believe that the precise definition of the coordinate geometry of copper at the active site will be crucial to understanding its functional operation. Also, the subtle changes that allow the dinuclear site to become catalytically active in, for example, tyrosinase and laccase[11,42-44] will also be an interesting outcome of gaining a more precise definition of the active-site structure of hemocyanin and its related binuclear copper proteins.[45]

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Registry No. **1**, 124020-98-0; **2**, 124021-00-7; **3**, 124021-02-9; **4**, 124021-05-2; **5**, 124021-07-4; $[Cu(MeCN)_4]CF_3SO_3$, 58452-28-1; $[Cu(MeCN)_4]ClO_4$, 14057-91-1; $[Cu(MeCN)_4]BF_4$, 15418-29-8.

Supplementary Material Available: Tables S1, S4, S7, and S10 (anisotropic thermal parameters), Tables S2, S5, S8, and S11 (bond distances), and Tables S3, S6, S9, and S12 (bond angles) for compounds **1–4**, respectively (15 pages). Ordering information is given on any current masthead page.

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Contribution from the Departments of Chemistry, University of Missouri—St. Louis, St. Louis, Missouri 63121, and University of Idaho, Moscow, Idaho 83843

Equilibrium Constants for the Binding of Aluminum to Human Serum Transferrin

Wesley R. Harris*,† and Jan Sheldon‡

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The binding of aluminum to human serum transferrin in 0.10 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid and 5 mM sodium bicarbonate at pH 7.4 has been studied by difference ultraviolet spectrophotometry. Aluminum binding produces peaks at 240 and 288 nm that are characteristic of binding at the transferrin specific metal binding sites. The more intense peak at 240 nm has a molar absorptivity of 14 800 ± 1600 M^{-1} cm^{-1} and has been used to determine two macroscopic aluminum binding constants of log K_1^* = 13.5 ± 0.2 and log K_2^* = 12.5 ± 0.3. Titrations of both forms of monoferric transferrin with Al^{3+} indicate that the larger K_1^* value is associated primarily with Al binding at the C-terminal site and the smaller K_2^* value is associated primarily with the N-terminal site. The Al–transferrin binding constants have been used to include transferrin in the equilibrium distribution of aluminum calculated by using a computer model of serum. This model has been used to evaluate the potential of several low molecular weight ligands for use in decorporation of aluminum.

Aluminum is found at relatively high concentrations in the Earth's crust, in drinking water, in several drugs, and in many processed foods.[1] Until the early 1970s most forms of inorganic aluminum were considered to be virtually nontoxic.[2,3] However, in the past 15 years, several toxic effects of aluminum have been discovered.[2-8] Normally the body maintains low aluminum concentrations through a combination of low intestinal absorption and effective renal clearance. Toxicity is most often observed when the intestinal barrier to aluminum uptake is somehow bypassed. For example, unusually high rates of encephalopathy[3,7-10] and an unusual type of osteomalacic osteodystrophy[2,4,7] among patients on long-term dialysis due to chronic kidney failure have been traced to high aluminum concentrations in the water used for dialysis. Aluminum toxicity is also observed in patients on chronic ambulatory dialysis[11] and on long-term total parenteral nutrition.[12] Aluminum-containing drugs, which are routinely given orally to patients with renal failure to control serum phosphate concentrations, are another source of toxic levels of aluminum in some patients.[4,7,8]

There is also an association between aluminum and Alzheimer's disease, although aluminum does not cause the disease.[13] There is an increase in the aluminum concentration in the brain cells that show the neurofibrillary degeneration characteristic of Alzheimer's disease.[14] The enhanced aluminum concentration may simply be a marker with no relationship to the clinical symptoms. However, it is also possible that the disease may involve metabolic defects that lead to an enhanced susceptibility of certain individuals to chronic aluminum toxicity.[13,15]

Aluminum-induced encephalopathy and osteomalacia associated with long-term dialysis can be treated with the chelating agent desferrioxamine B.[16-20] A primary consideration for any new chelating drug is its ability to remove aluminum from serum proteins, which reportedly bind about 80% of serum aluminum.[8,9,21-23] Fractionation of aluminum in serum has identified

† University of Missouri—St. Louis.
‡ University of Idaho.

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transferrin (Tf) as the major Al-binding serum protein.[24-26] Transferrin is the mammalian serum iron transport protein, which contains two similar high-affinity metal binding sites.[27,28] In vitro studies have shown that aluminum binding to transferrin (a) requires bicarbonate,[24] (b) produces the characteristic difference UV spectrum of metal–transferrin complexes,[29] and (c) competes directly with the binding of iron and gallium.[24,26] Thus there is no doubt that aluminum binds at the high-affinity metal-binding sites of this protein. However, there are conflicting reports on the magnitude of the Al–Tf binding constants.[30-32]

This paper reports new studies on the thermodynamics of aluminum binding to serum transferrin. The Al–Tf binding constants are used to include transferrin in an equilibrium model for the speciation of aluminum in serum. This model is then used to evaluate the potential efficacy of selected chelating agents as drugs for the selective binding of aluminum in serum.

