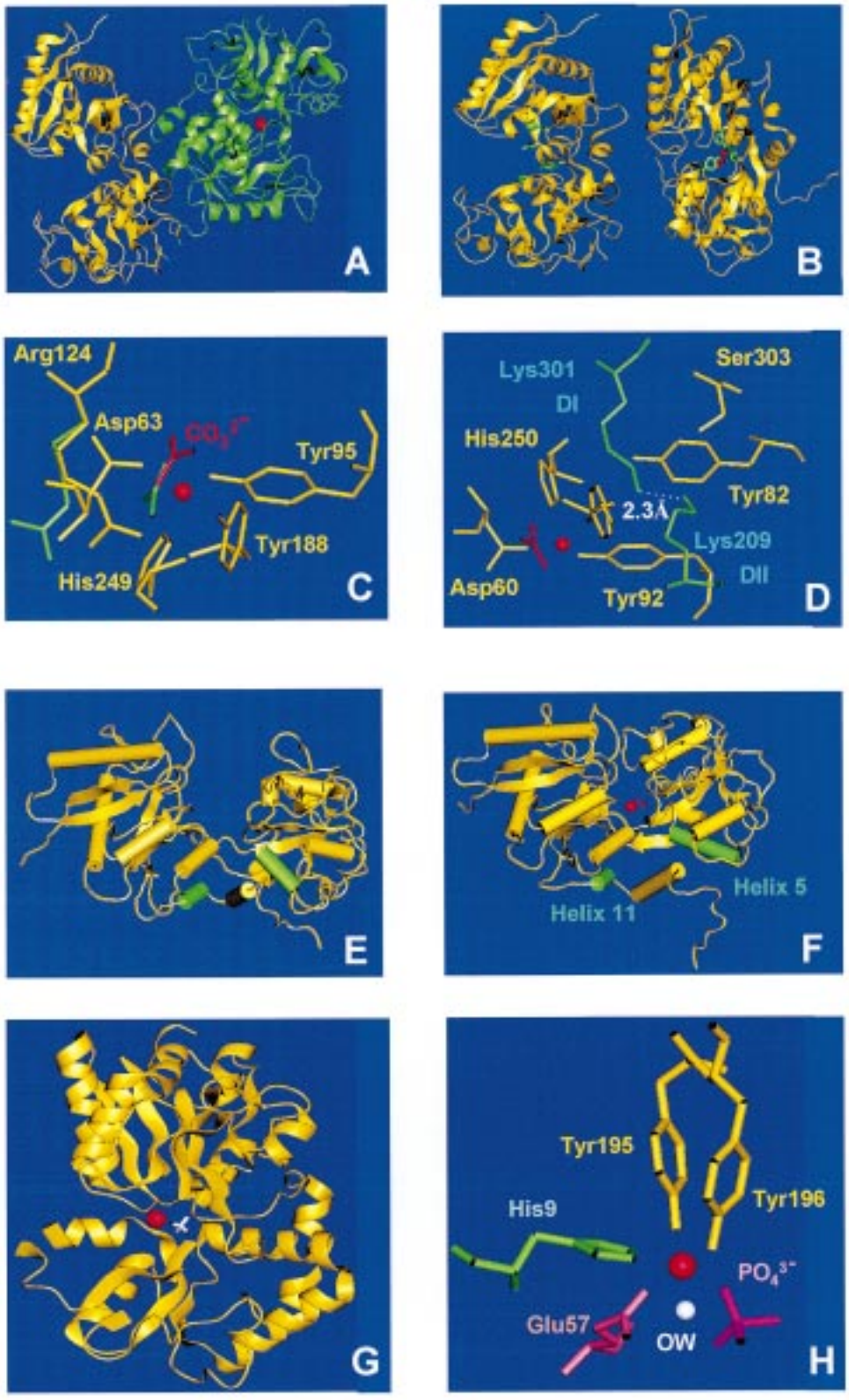


Figure 2. (A) X-ray crystal structure of human Fe_C-transferrin.[22] The C-lobe, which contains bound Fe^{3+}, is shown in a closed form (green) and the apo N-lobe in an open form (yellow). (B) X-ray crystal structures of recombinant apo (open) and holo Fe−hTF/2N (closed) N-lobe of transferrin. The side-chains of the binding amino acids are shown (green) (Adapted from refs 23 and 24). (C) Fe^{3+} site in human Fe−hTF/2N showing the two positions of Arg 124 and bound carbonate (Adapted from refs 23 and 24). (D) Dilysine trigger. Part of the iron-saturated N-lobe of ovotransferrin (Fe-oTF/2N) showing the proposed[55] H-bonding between the ϵ-amino groups of Lys209 and Lys301 (one protonated and one deprotonated). Protonation of this pair may act as a trigger for lobe opening and metal release (adapted from ref 55). (E and F) Conformational changes of the recombinant N-lobe of human transferrin induced by Fe^{3+}. Note the movement of helix 5 (domain NII) which appears to pivot about helix 11 (Adapted from refs 23 and 24). (G) X-ray crystal structure of the ferric ion binding protein (FBP) from *Haemophilus influenzae* (adapted from ref 9). (H) Fe^{3+} site of FBP (adapted from ref 9).

and VO^{2+} transferrin complexes. In the case of Cu^{2+} and VO^{2+} it is possible to distinguish between the N- and C-lobe sites on the basis of the *g* values and hyperfine coupling constants, but for the other ions spectra can be difficult to interpret fully.[36]

Extended X-ray absorption fine structure (EXAFS) spectroscopy can provide relatively accurate information on the metal environment and sometimes be used to study differences in the metal binding sites.[37−40] Average metal−ligand bond lengths and coordination numbers can be determined, but O cannot be distinguished from N as a ligand and the two metal sites in M_2−transferrin complexes cannot be distinguished.

Each lobe of transferrin contains a distorted octahedral Fe^{3+} binding site consisting of two Tyr, one His, one Asp, and one bidentate carbonate anion (the so-called "synergistic anion"). These two binding sites are remarkably similar, and the metal−ligand bond lengths are all about 1.9−2.2 Å, as judged from X-ray crystallography. The structures of complexes with Fe^{3+} bound to the isolated N-lobe of transferrin (half molecule) and NII domain (quarter molecule) have also been solved recently.[41] The structures of the N-lobe show that the overall protein structure and the metal and anion binding sites are preserved in the half molecule. Figure 2C shows the Fe^{3+} binding site of the recombinant N-lobe of human serum transferrin.[23,24] The ligands are from four different parts of the protein structure, one from domain 1 (Asp63 in human transferrin), one from domain 2 (Tyr188), and two from the two polypeptide strands (Tyr95 and His 249) that cross over between the two domains at the back of the iron site.[22] Thus, the domains can move apart to a more open conformation, hinged by the backbone strands. This allows release of Fe^{3+} from the site. The Asp ligand appears to play a crucial role in the metal site since it coordinates to the metal through one carboxylate oxygen while forming hydrogen bonds with the two domains of the N-lobe via its other oxygen. The metal binding site of bacterial FBP is remarkably similar to those of the two lobes of transferrins, although the evolution of FBP is different. FBP binds only one Fe^{3+}/molecule and is approximately the size of one lobe of transferrin. Iron is octahedrally coordinated to two oxygens from two Tyr residues, one nitrogen from an imidazole ring of His, one oxygen from Glu instead of Asp, a monodentate phosphate anion, and oxygen from a water molecule (Figure 2H). Carbonate can also serve as the synergistic anion.[8]

C. Anion Binding Sites

An intriguing aspect of the chemistry of transferrin is that Fe^{3+} cannot bind strongly without concomitant binding of a synergistic anion. The anion carbonate occupies a pocket in domain 2. The pocket is formed by positively charged groups: the side chain of an arginine residue (Arg121 in hLF, Arg124 in hTF) and the N-terminus of helix 5. The synergistic anion plays an important role in creating the metal binding site. Without this anion, the positively charged Arg side chain and the N-terminus of helix 5 may inhibit metal binding to the specific site. In the absence of the synergistic anion, metal binding is weak (except for VO^{2+}), and this presumably accounts for the weak, nonspecific metal binding. The synergistic anion may also play a role in iron release. Protonation of the carbonate ion could disrupt the hydrogen bonding pattern, allowing breakup of the binding site, which is essential to ensure reversibility of metal binding (see below). The common features of synergistic anions are the presence of a carboxylate donor and, one or two carbon atoms away, a second (proximal) electron donor group that can act as a potential ligand for metal binding.[80] Carbonate serves as the synergistic anion in vivo and has a higher affinity than most other anions, but oxalate is also efficient in promoting metal ion binding.[43] Both carbonate and oxalate bind to Fe^{3+} in a bidentate mode.[25−34,43]

The X-ray crystal structures of several transferrins with different bound metal ions and different anions have been solved[43−47] (Table 2). The polypeptide folding and domain closure for Fe^{3+} lactoferrin are similar with either carbonate or oxalate as the synergistic anion. Each lobe contains an approximately octahedral Fe^{3+} binding site. Oxalate binds to Fe^{3+} in a symmetric 1,2-bidentate mode (Fe−O distances 2.07 and 1.91 Å) in the C-lobe, whereas the coordination of this anion to iron is remarkably asymmetric (2.55 and 1.87 Å) in the N-lobe. The crystal structure of Cu^{2+}-substituted lactoferrin shows that although the overall structure of the protein is unchanged compared with diferric lactoferrin, the metal sites are subtly different. In the N-lobe Cu^{2+} ion is five-coordinate and approximately square pyramidal, with a long apical bond of 2.8 Å to Tyr92 and only a monodentate carbonate ion. In the C-lobe, however, Cu^{2+} is six-coordinate but more distorted from regular octahedral geometry. Carbonate can be displaced from the $Cu_2(CO_3)_2$LF ternary complex by addition of a large excess of oxalate, but no such displacement occurs for $Fe_2(CO_3)_2$LF.[46] In Cu_2(oxalate)-LF, Cu^{2+} is also five-coordinate in the N-lobe bound to a monodentate oxalate (2.0 Å). Multinuclear NMR can also be used to monitor carbonate or oxalate binding to the metal ion. The most clear evidence is the coupling $^2J(^{205}Tl-^{13}C)$ between ^{13}C from the anion and ^{205}Tl (see section IV).

D. Metal-Induced Conformational Changes

Transferrin has long been known to undergo conformational changes during Fe^{3+} uptake and release (Figure 2B). The conformational changes are likely to be of functional importance and to play a crucial role in receptor recognition. X-ray crystallographic studies of Fe_NFe_C−lactoferrin (iron in both sites) and Fe_C−lactoferrin (iron in C-lobe site only) have shown that binding of Fe^{3+} and carbonate causes the N-lobe to change its conformation from wide-open to closed.[25,29,47] This involves a 54° rotation of the NII domain relative to the NI domain and flexing of the two antiparallel extended polypeptide strands which run behind the iron binding site connecting domains NI and NII. Figures 2E and 2F show the open and closed forms of the N-lobe of recombinant N-lobe of transferrin. The most remarkable change is to helix 5 in domain NII, which appears to pivot about helix

11. The structures of duck apo and holo-oTF show that Fe^{3+} also induces C-lobe conformational changes from wide-open to closed.[32,33] The position of a possible equilibrium between the open and closed forms of the C-lobe for the apo- structure may be influenced by the crystallization conditions and by crystal packing forces. Indeed, both C-lobe open and closed forms of human lactoferrin have been crystallized.[47] Domain closure in transferrin is probably at least a two-step process, and "intermediate conformations" may represent stages of domain closure.[48] Small-angle X-ray scattering shows that loading of transferrin with Fe^{3+}, Cu^{2+}, or In^{3+} causes a decrease in the radius of gyration, consistent with lobe closure.[49−51] The isolated N- and C-lobes undergo structural changes in solution on iron binding and release, similar to those of the corresponding lobes of intact transferrin, despite the fact that the C-lobe of the apo-protein can open only to the equivalent of about 75% of the N-lobe due to the presence of an extra disulfide bridge.[50]

Isotopic labeling of transferrin can provide assignments of resonances for specific amino acid residues of transferrin, which enhances the usefulness of NMR spectroscopy in exploring conformational changes in the protein. By means of 2D [^{1}H,^{13}C] HMQC−NOESY and molecular modeling, the S−$^{13}CH_3$ resonances of all the five Met residues of the N-lobe and nine Met residues of intact transferrin have been tentatively assigned.[52,53] The advantage of labeling the ϵ-CH_3 group of Met is that each ^{1}H resonance is a singlet, and moreover, the Met residues are well spread throughout the protein and occupy several environments. Some of the Met resonances (e.g., Met464 and Met109) are sensitive to metal binding even though these residues are far away from the metal binding site (>10 Å). Similar changes in ^{1}H/^{13}C shifts of Met resonances are observed for Fe^{3+}, Al^{3+}, and Bi^{3+}, indicating that they induce similar conformational changes.[54] This provides a useful technique for investigating protein conformational changes induced by metals under biologically relevant conditions.

