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A comparative study of aluminum(III), gallium(III), indium(III), and thallium(III) binding to human serum transferrin

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Abstract

Group 13 cations exhibit an essentially similar chemical behavior in aqueous solution. Under physiological conditions these cations exist as metal complexes. They are known to bind tightly to human serum transferrin in the blood. Here, the numerous published studies on the interactions of Group 13 metals with transferrin are reviewed, particular attention being given to the comparative analysis of the binding constants and to the kinetics and mechanisms of metal ion uptake and release. The structural and functional information obtained on these metallotransferrins by advanced physicochemical methods, such as NMR spectroscopy, is presented in light of the recent crystal structures of ferric- and apotransferrin. The biological consequences of binding of aluminum(III), gallium(III) and indium(III) to transferrin are discussed in relation to the relevant roles played by these metal ions in pharmacology and toxicology.

Keywords: Transferrin; Aluminum; Gallium; Indium; Thallium; Nuclear magnetic resonance

1. Introduction

Serum transferrin is the protein that transports ferric ion through the blood between sites of uptake, utilization, and storage. It also serves a bacteriostatic role by denying iron to microorganisms. It is a member of a small family of iron-binding proteins that includes lactoferrin, ovotransferrin, and melanotransferrin [1]. Lactoferrin is found in milk, tears, and other secretions and in association with white blood cells; ovotransferrin is found in avian egg whites. Both proteins serve as bacteriostatic agents. Melanotransferrin is a membrane protein found in malignant melanoma cells that appears to facilitate cellular iron uptake [2].

Each of the transferrins consists of a single polypeptide chain of about 680 amino acids [3]. They all fold into two distinct lobes that are connected by a short polypeptide chain, as shown in Fig. 1. In human serum transferrin there is a 40% sequence identity between the two lobes [3], and it is believed that the modern transferrin molecules were produced by a gene duplication of an ancestral single-lobe protein [3,4]. The gene duplication must have occurred early in the evolution of higher animals, since bilobal transferrins are found in all vertebrates as well as in crabs, spiders, and insects [4]. There is a family of bacterial iron transport proteins that are similar to a single lobe of transferrin in terms of tertiary structure and iron-binding sites, but it appears that the bilobal transferrins and these single-lobe bacterial iron-binding proteins evolved independently from a common, single-lobe ancestor [5].

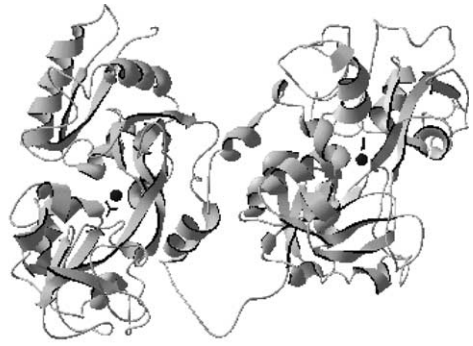


Fig. 1. Structure of diferric transferrin based on X-ray coordinates from Ref. [6].

Each lobe of transferrin is further divided into two domains. There is a single, high-affinity iron-binding site located within a cleft separating the two domains. In the apoprotein both lobes adopt an 'open' conformation, in which the cleft between the two domains is rather wide. Upon iron binding, the domains rotate towards each other by about 60° to produce the 'closed' structure of the ferric protein. The closed and open conformations of the transferrin N-terminal lobe are shown in Fig. 2. This conformational change is thought to play a significant role in regulating the binding and release of metal ions and in modulating the affinity of the protein for its receptor [2].

In both the N- and C-terminal binding sites, the ferric ion is found in a six-coordinate, distorted octahedral coordination environment as shown in Fig. 3. Two tyrosines, one histidine, and one aspartic acid provide four ligating groups to the metal. The fifth and sixth coordination sites are occupied by a bidentate carbonate ligand referred to as the synergistic anion [6,7]. Essentially no metal–protein binding occurs in the absence of this carbonate or some suitable substitute anion [8]. This requirement of a synergistic carbonate anion for metal binding is often considered to be the defining characteristic of the members of the transferrin family. The single-lobe bacterial iron-binding proteins have the same set of protein ligands, but utilize a phosphate anion rather than carbonate [5].

Blood contains about 30 μM serum transferrin [9]. In normal serum the transferrin binding sites are only about 30% saturated with ferric ion [9–11], which leaves a concentration of about 40 μM of vacant binding sites. Thus it is not necessary for another metal ion to displace the tightly-bound ferric ion in order to be transported in blood by transferrin. Transferrin is involved in the transport of a variety of tri- and tetravalent cations [12]. This review focuses on transferrin binding of the Group 13 metal ions, aluminum(III), gallium(III), indium(III), and thallium(III). Although, none of the

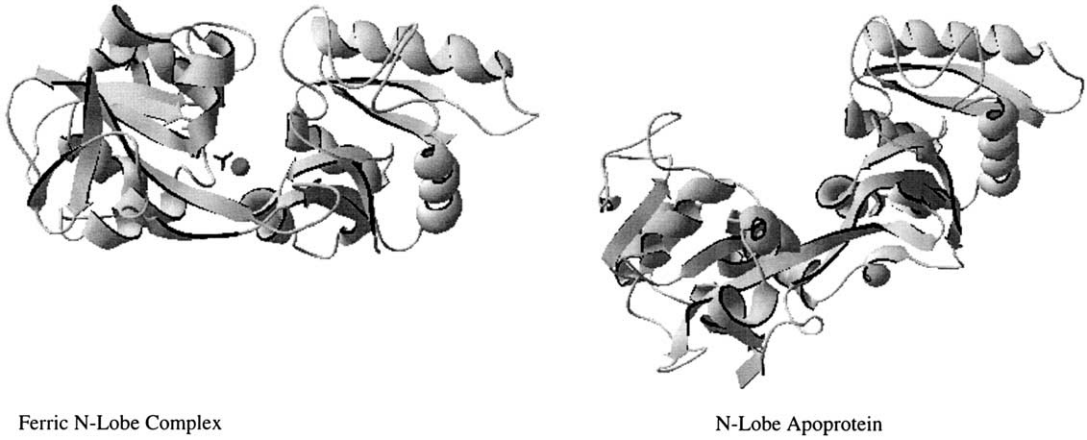


Fig. 2. The structures of the transferrin N-lobe half-molecule with and without ferric ion, based on coordinates from Refs. [36,231].

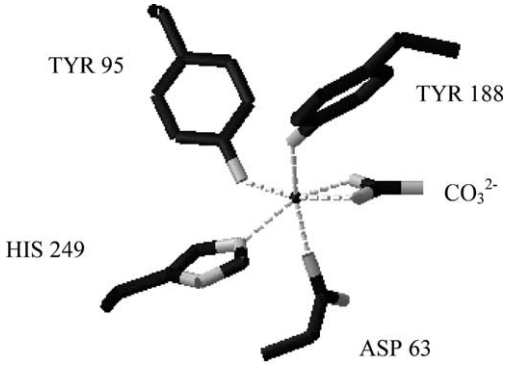


Fig. 3. Coordination geometry around the ferric ion in the protein complex with the N-terminal transferrin half-molecule. Based on coordinates from Ref. [36].

Group 13 elements qualifies as essential to life, Al(III), Ga(III) and In(III) are, nonetheless, of large biological interest. At variance, Tl(III) is an important spectroscopic probe, but has little biological relevance; indeed, thallium, in the oxidation state +3, is strongly oxidizing and does not exist in nature as such.

Al(III) is of great concern due to its large natural abundance and its possible toxic effects [13–19]; Ga(III) and In(III) are of interest for their widespread medical and radiodiagnostic applications [20–22]. The main biochemical features of these metals and of their interactions with transferrin are surveyed below. It is remarkable that all these metal ions, bearing a tripositive charge, show large affinities for transferrins. This suggests that all these metal ions, when entering the blood stream, will use transferrin as their biological carrier and will share the biochemical 'machinery' of iron, including the receptorial cell internalization mechanism and storage inside ferritin. Biochemical evidence tends to confirm this picture [23].

Table 1
Ionic radii and reduction potentials for the Group 13 metal ions and Fe^{3+}

Metal ion	$M^{3+} + 3e^- \rightarrow M$ [a]	Ionic radius M^{3+} (Å) [b]
Al^{3+}	−1.68	0.54
Ga^{3+}	−0.53	0.62
Fe^{3+}	+0.77	0.65
In^{3+}	−0.34	0.80
Tl^{3+}	+0.72	0.89
Tl^{3+}	+1.25 [c]	
Tl^{+}	−0.336 [d]	1.50

[a] Potentials from Ref. [232].
[b] Ionic radii for six-coordinate cations from Shannon [233].
[c] Potential for $Tl^{3+} + 2e^- \rightarrow Tl^+$.
[d] Potential for $Tl^+ + e \rightarrow Tl$.

2. Aqueous chemistry of the Group 13 metal ions

2.1. Aquo ions

The reduction potentials for the Group 13 metal ions are shown in Table 1. The reduction potentials for In^{3+}, Ga^{3+}, and especially Al^{3+} are negative, and under physiological conditions, these metals exist exclusively as complexes of the trivalent ions. The redox chemistry of thallium is significantly different from the other Group 13 metals. Under standard conditions the most stable oxidation state is Tl^+, but both Tl^+ and Tl^{3+} can exist under biological conditions. The redox chemistry of all the Group 13 ions is very sensitive to pH due to hydrolysis reactions, which are discussed in detail below.

The trivalent Group 13 metal ions have closed shell electron configurations, and thus they are all diamagnetic and lack any d–d electronic spectra. Unlike Fe^{3+}, they lack a stable divalent oxidation state and therefore do not show the strong charge-transfer bands that are characteristic of Fe^{3+}-binding to ligands such as phenol and hydroxide.

The ionic radii for the Group 13 metal ions are also listed in Table 1. As one expects, the ionic radius steadily increases going down the periodic table from Al(III) to

Tl(III). In hard–soft–acid–base theory, the hardness of a metal ion correlates with the metal ion charge/radius ratio, so the metal ions become softer as they get larger. The Al(III), Ga(III), and In(III) ions are all classified as hard [24], and show a preference for hard oxygen and nitrogen donors such as those that comprise the transferrin binding site. Both Tl(III) and especially Tl(I) are classified as soft [24], and would be expected to show a preference for softer donors such as thiols.

2.2. Hydrolysis

The chemistry of most trivalent metal ions in an aqueous solution is heavily influenced by hydrolysis reactions. The hydrolysis of an aquo metal ion can be described as an acid–base reaction in which one of the coordinated water molecules dissociates to form a bound hydroxo ligand and release a proton. For the trivalent Group 13 metal ions, there is a sequence of such deprotonation reactions that terminate in the formation of the anionic $M(OH)_4^-$ species. Each step in this hydrolysis sequence can be described by a K_a, defined as

$$K_{an} = \frac{[M(OH)_n^{3-n}][H^+]}{[M(OH)_{n-1}^{4-n}]} \quad (1)$$

where coordinated water molecules have been omitted. The sequential hydrolysis pK_as for the Group 13 ions are listed in Table 2. The values for the Fe^{3+} ion are included for comparison.

The trends in the stepwise pK_as reflect several different factors. Ionic bonding between the metal and the OH^- ligand is related to the charge to radius ratio of the metal ion, so that the ionic contribution to hydrolysis decreases going down the periodic table as the metal ions get larger. Covalent bonding tends to increase going down the periodic table. The covalent contribution is especially small for Al^{3+} and is particularly important for Tl^{3+}. Due to the differences in covalency in the metal–hydroxide bond, the smallest ion, Al^{3+}, is the least acidic (highest pK_{a1}) while the largest ion, Tl^{3+}, is the most acidic ion (smallest pK_{a1}). In addition, the Group 13 metal ions change coordination number between the hexacoordinate $M(H_2O)_6^{3+}$ ion and the four-coordinate $M(OH)_4^-$ ion, and this compresses the first three sequential pK_as into a span of only about 1 log unit [25]. In the case of Al^{3+} and In^{3+}, the second dissociable proton actually has a lower pK_a (i.e. is more acidic) than the first proton.

Table 2 also lists the total solubility of each metal ion at pH 7.4. Total solubility refers to the sum of the concentrations of the free aquo ion and all mononuclear hydroxo complexes. For all the ions in Table 2, the only significant species at pH 7.4 are $M(OH)_3$ and $M(OH)_4^-$. The total solubility of Al and Ga at neutral pH is relatively high because of the formation of significant amounts of the $M(OH)_4^-$ ions. Gallium in particular, exists almost exclusively as the $Ga(OH)_4^-$ gallate anion. This is a relatively well-behaved species, which easily equilibrates with other ligands in solution.

Al(III) behaves similarly to Ga(III). Based on the hydrolysis constants from Baes and Mesmer [26], the major species at physiological pH is $Al(OH)_3$. However, one should note that it is difficult to measure pK_as that involve the sparingly soluble $Al(OH)_3$, and the reported values for pK_{a4} vary widely. Based on the set of hydrolysis constants reported by Ohman et al. [27], $Al(OH)_4^-$ would be the dominant species, and the overall solubility would increase to about 8 μM [28]. The Fe^{3+}, In^{3+}, and Tl^{3+} ions exist in solution at pH 7.4 almost exclusively as the neutral $M(OH)_3$ species. This limits their overall solubility, especially for the ferric ion.

At higher concentrations, the trivalent Group 13 ions also form a large number of polynuclear hydroxo complexes. These complexes become less important in dilute solutions. At 10 μM metal ion, the only stable polynuclear complexes are $Al_{13}(OH)_{32}^{7+}$ and $In_3(OH)_4^{5+}$ [26]. Since physiological concentrations will be even lower than 10 μM, polynuclear hydroxo complexes are not likely to be important in vivo. However, at the higher concentrations characteristic of most in vitro studies, the neutral $M(OH)_3$ species tend to aggregate into colloidal particles, which do not form a visible precipitate, but nonetheless equilibrate very slowly with apotransferrin. The effects of metal hydrolysis on the kinetics of transferrin binding are discussed in more detail in Section 4.1.