Experimental Section

Materials. Human apotransferrin was purchased from Sigma and purified as previously described to remove chelating agents.[33] Monoferric transferrins were prepared as previously described.[34] A stock solution of $Al(NO_3)_3$ in 0.10 M HNO_3 was prepared from the reagent grade salt and standardized by complexiometric back-titration with ethylenediaminetetraacetic acid (EDTA) and copper.[35] Disodium salts of EDTA and nitrilotriacetic acid (NTA) were purchased and used as received.

Methods. Solutions of apotransferrin in 0.1 M *N*-(2-hydroxyethyl)-piperazine-*N'*-ethanesulfonic acid (hepes) containing 5 mM sodium bicarbonate were adjusted to pH 7.4 with sodium hydroxide. Difference UV spectra were recorded with a Varian 2290 spectrophotometer. Equal volumes of this solution were added to four dry sample cuvettes and one reference cuvette, and a base line of protein vs protein was recorded for each cuvette from 320 to 235 nm. The sample cuvettes were titrated with solutions of aluminum that contained various concentrations of NTA as a competing ligand. Equal volumes of water were added to the reference cuvette. During the titration the sample cuvettes were maintained at 25 °C by a thermostated cell holder connected to an external circulating water bath. One sample cuvette in each set was titrated with free Al^{3+} without added NTA to determine a value of the molar absorptivity ($\Delta\epsilon_M$) of the Al–Tf complex to be used in the calculations. It was assumed that both sites have the same molar absorptivity.

Binding constants were calculated as described previously[36] with the exception of the small adjustment to the molar absorptivities described below. Briefly, the system was described by mass balance equations for aluminum, transferrin, and NTA. A protonation constant for free NTA of $10^{9.67}$, an aluminum–NTA stability constant of $10^{11.37}$, and successive aluminum–NTA chelate hydrolysis constants of $10^{-5.09}$ and $10^{-8.28}$ were taken from Martell and Motekaitis.[37] Free aluminum hydrolysis constants were defined as

$$\beta_n = \frac{[Al(OH)_n][H^+]}{[Al(OH)_{n-1}]} \quad (1)$$

Log β_n values of −5.46, −10.04, −15.63, and −23.40 were calculated for 0.1 M ionic strength by using the empirical formula described by Baes and Mesmer.[38]

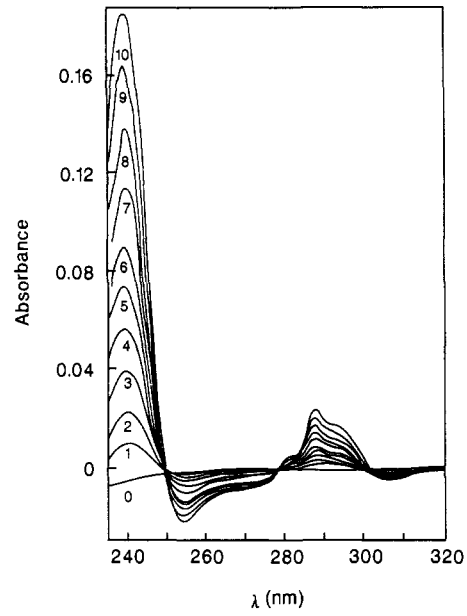

Figure 1. Difference ultraviolet spectra generated by the titration of 2.2 mL of 8.34×10^{-6} M apoTf with 2.20×10^{-4} M Al^{3+}. Key [(spectrum no.) volume of Al^{3+} solution, μL]: (0) 0; (1) 10; (2) 20; (3) 30; (4) 40; (5) 50; (6) 65; (7) 85; (8) 105; (9) 125; (10) 155.

The aluminum–NTA stability constants, the free aluminum hydrolysis constants, and initial guesses for the aluminum–transferrin binding constants were used to solve the mass balance equations for [Al–Tf] and [Al_2–Tf]. These concentrations were used with the appropriate value of $\Delta\epsilon_M$ to calculate an apparent absorptivity for each data point. The values of the aluminum–transferrin binding constants were then varied to minimize the squares of the residuals between observed and calculated absorptivities.

Titration of apoTf with NTA alone produced a negative absorbance at 240 nm as a result of a tail from a peak at lower wavelength. This change appeared to be biphasic, with a small rapid change from the addition of small amounts of NTA followed by a more gradual change that was evident only when more concentrated NTA titrants were used. In the forward titrations of apoTf with Al^{3+}–NTA solutions, the initial negative NTA–Tf absorbance was essentially saturated within the first one or two data points. Thus the change due to NTA appeared as a negative intercept in the titration curves.

