Difference Ultraviolet Spectroscopic Studies on the Binding of Lanthanides to Human Serum Transferrin

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Apotransferrin in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid at 25 °C and pH 7.4 has been titrated with Pr^{3+}, Gd^{3+}, Tb^{3+}, Ho^{3+}, Er^{3+}, and Lu^{3+}, and the metal binding has been monitored by difference ultraviolet spectroscopy. Molar absorptivities for the lanthanide–transferrin complexes of about 20 000 M^{-1} cm^{-1} per binding site have been calculated from the initial slopes of the titration curves. There is little change in molar absorptivity as a function of ionic radius between Lu and Gd. However, there is a consistent decrease in the number of metal ions bound at saturation from 1.9 for the smallest ion, Lu^{3+}, to 1.6 for Gd^{3+}. This decrease is attributed to competitive binding of the larger lanthanide ions by the ambient bicarbonate in the buffer. Titrations of both forms of monoferric transferrin indicate that lanthanide binding is consistently stronger at the vacant C-terminal binding site of N-terminal monoferric transferrin. Sequential macroscopic equilibrium constants of $\log K_1^* = 7.96 \pm 0.21$ and $\log K_2^* = 5.94 \pm 0.26$ have been determined for the binding of Gd^{3+} to the two transferrin metal-binding sites. The separation of 2.0 log units between the successive binding constants is unusually large compared to results for d-block metal ions.

Introduction

Human serum transferrin (Tf) is responsible for the transport of ferric ion among sites of uptake, utilization, and storage.[1-4] It also contributes to bacteriostasis by restricting serum concentrations of available iron to very low levels. Lanthanide–transferrin complexes have been studied repeatedly over the last 20 years, primarily because these metal ions are useful luminescent probes of the transferrin metal-binding sites.[5-11] With the recent interest in lanthanides, particularly Gd^{3+}, as magnetic resonance imaging contrast agents,[12] there is more interest in lanthanide–transferrin complexes.

There have been conflicting reports on the stoichiometry of lanthanide–transferrin complexes. Luk[5] reported difference UV titrations of apoTf at pH 8.5. He concluded that Tf binds 2 equiv of Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+} but that the protein binds only 1 equiv of the larger Nd^{3+} and Pr^{3+} ions. Subsequent difference UV studies appeared to confirm a 2:1 stoichiometry at higher pH for Er^{3+}, Eu^{3+}, Gd^{3+}, and Ho^{3+}.[13,14] Recent difference UV titrations at pH 7.4 with Nd and Sm contradicted Luk's original report by showing partial occupancy of the second binding site by these metal ions.[15] This discrepancy had been attributed to competition for the metal ions from the higher concentrations of bicarbonate used by Luk. However, recently it was reported that apoTf binds only one Gd^{3+} ion at pH 7.4, even at *ambient* bicarbonate concentrations.[16] If true, this would represent a highly unusual degree of site selectivity for Tf as well as an unusual inconsistency in the coordination chemistry of Gd compared to other lanthanides. Since we have a long-standing interest in the thermodynamics of metal binding to transferrin, we decided to investigate further the stoichiometry of the binding of lanthanides to transferrin. More extensive studies have been conducted to determine equilibrium constants for the binding of Gd^{3+}. The results show that there is a strong correlation between the ionic radius of the lanthanide and the degree of saturation of apoTf. Although it is not possible to saturate both Tf binding sites with Gd^{3+}, there is very clear evidence of binding at both the N- and C-terminal sites of the protein.

Experimental Section

Materials. Human apotransferrin was purchased from Calbiochem and purified as previously described to remove chelating agents.[15] Monoferric and diferric transferrins were prepared as previously described.[17] Stock solutions of each lanthanide were prepared from the reagent grade chloride salt and standardized by complexometric titration with ethylenediaminetetraacetic acid (EDTA).[18] Na_2EDTA, nitrilotriacetic acid (NTA), and ethylenediamine-*N*,*N'*-diacetic acid (EDDA) were purchased and used as received.