The mechanism for opening and closing the lobes of transferrin may involve a pH-sensitive interdomain interaction. Endocytosis into acidic endosomes (pH ca. 5.5) may result in the protonation of one or both of the residues Lys209 and Lys301, the so-called dilysine "trigger" (Figure 2D), based on the X-ray structure of the N-lobe of hen ovotransferrin.[55] The two resulting positive charges on opposite domains may then provide the driving force to push the two domains apart and hence allow release of metal ion. The crystal structure of diferric hen ovotransferrin shows that a related interdomain interaction, between the side chains of Gln541 and Lys638, is present in the C-lobe.[31] The distance between the oxygen atom of Gln541 and the nitrogen atom of Lys638 is ca. 3.0 Å. Both the Lys209−Lys301 and Gln541−Lys638 couples are near the iron binding sites and share the same position in each lobe, and both of them are also in hydrophobic environments. That the C-lobe of ovotransferrin retains Fe^{3+} to lower pH values than the N-lobe is probably relevant to the difference in the two "triggers". A similar trigger is also possible for the N-lobe of human transferrin.[55] For hTF, a Lys−Asp−Arg triplet in the C-lobe may represent another type of trigger, which provides an explanation for C-lobe retaining Fe^{3+} to lower pH values. Low-angle X-ray scattering studies of the N-lobe of recombinant human serum transferrin are consistent with Asp63, an iron binding ligand, serving as a trigger for the closure of the two domains upon Fe^{3+} uptake, since such a trigger might be abolished completely by the mutation of Asp63 to Ser or Cys (i.e., the lobe does not close).[56] However, recent studies of D63S lactoferrin by X-ray crystallography have shown that the N-lobe is completely closed, which has led the proposal of an equilibrium between open and closed forms in solution with a low-energy barrier.[57] In addition, the binding of another anion such as chloride is a prerequisite for Fe^{3+} release, even in the presence of receptor.[58]

E. Hydrophobic Patch in Helix 5

Hydrophobic patches centered on Trp128 in the N-lobe (hTF) and Trp460 in the C-lobe may play a role in the mechanism of uptake and release of Fe^{3+} and synergistic anion by transferrin. On either side of Trp128 in hTF are Leu122 and Ile132, and together they form the hydrophobic patch L122−W128−I132 within helix 5. This helix makes contact with anion and metal binding site via its N-terminal end and is H-bonded to the bound carbonate via the NH groups of Gly127 and Ala126.[31] The counterpart in the C-lobe is the Trp460 which is part of an analogous hydrophobic patch V454−W460−M464. Both Trp128 and Trp460 are almost totally conserved in known sequences of transferrins,[32] apart from two insect transferrins in which Trp128 is replaced by Tyr in cockroach transferrin and Trp460 is replaced by a leucine residue in hornworm transferrin. Other residues in these hydrophobic patches show a high degree of conservation as aliphatic hydrophobic groups.

Leu122 and Ile132 lie below and above the plane of the indole ring of Trp128, and the ^{1}H resonances of their side chains are shifted to high field in the NMR spectrum of hTF/2N due to the ring current of Trp128. Small movements of these side chains relative to Trp128 result in ^{1}H NMR chemical shift changes. Therefore, ^{1}H NMR can be used to monitor local structural changes induced by metal ions (Figure 3).[59,60] The shift changes induced by metals are slightly different for Al^{3+}, Ga^{3+}, Fe^{3+}, and Bi^{3+} and also different when different anions are used for the same metal (Ga^{3+}), which indicates that they may induce slightly different local structural changes.

IV. Metal Uptake and Release

A. Strength of Metal and Anion Binding

The transferrins are primarily iron binding proteins, but in human serum, transferrin is only about 30% saturated with iron, so there is potential capacity for binding to other metal ions which enter the body. Indeed, about 30 metal ions have been reported to bind to transferrin with either carbonate, oxalate, or other carboxylates as synergistic anions, although

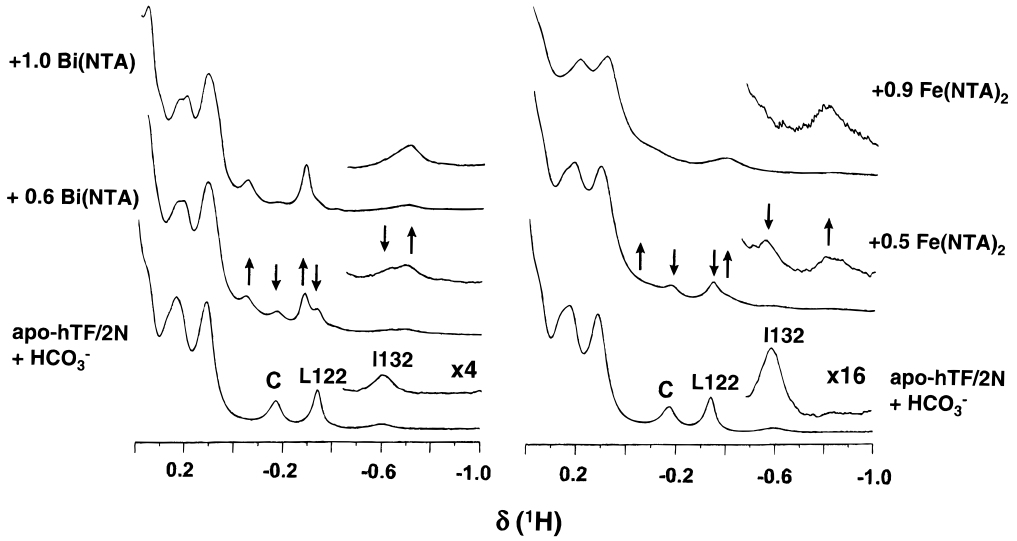


Figure 3. High-field region of 500 MHz ^{1}H NMR spectra of hTF/2N and after addition of various mole equivalents of Bi^{3+} (left) and Fe^{3+} (right). Leu122 and Ile132 are above and below Trp128 in a hydrophobic patch, which backs onto the metal binding site and H-bonds to the synergistic anion (Adapted from ref 60).

Fe^{3+} has a higher affinity than any other metal ion for which the binding constant has been determined.[41] Other ions which bind include main-group metal ions such as Bi^{3+},[61] Ga^{3+},[62] In^{3+},[63,92] Al^{3+},[64] and Tl^{3+},[111] transition-metal ions such as Mn^{2+},[65] Cu^{2+},[109] Ni^{2+},[66] Zn^{2+},[67] and Ru^{3+},[68] and lanthanide ions such as La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, and Gd^{3+}.[69−71] Such binding may play an important role in the transport and delivery of medical diagnostic radioisotopes such as $^{67}Ga^{3+}$ and $^{111}In^{3+}$,[72] toxic metal ions such as Al^{3+},[73] and therapeutic metal ions such as Ru^{3+} and Ti^{4+}.[68,74,75] Transferrin is also thought to transport toxic transuranium elements such as Pu^{4+} in the body.[76] In rabbit plasma, Sc^{3+} has been found to be present as a transferrin complex both in vivo and in vitro.[77] Many metal ions have also been exploited for their spectroscopic properties in investigations of transferrin structure and function, although even if the metal binds to the iron site the structure of the site may be perturbed to suit the metal ions.

Electronic absorption spectroscopy is frequently used to study metal binding to the specific iron sites of transferrins. Apo-transferrin is a colorless protein with an intense ultraviolet absorption near 280 nm with ϵ_{278} 93 000 M^{-1} cm^{-1} attributable to $\pi-\pi^*$ transitions of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. The binding of metal ions to the phenolic groups of the tyrosine residues in the specific metal binding sites of apo-transferrin leads to the production of two new absorption bands at ca. 240 and 295 nm in UV-difference spectra. This has been widely exploited for metal titration and thermodynamic studies. Typical difference spectra are shown in Figure 4, in which two new bands centered at 241 and 295 nm appear and increase in intensity with time after addition of 2 mol equiv of Bi^{3+} as [Bi(Hcit)], indicative of slow complexation of Bi^{3+} in the specific binding sites of apo-transferrin. When transition-metal ions bind to apo-transferrin, there are often additional intense tyrosinate-to-metal charge-transfer (LMCT) bands in the visible region of the spectrum (400−500 nm; ϵ ca. (4−9) × 10^3 M^{-1} cm^{-1}) which are also diagnostic of site-specific binding. For example, the Fe^{3+} complex is orange-red with a band at ca. 465 nm, the Cu^{2+} and Co^{3+} complexes are yellow, and the Mn^{3+} complex is brown.[78]

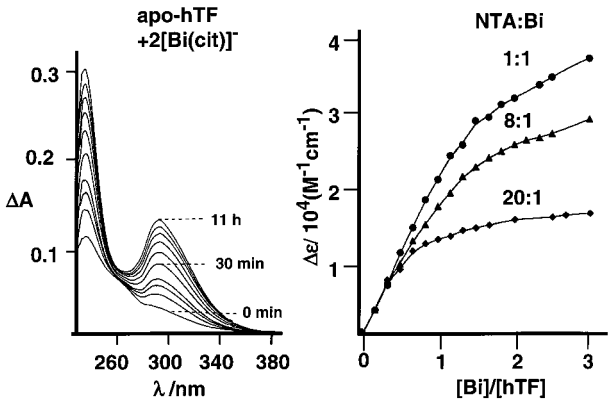


Figure 4. Detection of metal binding to transferrin by UV difference spectroscopy. The bands at 241 and 295 nm arise from the deprotonation of phenol groups of Tyr residues of hTF on metal binding. The slow uptake of Bi(III) citrate by apo-transferrin after addition of 2 mol equiv of Bi^{3+} is shown (left). Bi−hTF binding constants can be determined via titrations with various mole equivalents of [Bi(III)-$(NTA)_x$] (Adapted from ref 61).

Electronic absorption spectroscopy not only distinguishes between binding at the specific iron sites and at nonspecific sites (e.g., Pt^{2+}),[79] but also allows determination of the strength of metal binding to transferrin. Different anions can be distinguished due to slightly different effects on the absorption spectrum.[80] However, UV difference spectra do not provide any direct evidence for closed (after metal binding) or open (apo form) lobe conformations or information about whether Asp and His ligands are coordinated to bound metal ions.

Typical UV-difference spectra and metal−transferrin titration curves are shown in Figure 4. For some metal ions which are readily hydrolyzed at biological pH, such as Bi^{3+}, Ga^{3+}, In^{3+}, and Al^{3+}, chelating ligands such as NTA and EDDA are used as complexing ligands to maintain the metal ions in

Table 3. Stability Constants for Metal Binding to Human Serum Apo-Transferrin (log *K*), Metal Radii, and Molar Absorptivity per Bound Metal Ion ($\Delta\epsilon_1$)

metal ion	radius (Å)	$\Delta\epsilon_1$ (M^{-1} cm^{-1})	[HCO_3^-]	log K_1	log K_2	ref
Fe^{3+}	0.65	18 000	ambient	21.4	20.3	91
Bi^{3+}	1.03	21 900	5 mM	19.4	18.6	61
Bi^{3+} (N-lobe)[b]	1.03	22 000	5 mM		18.9	60
Ga^{3+}	0.62	20 000	5 mM	19.7	18.8	107
In^{3+}	0.80	17 200	5 mM	18.5	16.6	92
Sc^{3+}	0.75	22 000	5 mM	14.6	13.3	81
Al^{3+}	0.54	14 800	5 mM	13.5	12.5	73
Gd^{3+}	0.94		ambient	6.8		71
Sm^{3+}	0.96	21 000	ambient	8.4	6.6	69
Nd^{3+}	0.98	18 700	ambient	7.3	5.3	69
Cu^{2+}	0.73		15 mM	12.3	11.1	109
Ni^{2+}	0.69	14 800	5 mM	4.1	3.2	66
Zn^{2+}	0.74	13 300	ambient	5.7	4.3	67a
Fe^{2+}	0.78			6.7[b]	5.4	67a
Mn^{2+}	0.83	10 100		4.1		65
Cd^{2+}	0.95	11 600		6.2	5.1	107

[a] Recombinant N-lobe of transferrin. [b] Estimated from a linear-free-energy relationship (LFER) for the complexation of Zn^{2+} and Fe^{2+}.

solution and produce a concentration of free metal ion sufficient to partially load the two metal binding sites of the protein.

For metal ions with known stability constants for chelating ligands, a method utilizing a Hill plot is effective for the calculation of metal−transferrin binding constants, especially for K_1.[61] This is based on the calculation of equilibrium constants for a competition reaction between transferrin and chelating ligands (K_{a1} and K_{a2}) such as NTA and EDDA. Metal−transferrin stability constants are then obtained by simply multiplying the stability constant of the metal chelate complex (K_{ML}) by the equilibrium constant of metal complex and apo-transferrin. By assuming that the two binding sites of transferrin are independent and equivalent, the following equation can then be used to fit the experimental data:

$$1/Y = 1/n + \{1/(nK_a)\}[L]/[ML] \quad (1)$$

where K_a is the intrinsic binding constant, $K_{a1} = 2K_a$, $K_{a2} = K_a/2$, and $Y = [\text{M-bound}]/[\text{hTF}]_{total}$. The concentration of metal bound to transferrin is calculated by assuming that one site of transferrin fully loaded with metal ion has a molar absorptivity of $\Delta\epsilon_1$. By choosing different ratios of chelating ligands (L) to metal ions, a single metal site of transferrin can be loaded and eq 1 simplifies to

$$1/Y = 1 + (1/K_{a1})[L]/[ML] \quad (2)$$

This method has been used to calculate Bi−hTF, Sc−hTF, and Bi−hTF/2N binding constants (Table 3)[60,61,81] The advantage of this method is that the accuracy of the stability constants can be estimated not only from different titration curves but also from the intercepts of the plots. The second binding constant is then recalculated using the literature method,[62] giving rise to two binding constants for the binding sites of transferrin. After obtaining K_1, the method is extremely sensitive to K_2.

The method frequently used by Harris and coworkers[69] for calculation of metal−transferrin binding constants is to measure the absorbance at ca. 240 nm, fit the experimental data by an interactive procedure to minimize the difference between calculated and analytical values of $\Delta\epsilon$

$$\Delta\epsilon_{calcd} = \Delta\epsilon_M K_1[M][TF] + 2\Delta\epsilon_M K_1 K_2[M]^2[TF] \quad (3)$$

where $\Delta\epsilon_M$ is the molar absorptivity per bound metal ion for metal transferrin, obtained from the initial linear portion of the curve. For a given set of experiments, the concentrations of free metal ions, free competing ligands, and apo-transferrin are evaluated by iteration to minimize the difference between calculated and experimentally fixed values of total metal, total ligand, and total transferrin. Values of K_1, K_2, and $\Delta\epsilon_M$ can be calculated by minimizing the sum of the squares of the residuals between the observed and calculated $\Delta\epsilon$ values. Several metal−transferrin binding constants have been calculated in this way (Table 3).