Table 2
Sequential hydrolysis pK_as for the Group 13 metal ions [a]

	Al^{3+}	Ga^{3+}	In^{3+}	Tl^{3+}	Fe^{3+}
pK_{a1}	5.46	3.09	4.49	1.11	2.68
pK_{a2}	4.58	3.55	4.07	1.20	3.73
pK_{a3}	5.70	4.40	4.58	1.73	6.33
pK_{a4}	7.75	6.05	9.42	11.45	9.35
Total solubility at pH 7.4 (μM)	0.5	1	0.06	0.06	3×10^{-6}

[a] Values calculated from data in Baes and Mesmer [26] for 0.15 M ionic strength.

3. Transferrin binding constants

3.1. Transferrin equilibria

As discussed in Section 1, the binding of a metal ion at each of the two high-affinity transferrin binding sites involves the formation of a ternary complex of the metal ion, the protein, and a synergistic bicarbonate anion derived from the buffer. Kinetic studies have shown that the bicarbonate anion binds first [29], and the equilibrium constants for the binding of bicarbonate and several other anions to apotransferrin have been measured [30–34]. The overall metal-binding reaction can be described as two sequential equilibria, defined as

$$HCO_3^- + \text{apoTf} \overset{K_C}{\rightleftarrows} HCO_3\text{–Tf} \quad (2)$$

$$M^{3+} + HCO_3\text{–Tf} \overset{K_M}{\rightleftarrows} M\text{–}CO_3\text{–Tf} \quad (3)$$

Proton release studies have shown that the overall reaction for the binding of trivalent metal ions involves three released protons [35]. The magnitude of K_C indicates that the anion binds to apoTf as HCO_3^- [33], while the crystal structure of ferric transferrin shows that the synergistic anion in the final metal–transferrin complex is CO_3^{2-} [7,36,37]. This suggests that one of the three protons are derived from deprotonation of the synergistic anion. The coordination of two tyrosines would account for the other two protons released. It would be reasonable to assign all three released protons to Eq. (3), but this has not been definitively established.

In practice, metal binding equilibria are measured in buffered solutions at a single pH, and the reported binding constants are valid only for the pH at which they are measured. In defining these effective binding constants, hydrogen ions are omitted from the equilibrium expressions, so that

$$K_C = \frac{[HCO_3^- - \text{Tf}]}{[HCO_3^-][\text{apoTf}]} \quad (4)$$

$$K_M = \frac{[M^{3+} - CO_3^{2-} - \text{Tf}]}{[M^{3+}][HCO_3^- - \text{Tf}]} \quad (5)$$

Eqs. (4) and (5) can be used to describe how the effective transferrin binding constant varies with changes in the concentration of bicarbonate in the solution. The carbonate dependence is described by the equation

$$\log K^* = \log K_M + \log \alpha \quad (6)$$

where K^* is the effective transferrin binding constant at a specific bicarbonate concentration, and α is the degree of saturation of apoTf with bicarbonate, which is calculated as

Table 3
Selected macroscopic metal transferrin binding constants for pH 7.4

Metal ion	Log K_{M1}	Log K_{M2}	Δlog K	Reference
Al^{3+}	12.4±0.5	11.8±0.6	0.6	Martin et al. [58]
Al^{3+}	13.72±0.2	12.72±0.2	1.0	Harris and Sheldon, [46]
Fe^{3+}	21.91	20.62	1.3	Aisen et al. [40]
Ga^{3+}	19.75±0.25	18.80±0.25	0.95	Harris and Pecoraro, [49]
In^{3+}	18.30±0.1	16.44±0.13	1.86	Harris and Chen, [39]

$$\alpha = \frac{K_C[\text{apoTf}]}{1 + K_C[\text{apoTf}]} \quad (7)$$

Studies using Zn^{2+} have confirmed that the carbonate dependence of K^* is described by Eq. (6) [38].

3.2. Group 13 binding constants

Transferrin binding constants have been measured for all the Group 13 metal ions except for Tl(III). The original papers typically report values of K^* for pH 7.4 and various bicarbonate concentrations. These experimental values of K^* have been converted to K_M values using Eqs. (6) and (7) with a value of log $K_C = 2.48$ [30], and these K_M values are listed in Table 3.

Although the K_M values describe the equilibrium between the metallotransferrin and the free M^{3+} aquo ion, the actual equilibrium in solution is between the metallotransferrin and a mixture of the $M(OH)_3$ and $M(OH)_4^-$ hydroxo complexes. The apparent strength of metal binding reflects this competition between transferrin and hydroxide. When this competition is taken

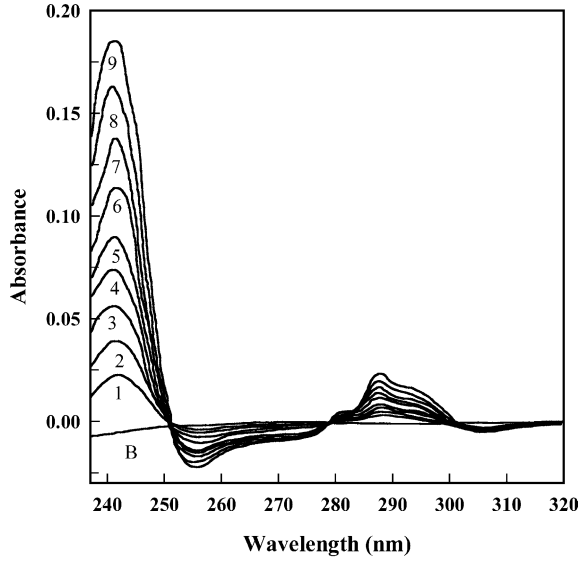


Fig. 4. Difference UV spectra produced by the addition of aliquots of Al^{3+} to apotransferrin in 0.1 M Hepes, pH 7.4, at 25 °C. Adapted from Ref. [46].

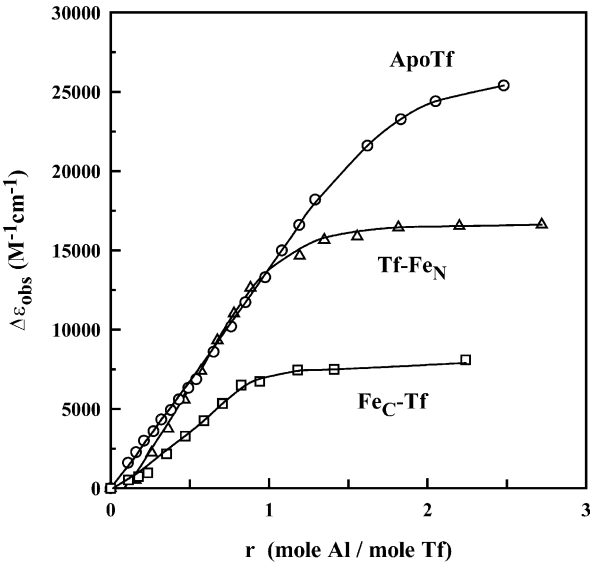


Fig. 5. Difference UV titrations curves for Al^{3+} binding to apotransferrin and to the vacant binding sites of both forms of monoferric transferrin in 0.1 M Hepes buffer, pH 7.4, at 25 °C. Adapted from Ref. [46].

into account, the order of effective binding constants is $Fe^{3+} > In^{3+} \gg Al^{3+} > Ga^{3+}$, where each > symbol corresponds to roughly 1 log unit difference in the effective binding constant at pH 7.4 [39]. This competition by hydroxide explains, for example, why Fe–Tf and In–Tf can be analyzed by gel electrophoresis [40–43], while Ga–Tf and Al–Tf dissociate during the analysis [44,45].

3.3. Al(III) binding

Al(III) binding has been investigated several different times. Harris and Sheldon used difference UV spectroscopy to follow metal binding [46]. This method follows the perturbation in the protein UV spectrum that results from metal binding to tyrosine residues by using an identical concentration of apoprotein as the reference solution. Fig. 4 shows the family of difference UV spectra generated by the addition of sequential aliquots of Al^{3+} to apotransferrin. The raw absorbance data are converted to an apparent absorptivity ($\Delta\varepsilon_{obs}$), which is independent of the protein concentration and allows a direct comparison of results from different experiments. A plot of $\Delta\varepsilon_{obs}$ versus the ratio of [Al]/[Tf] is shown in Fig. 5 for the titration of apoTf.

At low values of r where there is an excess of protein, transferrin binds essentially 100% of each aliquot of added Al^{3+}. As long as binding is complete, the plot is linear with a slope equal to the molar absorptivity ($\Delta\varepsilon_M$) of the Al–Tf complex. Fig. 5 shows that the plot is essentially linear from $r = 0$ to ca. 1.3 with a slope of 14,800 M^{-1} cm^{-1}. Above $r = 1.3$, the plot begins to curve and approaches a plateau at about 25,000 M^{-1} cm^{-1} for $r > 2.5$.

Methods have been developed to load ferric ion selectively into either the C- or N-terminal binding sites of apoTf to form monoferric transferrins [47,48]. Fig. 5 also shows titrations of both C- and N-terminal monoferric transferrin (Fe$_C$–Tf and Tf–Fe$_N$) with Al^{3+}. Iron binds so tightly that it blocks Al^{3+} binding at the occupied site and allows one to monitor binding at the remaining vacant site. The plots for the binding of Al^{3+} to apoTf and both forms of monoferric Tf have similar initial slopes, which indicates that the Al(III) complexes formed at the two transferrin binding sites have essentially the same molar absorptivity.

Given a molar absorptivity of 14,800 M^{-1} cm^{-1}, each monoferric Tf titration should level off at 14,800 M^{-1} cm^{-1}, and the apoTf titration curve should level off at 29,600 M^{-1} cm^{-1}. This result is observed only for the binding of Al^{3+} to the vacant C-terminal site of Tf–Fe$_N$. In contrast, the titration of the vacant N-terminal site levels off at a lower value of about 8000 M^{-1} cm^{-1}, which indicates that the N-terminal site does not saturate with Al^{3+}. This has been attributed to competition from the formation of the Al hydroxide complexes [46]. The titration curve for apoTf can be explained as the complete binding of Al^{3+} to the stronger C-terminal

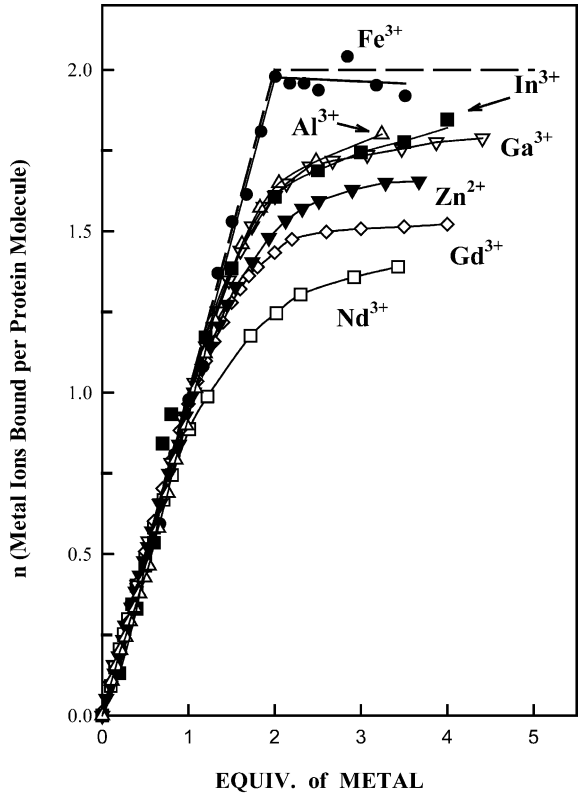


Fig. 6. Plots of n, the average number of bound metal ions, versus the equivalents of metal ion added to apotransferrin. Adapted from data in Ref. [12].

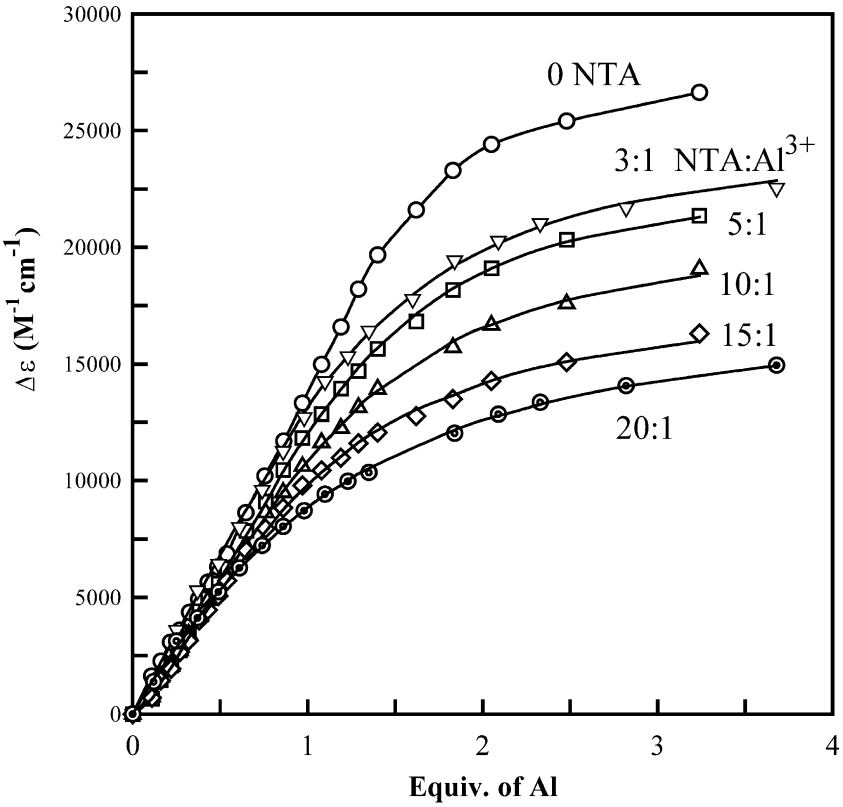


Fig. 7. Difference UV titration curves from the addition of aliquots of Al–NTA solutions to apotransferrin in 0.1 M Hepes buffer, pH 7.4, at 25 °C. The molar ratio of NTA–Al in each titrant is given in the plot. Adapted from Ref. [46].

site from $r = 0$ to 1, followed by the partial saturation of the weaker N-terminal site at $r > 1$.