Methods. Solutions of apotransferrin in 0.1 M *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (hepes) were adjusted to pH 7.4 with sodium hydroxide. No bicarbonate was added to the buffer. Equal volumes of this solution were added to dry sample and reference cuvettes, and a baseline of protein vs protein was recorded from 320 to 240 nm. The sample cuvette was titrated with an acidic solution of the selected lanthanide ion, while equal volumes of water were added to the reference cuvette. During the titration, the sample cuvette was maintained at 25 °C by a thermostated cell holder connected to an external circulating water bath. Difference UV spectra were recorded with a Varian 2290 spectrophotometer.

To determine binding constants for Gd^{3+}, apotransferrin samples were titrated with solutions of Gd^{3+} that also contained varying ratios of either NTA or EDDA as a competing ligand. Binding constants were calculated as described previously.[19]

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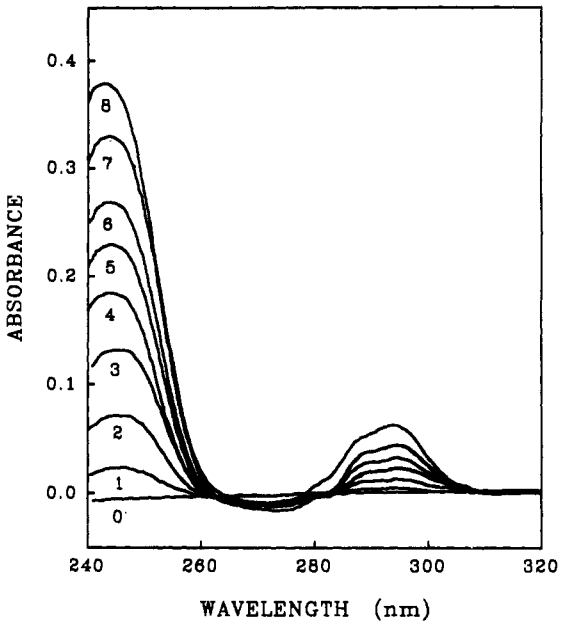

Figure 1. Difference UV spectra produced by the addition of aliquots of 2.81×10^{-4} M Nd^{3+} to 2.2 mL of 1.28×10^{-5} M apoTf in 0.1 M hepes buffer at pH 7.4 and 25 °C. Curve 0 is a baseline of protein versus protein. Spectra: (1) 10 μL of Gd; (2) 30 μL of Gd; (3) 50 μL of Gd; (4) 70 μL of Gd; (5) 90 μL of Gd; (6) 110 μL of Gd; (7) 150 μL of Gd; (8) 260 μL of Gd.

Urea polyacrylamide gel electrophoresis was conducted as previously described.[17] Briefly, slab gels were cast with 8% (w/v) of 19:1 polyacrylamide/*N,N'*-methylenebis(acrylamide) in a buffer containing 6.2 M urea, 1.6 mM EDTA, 10 mM boric acid, and 0.1 Tris at pH 8.4. The tank buffer was the same except that no urea was included. Gels were run at a constant current of 30 mA for approximately 15 h in a Bio-Rad Protean TM-11 16-cm vertical slab gel assembly cooled by water at 4 °C from an external circulating water bath. Gels were stained with Coomasie blue and scanned on a Bio-Rad Model 620 densitometer. Peak areas were obtained by integration using Bio-Rad 1D software.

Crystallographic coordinates for rabbit serum transferrin were obtained from Dr. Peter Lindley and the Birkbeck College transferrin group. The coordinates reflect a slightly better refinement than that previously reported,[20] such that the atomic positions of the synergistic bicarbonate anion are now resolved. The structure was examined in detail using a Silicon Graphics 4D-80GT graphics workstation running the program Insight.

Results

Molar Absorptivities. In the first series of experiments, apotransferrin (apoTf) was titrated with acidic solutions of the chloride salts of Lu^{3+}, Er^{3+}, Ho^{3+}, Tb^{3+}, Gd^{3+}, and Pr^{3+} with no added chelating agent. A typical family of difference spectra for Gd^{3+} is shown in Figure 1. The spectra are essentially identical for all the lanthanides studied.

To normalize data from run to run and to correct for dilution effects, the absorbance data are converted to apparent absorptivities by dividing the absorbance at 245 nm by the analytical concentration of transferrin. The resulting absorptivity is plotted versus the ratio of total lanthanide to total transferrin. Titrations of apoTf with Lu^{3+} and Gd^{3+} are shown in Figures 2 and 3, respectively.