The stability constants of metal−transferrins are affected by the concentration of the synergistic anion, usually bicarbonate. The binding constant of apo−hTF for Ga^{3+} (log K_1) is 18.7 at ambient bicarbonate concentration (0.14 mM) and 20.3 at the serum bicarbonate level (27 mM),[62] and similarly for Fe^{3+}, log K_1 is 20.7 and 22.8, respectively.[89] Binding, therefore, becomes stronger at higher bicarbonate concentrations. To compare metal−hTF binding constants which have been measured at a different bicarbonate concentrations, the following relationships have been verified:[82]

$$\log K^* = \log K_M + \log \alpha_c \quad (4)$$

where K^* is the bicarbonate-independent stability constant, $\alpha_c = K_c[HCO_3^-]/(1 + K_c[HCO_3^-])$, and K_C and K_M represent equilibrium constants for the following two reactions

$$K_C \qquad HCO_3^- + \text{apoTf} \rightleftharpoons HCO_3{-}TF \quad (5)$$

$$K_M \qquad M^{3+} + HCO_3TF \rightleftharpoons M{-}CO_3{-}TF \quad (6)$$

Other methods such as equilibrium dialysis and EPR have also been used to calculate stability

constants of metal−transferrin complexes (e.g., Fe^{3+}, Gd^{3+}, and Mn^{2+}).

Many factors will affect the strength of metal-binding, including pH and salt concentration. Although transferrin contains two metal-binding sites of remarkably similar thermodynamic and spectroscopic properties,[83−85] Fletcher and Huehns[86,87] and Harris and Aisen[88] have shown that the two sites differ in their ability to donate iron to reticulocytes. The equilibrium studies of Assa et al.[83] and Aisen and Leibman,[89] demonstrate the presence of two sites a and b on transferrin, which were identified as the C- and N-terminal sites, respectively,[90] and which differ kinetically and thermodynamically. Aisen et al.[91] have shown that the C-terminal site of transferrin binds Fe^{3+} more strongly than the N-terminal site, by a factor of about 20 at pH 7.4, with an ambient bicarbonate concentration. Similar behavior has also been found for other metal ions. The affinities of the two lobes for Ga^{3+} and Bi^{3+} differ by a factor of 10 and 6.8,[61,62] respectively, and for In^{3+}, the factor is 76.[92] Normally the two sites differ by approximately one unit in their log *K* values, which is beyond the purely statistical factor of 4, primarily due to a difference in the intrinsic binding affinities of the two lobes. In vitro, the two lobes of transferrin display somewhat different Fe^{3+} release properties. The N-lobe releases Fe^{3+} at pH about 5.7, while the C-lobe retains Fe^{3+} to pH values of about 4.8.[93−95] Binding of Fe,Co−transferrin to the transferrin receptor at pH 5.6 apparently reverses the order of metal stabilization: Fe^{3+} is labilized (by pyrophosphate) from the C-lobe site in preference to the N-lobe.[96] Cu^{2+} does not bind to serum transferrin below pH 6,[82] and binds only to the C-terminal site as the pH is raised from 6.0 to 7.2. At higher pH, Cu also binds to the N-terminal site, but on further increasing the pH to above 9.5, Cu^{2+} is lost from the specific metal binding sites.[97]

Nonsynergistic anions such as Cl^-, ClO_4^-, $HP_2O_7^{3-}$, and ATP^{3-} also affect the thermodynamic stability of metal−transferrin complexes. Chasteen and coworkers[98−100] proposed nonsynergistic anion binding based on the EPR spectral measurements. Williams et al.[101] reported that the thermodynamic stability of iron binding to the N-terminal site relative to the C-terminal site increased with increasing concentrations (0−0.5 M) of NaF, NaCl, NaBr, NaI, $NaNO_3$, Na_2SO_4 and $NaClO_4$ and that this effect was enhanced as the pH increased in the range pH 7−8.4. The binding of nonsynergistic anions to apo−hTF probably interferes with metal binding by competing directly with the binding of the synergistic anion.[102] Competition experiments have shown that the strength of nonsynergistic anion binding to transferrin follows the order $HPO_4^2 > SO_4^{2-} \cong F^- > ClO_4^- \cong Cl^- \cong Br^-$. Titration of anions with apo-transferrin leads to negative absorption bands centered at 245 and 290 nm in the difference UV spectra, which are remarkably similar to those produced by the binding of metal ions to apo-transferrin. The strength of binding of many anions (both synergistic and nonsynergistic) to transferrin was determined on this basis. Other methods such as EPR and NMR have also been used to study the thermodynamics of anion binding to transferrin (Table 4). The strength of anion binding to transferrin was rationalized and found to correlate with the charge-to-radius ratio.[102]

Table 4. Equilibrium Constants for the Binding of Various Anions to Transferrin

anion	protein	log *K*	methods	ref
HCO_3^-	hTF	2.66, 1.80	UV	143
$C_2O_4^{2-}$ (oxalate)	hTF/2N	4.04	NMR	128
		4.4	UV	144
malonate	hTF/2N	3.7	UV	144
ClO_4^-	hTF	1.64, 0.82	UV	102
	Fe_2−hTF	2.90	EPR	100
Cl^-	hTF	1.83, 0.91	UV	102
	Fe_2−hTF	1.97	EPR	100
Br^-	hTF	1.74, 0.87	UV	102
F^-	hTF	1.97, 1.35	UV	102
SCN^-	Fe_2−hTF	3.56	EPR	100
SO_4^{2-}	hTF	3.62, 2.76	UV	141
	hTF/2N	4.2	UV	144
VO_4^{3-}	hTF	7.45, 6.6	UV	143
PO_4^{3-}	hTF	4.19, 3.25	UV	143
ATP^{3-}	Fe_2−hTF	2.62	EPR	100
$HP_2O_7^{3-}$	Fe_2−hTF	2.86	EPR	100

1. Rationalization of the Strength of Metal Binding to Transferrin

The strength of binding of trivalent metal ions to transferrin was originally thought to be related to the size of the metal ion, being optimum for Fe^{3+} (ionic radius 0.65 Å),[103] weaker for slightly smaller (Ga^{3+}, 0.62 Å) or larger (In^{3+}, 0.80 Å) ions, and much weaker for very small (Al^{3+}, 0.54 Å) or very large ions (lanthanides, 0.86−1.03 Å).[81] It was assumed for this Venus fly-trap protein that for strong binding the size of the metal ion must be matched to the size of the interdomain binding cleft, and this argument can be used to rationalize the finding that lactoferrin promotes oxidation of the large metal ion Ce^{3+} (1.10 Å) to the smaller Ce^{4+} (0.87 Å).[160] However, the large metal ion Bi^{3+} (ionic radius 1.03 Å), an ion widely used in medicine as an antiulcer drug,[105,106] binds very tightly to human transferrin and recombinant N-lobe of transferrin (hTF/2N), whereas if the strength of binding of metal ions to transferrin is optimized to the size of the interdomain binding site, then Bi^{3+} should bind as weakly as the lanthanide ions. In fact, Bi^{3+} binds almost as strongly as Ga^{3+}, with log K_1 19.42, and log K_2 18.58.[61] There are good linear-free-energy-relationships (LFER) between the strengths of binding of Fe^{3+} and other metal ions to a range of oxygen and nitrogen donor ligands including transferrin.[61] It appears that the types of amino acid side-chain donors in transferrin determine the strengths of metal binding rather than the size of the binding cleft.[61]

There is a good correlation between the strength of metal binding to the first lobe of transferrin (log K_1)[81,118] and the stability constants (log $K_1(OH^-)$) for hydroxide binding to the same metal ions (Figure 5).[108] The second binding constant for transferrin is usually ca. one log unit lower than log K_1, and log $K_1(OH^-)$ values are related to pK_a values by log $K_1(OH^-) = 14 - pK_a$. Hence, the most readily hydrolyzed (most acidic) metal ions bind most strongly

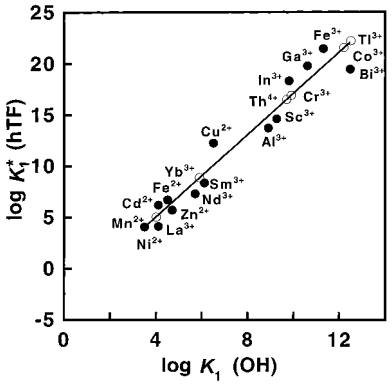


Figure 5. Correlation of the first metal binding constant of human transferrin for divalent and trivalent metal ions with that for hydroxide binding (or metal ion acidity: log $K_1(OH) = 14 - pK_a$, where K_a is the hydrolysis constant): (●) experimental data, (○) predicted values. The intercept of this correlation is ca. −3, a value consistent with a flexible ligand which becomes more organized on metal binding. (Adapted from ref 81).

to transferrin. In vivo, one function of transferrin is to protect against the deposition of insoluble Fe^{3+} hydroxide. Low M_r Fe^{3+} can catalyze the formation of the free radicals and hence damage cells. Such a correlation between metal binding to transferrin and to RO^- ligands also holds for complexes of model phenolate ligands. Therefore, it is not surprising that Fe^{3+} binds to transferrin more strongly than most other metal ions. Other highly acidic metal ions such as Bi^{3+} and Tl^{3+} also bind strongly to transferrin, and it is apparent why trivalent metal ions bind to transferrin more strongly than divalent metal ions and why Cu^{2+} binds to transferrin more strongly than other divalent metal ions.[109] Cu−hTF and Cu−hLF complexes have been used more than any other metal ion (except Fe^{3+}) for physicochemical studies of transferrins.[44]

The correlation between the strength of metal binding to transferrin (Figure 5) and metal acidity provides a basis for the prediction of unknown stability constants for metal−transferrin complexes. Thus, Tl^{3+} should bind to transferrin even more strongly than Fe^{3+}. Both ^{205}Tl NMR[110] and ^{13}C NMR[111] of the synergistic anions carbonate and oxalate have demonstrated that Tl^{3+} does indeed bind tightly, although the binding constants do not appear to have been determined. Similarly, Cr^{3+} is expected to bind strongly to transferrin with a predicted log K_1 17 ± 1.6. The binding of Co^{3+} to hTF (predicted log K_1 21.4 ± 1.6) is probably as strong as that for Fe^{3+}, and it is evident from reported work on Co^{3+}−transferrin complexes that binding is indeed strong even though the binding constant has not been reported.[112] Th^{4+} (ionic radius 0.94 Å) is known to bind to transferrin very strongly,[113,114] and the predicted binding constant is log K_1 16.5 ± 1.5. The predicted binding constant for Sc^{3+} has been verified,[81] and the expected strong binding of Ti^{4+} has now been demonstrated.[75]

The intercept in the above correlation can also be used as an indicator of metal-induced protein conformational changes. For small ligands, correlations of stability constants for binding of different metal ions to various ligands are well-known and have been discussed by Hancock and Martell.[115−117] A general relationship between the logarithms of the stability constants of complexes formed by ligands with n negatively charged oxygen atoms and log $K(OH^-)$ of the ligated metal has been derived and the intercepts in such log−log plots[117] discussed in terms of eq 7

$$\log K_1(\text{polydentate}) = \log K_1(OH^-) + (n - 1)\log 55.5 \quad (7)$$

where K_1(polydentate) refers to the stability of a complex with an n-dentate polydentate ligand and 55.5 is the molality of water. The term log 55.5 represents the entropy of translation of 1 mol of water generated at a concentration of 1 m. Therefore, for bidentate ligands such as oxalate, the intercept should be log 55.5 (1.74) and tridentate ligands such as citrate 2 log 55.5 (3.49).

The linear correlation between the strength of metal binding to transferrin and hydroxide (Figure 5) has an intercept of −3.4, which is much lower than expected for a multidentate ligand formed by the four protein donors: 2Tyr, 1Asp, and 1His. Plots with much smaller intercepts than expected have previously been found for ligands which form large chelate rings. For example, the correlation between desferrioxamine-B and hydroxide has an intercept of ca. −1.3 (instead of 5 log 55.5), octane-1,8-dihydroxamate an intercept of near 0 (instead of 3 log 55.5), and succinate −2.3 (instead of log 55.5). These data have been interpreted in terms of unfavorable entropy contributions associated with immobilizing the long chelating arms of the ligands.[117] For transferrin, one possible interpretation of the negative intercept is that transferrin structure is flexible and that after metal binding the protein conformation is more restricted with a resultant loss of entropy. Such an interpretation is in line with X-ray data: the conformation of the lobe changes from open to closed when the metal binds.[25,26] The interpretation of the intercept in terms of entropy effects can also be extended to the enzymes carboxypeptidase and carbonic anhydrase.[118]

2. Order of Lobe-Loading with Metal Ions

The order of lobe-loading can be determined by electrophoresis of transferrin in 6 M urea as described by Makey and Seal[119] (Table 5). This involves the separation of M_2−TF from M_C−TF and M_N−TF and works well provided that the metal ions remain bound to the protein under the conditions of electrophoresis, which is not always the case (e.g., for Al^{3+}). Another method is differential scanning calorimetry (DSC).[120] Apo−hTF shows two major thermal transitions with T_m values of 57.6 and 68.4 °C, whereas ovotransferrin shows only a single transition at 60 °C (Figure 6). Addition of 1 mol equiv of Fe^{3+} (as [Fe(NTA)$_2$]) to hTF perturbs the transition temperature of only one lobe. Addition of a second mol equiv of Fe^{3+} affects the transition temperature of the other lobe. By comparing these results with those for the N-lobe alone, it was determined that the C-lobe was preferentially loaded. Although promising, there are only two other studies of the binding of metal ions to