Similar negative difference spectra have been observed following the addition of inorganic anions to apoTf.[39,40] The negative absorbances observed here are attributed to the formation of a binary NTA–Tf complex. Anion binding is blocked when a metal ion occupies the binding site.[39,40] Thus the apparent $\Delta\epsilon_M$ for Al–Tf in the presence of NTA reflects both the positive absorbance due to Al–Tf binding and the loss of the negative absorbance of the NTA–Tf species. In the calculations of binding constants from the forward titration data, the molar absorptivity as measured in the absence of NTA has been increased by half the absolute value of the *y*-intercept of the titration curve. This correction was typically only 3–6% of $\Delta\epsilon_M$.

Reverse titrations were run in which free NTA was added to solutions of aluminum and transferrin. Higher concentrations of NTA were necessary for the reverse titrations, and the problem of negative absorbances due to NTA–Tf complexes was more severe. In titrations of apoTf with aluminum alone, there was an isosbestic point at 250 nm. Thus, any decrease in absorbance at 250 nm could be attributed to an NTA–Tf interaction. For the reverse titrations, each data point was corrected back to the original base line based on the decrease in absorbance at 250 nm.

Results

Solutions of apoTf were titrated with an acidic solution of aluminum nitrate to generate the family of spectra shown in Figure 1. The absorbance maxima at 240 and 288 nm reflect pertur-

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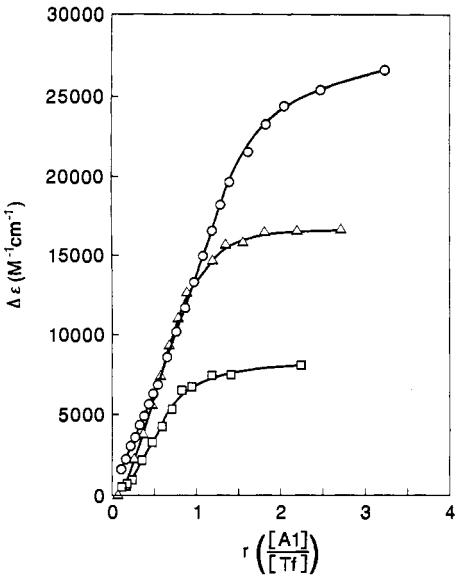

Figure 2. Plots of $\Delta\epsilon$ vs r for the titration of apoTf (○), N-terminal monoferric transferrin (△), and C-terminal monoferric transferrin (□) with Al^{3+}.

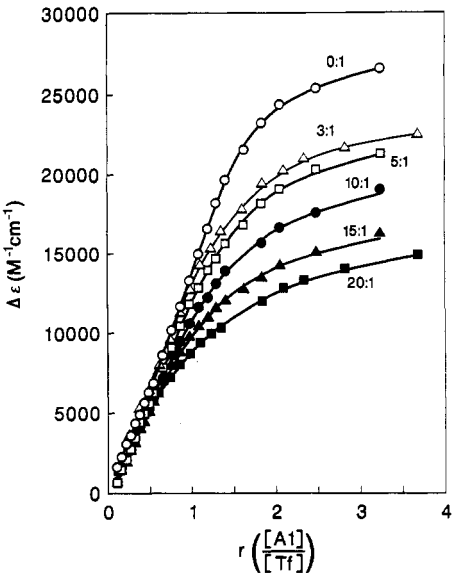

Figure 3. Plots of $\Delta\epsilon$ vs r for the titration of apoTf with Al^{3+} solutions that contained varying ratios of NTA:Al^{3+} as indicated in the figure.

bations in the aromatic bands of coordinated tyrosines and are characteristic of metal binding at the two high-affinity binding sites of Tf.[41-43] To normalize results from run to run, the absorbance at 240 nm was divided by the analytical Tf concentration to give an apparent absorptivity, $\Delta\epsilon$, for each titration point.

Values of $\Delta\epsilon$ were plotted vs r, the Al/Tf ratio, to give the titration curves shown in Figure 2. The titration of apoTf with Al^{3+} gave linear plots from $r = 0$ to $r \sim 1.0$. The plots then curved downward and eventually leveled off at a $\Delta\epsilon$ of about 26000 M^{-1} cm^{-1}. When no NTA was present, the slope of the initial, linear segment of the titration curve was equal to the molar absorptivity ($\Delta\epsilon_M$) of the Al–Tf complex. Several replicate titrations gave a value of $\Delta\epsilon_M = 14800 \pm 1600$ M^{-1} cm^{-1} per Tf binding site.