The initial slope of the apoTf titration curve is linear for both cations. This linearity indicates that, in the early stages of the titration, the transferrin is binding essentially 100% of the metal ion in each aliquot of titrant. Under these conditions the initial slope of the titration curve is equal to the molar absorptivity of the lanthanide–transferrin complex ($\Delta\epsilon_M$). Molar absorptivities

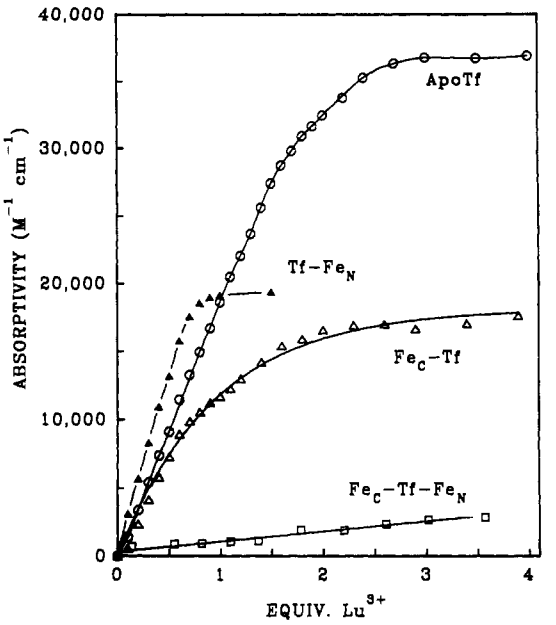

Figure 2. Titration curves for the addition of Lu^{3+} to approximately 15 μM solutions of apoTf, both forms of monoferric transferrin, and diferric transferrin in 0.1 M hepes buffer at 25 °C and pH 7.4.

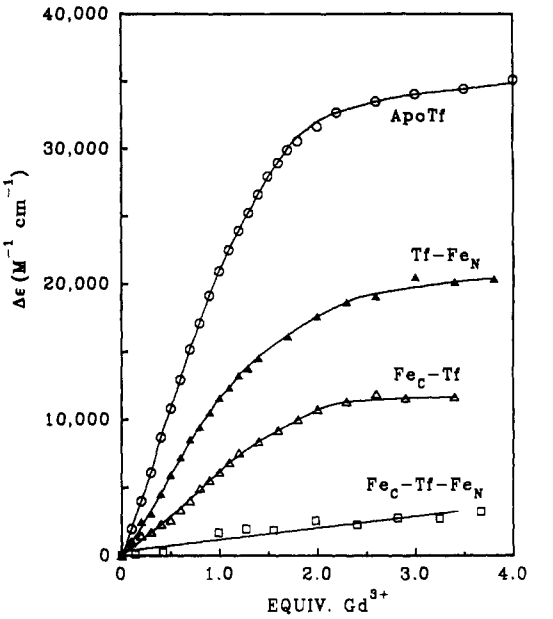

Figure 3. Titration curves for the addition of Gd^{3+} to approximately 15 μM solutions of apoTf, both forms of monoferric transferrin, and diferric transferrin in 0.1 M hepes buffer at 25 °C and pH 7.4.

Table I. Molar Absorptivities and Equivalents of Metal Ions Bound to Apotransferrin at Saturation

metal ion	$\Delta\epsilon_M$ (M^{-1} cm^{-1})	n_{max}[a]	ionic radius (Å)[b]
Lu^{3+}	19 900 ± 1200	1.9	0.861
Er^{3+}	20 800 ± 1000	1.8	0.890
Ho^{3+}	20 300 ± 1300	1.8	0.901
Tb^{3+}	20 600 ± 1600	1.7	0.923
Gd^{3+}	21 600 ± 1700	1.6	0.938
Sm^{3+}	21 000[c]	1.5	0.958
Nd^{3+}	18 700[c]	1.4	0.983
Pr^{3+}	≥12 300	0.9–1.2[d]	0.990

[a] Calculated by use of eq 1. [b] Radii for 6-coordinate complexes. Radii for 7-coordinate complexes are approximately 0.05 Å larger. [c] Results from ref 15. [d] Based on estimates of $\Delta\epsilon_M$ of 16 000–20 000 M^{-1} cm^{-1}.

calculated by this method are listed in Table I. The $\Delta\epsilon_M$ values for Lu^{3+}, Er^{3+}, Tb^{3+}, Ho^{3+}, and Gd^{3+} are all about 20 000 M^{-1} cm^{-1}.