Table 5. Order of Lobe Loading of Transferrin with Various Metal Ions Using Different Techniques

metal donor	protein	synergistic anion	pH	method	preference	ref
$(NH_4)_2Fe^{II}(SO_4)_2$	hTF	bicarbonate	7.4−8.5	electrophoresis	N-lobe	121
$Fe^{III}(NTA)_2$	hTF	bicarbonate	6−8.5	electrophoresis calorimetry; [^{1}H,^{13}C] NMR	C-lobe	90,91, 122,123
Fe^{III}−citrate	hTF	bicarbonate	7.4−8.5	electrophoresis	N-lobe	121
Fe^{III}−oxalate	hTF	bicarbonate	7.4−8.5	electrophoresis	N-lobe	121
Cr^{III}−citrate	hTF	bicarbonate	7.5	EPR	both	126
			5.9		N-lobe	126
$Al_2^{III}(SO_4)_3$	hTF	bicarbonate	8.8	^{1}H, [^{1}H,^{13}C] NMR	N-lobe	59
$Al^{III}(NO_3)_3$	oTF	bicarbonate	7.6	^{13}C, ^{27}Al NMR	N-lobe	131
	oTF	oxalate	7.6		C-lobe	
$Ga^{III}(NTA)_2$	hTF	oxalate	7.2	^{1}H, [^{1}H,^{13}C] NMR	C-lobe	52,54,128
		bicarbonate	7.2−7.4	NMR	both	
$Ga^{III}Cl_3$	oTF	bicarbonate	7.6	^{13}C, ^{71}Ga NMR	N-lobe	135
		oxalate	7.6		both	
In^{III}−citrate	hTF	bicarbonate	7.25	NMR	C-lobe	129
$Sc^{III}(NTA)_2$	hTF	bicarbonate	7.4	^{1}H NMR	C-lobe	81
$Sc^{III}Cl_3$	oTF	bicarbonate or oxalate	7.6	^{45}Sc, ^{13}C NMR	both	134
Bi^{III}(NTA)	hTF	bicarbonate	7.25	^{1}H, [^{1}H,^{13}C] NMR	C-lobe	61
ranitidine Bi^{III} citrate	hTF	bicarbonate	7.80	[^{1}H, ^{13}C] NMR	C-lobe	
$Co^{II}Cl_2+H_2O_2$	hTF	bicarbonate	7.4	NMR	N-lobe	125
$Gd^{III}Cl_3$	hTF	bicarbonate	7.4−8.5	EPR	C-lobe	71
$VOSO_4$	hTF	bicarbonate	6.0	EPR	C-lobe	124
VO_2^+	hTF	bicarbonate[a]	7.5−9.0	^{51}V NMR	N-lobe	136
$Tl^{III}Cl_3$	hTF	bicarbonate	7.3−7.8	^{205}Tl NMR	C-lobe	111
	oTF	bicarbonate	7.6		both	130

[a] VO_2^+ can bind without a synergistic anion.

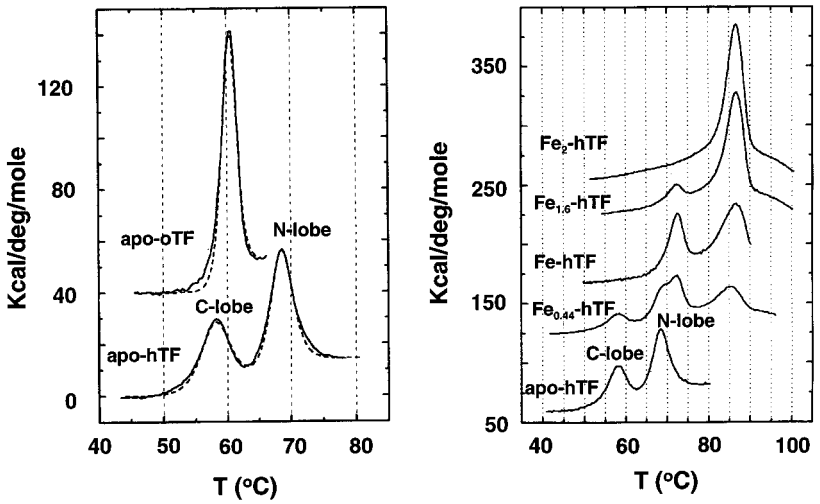


Figure 6. Differential scanning calorimetry of apo−hTF and apo-oTF (in 500 mM Hepes, 25 mM $NaHCO_3$ at pH 7.5, left) and with addition of different mole equivalents of Fe^{3+} (added as [$Fe(NTA)_3$]) to apo−hTF (right). Fe^{3+} binds preferentially to the C-lobe of hTF, followed by the N-lobe. The scan rate was 81.5 °C/h (Adapted from ref 123).

transferrin by this method.[122,123] EPR has been used to determine the order of lobe-loading with paramagnetic ions such as VO^{2+} [124] and Gd^{3+}.[125]

Various NMR methods have also been used to detect the order of lobe-loading with various metal ions. ^{1}H NMR studies of transferrin are complicated by the severe overlap of signals and the broadening of the resonances (slow tumbling due to large M_r). With the aid of resolution enhancement, resonances for most of the His residues can be resolved and pH titration curves have been established.[127] In the high-field region of the spectrum (ca. 0.5 to −2 ppm), peaks due to aromatic ring-current-shifted methyl groups can be resolved. The sharpest peaks in the spectrum (2.0−2.3 ppm) belong to the *N*-acetyls of the glycan chains, attached to Asn416 and Asn611 in the C-lobe. Metal ions bound to the C-lobe can cause chemical shift changes in this region, probably due to structural perturbations. Al^{3+}, Ga^{3+}, In^{3+}, and Bi^{3+} all bind tightly to apo-transferrin, and resonances for free and bound forms are usually in slow exchange on the NMR time scale when these metal ions are titrated into solutions of the protein. By comparing the pattern of shift changes for transferrin itself with those of the isolated recombinant N-lobe, the order of lobe-loading can be determined.[59,128,129]

Since the synergistic anion is bound directly to the metal, it is possible to detect lobe-loading via ^{13}C NMR studies of ^{13}C-labeled anions (e.g., $H^{13}CO_3^-$ and $^{13}C_2O_4^{2-}$). The ^{13}C chemical shift for bound anion in each lobe is slightly different, and by comparing the chemical shift with that of recombinant N-lobe transferrin or monoferric transferrin, the order of lobe-loading can be determined (Table 5). Sometimes

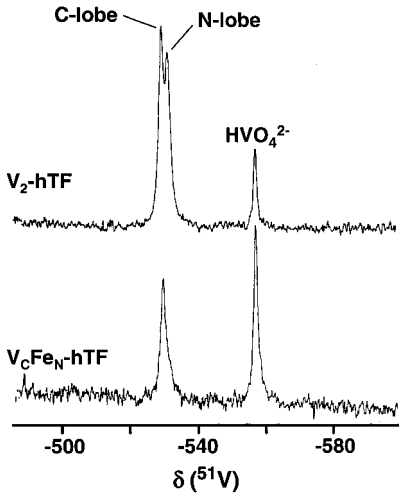


Figure 7. ^{51}V NMR spectra of human transferrin showing two vanadium(V) binding sites with chemical shifts of −529.5 and 531.5 ppm for V in the C-lobe and N-lobe, respectively. The protein concentrations used in these experiments were ca. 0.7 mM (in 100 mM Hepes, pH 7.4). (Adapted from ref 136a).

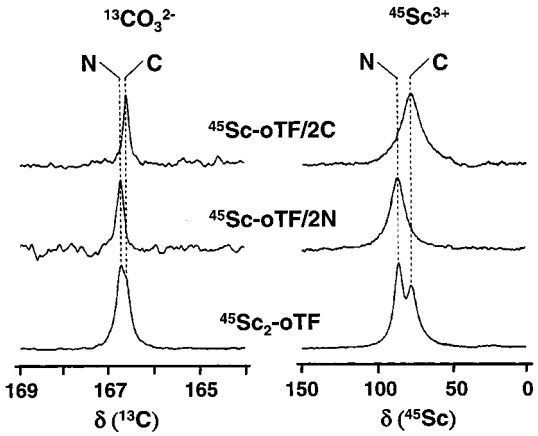


Figure 8. ^{13}C (125.7 MHz) and ^{45}Sc (121.5 MHz) NMR spectra of Sc_2^{III}-oTF, Sc^{III}-oTF/2N, and Sc^{III}-oTF/2C, showing the distinction between the two metal sites of chicken ovotransferrin (oTF). The ^{45}Sc chemical shift difference between the two lobes is 7 ppm (Adapted from ref 134).

spin−spin coupling between the metal and ^{13}C (from the synergistic anion) can be observed, such as $^2J(^{13}C-^{205}Tl)$ 270−290 ($H^{13}CO_3^-$) and 15−30 Hz ($^{13}C_2O_4^{2-}$). Nonequivalence of the ^{13}C atoms in oxalate results in a $^1J(^{13}C-^{13}C)$ value of ca. 70−75 Hz ($^-O_2$-$13C-^{13}CO_2^-$), and provides further evidence for oxalate being directly bound to the metal ion.[130,131]

Multinuclear NMR spectroscopy can provide a direct method for determining the order of lobe-loading.[132] ^{51}V NMR is a sensitive probe for studying the binding of vanadium(V) to human transferrin, and the two lobes can be easily distinguished (Figure 7). Interestingly, no synergistic anion is required for formation of VO_2^+−transferrin, presumably because the two oxygen atoms bound to V can take on the structural role normally played by carbonate oxygens.[136] Similarly, the quadrupolar nucleus ^{45}Sc ($I = 7/2$) can be used to investigate subtle differences between the two lobes and lobe-loading of chicken oTF with Sc^{3+}(Figure 8). Other nuclei such as ^{27}Al ($I = 5/2$), ^{71}Ga ($I = 3/2$), and ^{205}Tl and ^{113}Cd ($I = 1/2$) have also been used to monitor lobe-loading,[133,137] and these data are summarized in Table 5.

Resonances of half-integer quadrupolar nuclei bound to transferrin are much sharper when recorded at very high magnetic fields. In the limit of slow isotopic molecular motion, the line width ($\Delta\nu_{1/2}$) of the central transition ($m = 1/2$ to $-1/2$) decreases with increasing magnetic field

$$\Delta\nu_{1/2} = k[\chi^2/(\tau_c\nu_o^2)] \quad (8)$$

where χ is the quadrupolar coupling constant ($\chi = e^2qQ/h$), τ_c is the correlation time for fluctuations in the electric field gradient at the nucleus, and ν_o is the resonance frequency (proportional to the magnetic field). Decreasing temperature and increasing viscosity give rise to a decrease in line widths.[138] In these quadrupolar systems, the chemical shift is field-dependent, in contrast to $I = 1/2$ nuclei. The second-order dynamic frequency shift is given by[139,140]

$$\Delta\delta_d = k(\chi^2/\nu_o^2) \quad (9)$$

The chemical shift of ^{71}Ga in ovotransferrin is −103 ppm at 11.7 T but −57 ppm at 17.6 T.[135]

The order of lobe-loading can also be detected via studies of 2D [^{1}H,^{13}C] heteronuclear multiple quantum coherence (HMQC) spectra of isotopically labeled protein at concentrations of only ca. 0.3 mM. The 2D HMQC method involves inverse detection of ^{13}C, i.e., detection of ^{13}C via observation of ^{1}H resonances, and is much more sensitive (by up to $(\gamma_H/\gamma_{13C})^{5/2}$) than direct ^{13}C detection. Figure 9 shows the HMQC spectrum of hTF before and after addition of Fe^{3+}. This method has also been used to determine the order of lobe-loading of Ga^{3+}, Al^{3+}, and Bi^{3+}.[54] Due to the ^{13}C labeling of Met and the sensitivity enhancement by inverse detection, it is possible to detect lobe-loading at concentrations of biological relevance in blood pressure.[104]

B. Mechanism

1. Kinetics of Iron Uptake and Release

The mechanism of iron uptake and release is of crucial importance to understanding the biological activity of transferrin.[141] In vivo, iron is thought to be transferred on to transferrin from an Fe(III) complex to prevent hydrolytic attack on the ferric ion. Similarly, complexes such Fe(III) citrate and nitrilotriacetate (NTA) are commonly used in vitro. Several steps are involved in the formation of the specific anion−metal−transferrin ternary complex: binding of the synergistic anion (bicarbonate in vivo) to apotransferrin, displacement of one or two ligands from the added metal complex, formation of an intermediate "anion−metal−chelate−transferrin" complex, loss of the chelated ligands, and, finally, a conformational change of the protein, presumably from cleft open to closed form. The mechanism for iron uptake by lactoferrin is thought to be similar to that of serum transferrin.[142] The binding of synergistic anions to apo−hTF and apo−hLF has been confirmed by UV difference, ^{1}H NMR and fluorescence emission

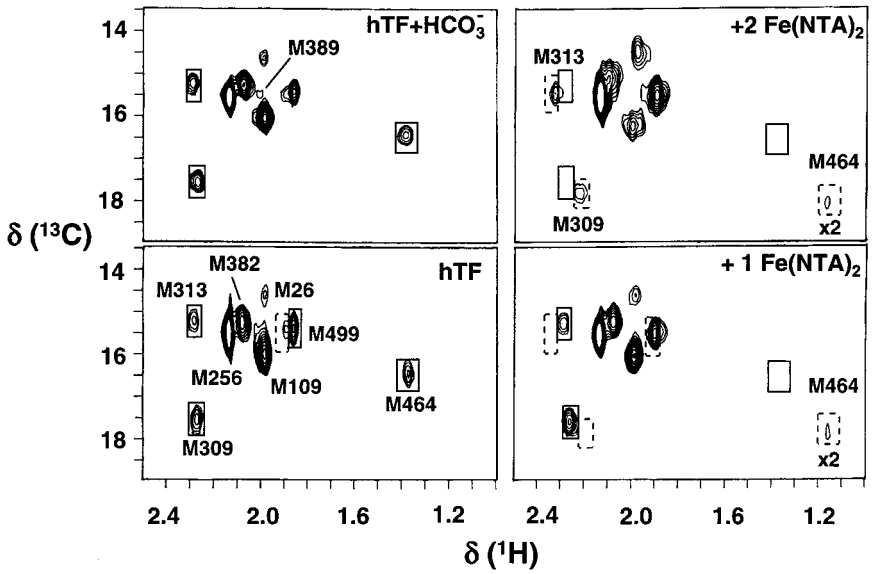


Figure 9. Use of ^{13}C-labeled transferrin to determine the order of lobe loading of transferrin with metal ions. 2D [^1H, ^{13}C] HMQC spectra of apo-[ϵ-^{13}C]Met−hTF before, after addition of 10 mM carbonate, followed by addition of 1 and 2 mol equiv of Fe^{3+}. The solid boxes indicate initial peaks, and the dotted boxes show new peaks. Specific shifts of cross-peaks on binding either the first or second equivalent of Fe^{3+} indicate the order of lobe-loading, e.g., peaks for Met464 and M499 in the C-lobe shift on binding the first equivalent, and Met309 and Met313 in the N-lobe shift only on binding the second equivalent (Adapted from ref 54). Recent data (He, Q. Y; Mason, A. B.; Tam, B. M; MacGillivray, R. T. A.; Woodworth, R. C. Unpublished results) suggest that the assignments for peaks for M26 and M109 should be reversed, but this does not affect the interpretation.

spectroscopy.[128,143−145] Apo-lactoferrin interacts strongly with this bicarbonate, possibly due to the binding of carbonate to apo-lactoferrin instead of bicarbonate as for apo-transferrin.