Since the titration curves level off at $\Delta\epsilon$ values well in excess of the molar absorptivity, it is clear that the aluminum is binding to both of the Tf binding sites. This is confirmed by titrations of both N-terminal and C-terminal monoferric transferrins. In each of these proteins, one of the two binding sites is blocked by the more tightly bound ferric ion. Thus one can measure the spectrum from the binding of aluminum to a single binding site. The resulting titration curves are also shown in Figure 2. The titration curve for C-terminal monoferric transferrin levels off at an absorptivity of about 8000 M^{-1} cm^{-1}. The titration curve for N-terminal monoferric transferrin levels off near 16000 M^{-1} cm^{-1}. Thus one can assign the larger log K_1* value primarily to the C-terminal site.

Effective Al^{3+}–Tf binding constants are described by eq 2 and 3. The formation of the specific metal–Tf complexes requires

$$K_1^* = \frac{[\text{Al–Tf}]}{[\text{Al}][\text{apoTf}]} \quad (2)$$

$$K_2^* = \frac{[\text{Al–Tf–Al}]}{[\text{Al}][\text{Al–Tf}]} \quad (3)$$

bicarbonate and releases two or three protons depending on the charge on the metal ion.[43] Since $[H^+]$ and $[HCO_3^-]$ terms are omitted from eq 2 and 3, the effective binding constants are valid only under the experimental conditions of pH 7.4 and 5 mM bicarbonate. The apoTf was titrated with solutions that contained both aluminum and NTA with ligand:metal ratios ranging from 3:1 to 20:1. A representative set of titration curves are shown in Figure 3. Values for K_1* and K_2* are determined by nonlinear least-squares fits of the titration curves. The average values for 18 replicate forward titrations are log $K_1^* = 13.6 \pm 0.2$ and log $K_2^* = 12.5 \pm 0.3$.

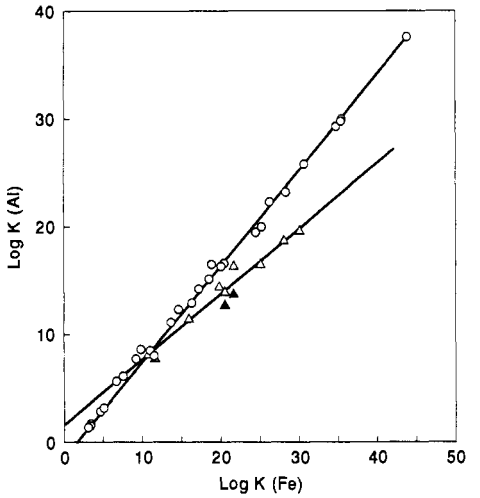

Figure 4. Linear free energy relationships for the complexation of Fe^{3+} and Al^{3+}. Each data point consists of the Fe^{3+} stability constant of a given ligand as the x coordinate and the Al^{3+} stability constant of the same ligand as the y coordinate. The open circles represent ligands that coordinate exclusively through oxygen donor groups. The open triangles represent ligands that coordinate through a combination of oxygen and nitrogen donor atoms. The points for transferrin are shown as the filled triangles.

Reverse titrations were also run by first adding approximately 0.8 equiv of Al^{3+} to apoTf and then titrating with free NTA. This procedure gave excellent consistency in log K_1* values from run to run, with an average value for six replicate titrations of log $K_1^* = 13.4 \pm 0.1$. However, the quality of the individual fits of $\Delta\epsilon_{obs}$ vs. $\Delta\epsilon_{calc}$ was much poorer than that observed for the forward titrations. When the binding constants from the forward and reverse titrations are averaged together, one obtains a value of log $K_1^* = 13.5 \pm 0.2$.

No value of log K_2* was determined by reverse titrations. It appeared that one could not add more than 1 equiv of Al to transferrin in the absence of a carrier ligand without producing essentially irreversible hydrolysis of a portion of the Al. Similar results were observed for transferrin binding of Cd(II),[44] Nd(III),[33] and Sm(III).[33]

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Table I. Constituents of the Model for Aluminum in Normal Serum

constituent	concn, mM	constituent	concn, mM
aluminum	0.005	histidine	0.085
albumin	0.630	lactate	1.820
bicarbonate	25	free magnesium	0.520
free calcium	1.16	phosphate	0.0381
cistine	0.0400	sulfate	1.20
citrate	0.113	transferrin	0.050
cysteine	0.0230	zinc	0.010
glycine	2.75		