The lack of saturation of both binding sites with metal ions is in fact a quite common feature of transferrin chemistry. Ga(III) binding, like that of Al(III), is limited by the formation of $Ga(OH)_4^-$ [49]. The binding of other metal ions such as Zn(II) [38,50], Cd^{2+} [51] and the lanthanides [52–54] is limited by the formation of metal–carbonate complexes. This phenomenon is illustrated in Fig. 6, in which the absorptivity data have been converted into values for n, defined as the average number of metal ions bound per transferrin molecule, and plotted as a function of the equivalents of metal ion added to the apoprotein. All the metal ions were added as the aquo ions except Fe(III) and In(III), which were added as complexes with nitrilotriacetic acid (NTA) to increase the rate of metal binding. Aluminum and gallium level off at n ca. 1.8, while the saturation levels are even lower for Zn^{2+}, Gd^{3+}, and Nd^{3+}. The indium plot also levels off at $n \sim 1.8$, although this value may have been lowered slightly by the presence of the NTA.

Al(III)–transferrin binding constants were measured by repeating the difference UV titrations of apoTf with Al(III) solutions containing different concentrations of the competitive chelating agent NTA. This produces the family of curves shown in Fig. 7. The symbols show the actual data and the lines are calculated by non-linear least squares using K_1 and K_2 as adjustable parameters. Similar experiments were conducted in the reverse direction, i.e. starting with the pre-formed Al–Tf complex and adding free NTA. The Group 13 binding constants listed in Table 3 were all measured using the same basic difference UV method.

There are several other reports of Al(III) binding constants involving different temperatures, bicarbonate concentrations, and ionic strengths [55–58]. The reported binding constants generally fall in the range 10^{12}–10^{14}. The variations in bicarbonate concentrations can account for a variation of only about 0.2 log units in the log K^* values. Martin et al. [58] conducted a careful difference UV study on Al(III) binding and reported values of $\log K_1^* = 12.2$ and $\log K_2^* = 11.6$ for 5 mM bicarbonate and 25 °C. The reason for the variation between these results and those of Harris and Sheldon [46] is not clear.

Fatemi et al. [57] also used difference UV spectroscopy to study Al(III) binding. However, the absorbance change from $r = 1$ to 2 is much larger than the change from $r = 0$ to 1, giving a sigmoidal titration curve, and there are serious problems in the methods used to calculate the binding constants. Cochran et al. [55] have reported values of $\log K_1^* = \log K_2^* = 13.72$ for 18 mM bicarbonate and 37 °C. This is the only study that

reports that the two binding sites have the same binding constant.

3.4. Ga(III), In(III), and Tl(III) binding

A wide range of Ga–Tf binding constants have been reported [49,59–61]. One distinctive feature of the Ga(III) system is that the $Ga(OH)_4^-$ is so well-behaved that one can titrate apoTf in the absence of any added chelating agent and calculate the Ga–Tf binding constant based on hydroxide as the competitive ligand. Larsen et al. [60] measured a binding constant of $\log K = 5.4$ for the stronger binding site, but these authors neglected to correct for the hydrolysis of the free Ga^{3+} ion. If this reported $\log K$ is corrected for hydrolysis, the result is $\log K_M = 19.6$, in excellent agreement with the value of 19.75 listed in Table 1.

Kulprathipanja et al. [59] have reported a Ga–Tf binding constant of $\log K = 23.7$. However, studies using linear free energy relationships for the binding of Fe(III) and Ga(III) indicate that this value is unrealistically high for transferrin [12]. One early study, which used a large excess of Ga(III), reported weak binding to 14 binding sites and should be disregarded [61].

Because of slow equilibration kinetics, the In–Tf binding constants listed in Table 3 were determined by a batchwise titration method rather than the continuous titration method used with Al(III) and Ga(III). Harris et al. [39] have reported linear free energy relationships that indicate that the In–Tf constants in Table 3 are consistent with the Fe–Tf binding constants. The In–Tf constants also conform to the linear free energy relationship reported by Li et al. [62] that uses the first hydrolysis constant of the M^{3+} ion to predict the Tf binding constants.

Two other In–Tf binding constants have been reported, but neither is considered to be reliable. Kulprathipanja et al. [59] report a value of $\log K = 30.5$ for In–Tf. This value, which is over 9 log units larger than the Fe^{3+}–Tf binding constant, is unrealistically high. Lurie et al. [63] used perturbed angular correlation (PAC) to measure a binding constant of 10^{24}, but it appears that these authors failed to correct for the degree of protonation of the competitive chelating agents used in this study. This study also reports a very large standard deviation for the In–Tf binding constant.

Difference UV titration curves for Tl(III) are linear with a sharp break at 2.0 equivalents of metal ion [64]. This is indicative of strong binding, with saturation of the two sites by the addition of a stoichiometric amount of Tl^{3+}. No binding constant for Tl(III) with transferrin has been reported, but there are methods for estimating a value. Li et al. [62] have observed a linear relationship between the first hydrolysis constant for a metal ion and its transferrin binding constant, and have estimated a Tl(III)–Tf binding constant of about 10^{22}.

Even though the formal $Tl^{3+} \rightarrow Tl^{+}$ reduction potential is quite positive, the Tl(III)–Tf complex appears to be relatively stable toward reduction [64]. This is consistent with the large Tl(III)–Tf binding constant. Complexation by Tf stabilizes the Tl(III) ion, while there is no indication that Tf binds Tl(I). Thus the reduction of Tl(III)–Tf corresponds to the reaction

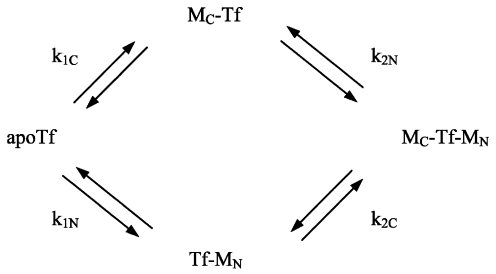
$$\text{Tl(III)–Tf} + 2e^- \leftrightarrows Tl^+ + \text{apoTf} \quad (8)$$

The selective binding of the trivalent ion makes the reduction to Tl^+ in Eq. (8) more difficult. Based on the formal Tl^{3+} reduction potential and the estimated Tl(III)–Tf binding constant, the calculated reduction potential for Eq. (8) is −0.53 V versus NHE. This is below the effective range of biological reductants [65], which explains the in vivo stability of Tl(III)–Tf. A similar stabilization by Tf binding of an otherwise unstable, higher oxidation state has been observed for Mn(III)–transferrin [66] and Ce(IV)–lactoferrin [67].

3.5. Site selectivity

The binding constants listed in Table 3 are macroscopic constants. Since there are two high-affinity binding constants, the equilibria between metal ions and transferrin involve four site-specific, microscopic binding constants defined for the equilibria shown in Scheme 1. The subscripts indicate whether the constant refers to the C- or N-terminal site and whether it is the first or second metal ion to bind to the protein.

For 2 equivalent, non-interacting binding sites, all four microscopic constants are the same. Nevertheless, statistical factors would lead to a separation of 0.6 log units between the macroscopic constants K_1^* and K_2^*. The observed $\Delta\log K$ values listed in Table 3 are in the range of 1–2, which is slightly larger than this statistical factor. One possible explanation for these larger $\Delta\log K$ values is that the sites are non-interacting, but intrinsically inequivalent, i.e. $k_{1C} = k_{2C} > k_{1N} = k_{2N}$. A second possibility is that the two sites in apoTf are equivalent, but there is negative cooperativity, so that the binding of the first metal ion to either site reduces the binding



Scheme 1.

affinity at the remaining empty site, i.e. $k_{1C} = k_{1N} > k_{2C} = k_{2N}$.

These issues have been addressed directly for the binding of Fe(III) using the Makey–Seal electrophoresis method that can quantify all four forms of transferrin shown in Scheme 1 [40,68]. Aisen et al. [40] reported that $\log k_{nC} - \log k_{nN} \sim 0.75$ for both $n = 1$ and 2. This inequivalence between the two sites accounts for most of the separation between the macroscopic binding constants. It is interesting that this inequivalence largely disappears for the C- and N-terminal transferrin half-molecules prepared by proteolytic cleavage of diferric transferrin. The isolated N-lobe has virtually the same binding affinity as the N-lobe of holotransferrin [69]. In contrast, the ferric binding constant of the C-lobe of holotransferrin is about 0.6 log units larger than the constant for the isolated C-lobe half-molecule [70]. In addition to the inequivalence of the two binding sites, there may be a small, negative cooperativity [40], which would act to slightly increase the separation between the two macroscopic binding constants.

The titrations of monoferric transferrins with Al^{3+} at pH 7.4 reported by Harris and Sheldon [46] are consistent with the data on ferric ion and indicate stronger binding to the C-terminal site. A preference for the C-terminal site has been observed in difference UV titrations of other trivalent ions such as the lanthanides [53,54] and Bi^{3+} [71], and there is clear NMR evidence that Tl^{3+} binds preferentially to the C-terminal lobe with carbonate as the synergistic anion [64,72]. There are no difference UV data on the site selectivity for Ga^{3+} or In^{3+}, but it seems likely that these metal ions would also show a preference for the C-terminal site.

New methodologies have been developed over the last few years that appear to be able to separate and quantify the monoaluminum forms of Tf, so that one can analyze a sample for the concentrations of Al_2–Tf, Al_C–Tf, Tf–Al_N, and apoTf [73–76]. de Jong et al. [76] report that at pH 8, aluminum (as unchelated Al^{3+}) binds preferentially to the C-terminal site in the presence of bicarbonate as the synergistic anion. Interestingly, the Al^{3+} binds preferentially to the N-terminal site in the presence of oxalate. de Jong et al. [76] also report that Al(III)-binding is actually stronger in the presence of oxalate than it is in the presence of bicarbonate. They have posed the question of whether an Al–oxalate–Tf species might be important in vivo.

There are conflicting reports on cooperativity between the two transferrin binding sites. Nagaoka and Maitani [77] suggest that loading Fe(III) into the C-terminal site increases the binding affinity of Fe(III) at the N-terminal site, while Van Landeghem et al. [75] report that loading Fe(III) into the C-terminal lobe decreases the binding affinity of Al(III) at the N-terminal lobe [75]. This issue deserves further attention in order to understand the speciation of Al(III) in serum.

The addition of Al(III)–citrate to apoTf leads to preferential loading of the N-terminal site [74], just as does the addition of Fe(III)–citrate [40,77]. There are also reports that the Al(III) in serum appears to be localized primarily in the N-terminal lobe [74], which would be consistent with loading of the protein in vivo by Al(III)–citrate. These results suggest that under certain conditions, the sequences in which the two lobes bind metal ions are under kinetic control. The sequence of metal binding to the two sites under various conditions is discussed further in Section 5.

3.6. Effect of ionic radius

There have been suggestions that there might be steric restrictions on the binding of larger metal ions. Based on the diminished peak heights in the difference UV spectra, Luk [78] concluded that Tf binds only a single metal ion of the larger lanthanides. Based on similar difference UV data, Harris et al. [79] suggested that Th(IV) could bind two tyrosine residues in the C-terminal site but only one at the N-terminal site. Both these studies made the erroneous assumption that the plateau in the difference UV spectrum reflected saturation of the available protein binding sites. As discussed above, the lack of saturation of both binding sites with metal ions is in fact a quite common feature of transferrin chemistry [38,39,46,50–54]. Harris et al. [54] have recently studied the issue of steric restrictions in some detail. Metal ions at least as large as Nd(III) (radius = 1.109 Å) clearly bind to both transferrin sites. However, linear–free-energy relationships indicate that there is a detectable decrease in the binding affinity at the N-lobe site for metal ions larger than Tb(III) (radius = 1.04 Å). The crystal structure of Sm_2–lactoferrin shows that there is a greater disruption of hydrogen bonding to the synergistic carbonate in the N-lobe [80]. Baker et al. [81] recently reported the crystal structure of dicerric transferrin, which shows that the Ce(IV) ion (radius = 0.87 Å) binds in the same coordination environment as Fe(III) in both the C- and N-terminal sites with little change in the overall protein structure. There do not appear to be any steric restrictions that would affect the trivalent Group 13 elements.

4. Kinetics

4.1. Metal binding kinetics

Water exchange rates are often used to represent the general lability of a metal ion for ligand exchange reactions in aqueous solutions. The reported rate constants for water exchange fall in the order $Al^{3+} <$

$Fe^{3+} < Ga^{3+} < In^{3+}$ [82]. No value was reported for Tl^{3+}, but based on the trends in the periodic table, one would expect that Tl^{3+} would have the fastest exchange rate. Qualitatively, the rates at which metal ions react with apoTf in neutral solution do not correlate very well with the water exchange rates. The Ga^{3+} and Al^{3+} ions react relatively rapidly, while the Fe^{3+} and In^{3+} ions react more slowly. The rates appear to be more closely related to the hydrolytic behavior of the metal ions. The unchelated ferric ion rapidly hydrolyzes and polymerizes, which leads to very slow iron donation from colloidal ferric hydroxide to apoTf [83]. It has long been common practice to add ferric ion as a metal chelate, often with NTA. This ligand can load the two transferrin binding sites with Fe(III) within a few seconds [84].