Evidence for an intermediate anion−metal−chelate−transferrin complex has been obtained from studies using Fe^{III}−acetohydroxamate in iron uptake experiments.[146] With bound anion, four of the six iron ligands are then in place on domain II (two carbonate oxygens and two oxygens from the side chains of Tyr residues) for metal binding. It can be proposed that the metal ion then binds to these oxygens, possibly with the chelate ligands still attached. Evidence for this mechanism is provided by studies of the 18 kDa 4NII fragment of duck oTF. The folding of this fragment is the same as for the NII domain of intact transferrin.[147] Fe^{3+} is bound to two oxygens from the side chain of two Tyr residues, a bidentate carbonate, with the remaining two coordination sites occupied by a nonprotein ligand, a bidentate glycine. This has been confirmed by the high-resolution X-ray structure of duck OTF/4NII (Lindley et al., unpublished data). The last step involves the closure of the two domains over the metal ion. Any remaining low M_r, chelating ligand on the metal will be dispersed by the Asp and His ligands from domain I followed by slow conformational changes to achieve the final state of equilibrium.

Most of the iron transported by transferrin in circulation is destined for hemoglobin, which gathers iron by the receptor-mediated pathway. Therefore, understanding the mechanism of iron release from the protein is crucial for the physiology of transferrin. Several factors influence iron release individually or together. These include the site from which release takes place, pH and ionic strength, the nature of the competitive chelators, and binding of transferrin to its receptor. The mechanism by which iron is released from transferrin and recombinant N-lobe transferrin to low molecular mass chelating agents has been investigated extensively.[148−152] In many cases, saturation kinetics with respect to the free ligand are observed and interpreted in terms of conformational gating. Iron removal by NTA and DTPA follows simple first-order kinetics, while several other ligands appear to follow a combination of both saturation and first-order components.[141,153,154] Both pH and salt affect the iron release kinetics, and metal removal is faster at pH 6 compared to at pH 7.4. The kinetics also depend on the chelator used, since most of them are anionic and bind quite strongly to apo-transferrin.[155]

Nonsynergistic anions and salts (ionic strength) may play a role in promoting and modulating iron release. With a synthetic tricatechol-based iron-sequestering agent, the rate of iron release from both lobes of transferrin extrapolates nearly to zero as the ionic strength of the medium nears zero.[156] Various anions accelerate iron release, attributable to the presence of cationic groups near the metal binding site (Arg and Lys), which form a specific effector or allosteric anion binding site. Nonsynergistic anions may even interfere directly with the synergistic anion and replace it when present in large excess. Iron removal by pyrophosphate from the two sites exhibits weak cooperatively. The release of iron from the C-lobe is accelerated by the presence of kinetically inert Co^{3+} in the N-lobe, but there is no significant effect of C-lobe occupation on iron release from the N-lobe.[157]

The transferrin receptor (TFR) can modulate the rate of iron removal from transferrin. At pH 7.4, the detergent-solubilized TFR and Fe_2−transferrin complex releases iron to pyrophosphate at only one-tenth

the rate of Fe_2−transferrin, whereas at the pH of the endosome (pH 5.6), the TF−TFR complex releases iron more readily than "free" transferrin.[158] The TFR has a differential effect on iron release from the N- and C-lobes of transferrin. At extracellular pH 7.4, TFR retards the rate and extent of iron removal from both lobes, whereas at endosomal pH 5.6, iron release from the N-lobe is substantially the same whether transferrin is free or bound to the TFR. In contrast, the rate of iron release from the C-lobe is much faster when the receptor is bound.[159] This may explain why circulating human transferrin contains iron bound predominantly to the N-lobe despite the apparently weaker binding.

2. Trigger Mechanism of Metal Release

The lobe-open to lobe-closed conformational change of transferrin appears to be crucial for iron release. However, little is known about how the protein conformational change is triggered. TFR binding, pH, and salt effects may all play roles in this process. To understand iron release, sites of protonation, anion binding and interaction with transferrin receptor (TFR) must be identified. The pH value has a different effect on metal release from the two lobes and is different for transferrin and lactoferrin. Iron is released from hTF over the range pH 6 to 4, preferentially from the more acid labile N-lobe. Lactoferrin is distinctly more stable in acid, iron release occurring two pH units lower,[160] and almost simultaneously from the two sites.

The initial step in metal release may involve protonation of the metal-bound carbonate ion giving bicarbonate. Such a reduction in charge on the anion could result in the movement of bicarbonate away from the iron site and detachment from helix 5 in the N-lobe or may promote a change from bidentate to monodentate coordination as in the case of Cu^{II}−hLF.[44] The recent high-resolution X-ray crystal structure of Fe−hTF/2N crystallized at pH 5.75, interpreted in terms of two conformations, provides evidence for this, since Arg124 is alternatively in two positions.[24] Arg124 is either bonded to Fe-bound carbonate or rotated away from bound bicarbonate. H-bonding between the anion and the protein is weak, and the affinity of the protein for the metal is lowered. There is an alternative site at the back of the iron site where protonation could stimulate iron release. Distinct differences between lactoferrin and serum transferrin have been found. In the X-ray structures of the N-lobe of rTF and chicken oTF, a pair of lysine residues, the so-called "dilysine trigger" (Lys206 and Lys296), is directly H-bonded, implying that one of them is neutral. Protonation of either Lys would break this interaction, destabilize the closed conformation of the protein, and lead to cleft opening. Cleft opening with associated removal of further metal ligands would allow the release of the iron to chelating ligands. In the C-lobe of transferrin, a different arrangement of charged residues is found and would be less easily protonated. In hLF/2N, the interaction is also different, one Lys is changed to Glu, giving a Glu−Lys ion pair instead of Lys−Lys. This could account for the different acid stabilities of lactoferrin and transferrin.

3. Nonsynergistic Anion Site

The binding of nonsynergistic anions to transferrin can have pronounced effects on the kinetics of iron release in vitro. UV titrations of anions with intact apo-transferrin and mutant recombinant N-lobe of transferrin have led to the conclusion that the anion binding sites are near to the metal binding sites.[143,144] The binding of anions to transferrin may occur at sites which are tri- and tetra-positively charged. Since the binding of bicarbonate leads directly to the metal transferrin complex, it seems likely that the anion binding site involves the essential guanidinium group of arginine (Arg124 and Arg121 in hTF and hLF, respectively) which is hydrogen bonded in the ternary complex. Lysines have also been suggested as possible anion binding resides. The titration of anions with K206Q, K296Q, and K296E mutants of hTF/2N has shown that Lys296 and Lys206 in the N-lobe, which are near the metal binding site, appear to play important roles in binding of both synergistic and nonsynergistic anions to apo-transferrin. Lys296 may be the initial site for divalent anion binding to hTF/2N.[144] The recent crystal structure of apo−hLF[32] has shown that chloride ions are bound in the anion binding sites of both lobes and H-bonded to the surrounding residues with distances of 3.39 (Thr117 OG1), 3.51 (Arg121 NE), 3.55 (Ala123), and 3.17 Å (Tyr192 OH) in the N-lobe and 2.95 (Thr461 OG1), 3.41 (Arg465 NE), 3.60 (Arg465 NH2), 3.38 (Gly468 N), and 3.61 Å (Tyr528 OH) in the C-lobe. There is no change in UV difference spectra on addition of anions to diferric transferrin, indicating that anion binding is blocked, in agreement with the crystal structure of apo−hLF. In contrast to UV difference spectra, EPR spectral changes are induced by addition of anions to diferric transferrin.[100] Thus, it is likely that other anion binding sites also exist in addition to those for the synergistic anion.

V. Transferrin Receptor

The primary structure of the human transferrin receptor (hTFR) has been deduced from the nucleotide sequence of its cDNA.[161,162] It is a disulfide-bonded dimer consisting of two identical monomers with molecular mass 95 kDa (Figure 10).[163] Each receptor monomer binds one molecule of transferrin. The bulk of the receptor is in the extracellular environment with a molecular mass of ca. 70 kDa. This soluble fragment bears a trypsin-sensitive site and retains the transferrin binding activity of intact transferrin receptor. A complex of this fragment (residues from 121 to 760) with transferrin has been crystallized at pH 6.2 with a resolution of only 3.8 Å.[164] There are three N-linked carbohydrate chains attached to three asparagine residues (Asn251, Asn317, and Asn727)[165] and one O-linked at threonine (Thr104).[166] The presence of these carbohydrates is necessary for normal receptor function. Mutated receptor without asparagine carbohydrate sites has

Table 6. Binding of Transferrin and Lactoferrin to Various Transferrin Receptors

transferrin	receptor type	affinity constant (K_a, M^{-1})	receptor M_r (kDa)	site density	ref
rTF	rabbit reticulocytes	4.6×10^6 (apo-rTF) 2.5×10^7 (Fe_C-rTF) 2.8×10^7 (Fe_C-rTF) 1.1×10^8 (Fe_2-rTF)			183
hTF	platelets (human)	3.58×10^9; 1.92×10^9	105		171
	insect-form trypanosome 221	3.3×10^7 (Fe_2−hTF)	52, 42		181
	insect-form trypanosome 118	1.9×10^8 (Fe_2−hTF)	52, 42		181
	insect-form trypanosome VO_2	1.1×10^7 (Fe_2−hTF)	52, 42		181
hLF	intestinal mucosa (human)	3×10^5	110 (trimer)	4.3×10^{12} sites/mg protein	172
	intestinal mucosa (mouse)	3.5×10^6	130	0.5×10^{12} sites/mg protein	173
	monocytes (human)	2.2×10^8 (Fe_2−hLF) 1.3×10^8 (20% Fe saturated hLF)		1.6×10^6/cell	174
	macrophages	5.9×10^5			174,175
	platelets (human)	1.67×10^{10}, 1.23×10^9	105		171
	T-lymphocytes (human)	1.2×10^7	107, 115	2×10^6/cell	176
	piglet brush-border membrane	3.3×10^5			177
bTF	insect-form trypanosome 221	3.85×10^8 (Fe_2-bTF)	52, 42		181
	insect-form trypanosome 118	3.85×10^8 (Fe_2-bTF)	52, 42		181
	insect-form trypanosome VO_2	7.7×10^7 (Fe_2-bTF)	52, 42		181
	aortic endothelial cells (bovine)	1×10^{10}	35	3×10^4/cell	178
	intestinal mucosa (mouse)	2.6×10^6	130	0.5×10^{12} sites/mg of protein	173
	hepatocytes (rat)	5×10^7	45	1×10^6/cell	179,180

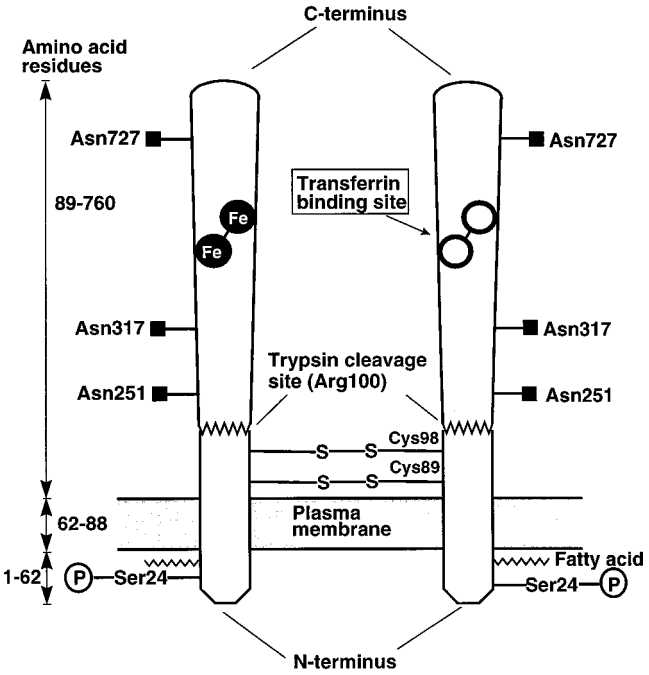


Figure 10. Schematic representation of the transferrin receptor (TFR), which is formed by two identical monomers linked by two disulfide bonds (at Cys89 and Cys98). The N-terminal domain contains 61 residues and is in the hydrophobic intracellular region. The extracellular hydrophilic C-terminal domain contains 672 residues. The transferrin binding sites and glycan chains (attached to Asn) are located in this domain (Adapted from ref 163).

impaired transferrin binding activity, less intersubunit disulfide bonding, and a diminished surface expression.[167] Elimination of Thr104 increases the susceptibility of the receptor to cleavage.[168] The receptor also has an intracellular domain with the N-terminal domain facing the cytoplasm and a hydrophobic transmembrane region (26 residues). The cytoplasmic tail contains four potential phosphorylation sites, but only Ser24 appears to be a target for protein kinase-C-mediated phosphorylation. The role of receptor phosphorylation is not understood. Phosphorylation may be involved in receptor endocytosis since agents such as phorbol esters that enhance receptor phosphorylation also stimulate internalization.[169,170]

Several lactoferrin receptors (LFR) have been characterized (Table 6) as monomers, dimers, or even trimers. The molecular mass of LFR depends largely on the species and the types of cells, ranging from 42 to 110 kDa. The binding affinity of transferrin and lactoferrin for various receptors is very high, from 10^5 to 10^{10} M^{-1} (Table 6).[171−180] Small differences between transferrin receptors can have large effects on the binding affinity for transferrin from different mammals and different cells, as shown in Table 6.[181] The diferric protein always has a higher affinity for the transferrin receptor than its monoferric and apo forms. For example, the binding of diferric rabbit serum transferrin to rabbit reticulocytes is 22-fold stronger than that of its apo form, and human diferric transferrin[182] has a 7-fold advantage over monoferric transferrin in delivering iron to reticulocytes.[183] All nucleated cells in the body appear to express the TFR which has been found in red blood cells, erythroid cells, hepatocytes, intestinal cells, monocytes (macrophages), brain, the blood-brain barrier (also blood-testis and blood-placenta barriers), and also some insects and certain bacteria.[184−186] TFRs have been localized on the endothelial surfaces of brain capillaries that comprise the blood-brain barrier.[187] Generally, malignant cells have a very high level of TFR expression, and all these cell types require high iron to grow.[188,189] Proliferating cells require iron especially for ribonucleotide reductase production, which is essential for DNA synthesis and for Fe proteins such as hemoglobin.