Linear Free Energy Relationships. Two linear free energy relationships for the complexation of Al^{3+} and Fe^{3+} are shown in Figure 4. Each data point represents a ligand. The x coordinate is the stability constant of the ligand with Fe^{3+}, while the y coordinate is the stability constant of the same ligand with Al^{3+}. Data on low molecular weight ligands were taken primarily from the critical compilations of Martell and Smith.[45] One set of data in Figure 4 includes ligands that bind through a combination of aliphatic amines and oxygen donors. The second data set includes ligands that bind exclusively through oxygen donors. Equations 4 and 5 describe these two plots. The higher slope of the oxygen-donor LFER reflects the "hardness" of the small Al^{3+} ion. Within each data set there is an excellent correlation between the Fe^{3+} and Al^{3+} binding constants. The Pearson correlation coefficients are $r = 0.986$ for the amine–oxygen data set and $r = 0.999$ for the oxygen-only data set.

$$\text{O donors} \quad \log K_{Al} = 0.879(\log K_{Fe}) - 1.055 \qquad (4)$$

$$\text{N,O donors} \quad \log K_{Al} = 0.625(\log K_{Fe}) + 1.364 \qquad (5)$$

It has been shown that the transferrin binding constants for several metal ions conform to this type of LFER.[33,42,44,46] Thus the LFER provides a basis for using the Fe^{3+}–Tf binding constants to estimate the values for the Al^{3+}–Tf binding constants. The data points for the log K_1* and log K_2* values for transferrin are shown as the solid triangles in Figure 4. The iron–Tf binding constants used were based on the data from Aisen et al.[47] as corrected by Martin[31] to incorporate an improved set of stability constants for citrate, which was used as the competing ligand. The aluminum constants fall about 1.2 log units below the LFER for ligands that bind through a combination of amine and oxygen donors. A similar LFER based on fewer data points was prepared for the binding of Ga^{3+} and Al^{3+}. The observed Al–Tf binding constants are about 1.3 log units below the values predicted by the Ga^{3+}–Al^{3+} LFER.

Speciation of Aluminum. A multicomponent equilibrium model was used to calculate the speciation of aluminum in serum. The model consisted of the concentrations of relevant components in serum and the stability constants of all metal complexes. Concentrations of most low molecular weight components were taken from the study of May et al.[48] on the speciation of various divalent cations in serum. Concentrations of total sulfate, total phosphate, and free calcium were taken from Shepard.[49] The average Tf concentration in serum is 37 μM.[27] In normal serum the protein is only 30% saturated with iron,[28] which is preferentially bound to the N-terminal binding site. Therefore, the model included a total of 50 μM Tf binding sites. The serum albumin concentration was fixed at 6.3×10^{-4} M.[50] The total aluminum concentration was set at 5 μM, which is in the range of serum concentrations observed for patients on hemodialysis.[9,10,19,20,23,25] The model included calcium, magnesium, and zinc as competitive metal ions. The concentration of each component is shown in Table I.

Table II. Calculated Speciation for 5 μM Al^{3+} in the Computer Model for Normal Serum

species	% tot. Al	species	% tot. Tf
Al–HCO_3–Tf	94.2	HCO_3–Tf	30.9
Al(H_{-1}citrate)	4.9	HPO_4–Tf	32.9
$Al(OH)_3$	0.4	SO_4–Tf	21.2
Al(H_{-1}lactate)	0.2	Al–HCO_3–Tf	9.4
$Al(OH)_4$	0.2	apoTf	4.4
		Zn–HCO_3–Tf	1.2

Table III. Values and Sources of the Aluminum Stability Constants Used in Speciation Calculations[a]

ligand	Al^{3+} log β_{ijk} (i, j, k)	log $K_{ML}(Fe^{3+})$	log $K_{ML}(Zn^{2+})$	log $K_{ML}(Ca^{2+})$
DFO-B	24.15 (1, 1, 1)[b]	30.6	10.07	2.64
NTA	20.08 (1, 1, −1)[c]		10.66	6.39
EDTA	24.23 (1, 1, −1)[c]		16.44	10.61
HBED	26.10 (1, 1, 0)[d]	39.68	18.37	9.29
MECAMS	39 (1, 1, 0)[d,e]	41[e]	30.2[f]	
tiron	16.6 (1, 1, 0)[c]		10.41	4.40
	30.0 (1, 2, 0)		18.52	7.99
	39.4 (1, 3, 0)			
AHA	7.95 (1, 1, 0)		5.40	2.40
	15.29 (1, 2, 0)		9.60	
	21.47 (1, 3, 0)			

[a] Values taken from ref 45 unless otherwise noted. [b] Value taken from ref 54. [c] Value taken from ref 37. [d] Value determined from corresponding ferric ion stability constant and the Fe–Al LFER shown in Figure 4. [e] Estimated from the corresponding β_{113} value using an average ligand pK_a of 11.5 for the first deprotonation of each catecholate group. [f] Value for β_{111} taken from ref 53.