Like ferric ion, In(III) exists at neutral pH almost exclusively as the sparingly soluble $In(OH)_3$ species. The binding of unchelated In(III) to apoTf is also relatively slow, requiring 1–2 h to reach completion [85]. In contrast to the iron system, the use of NTA does not result in rapid binding of In(III). Several hours are required for equilibration between In–NTA solutions and apoTf [39].

Unlike In^{3+} and Fe^{3+}, Ga^{3+} is relatively soluble at neutral pH due to the formation of the $Ga(OH)_4^-$ anion. The addition of the unchelated Ga^{3+} ion to apoTf leads to relatively rapid and reversible binding to the protein. Nevertheless, it appears that the kinetics of Ga^{3+}-binding to transferrin can still be affected by hydrolytic polymerization. Transferrin binding is complete within 10–40 min following the addition of substoichiometric aliquots of Ga^{3+} [49]. When higher concentrations of gallium are added, the rate of protein binding slows down [49,86,87].

Because of the widespread pharmaceutical use of radioisotopes of Ga(III) and In(III), there are numerous studies on the binding of trace amounts of these ions to transferrin in serum. Under these conditions, transferrin binding is complete within a few minutes for both Ga(III) [22,88–91] and In(III) [92,93]. The more rapid binding is presumably due to a combination of a very low metal ion concentration and the presence of serum chelating agents such as citric acid. Both these factors reduce hydrolytic polymerization and allow the protein to react rapidly with monomeric metal species.

The rate of transferrin binding to Al^{3+} appears to be similar to the rate for Ga^{3+}. One can readily add unchelated Al^{3+} to apoTf and observe binding within a few minutes [46]. Since the water exchange rate for Ga^{3+} is two orders of magnitude greater than that for Al^{3+} [94], the qualitative similarity in the rate of transferrin binding is consistent with the model in which the reaction is controlled primarily by the hydrolytic properties of the metal ion.

4.2. Metal release kinetics

The release of ferric ion from transferrin has been studied in some detail [95–107]. In the presence of a large excess of ligand, the reaction is pseudo first-order with respect to the ferric–transferrin concentration. The variation in the apparent first-order rate constant as a function of the ligand concentration can be described by the equation

$$k_{obs} = \frac{k_{max}[L]}{k_d + [L]} + k'[L] \quad (9)$$

The form of Eq. (9) suggests that there are two parallel pathways for metal removal. The first term represents a saturation pathway, while the second term represents a first-order pathway. The saturation pathway appears to be governed in large part by a gating conformational change in the protein [98,105,106]. For many ligands, especially catechols [86,104,108,109], hydroxypyridones [86,106,110,111] and hydroxamates [98,106,112], $k' = 0$ and the systems follow simple saturation kinetics. For pyrophosphate and phosphonic acids [48,95–97,100,111], as well as some aminocarboxylic acids [96,100,111], k' is large enough that a significant fraction of the iron is removed via the first-order pathway.

There are relatively few kinetic studies on the release of metal ions other than Fe(III) from transferrin. Li et al. [111] studied the release of Al(III) from the recombinant N-lobe half-molecule of Tf by pyrophosphate. The apparent first-order rate constants for Al(III) as a function of the pyrophosphate concentration are shown

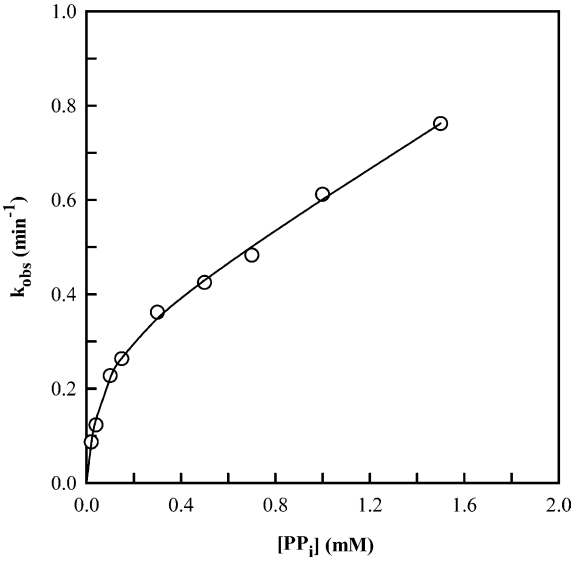


Fig. 8. Values of k_{obs}, the apparent first-order rate constant for the removal of Al^{3+} from the N-lobe transferrin half-molecule by pyrophosphate, plotted as a function of the solution pyrophosphate concentration. The rate constants were measured in 0.1 M Hepes buffer, pH 7.4, at 25 °C. Adapted from Ref. [111].

Table 4
Kinetic parameters for the release of Fe^{3+} and Al^{3+} from the N-lobe of transferrin by pyrophosphate [a]

	k_{max} (min^{-1})	k_d (mM)	k' (M^{-1} min^{-1})
Al^{3+}	0.31	0.061	311
Fe^{3+}	0.051	16.9	0.25

[a] Values from Ref. [111].

in Fig. 8. The curve for Al(III) release has the same basic shape as the corresponding curve for Fe(III). The data in Fig. 8 have been fit to Eq. (9), and the kinetic parameters for both Fe(III) and Al(III) are listed in Table 4. There is a modest difference in k_{max} values between Fe(III) and Al(III), but there are much greater differences in both k_d and k'. For the Al(III) ion, the reaction reaches saturation at much lower ligand concentrations, and the k' for Al(III) removal by pyrophosphate is over 1000-times greater than the k' for Fe(III). For 50 mM PP_i the overall rate of Al(III) removal through both pathways is about 200-times faster than the rate of Fe(III) removal.

Marques and coworkers [113,114] have observed a similar ratio between the rates for Al(III) and Fe(III) release from native transferrin by citrate, although they interpret the kinetic parameters in Eq. (9) differently. Turcot et al. [86] compared the release of Ga(III) and Fe(III) by hexadentate catechol and hydroxypyridone ligands. They observed essentially no difference in the k_{max} values for Fe(III) and Ga(III) and a much smaller k_d for Ga(III). It is difficult to draw general conclusions from limited data. Nevertheless, it appears that the conformational change in the protein that is rate-limiting for the saturation pathway is relatively independent of the metal ion, but that Al(III) and Ga(III) are lost much more rapidly than Fe(III) after the protein has adopted its 'open' conformation.

5. NMR studies

NMR spectroscopy is extremely powerful for investigating the solution chemistry of proteins and has been, so far, largely used to analyze apotransferrin and its metallosubstituted derivatives. Unfortunately transferrin, with a molecular weight of ca. 80 kDa, is too big for complete structural determination in solution by current multidimensional NMR techniques [115]. In particular, ^{1}H-NMR studies of transferrins are hampered by the large natural signal linewidths, related to slow molecular tumbling, which make individual resonances difficult to resolve [116]. In spite of this, ^{1}H-NMR spectroscopy has been successfully employed to highlight some features of the solution chemistry of transferrins [71,116–125]. To reduce the mass of the system and improve the quality of the ^{1}H-NMR spectra, some researchers preferred working on the purified N-terminal fragment of the protein, with a mass of ca. 40 kDa, rather than on the entire protein [117,119]. Specific information on the residues located near to the metal binding sites was obtained by ^{1}H-NMR studies of transferrins loaded with paramagnetic probes such as cobalt(II) and lanthanides [126–129]. In fact, the hyperfine shift permits extraction of the ^{1}H-NMR signals close to the paramagnetic center out of the envelope of the diamagnetic signals. Alternatively, ^{13}C-NMR spectroscopy has been used to monitor the behavior of the ^{13}C enriched synergistic anion, which is tightly bound in the metal–carbonate–protein ternary complex [85,130–136]. Finally, some authors exploited multinuclear NMR techniques (namely ^{27}Al- and ^{205}Tl-NMR) to monitor directly the metals in their specific binding sites and to reveal even very slight intersite differences [64,72,137–142]. Below, the information obtained through the years by the NMR approach on transferrin derivatives of Group 13 elements is reviewed.

5.1. ^{1}H-NMR spectroscopy

As mentioned above, transferrin is too large for complete structural determination in solution by NMR: ^{1}H-NMR lines are intrinsically broad so that it is extremely difficult to resolve the individual resonances in crowded spectral regions. In spite of this, mainly owing to the substantial contributions of the groups of Woodworth and Sadler, valuable information has been gained on the solution behavior of transferrins and their interactions with Group 13 cations, by application of ^{1}H-NMR spectroscopy. Apart from pioneering studies in collaboration with Williams dating to the early 70s [118], Woodworth and coworkers published, in 1987, an interesting ^{1}H-NMR study on the binding of Ga(III) to the proteolytically derived N-terminal half-molecule of transferrin [119]. They noticed that the pH dependent titration curves of the histidinyl C2 proton chemical shifts are significantly altered upon formation of the ternary gallium–oxalate–transferrin complex. Specifically, two high pK_a histidines failed to titrate when the metal and the synergistic anion formed the ternary complex, suggesting involvement of these residues in the metal binding sites [119].

Later on Sadler and coworkers, with the help of specific resolution enhancement techniques, were able to follow sequential binding of Al(III) to the N and C lobes of apoTf at pH 8.8 [116,120]. Indeed, several resonances could be resolved, in both the aliphatic and aromatic regions of the 500 MHz ^{1}H-NMR spectra of apotransferrin, when enhanced by combined application of exponential and sine bell functions to the free induction decay. This procedure removes broad resonances from the ^{1}H-NMR spectrum leaving sharp peaks from protons located in the most mobile regions of the protein. Apart from peaks assignable to glycan *N*-acetyl and

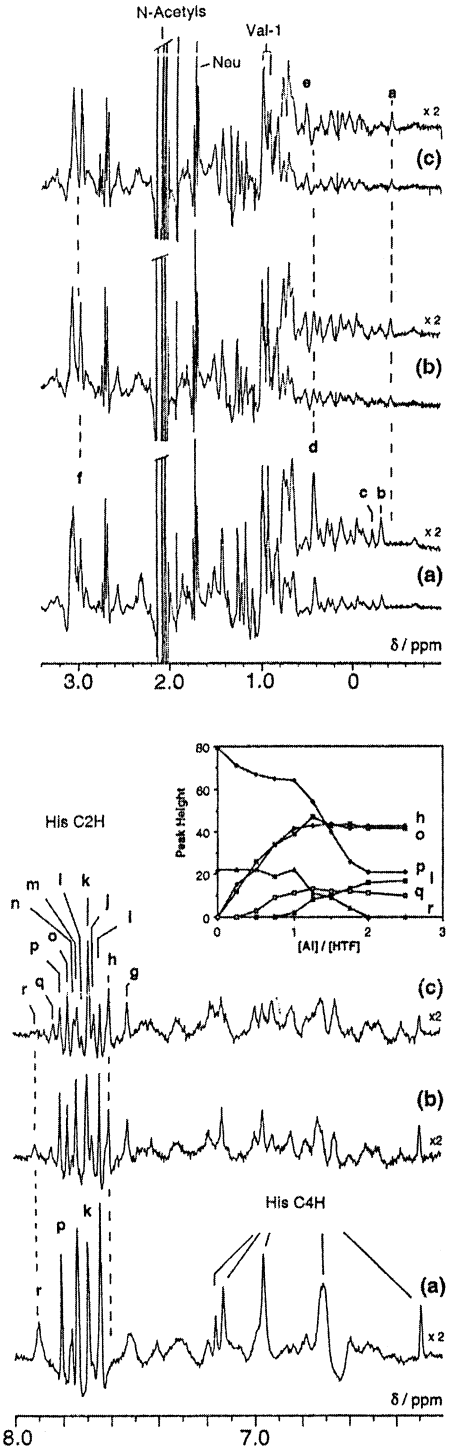


Fig. 9. Resolution enhanced 500 MHz ^{1}H-NMR spectra of 0.56 mM apoTf in 0.1 M $NaDCO_3$ pH 8.8. (A) Aliphatic region; and (B) aromatic region: (a) before; and (b) after addition of 1.25; and (c) 2.5 molar equivalents of Al^{3+}. The intensities of His C2H peaks versus the Al/apoTf molar ratio are plotted in the inset. Adapted from Ref. [116].

sugar ring protons, some high field shifted resonances were observed which are likely to arise from methyls close to the faces of aromatic side chains as well as a number of peaks in the aromatic region assignable to His C2H protons. Notably some of these signals undergo peculiar changes following addition of increasing amounts of Al(III) (see the spectra reported in Fig. 9). Detailed analysis of the ^{1}H-NMR spectral changes upon metal titration allowed these authors to propose that Al(III), at pH 8.8, first binds to the N-terminal site, then to the C-terminal site, in an order that is opposite to that of iron(III) ions at pH 7.4 [116,120]. Complete reversal of the ^{1}H-NMR spectral changes was observed by addition of desferrioxamine (DFO), a strong chelating agent for Al(III).