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Titrations were also conducted with Pr^{3+}. The binding of Pr^{3+} appears to be much weaker than that of the other lanthanides. The initial slope of the Pr^{3+} titration curve is quite low ($\sim$12 000 M^{-1} cm^{-1}), presumably due to only partial binding of the metal ion, even in the beginning of the titration. Thus one cannot calculate a reliable value of the molar absorptivity for this metal ion.

Isosbestic points are observed in the lanthanide–Tf difference UV spectra at 262 and 282 nm. In Gd titrations, these points are maintained until one has added approximately 1.5 equiv of metal ion, after which there is a gradual increase in the absorbance at 262 nm. There have been previous reports that bicarbonate interferes with lanthanide binding to apoTf.[14-16] The solubility product of $Gd_2(CO_3)_3$ is $10^{-32.2}$.[21] At ambient bicarbonate concentrations at pH 7.4, this K_{sp} is exceeded at 2.2×10^{-7} M free Gd^{3+}. In the apoTf titrations, this corresponds to only 0.02 equiv of free Gd. Thus the K_{sp} must be exceeded as soon as there is any detectable deviation from linearity in the titration curves.

Diferric transferrin was titrated with both Lu^{3+} and Gd^{3+}, as shown in Figures 2 and 3. One would not expect the weakly binding lanthanides to displace the much more strongly bound ferric ion from its transferrin binding site. For both metal ions, there was a gradual increase in the baseline from 240 to 320 nm, but there were no traces of the peaks near 245 and 290 nm that are characteristic of metal binding to the specific Tf binding sites.[5,13,15,22] The linear increase in absorbance at 245 nm, the absorbance maximum of the lanthanide–Tf complexes, had slopes of 700 M^{-1} cm^{-1}/equiv of Lu^{3+} and 800 M^{-1} cm^{-1}/equiv of Gd^{3+}. The same featureless increase in the baseline was observed when either Gd or Lu was titrated into pH 7.4 hepes buffer. Although no precipitation was visible in any of the titration solutions, this gradual shift in the baseline and the loss of the isosbestic point at higher metal:Tf ratios were attributed to light scattering from Gd–carbonate polymers.

One can calculate n, the number of metal ions bound at any point in the apoTf titration curve, from

$$n = \frac{\Delta\epsilon_{obs} - \epsilon_{La}\, eq_{La}}{\Delta\epsilon_{M} - \epsilon_{La}} \qquad (1)$$

where $\Delta\epsilon_{obs}$ is the observed absorptivity at any point, $\Delta\epsilon_M$ is the molar absorptivity of the lanthanide–Tf complex, ϵ_{La} is the absorptivity of the free lanthanide ion determined from titrations of diferric Tf, and eq_{La} is the molar ratio of lanthanide to Tf. Titration curves for apoTf have been converted to plots of n vs equivalents of metal as shown in Figure 4. Data for Nd^{3+} and Sm^{3+} from ref 15 are also shown in Figure 4. There is essentially complete binding of the first equivalent of Lu^{3+}, Er^{3+}, Ho^{3+}, Tb^{3+}, Gd^{3+}, and Sm^{3+}. The Nd^{3+} plot begins to curve at about 0.8 equiv. The plot for Pr^{3+} is calculated using a molar absorptivity of 20 400 M^{-1} cm^{-1}, which is the average value for the other lanthanide. However, this is only an estimate, and the data in Figure 4 are meant only to show that significantly less Pr^{3+} binds to apoTf.

All the apoTf titration curves reach a plateau after the addition of 2.5–3.0 equiv of metal ion. The value of n corresponding to this plateau is designated n_{max}, the maximum number of bound metal ions per transferrin molecule. Ionic radii and n_{max} values are listed in Table I. Lu^{3+} has the largest n_{max} value of 1.9. There is a steady decrease in n_{max} with increasing ionic radius. However, it is clear that both binding sites must be involved for all metal ions except Pr^{3+}. The appearance of the plateau at $n = 1$ for Pr^{3+} may be fortuitous and does not necessarily reflect any unusual differentiation between metal binding at the two sites.