Metal binding to transferrin is described by the sequential equilibria

$$HCO_3^- + \text{apoTf} \overset{K_C}{\rightleftharpoons} HCO_3\text{–Tf} \qquad (6)$$

$$HCO_3\text{–Tf} + M^{n+} \overset{K_M}{\rightleftharpoons} M\text{–}HCO_3\text{–Tf} \qquad (7)$$

On the basis of these equations, one can describe the bicarbonate dependence of the effective transferrin binding constant by

$$\log K^* = \log K_M + \log \alpha \qquad (8)$$

where α is the fraction of apoTf binding sites occupied by bicarbonate and K_M is the bicarbonate-independent binding constant corresponding to eq 7.[39] Values of log $K_C = 2.49$ and log K_M (Zn^{2+}) = 7.42 were taken from Harris and Stenback.[39] Equation 8 was used to calculate a log K_M value for Al^{3+} of 13.72. A value of 10^7 was used for the zinc–albumin binding constant.[51] The serum model did not include Al^{3+}–albumin or protein complexes of calcium and magnesium.

The Al speciation was calculated by using the well-established computer program ECCLES.[48] The calculated distribution of 5 μM Al in the basic model is shown in Table II. The Al is bound almost exclusively to Tf. The most important low molecular weight serum chelating agent is citrate, which binds only about 5% of the Al. The distribution of Tf is also shown in Table II. The aluminum binds only about 9% of the Tf and thus causes a rather small perturbation in the distribution of Tf species that one observes in the absence of this metal ion.[52] Most of the Tf exists as a mixture of binary Tf–bicarbonate and Tf–phosphate complexes. The aluminum has virtually no effect on the zinc or calcium distributions.

The speciation calculations were repeated by using the bicarbonate concentration of air-saturated pH 7.4 buffer, rather than the physiological bicarbonate concentration. Under these conditions there is a large increase in the concentration of low molecular weight Al complexes. Citrate and Tf bind approxi-

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Table IV. Speciation of Aluminum and Zinc in Serum Containing 5 μM Al^{3+} and 10 μM Exogenous Chelating Agent[a]

	% tot. Al		% tot. Zn			
ligand	Al-Tf	Al-L	Zn-Tf	Zn-Alb	Zn-L	[CaL], M
none	94		6	93		
DFO-B	0	100	7	92	0	10^{-11}
NTA	94	0	6	91	2	10^{-5}
EDTA	94	0	4	60	36	10^{-5}
HBED	3	97	4	54	41	10^{-8}
MECAMS	0	100	4	49	47	
tiron	65	30 (2:1) 2 (3:1)	6	93	0	10^{-9}
AHA	94	0	6	93	0	10^{-8}

[a] Abbreviations: Tf = transferrin, Alb = albumin, and L = exogenous ligand.

mately 70% and 18% of the total Al, respectively, with 6% of the Al existing as the $Al(OH)_3$ species.

Speciation calculations were used to evaluate the potential efficacy of various ligands for the clinical removal of serum aluminum. The serum model was modified by the inclusion of 10 μM concentrations of desferrioxamine B, NTA, EDTA, 1,4-bis(*o*-hydroxybenzyl)-1,4-bis(carboxymethyl)-1,4-diazabutane (HBED), 1,2-dihydroxy-3,5-disulfobenzene (tiron), 1,3,5-tris-(((2,3-dihydroxy-5-sulfobenzoyl)amino)methyl)benzene (MECAMS), or acetohydroxamic acid (AHA). Available binding constants for these ligands for Al^{3+}, Fe^{3+}, Zn^{2+}, and Ca^{2+} were taken from the literature.[37,45,53,54] When no Al^{3+} binding constant was available, one was estimated based on the ferric ion binding constant using the appropriate LFER from Figure 4. The complete set of equilibrium constants for these ligands is shown in Table III. Table IV lists the speciation results in the presence of the exogenous chelating agents.

Discussion

There have been several previous reports of Al-Tf binding constants. Cochran et al.[32] initially used a competitive equilibrium between Al^{3+} and Fe^{3+} and reported an average log K value of 15.4. However, these results have been challenged[31,55] on the basis that there was insufficient time for equilibration. Cochran et al.[30] have recently published a second study using difference UV titrations and report log K_1^* = log K_2^* = 13.5 in a buffer containing 25 mM Tris, 100 mM NaCl, and 10 mM bicarbonate at pH 7.4 and 37 °C. The results are suspect considering that statistical effects would lead to a separation of 0.6 log units between the successive binding constants for identical binding sites, and observed separations of about 1 log unit are typical.[42] In addition, this paper[30] reports complete saturation of apotransferrin at Al:Tf ratios of 3:1. This is directly contradicted by the titrations reported here for both apoTf and C-terminal monoferric transferrin.

Martin[31] reported much lower Al-Tf binding constants of log K_1^* = 12.2 and log K_2^* = 11.6 for 0.1 M Tris, 5 mM bicarbonate buffer at pH 7.4 and 25 °C. These values show a reasonable separation between K_1^* and K_2^*. Other than using Tris instead of hepes, the experimental conditions are the same as those used in this study. It is not clear why we obtain higher binding constants.