In a subsequent paper Sadler and coworkers addressed, again, binding of the synergistic anion oxalate and of Ga(III) to human serum transferrin and to its recombinant N-lobe at pH 7.25 [117]. They found that specific protein resonances are sensitive to oxalate binding under fast exchange conditions on the NMR time scale; at variance, slow exchange between apoTf and Ga(III)-loaded transferrin was observed, as expected. Binding of Ga(III) is accompanied by small changes in the orientations of some residues in the interdomain hinge region close to the metal binding site. Preferential binding of Ga(III) to the C-terminal site was shown. Notably, Ga(III)-binding to the C-lobe markedly perturbed resonances in the glycan *N*-acetyl region suggesting that metal-binding is somehow communicated to the surface of the protein. In addition, it was reported that Ga(III) is slowly displaced by Fe(III), with half life ca. 4.5 h: the paramagnetic broadening effects induced by Fe(III) allowed identification of resonances belonging to residues that are close to the metal sites. Some representative ^{1}H-NMR spectra of the Ga(III) transferrin system are reported in Fig. 10.

In 1995, Battistuzzi et al. published a study where the 600 MHz ^{1}H-NMR spectra of Al(III), Ga(III) and In(III) transferrin were reported and compared. Analysis of some specific spectral regions (namely 8.2/7.5 and 0/−0.7 ppm) pointed out a substantial ^{1}H-NMR similarity among these Group 13 metallotransferrins; at variance significant differences with apo and zinc(II) transferrin were observed [85].

Later on, important ^{1}H-NMR results were obtained jointly by the groups of Sadler and Woodworth. Indeed, concomitant application of heteronuclear multidimensional NMR and site directed mutagenesis led to the specific assignments of the five methionine residues in the transferrin N-lobe and of all nine methionines in the full protein [71,122–124]. Representative 2D [^{1}H,^{13}C] HMQC spectra of ^{13}C methionine labeled transferrin samples are shown in Fig. 11. By using methionine $^{13}CH_3$ resonances as probes, it was shown that Ga(III) binds preferentially to the C-lobe and subsequently to the N-lobe when oxalate is the synergistic anion. The ^{1}H-NMR shifts of Met 464, which is in the hydrophobic patch of helix 5 (C-lobe), in contact with the anion and

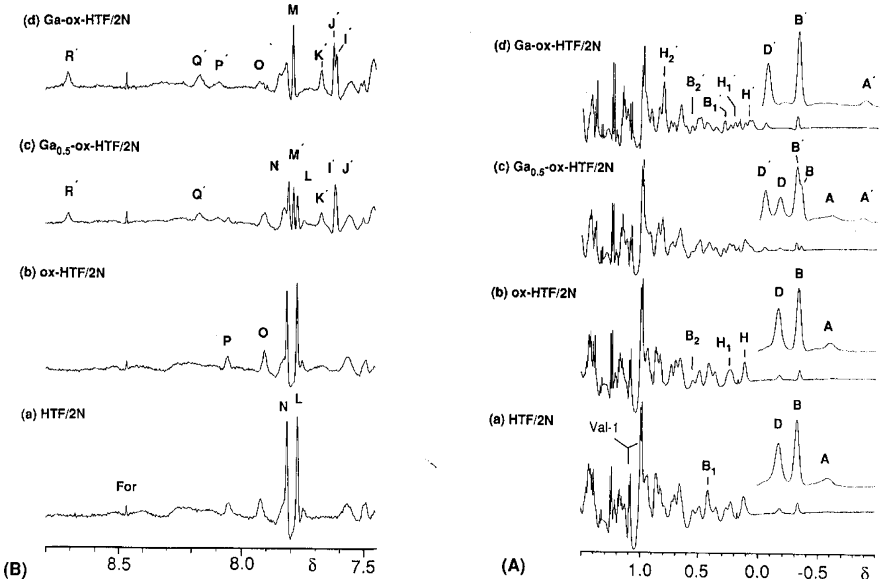


Fig. 10. Resolution enhanced 500 MHz ^{1}H-NMR spectra of: (A) the high field region; and (B) the histidine C2H region of the N-terminal lobe of apoTf and its derivatives with Ga(III). Adapted from Ref. [117].

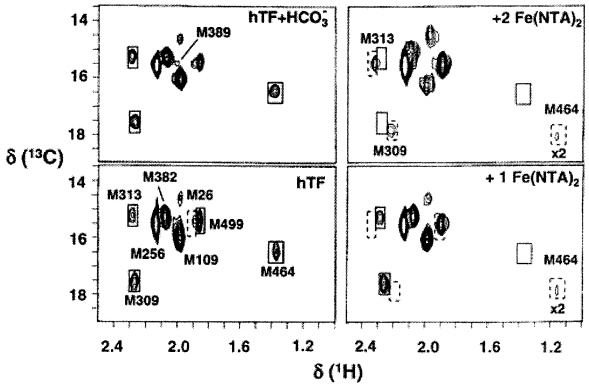


Fig. 11. 2D [^{1}H,^{13}C] HMQC spectra of ε-[^{13}C] Met–apoTf and after addition of 10 mM bicarbonate at pH 7.4, and 1.0 and 2.0 molar equivalents of Fe^{3+} as the NTA complex. Assignments of Met residues are reported. Adapted from Ref. [123].

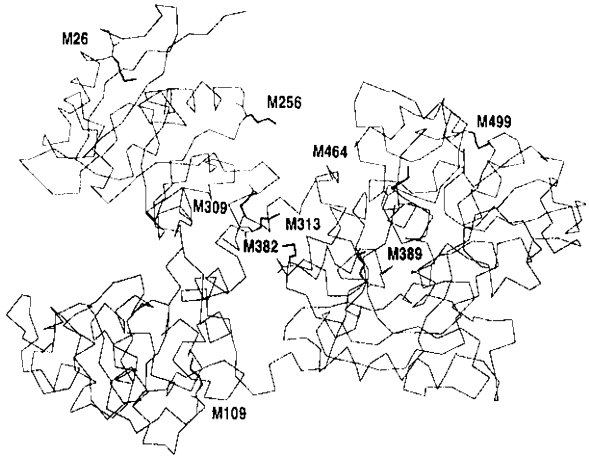


Fig. 12. Distribution of the nine Met residues in human serum transferrin based on X-ray crystal structure data (Ref. [123]).

the metal binding site, show that Ga(III) binding causes appreciable movements within this helix.

The C-lobe residue Met 382, which contacts the N-lobe hinge region, is perturbed when Ga(III) binds to the N-lobe, indicative of interlobe communication. This finding may be relevant in relation to recognition of fully metallated transferrin by its receptor. The availability of high-resolution crystallographic data of serum transferrin has been of great help for these ^{1}H-NMR studies. Remarkably, methionines are well distributed through the entire three-dimensional structure of transferrin implying that these assigned Met residues are good probes for the overall conformation of the protein in solution (Fig. 12).

In a recent study by He et al., the previously identified and assigned Met residues were monitored by ^{1}H-NMR spectroscopy to reveal conformational changes induced by metal binding to the N-terminal fragment. It was found that six different metal ions, namely Fe(III), Cu(II), Cr(III), Co(III), Ga(III) and In(III), produce essentially the same pattern of spectral changes implying that, in all cases, the protein undergoes a similar metal-dependent conformational rearrangement [124].

Moreover, the assignment of the methionine residues has been used to determine the order of lobe loading by Fe(III), Ga(III), Al(III) and bismuth(III) [123]; in particular Met 464 turned out to be particularly important to monitor the C-terminal site. In the case of Fe(III), preferential binding to the C-terminal lobe, at pH 7, was demonstrated, as expected. For Ga(III) no clear preference for either lobe was detected. Under the experimental conditions used in this study Al(III) was

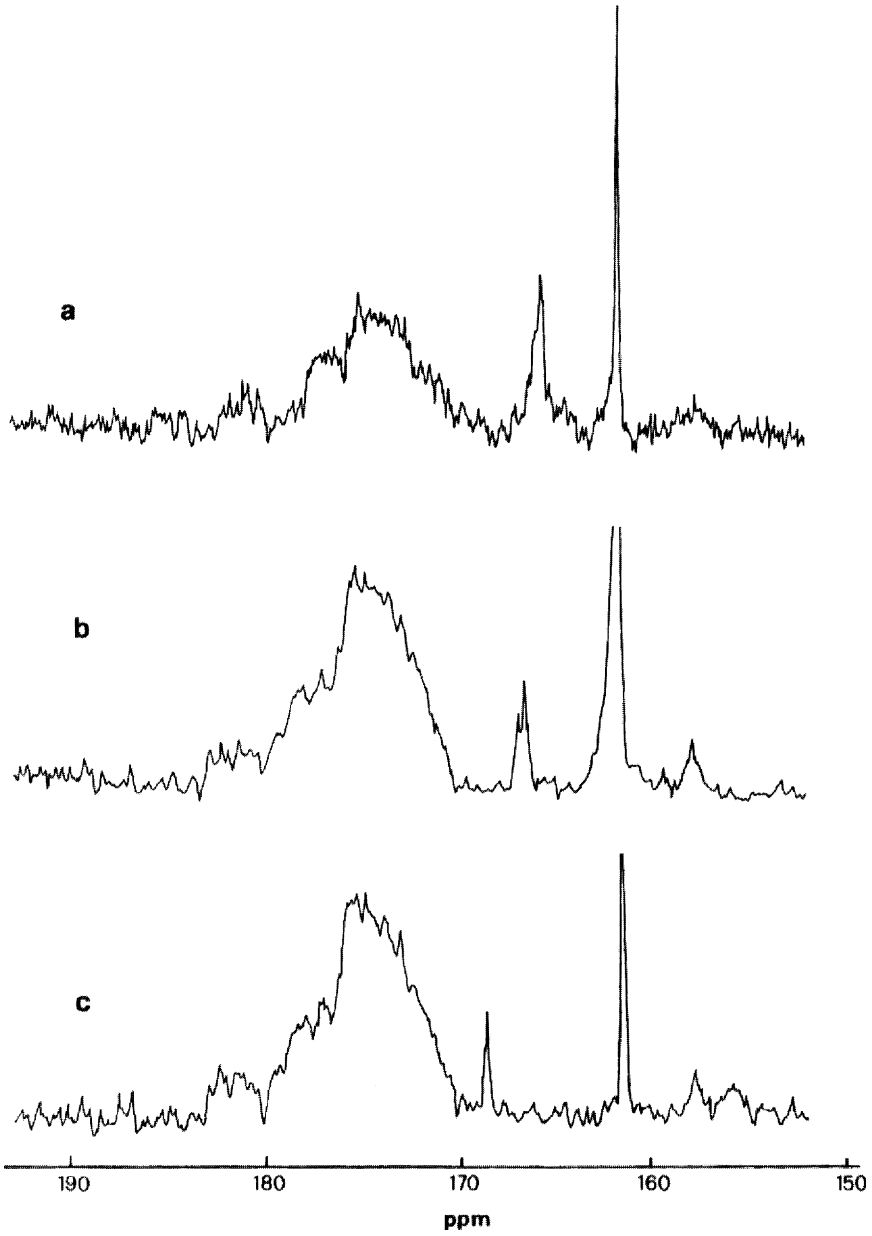


Fig. 13. 75.4 MHz ^{13}C-NMR spectra of the carbonyl region of various Tf–carbonate–metal complexes: (a) Al_2Tf; (b) Ga_2Tf; (c) Zn_2Tf. Adapted from Ref. [133].

shown not to bind apotransferrin at pH 7; in contrast, at pH 8.8, preferential loading of Al(III) to the N-terminal site was demonstrated, as previously suggested.

5.2. *^{13}C-NMR spectroscopy*

Monodimensional ^{13}C-NMR spectroscopy has been largely employed to gain specific information on the binding of the synergistic anion to the protein in the case of various metallotransferrins. When the bound metal is paramagnetic, the ^{13}C signal of the bound synergistic anion is broadened beyond detection [130,131]. In contrast, in the case of diamagnetic metallotransferrins, the ^{13}C-NMR signal is easily observed and direct information on the synergistic anion is drawn. ^{13}C-NMR studies of the synergistic anion in diamagnetic metallotransferrins were carried out on several derivatives. Specifically, Co(III) [132], Al(III) [133], Zn(II) [133], cadmium(II) [134], Ga(III) [133], In(III) [85], Tl(III) [137], titanium(IV) [135,136], scandium(III) [141], and Bi(III) [125] transferrins were investigated. Some representative ^{13}C-NMR spectra are shown in Fig. 13.