Recently, the lactoferrin receptor (LFR), MTF, and a divalent cation transporter[190] have been implicated in brain iron transport, and disruption of the expression of these proteins in the brain is probably one of

the main causes of altered brain iron metabolism in age-related neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease.[191] The overexpression of LFR may be linked to increased intraneuronal iron levels and degeneration of nigral dopaminergic neurons in Parkinson's disease.[192]

1. Regulation of TFR Expression

Precise modulation of cellular iron is required to provide iron for the synthesis of iron proteins such as ribonucleotide reductase and cytochromes and also to prevent cell damage from free radicals catalyzed by free Fe^{3+}. The expression of surface TFR is modulated through a negative feedback mechanism dependent on the intracellular iron and heme. Several studies have shown that in the presence of ferric ion, Hela cells,[193] human fibroblasts,[194] human leukemic cells lines,[195,196] T-acute lymphocyte leukemic cells,[197] and hepatocytes[198] exhibit concentration- and time-dependent decreases of their transferrin binding capacity, indicative of reduction of surface TFR. Conversely, incubation of cells with iron chelators leads to a marked increase in TFR.[199,200]

The TFR controls the access of transferrin-bound iron to most cells. Regulation of receptor expression is a key process in controlling cellular iron metabolism. This is self-regulated through iron-dependent changes in the abundance of TFRs, which control iron uptake, and of ferritin, which sequesters iron within the cell (Figure 11). The mechanisms for the regulation of TFR and ferritin expression are similar and largely posttranscriptional.[201,202] The 3′-untranslated region of receptor messenger ribonucleic acid (mRNA) contains a series of five iron-regulatory elements (IRE). Such specific mRNA elements (IRE) bind to a cytoplasmic iron-regulatory protein (IRE-BP, 90 kDa, now usually referred to as iron regulatory protein, IRP-1), which substantially increases the half-life of receptor mRNA and, hence, increases the rate of TFR synthesis. In contrast, the interaction of IRP-l with the IRE of ferritin mRNA in the 5′-untranslated region leads to inhibition of ferritin mRNA translation and, therefore, decreases the rate of ferritin synthesis. IRP-1 is an iron sensor which loses its aconitase activity when depleted of iron. Treatment of the cells with ferric ions results in a switch to a low-affinity IRP-1, which facilitates ferritin mRNA translation. An opposite effect is induced by treatment of cells with iron chelators (e.g., desferrioxamine). A second IRE-BP is known, IRP-2, but this is not an aconitase-like iron sensor. Nothing appears to be known about the regulation of the LFR.

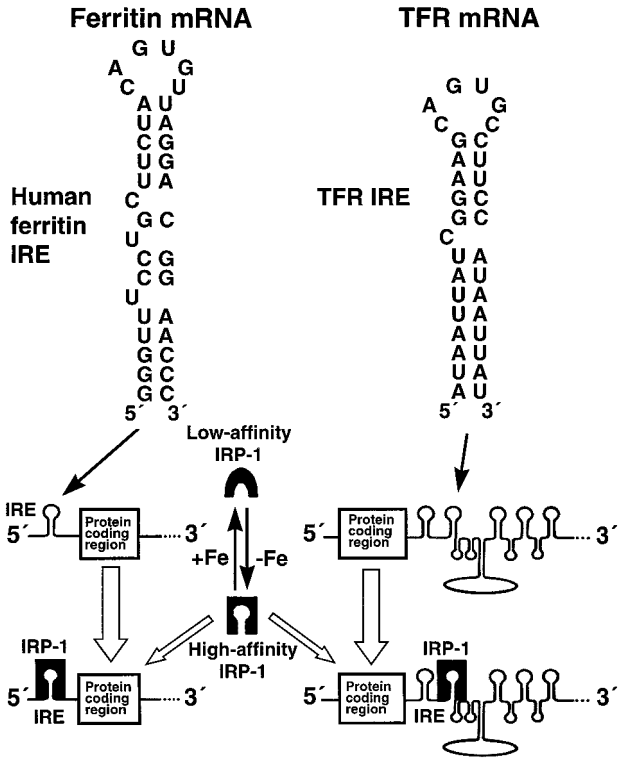


Figure 11. Regulation of TFR and ferritin expression by the iron-regulatory elements (IRE) and binding to iron-regulatory protein (IRP-1). Top shows the sequence and the proposed secondary structure of the human ferritin H chain IRE and one of the five IREs of TFR mRNA. The center shows the localization of IRE sequences in the 5′-untranslated region of ferritin H mRNA and in the 3′-untranslated region of TFR mRNA. At high iron levels, IRP-1 has bound iron and a lower affinity for IREs, which facilitates ferritin mRNA translation and TFR mRNA degradation. At low iron levels, the affinity of IRP-1 for IRE is increased and IRP-1 is bound to TFR IREs, blocking ferritin mRNA translation and increasing the rate of TFR synthesis.

VI. Metal Delivery in Biomedical Processes

A. Iron and Manganese: Implications for Neurochemistry

Iron is essential for the growth of mammalian cells, including the proliferation of tumor and immune cells and microorganisms (except lactic acid bacteria). Considerable efforts have been devoted toward understanding the mechanism of iron uptake by cells. Most cells of vertebrate animals obtain iron from the plasma iron transport protein, transferrin, via receptor-mediated endocytosis. This involves six main steps: binding of Fe_2−hTF to its receptor, internalization (endocytosis), acidification (pH 5.5), dissociation and possible reduction from Fe^{3+} to Fe^{2+}, translocation, and cytosolic transfer of iron to intracellular compounds such as ferritin and heme (Figure 12).

1. Receptor Recognition of Iron

Binding of transferrin to its receptor on the surface of cells is apparently a simple chemical event not dependent on metabolic energy. Each Fe_2−hTF binds to a monomer of the receptor. Receptor-mediated endocytosis is a complex process involving several biochemical stages (Figure 12).[203] Cellular uptake of iron from transferrin is initiated by the binding of diferric-transferrin to the TFR on the cell surface. After endocytosis via clathrin-coated pits, which bud from the plasma membrane as membrane-bound vesicles or endosomes, the transferrin−TFR complex is routed into the endosomal compartment (Figure 12). Upon maturation and loss of the clathrin coat, the endosome becomes competent to pump protons in a process energized by ATPase and the endosomal lumen is rapidly acidified to a pH of about 5.5.[204−206]

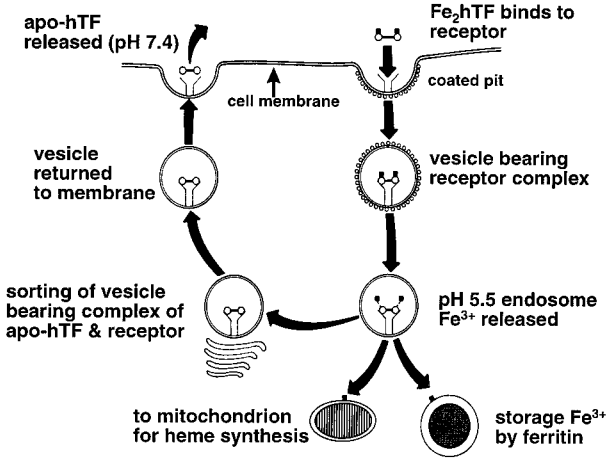


Figure 12. Diagram showing the pathway of cellular uptake of iron from transferrin via a cell receptor (Adapted from ref 203).

At this pH, the binding of iron to transferrin is weakened, leading to iron release from the protein. A recent report has shown that TFR can facilitate the release of iron from Fe^{3+}−hTF at low pH (5.5).[158] Reduction of Fe^{3+} to Fe^{2+} may occur after Fe^{3+} release, prior to traversal from the endosomal membrane into the cytosol. Transferrin remains bound to the receptor throughout this process, and the affinity of apo-transferrin for the TFR at low pH (5.5) is much higher than the diferric form, whereas the reverse is true at extracellular pH (7.4). The apo-transferrin−TFR complex is then recycled through exocytic vesicles back to the cell surface. At extracellular physiological pH, apo-transferrin dissociates from its receptor due to its low affinity at pH 7.4, released into the circulation, and reutilized. ATP-mediated energy is necessary for sustaining TFR-mediated endocytosis and recycling.[207−209]

The primary receptor recognition site of human transferrin is mainly on the C-lobe of the protein,[210] and it might therefore be expected that only Fe_C−hTF, not the Fe_N−hTF, can donate iron to cells. Similarly, the recognition site for the human transferrin bacterial transferrin receptor is thought to be primarily in the C-lobe.[211] However, this has been challenged by recent studies which show that receptor recognition sites reside both in the C- and the N-lobe of human serum transferrin.[212] Bacterial transferrin receptors (transferrin binding protein II, Tbp2) from *Haemophilus paragallinarum* and *Haemophilus avium* interact with both the C- and N-lobes of ovotransferrin, which indicates that both interactions may be necessary to acquire iron efficiently.[213] Further work is needed to identify the specific interaction sites of transferrins under different conditions.

One of the mechanisms for iron transport across the blood-brain barrier (BBB) involves the transferrin receptor, which is localized on the brain capillary endothelium. There seems little doubt that ferric iron−transferrin is taken up into isolated and cultured cerebral endothelial cells across their apical membranes by receptor-mediated endocytosis, which is the same or very similar to that shown by other cell types.[214,215] The TFR density in primary cultures of endothelium from bovine brain is 10^5 per cell. However, iron is finally transported into the brain largely without accompanying transferrin, which suggests that removal of iron from transferrin and exit from the endosome is probably important. Neurones and glial cells also have the same mechanism of receptor-mediated uptake. Iron is abnormally accumulated in the substantia nigra in Parkinson's disease. The mechanism is still unclear, although the LFR and MTF may play roles in iron transport.

A number of studies have shown that non-transferrin-bound iron can enter cells by a different mechanism, the so-called transferrin-independent iron uptake.[17,216] Many virulent bacteria, such as *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria gonorrheae*, acquire Fe^{3+} from the host proteins lactoferrin and transferrin using surface receptors. Free Fe^{3+} is released from these proteins and transported across the bacterial outer membrane into the periplasmic space. Fe^{3+} is transported across the periplasmic space by a ferric-iron binding protein (FBP). Recently, a soluble FBP from the periplasmic space of Gram-negative *Neisseria* has been isolated and characterized. It has a molecular mass of about one-half that of transferrin.[8] The X-ray crystal structure of a FBP from *H. influenzae* shows that the Fe^{3+} binding site is similar to that of transferrin and lactoferrin, with two oxygens from Tyr residues, one nitrogen from His, and one oxygen from Glu (instead of Asp as in TF and LF), a bound water and a monodentate phosphate instead of a bidentate carbonate (Figure 2H).[9] Since FBP is highly conserved, required for virulence, and is a nodal point for iron uptake, it provides a potential target for antibacterial drug design.

2. Manganese Transferrin

Manganese is an essential trace element and is present in several enzymes (e.g., superoxide dismutase, glutamine synthase) but at higher concentrations is neurotoxic. There are chemical and biochemical similarities between Fe^{3+} and Mn^{3+}, and a linear-free-energy-relationship (LFER) between Mn^{3+} and Fe^{3+} binding to low M_r ligands has been established[217]

$$\log K_{Mn} = 1.09(\log K_{Fe}) + 1.09 \qquad (10)$$

The binding of Mn^{3+} to apo−hTF bears resemblance to the ferric iron transferrin system. Due to the high acidity of Mn^{3+}, it binds to apo−hTF much stronger than Mn^{2+}, with log K_1 values of ca. 23 (predicted on the basis of the eq 10) and 4 for Mn^{3+} and Mn^{2+}, respectively.[65,217]

Mn^{III}−hTF is normally prepared by air-oxidation of Mn^{II}−hTF in the presence of bicarbonate. This oxidation is a very slow process and takes days to complete, in contrast to the oxidation of Fe^{II}−hTF to Fe^{III}−hTF. However, this process may be faster in serum, and in vitro experiments have shown that the binding of Mn^{2+} to transferrin and oxidation to Mn^{III}−hTF is facilitated by the serum protein ceruloplasmin.[218,219] There has been confusion regarding the distribution of manganese in plasma. This largely depends on the oxidation state of manganese in

plasma: almost all Mn^{3+} binds to transferrin in rats,[220] whereas only ca. 1% of Mn^{2+} binds to transferrin and over 80% binds to albumin, as calculated from a serum model.[65]

Numerous reports have shown that cellular uptake of Mn^{3+} is related to its binding to transferrin, presumably via receptor-mediated-endocytosis of Mn^{III}−hTF.[221−224] Manganese binds to transferrin as Mn^{3+} after exposure of serum to manganese oxide. The binding of Mn_2−hTF to human neuroblastoma cells (SHSH5Y) is extremely high, with a binding constant K_a of 8×10^8 M^{-1} and a density of 11 000 binding sites per cell.[224] This complex is internalized and reaches saturation after 2 h. Most of the internalized manganese is ferritin-bound after 24 h of exposure. There is evidence for metabolic interaction between manganese and iron, especially at the level of absorption from the intestine. Mn^{3+} and Fe^{3+} also interfere with each other during transfer from the plasma to the brain, liver, and kidneys. The distribution of iron and manganese in wild-type, hypotransferrinemic (produce <1% transferrin level), and heterozygous mice (50% transferrin levels) is similar in brain, heart, plasma, and blood. This indicates that transferrin is required for proper targeting of iron and manganese, particularly transfer from the liver to other organs, but non-transferrin transport mechanisms are also present. Free Mn^{2+}, when injected or infused as $MnCl_2$, is rapidly transported to other tissues, with K_{in} values of 1.0−1.7 $mL \cdot g^{-1} \cdot min^{-1}$ for entry into the brain across the blood-brain barrier, a similar mechanism to Fe^{2+} transport.[225,226] Once it is firmly bound to transferrin (probably as Mn^{III}−transferrin), entry of Mn into the brain, as for iron, is dramatically slowed.