One objective of this study was to evaluate the effect of the rather small ionic radius of Al^{3+} on its binding to transferrin. The size does not appear to have any impact on the selectivity between the two sites. The separation between successive Al^{3+} binding constants is 1.0 log units, which is typical of the Δ log K values for a variety of other metal ions.[42] The titrations of the monoferric transferrins show that Al^{3+} is bound more tightly at the C-terminal binding site. The same site preference has been observed for Zn^{2+}, Nd^{3+}, Sm^{3+}, Fe^{3+}, and Cd^{2+}.[33,44,47,56]

Although the stability constants for Al-Tf appear to be quite large, the most important equilibrium in vivo is between Al-Tf and $Al(OH)_3$, which has an overall formation constant of 10^{26}. Thus the effective stability constant for Al-Tf at pH 7.4 is actually about 10^7. The competition from hydroxide accounts for the leveling off of the Al titration curves at less than complete saturation of Tf, even in the absence of any added chelating agent. One would expect even greater dissociation of the Al-Tf complex during separations in which the solution bicarbonate concentration drops as low molecular weight Al species are separated from Al-Tf. This may account for the tendency of fractionation studies to show 15–20% low molecular weight Al in serum compared with the 5% indicated by the speciation calculations.

The LFER was used to evaluate the effect of size on the absolute magnitude of the Al binding constants. Al^{3+} complexes are generally less stable than the corresponding Fe^{3+} complexes, as indicated by the slope of 0.64 for the Fe–Al LFER constructed from nitrogen–oxygen ligands. Thus one expects the Al-Tf constant to be less than the Fe-Tf binding constant. The Al-Tf binding constants fall about 1.2 log units below the line corresponding to eq 5. Since the standard deviation in the Al binding constants calculated from eq 5 is 1.1 log units, it is not clear that the deviation of the Al-Tf data from the LFER is significant. Similar results are obtained from the Ga–Al LFER, although these results are based on fewer data points in the LFER. An LFER between Zn^{2+} and Cd^{2+} indicates no size effect for binding of the large Cd^{2+} ion.[44] The small size of the Al^{3+} ion may be responsible for a slight decrease in the Al-Tf binding constants, but the transferrin binding sites appear to adapt quite well to changes in ionic radius of the metal ion.

Martin[55] also prepared an Al–Fe LFER and suggested that one should correct the effective Tf binding constants for protonation of the tyrosine groups and plot true formation constants. This adds approximately 5 log units to the log K values. For most LFER the slope is so close to 1.0 that this correction has virtually no effect on the fit of the transferrin data to the least squares line. Because of the lower slopes of the Al–Fe LFER, this correction shifts the transferrin data points upward to about 0.6 log units above the line for amine–oxygen donors. This would indicate that the smaller size of the Al^{3+} ion results in no detectable loss in stability for the Al-Tf complex.

The speciation calculations clearly show why desferrioxamine B is an effective agent for removing Al from blood. A serum concentration of only 10 μM should be enough to completely remove all Al from transferrin, while having no significant effect on either Zn or Ca. At a 10 μM serum concentration, neither NTA or EDTA binds significant amounts of Al. As one might expect based on biological studies,[57,58] the calculations show that 64% of the EDTA and 92% of the NTA exist in serum as the calcium complexes. The remaining 36% of the EDTA binds zinc. The competition from these two metal ions is so severe that EDTA is not expected to bind Al even though it has an Al stability constant much greater than that of Al-Tf. This result demonstrates the importance of using as complete a model as possible for predicting in vivo metal ion speciation.

An obvious way to enhance the selectivity of amino carboxylates for Al^{3+} is to replace carboxylates with phenols, which have a well-established tendency to bind trivalent metal ions.[59] Therefore, speciation calculations have been performed for the diphenolic ligand HBED. As expected, this ligand binds Al^{3+} much more strongly than does Tf and might appear to be a strong candidate for Al decorporation. However, it binds as much Zn as does EDTA. Although the selectivity for Al^{3+} over Zn^{2+} has been improved relative to that of EDTA, the high absolute binding affinities for this ligand coupled with the absence of competition

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from Ca lead to greater Zn binding.

We also evaluated two dihydroxyphenol ligands, the bidentate tiron and the hexadentate MECAMS. The results for MECAMS were similar to those for HBED. MECAMS was clearly superior to Tf in its ability to bind Al, but it also bound significant amounts of Zn. Tiron was considered next because it was expected to have lower absolute binding affinities than MECAMS and thus should complex less zinc. A 10 μM concentration of tiron complexed about 30% of the Al, but did not bind either Ca or Zn. Thus it appeared that one should be able to synthesize catecholate ligands which would bind a large fraction of serum Al without disturbing the metabolism of either Zn or Ca. We would note that replacement of desferrioxamine B by its bidentate analogue acetohydroxamic acid gave quite different results. In this case, the loss in absolute binding affinity was enough to eliminate completely the binding of Al.