In most cases a single resonance for bound bicarbonate was detected. Detection of a signal for ^{13}C enriched protein-bound bicarbonate, well distinct from that of

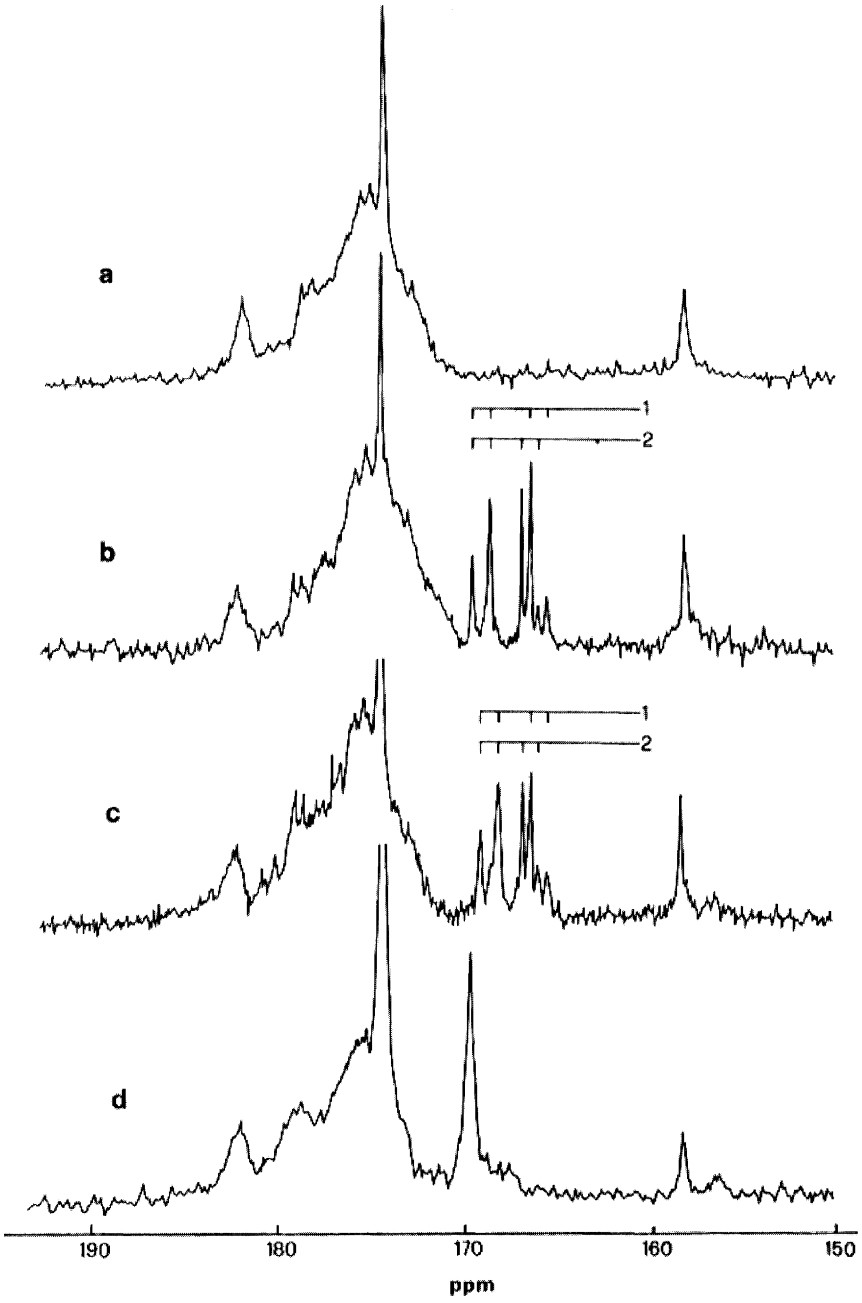


Fig. 14. 75.4 MHz ^{13}C-NMR spectra of the carbonyl region of various ovotransferrin–oxalate–metal complexes. (a) apoOTf; (b) Al_2Otf; (c) Ga_2Otf; (d) Zn_2Otf. Adapted from Ref. [133].

free bicarbonate, points out that the exchange process between the free and the bound species is slow on the NMR time scale. The ^{13}C-NMR chemical shift of the bound anion is more similar to carbonate than bicarbonate implying that the bound anion exists in a carbonate-like form; notably, there is a clear correlation between the chemical shift value and the charge of the bound metal [133]. In a few cases some small differences between carbonate loaded in the C-terminal site and carbonate loaded in the N-terminal site could be detected suggesting the presence of slight intersite differences [137,139].

Additional studies were carried out on ternary complexes of transferrins with Al(III) and ^{13}C enriched oxalate. Representative ^{13}C-NMR spectra of the aluminum–oxalate–ovotransferrin system are shown in Fig. 14. The complicate pattern of the observed ^{13}C-NMR signals suggested monodentate binding of oxalate to the metal and the existence of minor intersite differences [133]. Similar results were obtained by

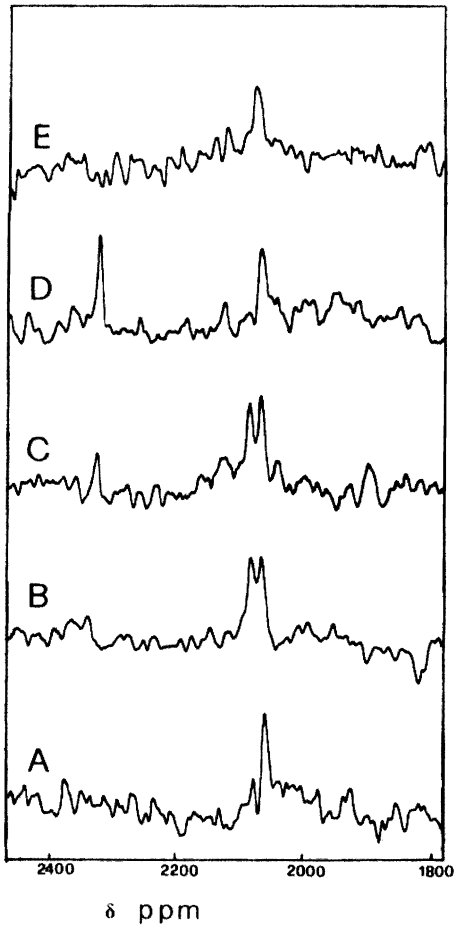


Fig. 15. ^{205}Tl-NMR spectra of various Tl(III) transferrin derivatives: (A) TlTf, pH 7.2; (B) Tl_2Tf, pH 7.2; (C) Tl_2Tf, pH 8.5; (D) Tl_2Tf brought to pH 6.4; (E) Tl_2Tf , pH 8.5 after addition of 1 equivalent Fe(III). Adapted from Ref. [64].

Aramini et al. on the oxalate derivatives of thallium transferrins [72] (vide infra).

5.3. Direct NMR studies of the bound metal

5.3.1. ^{205}Tl-NMR

The first ^{205}Tl-NMR study of human serum transferrin was reported in the early 80s [64]. The ^{205}Tl nucleus has nuclear spin 1/2 and is characterized by an excellent NMR sensitivity. Thus ^{205}Tl-NMR signals for protein-bound Tl(III) ions could be observed even at millimolar concentrations. ^{205}Tl-NMR studies clearly revealed two distinct resonances, of similar shape, for Tl(III) bound to apotransferrin in the $Tl(III)_2Tf$ derivative at physiological pH (Fig. 15). The two signals are relatively broad and separated by about 20 ppm on the very large ^{205}Tl chemical shift scale. This means that ^{205}Tl-NMR spectroscopy permits easy discrimination of the metal binding sites of transferrins, and that small but detectable intersite differences do exist.

Notably, the two ^{205}Tl-NMR signals were shown to exhibit a different pattern of pH dependence. The high field signal turned out to be more resistant to acidification than the low field signal. Moreover, at pH 7.4, Tl(III) binding to the two sites is sequential, and the high field signal appears first. By analogy with the behavior of Fe(III), the high field signal was assigned to Tl(III) bound to the acid-resistant C-terminal site and the low field signal to Tl(III) bound to the N-terminal site. This assignment was confirmed by additional experimental evidence including displacement experiments by Fe(III). A subsequent study by the same authors on the $Tl^{13}CO_3Tf$ derivative provided direct evidence of carbonate coordination to the metal, through the observation of ^{205}Tl–^{13}C magnetic coupling, as shown in Fig. 16 [137].

Later on, Aramini and Vogel reconsidered binding of Tl(III) to human serotransferrin and chicken ovotransferrin, in the presence of carbonate and oxalate, by use of ^{205}Tl- and ^{13}C-NMR spectroscopies [72]. Again, with carbonate as the synergistic anion, these authors observed two distinct ^{205}Tl-NMR signals for $Tl(III)_2Tf$. When the same adduct was prepared with ^{13}C-labeled carbonate, two closely spaced doublets in the carbonyl region of the ^{13}C-NMR spectrum of serotransferrin were observed; these signals correspond to the labeled anion directly bound to the metal ion in both sites of the protein. The analogous resonances in ovotransferrin are completely degenerate, and only one doublet is detected. The magnitudes of the spin–spin coupling between the bound metal ion and carbonate—$2J(^{205}Tl–^{13}C)$—range from ca. 270 to 290 Hz. Use of the proteolytic half-molecules of ovotransferrin and of the recombinant N-terminal half-molecule of serotransferrin led to unambiguous site specific assignment of the ^{205}Tl-and ^{13}C-NMR signals in both proteins. Titration studies showed that Tl(III) is bound with a greater affinity to the C-terminal site of serotransferrin, whereas no site preference was noted for ovotransferrin. When oxalate is used as the anion, instead of carbonate, the ^{205}Tl-NMR signals arising from the bound metal ion in the sites of ovotransferrin are shifted downfield and become almost degenerate. As mentioned above, a very complex pattern of resonances was observed for bound $^{13}C_2O_4^{2-}$ in the ^{13}C-NMR spectra of both proteins, in agreement with monodentate binding of oxalate to the metal [72].

5.3.2. ^{27}Al-NMR

In the early 90s Vogel and Aramini reported a series of ^{27}Al-NMR studies that demonstrated the feasibility of detecting quadrupolar metal ions bound tightly to rather large proteins via the quadrupolar central transition NMR approach [138–140]. Several interesting properties of ^{27}Al-NMR signals of transferrin-bound Al(III) were documented, such as their dependence on temperature, viscosity and molecular size. It was shown

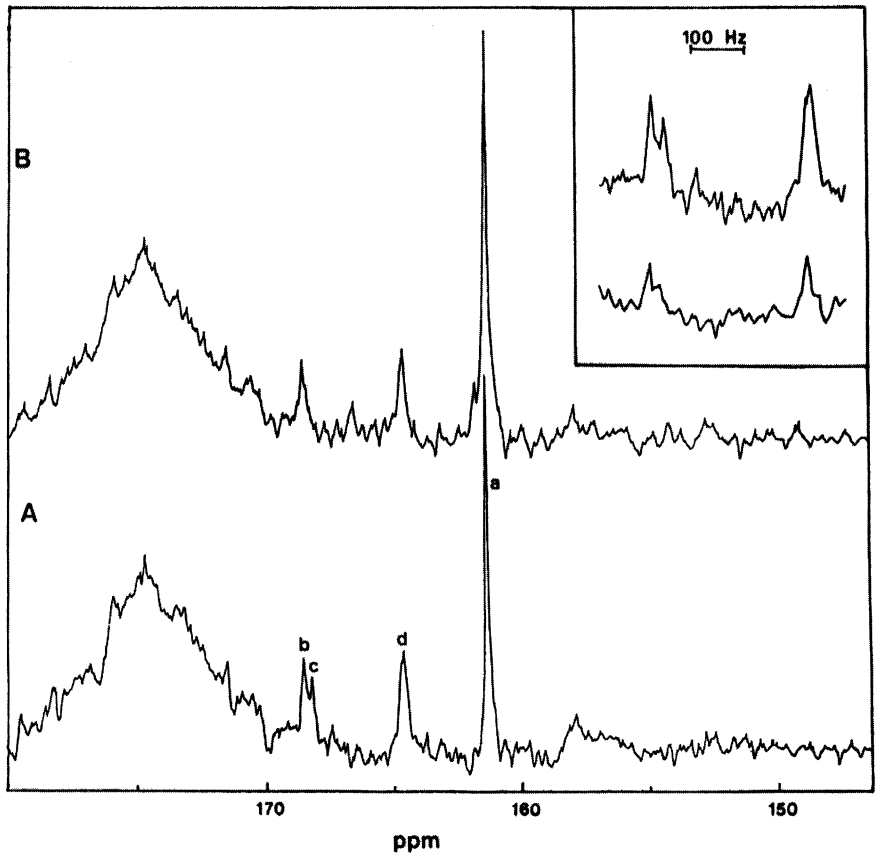


Fig. 16. Proton decoupled 75.4 MHz ^{13}C-NMR spectra of the carbonyl region of Tl(III) transferrin derivatives in the presence of a twofold excess of ^{13}C enriched sodium bicarbonate; Tris buffer, pH 8.3. (A) Tl_2Tf; (B) Fe_CTl_N Tf. The inset shows the detail of the signals typical of the bound anion. Adapted from Ref. [137].

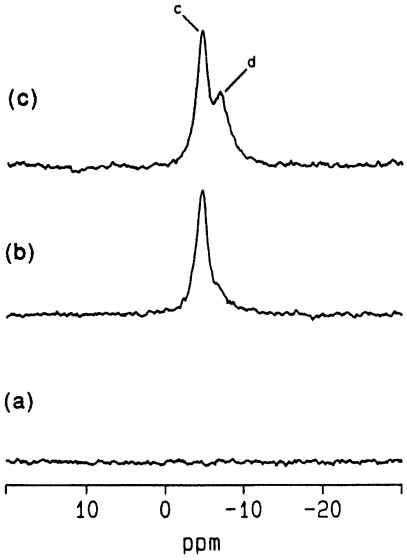


Fig. 17. ^{27}Al-NMR spectra (104.3 MHz) of 1.13 mM ovotransferrin in the presence of sodium carbonate and various amounts of Al^{3+}: (a) 0; (b) 1.0; (c) 2.0 equivalents Adapted from Ref. [139].

that both decreasing temperature and increasing viscosity produced a significant decrease in signal linewidth. These effects are in accord with quadrupolar relaxation theory that predicts that the linewidth of the central transition of a half-integer quadrupolar nucleus should decrease with increasing correlation time of the protein under non-extreme narrowing conditions. Specific information on the chemistry of Al(III)–transferrin was derived; remarkably the two sites are slightly different in terms of ^{27}Al-NMR chemical shift and signal shape. Analogous studies, carried out by the same authors on the Al(III) derivatives of ovotransferrin, led to similar results. Representative ^{27}Al-NMR spectra of the Al(III)–ovotransferrin system are shown in Fig. 17.

6. Other physicochemical methods

Other methods, beyond NMR, have been fruitfully applied to monitor binding of Group 13 cations to transferrins and to reveal the associated conformational changes. Interesting results have been obtained from circular dichroism (CD), from fluorescence spectroscopy, from small angle X-ray scattering (SAXS) and from PAC spectroscopy.