B. Radioisotopes: Ga, In, Actinides

1. Gallium Transferrin

In the last two decades, several gallium complexes have been extensively used in medicine as diagnostic and therapeutic agents (e.g., ^{67}Ga citrate).[227] ^{67}Ga, a low-energy γ-emitting radionuclide with a half-life of 78 h, is one of the most useful diagnostic agents. ^{68}Ga is of increasing interest in three-dimensional imaging by position emission tomography. Recently, Ga^{3+} nitrate has been approved in the United States for the treatment of hypercalcemia of malignancy, and Ga^{3+} maltolate has recently entered clinical trials for the treatment of bone disease and related conditions.[228]

^{67}Ga is normally injected as a solution containing excess of sodium citrate to prevent the hydrolysis of gallium, and in the X-ray crystal structure of gallium citrate ($[Ga(Hcit)_2]^{3-}$), citrate forms a tridentate chelate with Ga^{3+} via two deprotonated carboxylates and one deprotonated alkoxide.[229] Once it enters the circulation, ^{67}Ga transfers from citrate to serum transferrin and binds in the iron binding sites. This is in agreement with speciation models of serum and serum fractionation studies which have shown that essentially all of the ^{67}Ga is bound to transferrin at low gallium concentrations (99.9% and 95% of Ga bound to transferrin at Ga concentrations of 20 and 50 μM, respectively).[230−232] This is not surprising considering its high acidity, same charge (3+), and similar size to Fe^{3+} (Table 3). Gallium binds to lactoferrin 90-fold stronger than to transferrin, and lactoferrin can remove gallium bound to transferrin.[233] It also binds to the iron storage protein, ferritin.

In most cases, gallium enters cells (including tumor cells) via transferrin receptor-mediated endocytosis. Binding of gallium to transferrin is crucial for its anticancer activity. Gallium transferrin is a much more effective cytotoxic agent than gallium nitrate alone. Ga_2−hTF is recognized by EMT-6 cells with a binding constant of 5×10^6 M^{-1}, and transferrin enhances uptake of ^{67}Ga into EMT-6 tumor cells.[234] Similarly, gallium transferrin is taken up by human leukemic HL60 cells, and this complex inhibits TFR-mediated cellular uptake of iron and, hence, blocks the activity of the iron-dependent enzyme ribonucleotide reductase.[235−237] Gallium is expected to accumulate in tissues having high levels of transferrin, TFR, lactoferrin, and ferritin. Indeed, gallium is found to concentrate in malignant cells, where a large number of TFRs is expressed. It also accumulates in areas of inflammation that contain higher concentrations of lactoferrin.[232] The concentration of gallium in malignant tissue correlates with that of the TFR: for example, for tumor cells from a variety of lymphomas.[238,239] Similarly, there is a high correlation between TFR and ^{67}Ga uptake in lung tumors: TFR is expressed in lung cancer tissues with positive ^{67}Ga scans but not those with negative ^{67}Ga scans.[240] Therefore, cancer cells, clusters of macrophages, and epithelioid cells should be observable through imaging of ^{67}Ga in lung cancers and diffuse interstitial lung diseases.

Gallium can also accumulate in bacteria. The antimicrobial activity of gallium has been demonstrated in vitro against pathogens responsible for tuberculosis in humans, *Mycobacterium tuberculosis* and *Mycobacterium avium complex*.[241] Little is known about its mechanism of action. It is likely to enter bacteria through iron transport systems such as receptor-mediated uptake, ferritin, and siderophores and to interfere with iron metabolism, affecting DNA and protein synthesis. Transferrin, TFR (LFR) and FBP have been found in several Gram-negative bacteria.[242]

In addition to receptor-mediated uptake of gallium, there is a non-transferrin uptake mechanism, which may be similar to that for iron and manganese.[17,216] Sohn et al. implanted two tumor cell lines into hypotransferrinemic mice and found that the reduced transferrin level markedly reduced Ga uptake in one tumor type but had little effect on the other.[243] The in vivo uptake of ^{67}Ga is significantly enhanced in tumors in which the TFR is overexpressed. However, ^{67}Ga accumulation occurs via both receptor-mediated and non-transferrin uptake routes. In vitro non-transferrin uptake can be enhanced to levels that equal or even exceed receptor-mediated uptake simply by increasing the level of calcium or iron salts in the medium.[244] The relative importance of the two mechanisms for a specific cell type depends on factors

such as the density of TFRs and also on the extent to which the cell relies on reduction of Fe^{3+} to Fe^{2+} to release Fe from transferrin intracellularly, since Ga^{3+}−transferrin cannot be reduced.

2. Indium Transferrin

In^{3+} is also widely used in radiopharmaceuticals, for example, as In−bleomycin (BLM) and In−oxine (8-hydroxyquinoline). The major isotope in clinical use is ^{111}In ($t_{1/2}$ = 2.8 days) although ^{113m}In ($t_{1/2}$ = 1.7 h) is also a γ-emitting isotope. Much of the discussion on gallium above, relating to ease of hydrolysis, binding to transferrin, use as a tumor imager, is also applicable to indium.

Transferrin appears to be the primary target for In^{3+} in serum.[92] When ^{111}In-labeled compounds are administered with weakly bound ligands such as In−citrate or as acidic solutions, more than 95% of the In binds to macromolecules and essentially all of this is associated with transferrin.[245−247] However, if ^{111}In is present as a strong chelate with little or no binding to transferrin, the biodistribution characteristics are those of the non-transferrin form and ^{111}In thereby functions as a radiolabel. The binding affinities of In_2−hTF and Fe_2−hTF for the TFR on both human and rat reticulocytes are very similar.[248] The clearance of In_2−transferrin is slower than that of iron and gallium, which indicates that the receptor-mediated endocytosis uptake system is less effective.[249] There appears to be little transport of indium across the cell membrane, although In_2−TF binds to the TFR.[250]

There are inconsistent spectroscopic studies of In^{3+} transferrin. Small-angle X-ray scattering studies of chicken ovotransferrin metal complexes (M_2−oTF) have shown that In^{3+} (and Cu^{2+}) induces essentially the same domain closure in the protein as Fe^{3+},[50] whereas circular dichroism studies indicate that the conformational change induced by In^{3+} differs from that induced by Ga^{3+} and Al^{3+}.[251] However, In^{3+} still tends to accumulate in tissues which have high levels of TFR,[252] and, although it has not been proved, it seems likely that transferrin is a mediator for the delivery of In^{3+} into tumors.

3. Actinide Complexes

The half-lives of actinide isotopes range from 1.4 × 10^{10} years for ^{232}Th to 0.38 ms for ^{258}Fm.[253] The presence of actinides such as Th, U, Np, Pu, and Am in radioactive wastes is a major concern due to their potential for migration from the waste repositories and long-term contamination of the environment. Greatest attention has focused on plutonium (^{239}Pu, an α emitter with $t_{1/2}$ = 2.44 × 10^4 years), which is present in large amounts in nuclear reactors. The actinides exist in various oxidation states, for example, for Pu, Np, and Am from +3 to +7, U from +3 to +6, and Th +4. Under biological conditions, the stable oxidation states for these elements are +3 (Am) and +4 (Pu, Np, Th, and U).

Many actinide ions (Pu^{4+}, Th^{4+}, Pa^{4+}, U^{4+}, and Np^{4+}; Ac^{3+}, Am^{3+}, Cm^{3+}, and Cf^{3+}) are known to bind to the iron sites of transferrin, although few binding constants are known. The binding of Pu^{4+} to apo−hTF has been investigated by difference UV spectroscopy and ultrafiltration. The stability constant determined for Pu^{IV}−hTF (log K_1 21.3) is even slightly higher than that for Fe^{III}−hTF (log K_1 20.7).[254] It is known that Fe^{3+} readily replaces Pu^{4+} from transferrin,[255,256] indicative of a lower conditional stability constant than for Fe^{3+}. The complexation of U^{4+} to transferrin has been studied by time-resolved laser-induced fluorescence. The binding of U^{4+} to transferrin is very strong with log K = 16 under physiological conditions and low U^{4+} concentrations (μM).[257] The titration data provide clear evidence for the binding of two U^{4+} ions per transferrin molecule. The formation constants (log K_1) for binding of Am^{3+} and Cm^{3+} to hTF have been estimated to be 6.5 based on linear-free-energy relationships with the lanthanides Nd^{3+} and Sm^{3+}.[69] Small-angle X-ray scattering studies show that radius of gyration of chicken oTF decreases markedly on binding of Fe^{3+}, In^{3+}, Al^{3+}, and Cu^{2+} but not on binding of Hf^{4+} or Th^{4+}.[50,258] This suggests that the conformational changes in oTF induced by Hf^{4+} or Th^{4+} are much smaller than for Fe^{3+}, Al^{3+}, and Cu^{2+}. The lack of normal domain closure of transferrin after Hf^{4+} or Th^{4+} binding may be due to the preference of these tetravalent cations for a coordination number of eight.[50] Since Hf^{4+} shares the same oxidation state as Pu^{4+}, it is often studied as a replacement to transferrin for Pu^{4+}, which may also be unable to trigger domain closure.

The distribution of actinides between the cells and blood plasma has not been investigated systematically. The data available from both humans and animals have shown that more than 90% of Pu^{4+}, Am^{3+}, Cm^{3+}, and Cf^{3+} in blood is in the plasma.[258] In contrast, the majority of the U^{4+} and Th^{4+} is associated with red blood cells.[259,260] Numerous studies have demonstrated that Pu^{4+}, Th^{4+}, Pa^{5+}, U^{4+}, and Np^{5+} bind strongly to serum transferrin, which may plays a role in the transport of these metal ions in blood plasma. The trivalent actinides such as Ac^{3+}, Am^{3+}, Cm^{3+}, and Cf^{3+} also bind to other proteins in plasma. Apo-transferin may inhibit cell uptake of Pu^{4+}, and some Pu-transferrin complexes bind to the cell membrane.[261,262] However, they are not internalized and Pu is not released from transferrin for incorporation into cells. Liver and bone are the two major organs for Pu^{4+} toxicity, with ca. 70% of Pu deposited in the skeleton and the rest in the liver.[263] Uptake of Pu^{4+} into the liver is mediated by low molecular mass complexes such as Pu−citrate. The inability of transferrin to facilitate the uptake of Pu and Am into cells may result from actinide transferrin complexes not being in the correct conformation (closure) to bind to the TFR. Further work is needed to clarify this.

C. Therapeutic Metal Ions: Ru, Ti, and Pt

1. Ruthenium Complexes

Ruthenium(III) complexes are of current interest as potential anticancer agents. They are often active against metastases and not against the primary tumors.[68] Promising complexes are of the type *trans*-

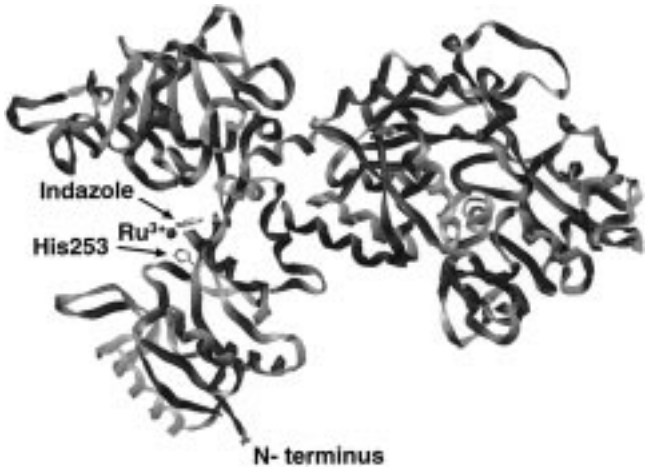
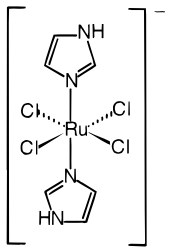


Figure 13. Crystal structure of indazole−Ru^{III}−hLF, the product from reaction of the anticancer complex $[RuCl_4(Ind)_2]^-$ with hLF, showing the Ru^{III}−indazole complex directly bound to His253, one of the iron binding ligands in the N-lobe. Some lower-affinity sites were identified as exposed His residues, primarily His590 and His654 in the C-lobe (Adapted from ref 74).