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Registry No. DFO-B, 70-51-9; NTA, 139-13-9; EDTA, 60-00-4; HBED, 35998-29-9; MECAMS, 71353-06-5; Tiron, 149-45-1; AHA, 546-88-3; Al, 7429-90-5; Fe, 7439-89-6; Zn, 7440-66-6.

Contribution from the Departments of Chemistry, University of Siena, 53100 Siena, Italy, and University of Florence, 50132 Florence, Italy, and Department of Physics, University of Washington, Seattle, Washington 98195

EXAFS Investigation on the Iron(III) Binding Sites of Hen Phosvitin

S. Mangani,*,† P. Orioli,‡ A. Scozzafava,‡ L. Messori,‡ and E. A. Stern§

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EXAFS spectroscopy has been applied to investigate the nature and the stereochemistry of the iron(III) binding sites in the protein phosvitin from chicken eggs in water solution with an iron/protein molar ratio of 10/1 at pH 7.2. The main result is that the iron atoms are bound to the protein in an octahedral environment and that the main binding sites are provided by the oxygen atoms of serine-bound phosphate groups at 1.93 (2) Å from the metal. The average number of bound phosphate groups is 4.4 (9) per iron atom, hexacoordination being achieved upon binding of other possible donors from the protein or solvent water molecules. No evidence has been found of short Fe-Fe interactions, so that, on the basis of our data, it can be stated that iron is not essentially involved in a polynuclear structure with Fe-O-Fe bridges.

Introduction

Phosvitins are a group of small phosphoglycoproteins that are the major components of the highly structured granules of the vertebrate egg yolk. All the phosvitins from different ovarian species share a very unusual amino acid composition characterized by an extraordinarily high serine content (about 50%) with extensive phosphorylation of these residues and by the relatively dominant presence of basic amino acids such as lysine and arginine, while those with nonpolar or sulfur-containing side chains are nearly or completely absent.[1] The biological role of phosvitins is not yet fully understood, although in view of the fact that essentially all the yolk metal is bound by this protein, it has been proposed that they can act as metal depositories for the embryo.[2] However, it is possible that they play a role as metal carriers in the blood of the maternal organism in the form of their precursors, the vitellogenins.[1,2]

Their ability to bind metals is certainly connected with the large number of serine-bound phosphoryl groups; however, in certain circumstances the imidazole nitrogens of the histidine residues and the peptide amide nitrogens can also act as donor atoms.[3]

The primary structure of hen phosvitin (PST) consists of a single chain of 216 amino acids for a molecular weight of ~35 000. There are 123 serine residues, of which 80 are located in a core region (residues 56-154) in blocks of up to 14 alternated with arginines, lysines, and more rarely asparagines. The C-terminal portion (residues 155-216) is particularly rich in histidines and contains another 27 serines.[4] A total of 98% of the serine residues are phosphorylated.[1]

Hen phosvitin in solution appears to be extremely flexible and unfolded, behaving essentially like a polyelectrolyte, the appearance of some secondary structure depending on the protonation of the phosphate groups. In fact only at very acidic pH (<3) does an extensively ordered conformation of a β-sheet type appear,[5] which is due to the presence of hydrogen bonds and salt linkages between the phosphate groups and the positively charged groups of basic amino acids. In the pH range 7-10, ORD[6,7] and resonance Raman studies[8] indicate the presence of an unordered structure. The binding of Ca^{2+}, Mg^{2+}, or Fe^{3+} does not induce a transition toward a β-type structure.[9,10]

Several reports on the interaction of phosvitin with metal ions such as Fe(II), Fe(III), Co(II), Mn(II), Ca(II), and Mg(II) have appeared in the literature;[1,10-12] however, the structure of the metal chromophore and the nature of the metal ligands remain to be fully understood. Early spectroscopic and magnetic studies on two forms of Fe(III) phosvitin at an Fe/protein ratio of 50/1 showed the iron atom coordinated by the oxygen atoms of the serine phosphate groups. It was suggested that in the green form the bound iron(III) ions have a tetrahedral coordination and magnetic susceptibility data indicated the formation of polynuclear iron clusters in which the metal ions are antiferromagnetically coupled.[13]

Because of the unordered conformation of phosvitin and its unique ability to bind metal ions, EXAFS spectroscopy seems to be exceptionally well suited to provide detailed structural information on the metal binding sites of this protein. We have recently investigated by CD and EXAFS techniques the interaction of

† University of Siena.
‡ University of Florence.
§ University of Washington.

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