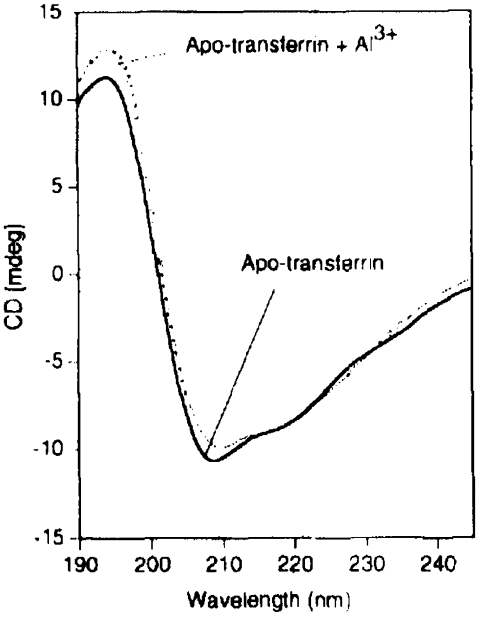


Fig. 18. CD spectra of apotransferrin in the far UV region in the absence and presence of Al^{3+}, sodium bicarbonate 1 mM, pH 7.4. Adapted from Ref. [148].

6.1. Circular dichroism

The CD technique is particularly suited to monitor the conformational state of proteins in solution; specifically CD provides direct information on the secondary structure of proteins [143]. By using the method described by Woody et al., the percentage of α *helical* and β *sheet* structure in proteins can be quantitated with a certain accuracy by analysis of the CD spectra in the UV [144]. In the early 70s a few studies were published reporting CD investigations of transferrins [145,146]. Much interest was directed to the analysis of CD effects related to metal binding, both in the so called *intrinsic* region (190–245 m), diagnostic of the protein secondary structure, and in the *aromatic* region (245–320 nm). Generally no large changes were detected upon metal binding, implying that domain closure around the metal does not bring about significant modifications in the secondary structure.

Later on, Tang et al. noticed that Al(III) causes some significant CD spectral modifications in the intrinsic region, consistent with a small decrease of alpha helix content (Fig. 18) [93]. Battistuzzi et al. analysed the CD spectral changes in the aromatic region, induced by addition of Ga(III), Al(III) and In(III), and observed that the effects of In(III) are somewhat different from those induced by the other cations. In principle, these changes may be related to the existence of minor conformational differences among these metallotransferrins [85].

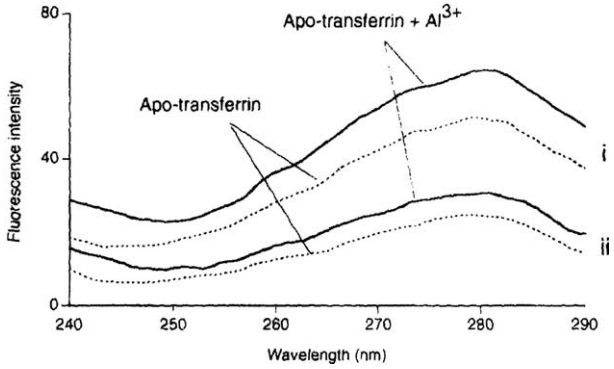


Fig. 19. Fluorescence emission spectra of (a) apotransferrin in the presence and absence of Al(III). Samples were excited at 280 and 295 nm, respectively. Adapted from Ref. [148].

6.2. Fluorescence spectroscopy

Fluorescence studies of proteins monitor directly the environment of tryptophan (Trp) residues buried inside the protein [147], although important contributions may also originate from tyrosine (Tyr) residues. Transferrin has a limited number of Trp residues so that its fluorescence spectra can be interpreted more easily. Notably, metal binding produces large modifications of the fluorescence spectra of transferrin. Tang et al. analyzed the changes of the fluorescence spectrum of apotransferrin caused by addition of Al(III) [148]. A large change in tryptophan fluorescence, with a maximum at 330 nm, was observed following Al(III) binding to apotransferrin, by excitation at either 280 or 295 nm, as shown in Fig. 19. There was no shift in wavelength of the fluorescence band of apotransferrin, but the intensity did increase significantly. Since excitation at 295 nm is specific for tryptophan residues, tryptophans—but not tyrosines—must be responsible for these spectral modifications. At variance a decreased fluorescence is the result of Fe(III) binding to apotransferrin, possibly due to energy transfer effects. Very recently a paper that describes the effects of a series of selective Trp mutations in the N-lobe on the fluorescence spectra has appeared [149]; these interesting results will probably prompt further fluorescence studies of transferrins and of the effects of metal binding.

6.3. Small angle X-ray scattering

Valuable information has been obtained by application of SAXS to transferrins [150–152]. This technique specifically monitors the overall conformation of the protein in solution and provides information on protein shape and size. In a first series of experiments Grossmann et al. used X-ray solution scattering to analyze the structural changes that take place following uptake or

release of Fe(III) from serum and chicken ovotransferrin and from human lactoferrin [150]. In the case of chicken ovotransferrin, data were obtained for both the intact protein and the isolated N- and C-lobes, with and without Fe(III). These studies revealed that both lobes undergo a change that is consistent with an opening of the inter-domain cleft when Fe(III) is removed from the protein. It was suggested that the conformational change of the protein increases the specificity of receptor binding and that the closed configuration of the iron-loaded protein is one crucial step in the mechanism of receptor-mediated endocytosis. In a subsequent study the same Group analyzed conformational changes of transferrins that are associated with the binding and release of different metals [151]. Data showed that In(III) and Cu(II) induce the same domain closure as Fe(III); Al(III) causes a conformational change of somewhat smaller magnitude, while Hf(IV) does not induce any conformational change. These findings are of potential interest to better understand molecular recognition of metal-loaded transferrins by the receptor.

6.4. Perturbed angular correlation

The PAC technique is based on the observation of an anisotropic correlation between the emission direction of two successive gamma rays while the nucleus is exposed to an electromagnetic perturbation [153]. The method is highly sensitive to the structure of the metal binding sites as well as to the dynamic behavior of molecules. Proteins can be investigated under nearly physiological conditions i.e. with liquid samples and low protein concentrations. This approach was applied to In(III) derivatives of serum transferrin in which indium had been selectively loaded in the C- or in the N-terminal site [154,155]. These derivatives were prepared by adding substoichiometric amounts of ^{111}In to C- or N-terminal monoferric human serum transferrin samples; a site occupancy by In(III), in either site, of ca. 60–70% was reached. Time differential PAC spectra showed a transition in the quadrupole frequency that took place at different temperatures, ca. 275 K in Fe_CIn_NTf and between 290 and 305 K in In_CFe_NTf. Debye and Arrhenius plots of the temperature dependence of the correlation time associated with molecular reorientation indicated, for In(III)–transferrins, an effective molecular volume about 50% larger than that of the hydrated diferric molecule determined by 'biochemical' methods, and an activation energy for re-orientation of ca. 0.065 eV. Thus, these findings implied the existence of relevant conformational differences between Fe(III)– and In(III)–transferrins [155].

7. Serum speciation

7.1. Protein binding

Transferrin is the predominant chelating agent for the binding and transport of Ga(III) and In(III). There appears to be a reasonable consensus that transferrin binds essentially all the In(III) in serum except at high In(III) concentrations [22,42–44,156], and there is little evidence for the involvement of any other serum protein in the transport of In(III). The reported values for the percentage of Ga(III) bound to transferrin appear to vary somewhat depending, in part, on the experimental method being used to separate the high and low molecular mass fractions of serum. There is obvious dissociation of Ga(III) from transferrin during typical gel electrophoresis [45,157,158]. Reports based on gel filtration, ultrafiltration, and dialysis range from 85 to 100% of Ga(III) bound to transferrin [22,44,45,88–90,156]. Ultrafiltration studies on Ga(III) in serum tend to show $>95\%$ binding to transferrin, while gel filtration and dialysis tend to give lower values.

It appears that much of this variability can be traced back to the effect of the bicarbonate concentration on the effective transferrin binding affinity as shown in Eqs. (6) and (7). In gel filtration and dialysis of serum samples, there is a drop in the bicarbonate concentration as the analysis proceeds, and this appears to promote the dissociation of Ga(III) from the protein. There is little change in the bicarbonate concentration during ultrafiltration, so this method tends to show a higher percentage of serum Ga(III) bound to proteins. Staker et al. [88] have addressed this issue directly by conducting ultrafiltration studies on solutions of Ga(III) and transferrin containing varying concentrations of bicarbonate. They report complete binding to transferrin only when the bicarbonate concentration is above 13 mM. The percentage of transferrin binding drops to only 75% at 5 mM bicarbonate. Harris [159] has pointed out that there is very good agreement between the percentage of Ga(III) bound to transferrin as determined by ultrafiltration [88] and the percentage calculated using K_C and the Ga–transferrin binding constants in Table 3. Taken together, the literature data are inconsistent with a small pool of ca. 3% low-molecular-mass Ga(III) in equilibrium with Ga–transferrin in serum.

There are even more serious experimental problems in the determination of the speciation of Al(III) in serum (see Chapter 10 and Ref. [160] for a more complete discussion of these issues). There is no convenient radioisotope available for assessing the serum speciation of Al(III). This fact, combined with the ubiquitous presence of environmental Al, leads to a serious problem of sample contamination. Ganrot [161] and Sanz–Medel [162] have shown that between 1960 and 1990 the

reported values for the concentration of Al(III) in normal human serum dropped from about 10 to about 0.4 μM due to better analytical methods and the growing awareness of the risk of sample contamination.

The problem of sample integrity is particularly serious for chromatographic separations, where the columns can either adsorb or release Al(III) [160,163–168]. Al–Tf is less stable than Ga–Tf and thus exhibits an even greater tendency to partially dissociate during separation procedures. There is a consistent difference in the results from size exclusion chromatography depending on whether bicarbonate was included in the eluting buffer. The addition of carbonate produces fewer peaks and a greater fraction of protein-bound Al(III) [163–165,169–175].

The most reliable data on the percentage of protein-bound Al(III) in serum have emerged from studies using micro-ultrafiltration. This method involves a minimal perturbation of the Al(III) and carbonate concentrations in the sample, and poses a lower risk of sample contamination. Ultrafiltration studies have been conducted on unspiked serum samples, which contain about 0.3 μM Al(III) [169,176,177], and on serum samples spiked with 2–7 μM Al(III) [178–180]. The results from these papers give an average of 12% low-molecular-mass Al(III). Recent chromatographic studies, where careful attention has been paid to sample contamination and column recovery, report 10% low-molecular-mass Al(III) [181,182]. Even though a few earlier ultrafiltration studies report higher values [165,183,184], there appears to be a growing consensus that serum contains about 10 % low-molecular-mass Al(III) [160,185].

Serum aluminum levels are elevated in patients on long-term renal dialysis, and there have been several studies on the distribution of Al(III) in uremic serum [169,176,178,186–189]. The average result from these studies is $19 \pm 9\%$ low-molecular-mass Al, which appears to be significantly higher than the value for normal serum. Two studies report a direct comparison between normal and uremic serum and, in each case, the percentage of low-molecular-mass Al(III) was about 5% higher in the uremic serum (8 vs. 13% [178] and 15 vs. 20% [169]).

Serum from dialysis patients typically contains about 4 μM total Al. A higher total Al concentration favors formation of polynuclear complexes, which could contribute to a larger low-molecular-mass fraction. A trimeric Al(III)–citrate complex ($Al_3(H_{-1}cta)_3(OH^-)^{4-}$) and a binuclear Al(III)–phosphate complex ($Al_2(PO_4)(OH)_2^+$) have been proposed as important Al(III) species [27,190,191]. However, the ultrafiltration studies discussed above do not reveal a consistent difference in the ultrafilterable fraction between normal and spiked serum samples. Another possibility is that the higher concentration of phosphate (and possibly other ligands) found in uremic serum leads to a higher low-molecular-mass fraction. It has also been suggested that Al(III) binding to transferrin can be affected by changes in the degree of iron saturation of transferrin in dialysis patients [75].

Some early studies attempted to resolve Al-binding serum proteins by size exclusion chromatography, which does not effectively separate transferrin and albumin [58,192]. This led to reports that albumin was a major aluminum-binding protein [172,175,193]. Ion exchange chromatography cleanly separates albumin and transferrin, and studies using this method consistently show Al(III) binding exclusively to transferrin [166,168,181,182,194]. This result has been confirmed by immunoaffinity chromatography [165,195]. Despite occasional reports to the contrary [57,196–198], there now appears to be a general consensus that transferrin is the only significant Al-binding protein in serum [58,192,199].

There are conflicting reports on the binding of Al(III) to purified albumin. Cochran et al. [164] could detect no significant binding, but Fatemi et al. [196] assign an ^{27}Al-NMR peak to an Al–albumin complex [196]. Based on the aluminum chemical shift, the binding is attributed to the Ca(II) binding sites on albumin [196]. Fatemi et al. [57] also report difference UV data that appear to indicate that albumin can compete with transferrin for Al(III) at physiological concentrations, but we feel these results are flawed by the use of protein concentrations that are much too high to allow accurate UV absorbance measurements. Martin [199] has argued that Al(III) binding to the Ca(II) sites on albumin is too weak to compete with other serum chelating agents, so that binding to albumin, under physiological conditions, is very unlikely, and we agree with that assessment.