$[Ru^{III}Cl_4L^1L^{11}]Y$, where $L^1 = L^{11} =$ N-heterocycle, or $L^1 =$ N-heterocycle, $L^{11} =$ DMSO, and Y = e.g., Na^+ or protonated heterocycle. For example, the imidazole (Im) complex $[Ru^{III}Cl_4(Im_2)]$HIm (**1**) and indazole (Ind) complex $[Ru^{III}Cl_4(Ind_2)]$HInd (**2**) exhibit good antitumor activity versus several human colon carcinoma cell lines.[264]



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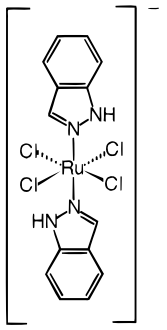

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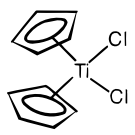
The latter is less toxic in chronic applications and can be used at higher doses. In contrast to square-planar platinum-based anticancer drugs, these Ru^{3+} complexes are octahedral and can undergo facile redox chemistry.[265] Ultrafiltration experiments have shown that there is little binding of these Ru^{3+} complexes to low mass ligands (less than 30 kDa) in serum. HPLC studies of $[Ru^{III}Cl_4(Ind_2)]$HInd have shown that most of the complex is bound to albumin (80%) and the remainder to transferrin. Binding of HInd$[Ru^{III}Cl_4(Ind_2)]$ to albumin is nonspecific, requiring 5 mol equiv for saturation whereas saturation of apo−hTF is reached after 2 mol equiv.[68]

X-ray crystallographic studies of human lactoferrin have demonstrated that Ru^{3+} coordinates directly to the imidazole nitrogen of His253, one of the Fe^{3+} ligands in the iron binding cleft of the N-lobe, with displacement of a chloride ligand (Figure 13). At least one indazole ligand remains coordinated to the Ru^{3+}.[74] Significantly, the binding of the two ruthenium(III) complexes, $[Ru^{III}Cl_4(Im_2)]$Him and $[Ru^{III}Cl_4(Ind_2)]$HInd, to transferrin is reversible. The metal complexes are released at pH 4−5 in the presence of a large excess of citrate or adenosine triphosphate (ATP). This suggests that transferrin can serve as a mediator to deliver ruthenium(III) anticancer complexes. The transferrin-bound complex may be taken up by tumor cells via the receptor-mediated transport system and enter cells. Many solid tumors express more TFRs than normal cells. The transferrin-bound complex appears to exhibit a significantly higher antitumor activity against human colon cancer cells than the albumin-bound complex or the Ru complex itself.[68,266] The released Ru^{3+} complex may target DNA and inhibit replication.[265]

2. Titanium Binding

Biological interest in titanium arises from the two titanium complexes, titanocene dichloride ($Ti^{IV}Cp_2Cl_2$, **3**) and budotitane ($[Ti^{IV}(bzac)_2(OEt)_2]$, where bzac is 1-phenylbutane-1,3-diketonate, **4** predominant *cis*,*cis*,*cis* isomer shown), which are on clinical trial as anticancer agents.[267,268]



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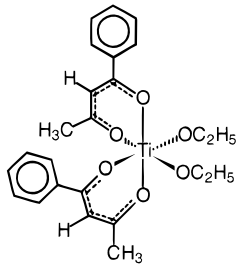

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$Ti^{IV}Cp_2Cl_2$ is active against a diverse range of human carcinomas, including gastrointestinal and breast carcinomas, but not against head and neck cancers and is now in phase II clinical trials (1997). Budotitane entered phase I clinical trials in Germany in 1986 for the treatment of colon cancer[269] and entered phase II in 1989. In addition, there is an enormous amount of titanium in a variety of biomaterials (artificial teeth, bones, etc.), and Ti is present in many foods as a whitening pigment. There are, therefore, many routes by which Ti could enter into biological systems. Recently, it has been shown that ^{45}Ti ($t_{1/2} = 3.1$ h) has potential use in radiopharmaceuticals.[270] However, little is known about the biological chemistry of titanium and its mechanism of action as an anticancer agent is poorly understood.[271] Ti^{4+} complexes, including the anticancer complexes budotitane and titanocene dichloride, hydrolyze rapidly in aqueous media and special formulations are required for their use. A coprecipitate of Cremophor EL, 1,2-propylene glycol in water-free ethanol, and the budotitane in a ratio of 9:1:1 is normally made up prior to administration.

In agreement with predictions based on metal ion acidity, Ti^{4+} has been shown to form a strong complex with human apo-transferrin by binding to the specific Fe^{3+} binding sites.[75] The uptake of titanium by transferrin is relatively slow and requires several hours for completion. Each transferrin molecule binds

to two Ti^{4+} ions, one in each lobe. Such binding may be important for titanium anticancer activity if transferrin delivers Ti^{4+} to tumor cells.[283] Ti^{4+} binding to transferrin is reversible, and Ti is released at low pH (ca. 5.5). At this pH value, Ti^{4+} can bind to DNA. Ti−DNA adducts once formed are stable at pH values of up to about 7 for about 2 days.[272] Binding of Cp_2TiX_2 (where X_2 is Cl_2 or glycine) to 5′-dAMP appears to involve an oxygen of phosphate and a nitrogen from the base (N7).[273] Both serum fractionation studies and ^{45}Ti radiolabeling experiments have shown that Ti^{4+} is associated only with transferrin both in vivo and in vitro,[270] an indication that Ti^{4+} is probably transported by transferrin in blood in a manner similar to Ga^{3+} and Ru^{3+}. Further work is needed to establish this.

3. Platinum Binding Sites

Despite previous suggestions that Pt anticancer complexes can bind to the specific Fe sites of transferrin, the major binding sites appear to be the solvent-accessible sulfurs of Met256 in the N-lobe and Met499 in the C-lobe.[79]

D. Toxic Metal Ions: Al

Aluminum is present at relatively high concentrations in the earth's crust, in drinking water, and foods (especially herbs, tea, some baking powders), and $Al(OH)_3$ is used in antacids for the treatment of peptic ulcers and gastritis. Aluminum has long been regarded as a relatively nontoxic element, but there is now concern over the use of orally dosed aluminum compounds.[274] Aluminum neurotoxicity has been demonstrated in long-term renal dialysis patients,[275] and there is a possible link between the deposition of Al^{3+} in the brain and Alzheimer's disease (AD).[276−278] The concentration of aluminum in the brains of AD patients is increased and Al appears to accumulate in specific regions of the brain.[279,280] Aluminum-induced encephalopathy can be treated with the iron chelating agent, desferrioxamine B,[281] which appears to be capable of removing excess of Al from specific tissues (e.g., serum and brain).

The binding of Al^{3+} to transferrin has been investigated by various spectroscopic techniques.[282] ^{27}Al, ^{13}C, and 2D [^1H,^{13}C] NMR spectroscopies have been used to detect uptake of Al^{3+} into the two lobes of several transferrins. At pH 8.8, Al^{3+} binds preferentially to the N-lobe of hTF, in contrast to most of other metal ions (Table 3). The binding constants of Al^{3+}−hTF based on UV difference titrations are log $K_1 = 13.5$, log $K_2 = 12.5$,[73] and computer-modeling suggests that Al^{3+} is almost exclusively bound to hTF (94%) in serum. However, considerable variation in the distribution of Al^{3+} in serum has been reported.[284] Citrate is the most probable low molecular mass ligand for the nonprotein bound $A1^{3+}$ in serum.[285,286] Both the ionic strength and concentration of bicarbonate will dramatically affect the final results.[217] For example, Al^{3+} may dissociate from Al_2−hTF in 6 M urea, a normal salt concentration used in gel electrophoresis, and in the absence of bicarbonate in some of the elution buffers, the binding of Al^{3+} to transferrin becomes weaker and the complex can dissociate on the column.[287] The binding of Al^{3+} to albumin has also been reported,[288,289] but seems to be weak and nonspecific. Albumin is unlikely to compete with transferrin, citrate, and phosphate in serum.

The chemical shifts of $^{27}Al^{3+}$ bound to ovotransferrin fall within the region 40 to −46 ppm, which is a characteristic of six-coordinate Al complexes,[290] suggesting Al^{3+} is six-coordinate in ovotransferrin, consistent with binding to the iron site with octahedral geometry. NMR chemical shift changes[54] suggest that Al^{3+} binding to transferrin induces similar conformational changes to Fe^{3+} and Ga^{3+}, and both iron and gallium transferrins are recognized by the receptor and taken up by cells. X-ray scattering studies, however, suggest that Al^{3+} causes a smaller conformational change than Fe^{3+}.[49]

Numerous studies have reported that Al^{3+} transferrin is recognized by cell receptors in a manner similar to Fe_2−TF.[291−293] Uptake of Al^{3+} by cells is probably via the receptor-mediator system and is enhanced by addition of apo-transferrin.[294,295] Al^{3+}, once bound to transferrin, inhibits iron uptake via the down regulation of TFR expression in K562 cells and interferes with intracellular release of iron from transferrin.[296] Al^{3+} passage across the blood-brain barrier appears to be mediated by this TF and TFR route and may interfere with normal cellular iron homeostasis and disrupt iron-dependent cellular processes in the central nervous system (CNS). The rate of uptake of Al^{3+} appears to be high enough to account for the Al^{3+} found in the brains of AD patients.[297] The aluminum content in the major iron storage protein ferritin isolated from the brains of AD subjects is 6-fold higher than that of normal age-matched controls.[298]

Aluminum overload in dialysis patients and experimental animals can also result in anemia and disorders of bone metabolism. Reports of Al_2−TF inhibition of cellular uptake of iron are conflicting.[292,296,299] The primary effect of Al^{3+} on Fe^{3+} metabolism is related more directly to inhibition of iron release from transferrin and to changes in iron distribution. The release of Al^{3+} from transferrin is much faster than Fe^{3+} despite the similar kinetic scheme.[300] When efficient excretion through the kidneys is no longer possible, Al^{3+} forms small, less-highly charged complexes which cross membrane barriers that usually prevent a highly charged ion from reaching regions such as the nervous system.

VII. Future Perspectives

There is now a growing family of transferrin proteins which have specific mechanisms for the uptake and release of Fe^{3+}. They are of the Venus fly-trap type, undergoing large conformational changes when the metal binds, and are related to periplasmic anion binding proteins which are well-known for bacteria. The iron transferrins range from serum transferrin to lactoferrin, ovotransferrin, and melanotransferrin to the bacterial half molecule FBP. Metal-loaded transferrins are recognized by specific receptors, not only those on mammalian cells but also on bacteria, including virulent pathogenic bacteria.

The detailed nature of interactions between transferrin and its receptor is currently unknown but can be expected to be revealed soon by ongoing X-ray crystallographic and other studies. There is much to be learned about how receptor binding alters both the thermodynamics and kinetics of metal uptake and release and about the role of the glycan chains, both those present on transferrin itself and those on the receptor. Transferrin has the unusual requirement for a synergistic anion to promote strong metal binding. In vivo, this is thought to be (bi)carbonate. It would be interesting to know more about the roles of other potential synergistic anions in vivo.

Transferrin mediation provides a specific pathway for the delivery not only of Fe^{3+} but also for other metal ions into cells. Since circulating serum transferrin is only partially loaded with Fe^{3+}, there is ample spare binding capacity. An important example is Mn^{3+}, but in vitro, oxidation-coupled uptake of Mn^{2+} is very slow—is there a catalyst in vivo? Although Fe^{3+} is bound very strongly to transferrin, uptake and release processes and competitive displacements of metal ions may be kinetically controlled. More work is needed in this area. In general, it might be possible to fine-tune ligands for metal ions to achieve kinetic control over transferrin uptake.

Serum transferrin is known to deliver therapeutic Ga^{3+} complexes to cells as well as radioactive ^{67}Ga (and ^{111}In) for diagnostic X-ray imaging. Similarly Ru^{3+} anticancer complexes can be delivered to cells by transferrin, which is also a strong candidate for binding the large metal ion Bi^{3+} from antiulcer complexes in the blood or in bacterial membranes. Ti^{4+} can also bind strongly, but whether Ti−transferrin plays a role in cell uptake of Ti anticancer complexes remains to be seen. It seems likely that M^{4+} ions such as VO^{2+} and Ti^{4+} impose different electronic and structural requirements on the metal binding sites compared to Fe^{3+}, and this may result in different recognition properties. There is a need to develop a better understanding of the coordination chemistry of transferrin (cleft open/closed, geometry, coordination number, dynamics, and architecture of ion sites etc).

A major challenge is to understand the role of transferrin in neurochemistry, in the transfer of iron across the blood-brain barrier, and its redistribution within the brain. It is not clear whether iron is continually transported in and out of the brain or whether the same pool of iron remains there and is reutilized. There are possible links between changes in iron metabolism and the neurotoxicity of Al^{3+} and with neurodisorders such as Alzheimer's disease. Fine control over iron metabolism at the pharmacological level can involve regulation of the levels of transferrin, transferrin receptors, and the iron storage protein ferritin at the DNA or mRNA levels. It would be interesting to know more about possible metal-induced damage to transferrin, e.g., redox reactions around the metal binding site, and how this affects its lifetime in circulation.

There is scope for (both organic and inorganic) drug design based on altering the metal mediation processes regulated by lactoferrin and by membrane-bound transferrins such as melanotransferrin and FBP, but these mediation processes are poorly understood at present. Indeed, a more detailed understanding of metal mediation processes in general is likely to revolutionize our use of metal-based drugs and therapeutic agents. It should be possible to move away from the empirical administration of metal compounds to designs based on specific uptake and delivery mechanisms and hence to the more effective and safer use of metals. The hope too is that new therapeutic leads for currently intractable diseases will emerge from such an approach.

VIII. Abbreviations

BBB	blood-brain barrier
bLF	bovine lactoferrin
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
EDDA	*N*,*N*′-ethylenediaminediacetate
hTF	human serum transferrin
hTF/2N	N-terminal half-molecule of human serum transferrin
hLF	human lactoferrin
HMQC	heteronuclear multiple-quantum coherence
IRE	iron-regulatory element
IRE-BP	iron-regulatory element binding protein
IRP	iron-regulatory protein
LFR	lactoferrin receptor
mRNA	messenger ribonucleic acid
MTF	melanotransferrin
*K**	bicarbonate-independent binding constant
NTA	nitrilotriacetate
NZ	ϵ-amino nitrogen of lysine (designation used in Protein Data Bank files)
oTF	ovotransferrin
oTF/2N	N-terminal half-molecule of chicken ovotransferrin
oTF/2C	C-terminal half-molecule of chicken ovotransferrin
rTF	rabbit serum transferrin
TFR	transferrin receptor

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