McLachlan and coworkers fractionated serum by size exclusion chromatography and assigned a peak, at an apparent molecular weight of about 15 kDa, to a new aluminum-binding protein that they named albindin [173,200]. The peak for this purported protein was quite small in untreated serum, but increased markedly upon the addition of high concentrations of Al(III) to the serum in vitro or upon treatment of the patient with DFO prior to taking the serum sample. Others had previously reported that a new Al–protein complex with a molecular weight of about 8 kDa was produced by treatment of patients with DFO [201,202]. The assignment of these chromatographic peaks as proteins is questionable because the Al-containing fractions do not show an appreciable absorbance at 280 nm. It is well established that administration of DFO leads to a large increase in ultrafilterable Al(III) [166,169,174,176,178,186,203,204], and several studies have identified the new low-molecular-mass serum component as the Al–DFO complex [166,168,169,174,181,182]. It seems likely that the reports of small aluminum-binding proteins result from the incorrect assignment of chro-

matographic peaks that are actually due either to Al–DFO, in the case of serum from patients treated with DFO, or to colloidal Al(III) hydroxide in the case of serum treated in vitro with high concentrations of Al(III) compounds. The analysis of Al(III) species following in vivo treatment with DFO may be complicated further by the appearance of additional chromatographic peaks due to the binding of Al(III) to metabolites of DFO [205].

7.2. Low-molecular-mass complexes

The identification of the low-molecular-mass aluminum complexes in normal serum is difficult. Al(III) is difficult to detect spectroscopically and the complexes are labile and tend to dissociate during separation procedures [160]. Although phosphate, hydroxide, and silicate have all been proposed as Al(III) ligands in serum [160,191,206,207], the ligand most often mentioned as a serum chelating agent for Al(III) is citrate [160,162,163,181,185,192,208–215].

The support for citrate binding of Al(III) in serum has often come from a consideration of binding constants [185,208,209,212,213], but some direct experimental evidence indicating the formation of Al–citrate in serum has recently been reported. Bantan et al. [214,215] have used anion exchange fast protein liquid chromatography to separate the anionic Al-complexes in serum and find that a significant fraction of the Al(III) co-elutes with citrate. Bell et al. [211] detected Al(III) binding to citrate in serum by ^{1}H-NMR, although it was necessary to use a very high (50 μM) total Al concentration.

Others have suggested, based on computer simulations of Al(III) in serum, that a significant fraction of the low-molecular-mass aluminum is bound to phosphate [191,206,207]. The recent report of somewhat lower Al–phosphate binding constant would appear to make it less likely that phosphate is the major Al-binding ligand in serum [216]. However, Bantan et al. [215] have analyzed serum from eight individuals by a combination of fast protein liquid chromatography and electrospray mass spectrometry and have detected Al(III) complexes with hydroxide, phosphate, and citrate. They report that citrate binds an average of about 50% of the total Al, while phosphate binds an average of about 25%. However, the authors stress that there is a high individual variability, and no Al–phosphate was detected in two of the eight individuals.

Bantan et al. [215] also proposed that some chromatographic samples contained mixed-ligand Al–citrate–phosphate complexes. The possible existence of such complexes has been raised previously by Atkári et al. [216]. It is clear that additional studies are needed on the speciation of low-molecular-mass Al(III) in serum.

8. Biological effects related to transferrin binding

8.1. Main features of Al(III), Ga(III) and In(III) in biological systems

Aluminum is found at relatively high concentrations in the earth's crust, in drinking water, and in foods; it is present as hydroxide in several pharmaceutical preparations. Although aluminum has long been considered as a relatively non-toxic element, there is now concern about its possible neurotoxic effects [13–16]. For instance, Al(III) has been implicated in the pathogenesis of Alzheimer's disease (AD) [17,18]. The concentration of Al(III) in the brains of AD patients is increased, and accumulation of Al(III) in specific regions of the brain has been reported [13]. Accordingly, the Al(III) content of ferritin isolated from the brains of AD subjects is on the average sixfold higher than that of controls [19]. These observations raised large interest into the investigation of the bioinorganic chemistry of Al(III) to unravel possible links to the etiopathogenesis of AD.

Ga(III) compounds find application in medicine as radiodiagnostic and therapeutic agents [20,21]. ^{67}Ga, a low-energy γ-emitting radionuclide with a half-life of 78 h, is one of the most useful diagnostic agents in the form of Ga(III) citrate. ^{68}Ga is of increasing interest for positron emission tomography. Ga(III) nitrate has been approved for the treatment of malignancy-associated hypercalcemia [20]. In(III) is also widely used as a radiopharmaceutical, the major isotope in clinical use being ^{111}In ($t_{1/2} = 2.8$ days) [22].

8.2. Speciation studies of Al(III), Ga(III) and In(III) in the blood

As stated above (Section 7.1), Group 13 cations, in the plasma, will tend to form the corresponding metallotransferrins unless they are chelated to strong,

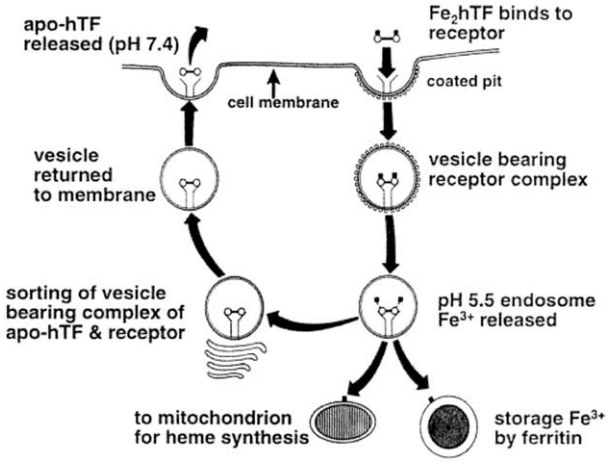


Fig. 20. The pathway of receptor-mediated cellular uptake of iron from transferrin. Adapted from Ref. [23].

low-molecular-mass ligands. Now, there is considerable evidence that these cations are indeed tightly associated to plasma proteins, mainly transferrin [23]. Speciation studies point out that Al(III) is predominantly bound to transferrin in serum; citrate and phosphate being the most important low-molecular-mass ligands [18]. In the case of Ga(III), speciation models of serum have shown that essentially all ^{67}Ga is bound to transferrin at low gallium concentrations [20]. When ^{111}In-labeled compounds are administered with weakly bound ligands or as acidic solutions, more than 95% of the In(III) binds to proteins, mainly transferrin. In contrast, if ^{111}In is given as a strong chelate, little or no binding to transferrin is observed, and the biodistribution profiles are deeply different [56].

8.3. Recognition of Group 13 metallotransferrins by the transferrin receptor

Transferrin transports its metal load into cells via the Tf receptor (TFR), a protein that can bind two transferrin molecules [23,217,218]. This receptor, at neutral pH, binds most strongly to diferric transferrin, less strongly to monoferric transferrin and weakly to apotransferrin. The complex of Fe_2Tf and TFR is taken into the cell by endocytosis; the endosome is then acidified to release the metal (around pH 5.5), and both apoTf and TFR are used again (Fig. 20).

This receptorial mechanism accounts for the entrance of most iron inside cells although TFR independent mechanisms have been described. The primary structure of the human transferrin receptor (hTFR) and its

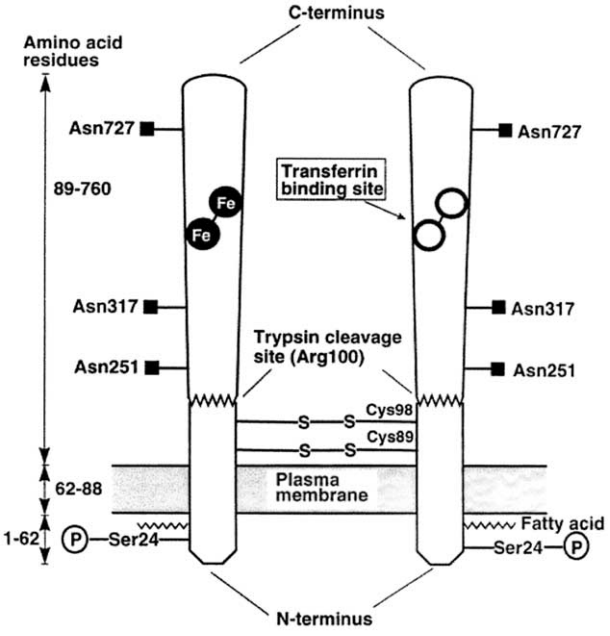


Fig. 21. Schematic representation of the transferrin receptor. Adapted from Ref. [23].

general organization are now known. It consists of two identical monomers linked by two disulfide bridges with a total mass of 190 kDa. Each monomer binds a transferrin molecule. The receptor monomer is essentially extracellular with a mass of ca. 70 kDa. A schematic diagram of the TFR is shown in Fig. 21. The soluble fragment retains the transferrin binding activity of the intact receptor. Very recently the X-ray structure of transferrin bound to its receptor has been reported, although to a relatively low resolution [219].

All nucleated cells of the body appear to express TFR but concentrations vary widely. In normal tissues the greatest amounts of TFR are expressed by hepatocytes, Kupfer cells, erythroid precursors and cells of the placenta. Malignant cells generally have very high TFR expression. In most cases there is a good correlation between expression levels of TFR and patterns of metal biodistribution [217].

Thus, to understand the bioinorganic chemistry of the Group 13 cations, it is necessary to determine if the receptorial mechanism of diferric transferrin holds also for Group 13 metallotransferrins. To the present state of knowledge, one can reasonably state that Group 13 transferrins are recognized by the specific transferrin receptor similarly to diferric transferrin, although precise comparative data are not yet available.

Indeed, numerous studies reported that Al(III)–transferrin is recognized by transferrin receptors and suggested that uptake of Al(III) by cells most probably occurs via the classical receptorial mechanism of iron [220,221]. Also, it has been reported that Al(III), once bound to transferrin, inhibits iron uptake and interferes with intracellular release of Fe(III) from transferrin [222]. Thus, the transfer of Al(III) across the blood–brain barrier is probably mediated by the transferrin receptorial mechanism, this representing the likely molecular basis for Al(III) accumulation in the brain and for the associated neurotoxic effects.

In most cases, Ga(III) enters cells via transferrin receptor-mediated endocytosis [223–229]. It has been reported that Ga(III)–transferrin is recognized by EMT-6 cells with a binding constant of 5×10^6 M^{-1}; similarly, Ga(III)–transferrin is taken up by human leukemic HL60 cells, and this complex inhibits TFR-mediated cellular uptake of iron [23].

The binding affinities of In(III) and diferric transferrin for the TFR on human reticulocytes are very similar; however, the clearance of In(III) transferrin is slower than that of Fe(III) and Ga(III), suggesting that entrance of In(III) transferrin inside cells is much less effective [230].

8.4. Biodistribution studies

Since Group 13 cations form stable adducts with transferrin and roughly follow the initial steps of the

iron pathway, one might expect, for these elements, a pattern of biodistribution similar to iron. This is essentially true for Ga(III): indeed, Ga(III) tends to accumulate in tissues having high levels of transferrin, TFR, lactoferrin, and ferritin. Also, Ga(III) 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 [21].

Although In(III) transferrin binds the transferrin receptor as well as diferric transferrin, the transport of In(III) across the cell membrane is much slower than in the case of Ga(III). Nonetheless, In(III) still tends to accumulate in tissues that have high levels of TFR, and it seems likely that transferrin is a mediator for the delivery of In(III) to tumors [230].

The profile of biodistribution of Al(III) in the case of aluminum overload differs significantly from that of iron. In particular accumulation of Al(III) in the brain and in bones has been demonstrated; this tissue specific accumulation is probably associated with the major toxic effects of aluminum overload, namely dementia, anemia and osteomalacia [14].

The fact that the profiles of biodistribution and accumulation of Group 13 elements are somewhat different from one another suggests the occurrence of distinct biochemical pathways such as different routes after intracellular release from transferrin and the existence of important transferrin-independent transport mechanisms.

9. Conclusions

We have shown that Group 13 cations exhibit an essentially similar aqueous chemistry. Notably, these cations do not exist as such at physiological pH but are present as complexes of the trivalent ions. Common low-molecular-mass ligands are hydroxide, citrate and phosphate. Notably transferrin, the Fe(III) binding protein of plasma, is a very strong ligand for these cations, so that Group 13 elements in the blood predominantly exist as transferrin complexes. Metal binding to transferrin shows peculiar features, such as the requirement of the synergystic anion, the presence of two similar but not identical binding sites, and a characteristic conformational transition from the 'open' to the 'closed' form. Detailed knowledge of structure–function relationships in transferrin is now substantiated by extensive crystallographic data obtained on ferric- and apotransferrins. Derivatives of transferrins with Group 13 cations were mainly investigated by spectroscopic techniques such as UV difference spectroscopy and NMR. On the grounds of the extensive experimental data collected so far, it can be proposed that Al(III), Ga(III), In(III) and Tl(III) transferrins exhibit very similar structural and functional features. Indeed, all these cations form stable ternary complexes with transferrin and carbonate. As documented by recent high resolution NMR results, the conformations of the resulting metallotransferrins are very similar to one another, so that all of them are efficiently recognized by the specific transferrin receptor. It follows that compounds of Group 13 metals in biological fluids, with the exception of a few strong chelates, will eventually give rise to the respective metallotransferrins, and that these metal ions will be internalized in cells by the transferrin receptor mechanism. Due to the important chemical differences existing between Fe(III) and Group 13 cations it is likely that after intracellular metal release from transferrin, their biological fates will diverge, and partially different biodistribution profiles will emerge. In any case it is clear that Al(III), Ga(III) and In(III) compounds, after reaching the blood stream, will use the first steps of the classical iron pathway, and this may have important consequences in relation to the toxicological or radiopharmacological roles of these compounds. For instance, important neurotoxic effects have been described for Al(III). Binding to transferrin and cellular uptake of aluminum by the receptorial mechanism is probably the molecular basis for Al(III) neurotoxicity. Conversely, accumulation of Ga 67 compounds inside tumors and their favorable antitumor properties are most likely a consequence of the large expression of transferrin receptors on tumor cells. Additional studies are still needed to fully exploit the chemistry and the biological properties of transferrin derivatives of Group 13 cations.

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