Ferric ion can be used to selectively block one Tf binding site while one measures lanthanide binding at the remaining site.

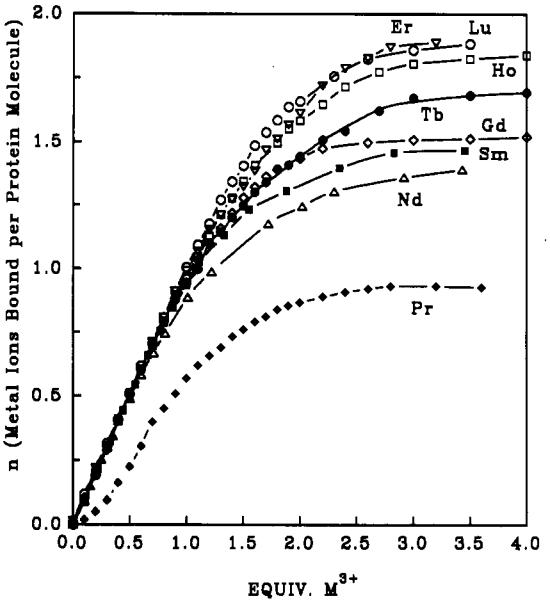

Figure 4. Plots of n, the number of metal ions bound per transferrin molecule, vs the equivalents of metal ion added to the protein, based on titrations of approximately 15 μM apoTf in 0.1 M hepes at 25 °C and pH 7.4 with aliquots of acidic solutions of Lu^{3+} (○), Er^{3+} (▽), Ho^{3+} (□), Tb^{3+} (●), Gd^{3+} (◇), Sm^{3+} (■), Nd^{3+} (△), and Pr^{3+} (◆). For comparison, data for Nd^{3+} and Sm^{3+} have been taken from ref 15.

Table II. Number of Metal Ions Bound per Mole of Vacant Binding Site in Monoferric Transferrins

metal ion	n_{max} for Tf–Fe_N	n_{max} for Fe_C–Tf	metal ion	n_{max} for Tf–Fe_N	n_{max} for Fe_C–Tf
Lu^{3+}	0.9	0.9	Tb^{3+}	1.0	0.6
Er^{3+}	1.0	0.8	Gd^{3+}	0.9	0.5
Ho^{3+}	1.2	0.7			

Both forms of monoferric transferrin have been prepared and have been characterized by polyacrylamide gel electrophoresis. Densitometry scans indicate that these samples are 80–90% pure C- or N-terminal monoferric with some diferric Tf present. It is particularly important that the samples contain little apoTf, which would also bind the metal and complicate the assessment of site-specific binding.

The spectra generated by titrations of monoferric transferrins are essentially identical to those for apoTf except for a lower intensity. Titrations of both forms of monoferric transferrin for Lu^{3+} and Gd^{3+} are shown in Figures 2 and 3, respectively. There is almost complete saturation of both forms of monoferric Tf by Lu^{3+}. This is consistent with the observation of $n_{max} = 1.9$ for the titration of apoTf with Lu^{3+}. The titrations clearly indicate weaker binding of Lu at the vacant N-terminal site of Fe_C–Tf.

Titration of Tf–Fe_N with Gd^{3+} eventually produces an absorptivity of approximately 20 000 M^{-1} cm^{-1}, indicating that the vacant C-terminal site has been saturated. However, the vacant N-terminal site of Fe_C–Ft is not saturated by Gd^{3+}. This is consistent with the value of $n_{max} = 1.6$ observed for the titration of apoTf.

Using the molar absorptivities discussed above for the free lanthanides and the lanthanide–transferrin complexes, n_{max} values for saturation of the vacant binding sites of both forms of monoferric transferrin have been calculated. These site-specific n_{max} values are listed in Table II. In all cases, n_{max} is $\sim$1.0 for the vacant C-terminal site of N-terminal monoferric transferrin. The n_{max} values for lanthanide binding to the vacant N-terminal site of Fe_C–Tf are smaller and show the same decrease with ionic radius observed for the n_{max} values of apoTf. There is reasonably good agreement between the n_{max} values for apoTf in Table I and the sums of the two site-specific n_{max} values listed in Table II.

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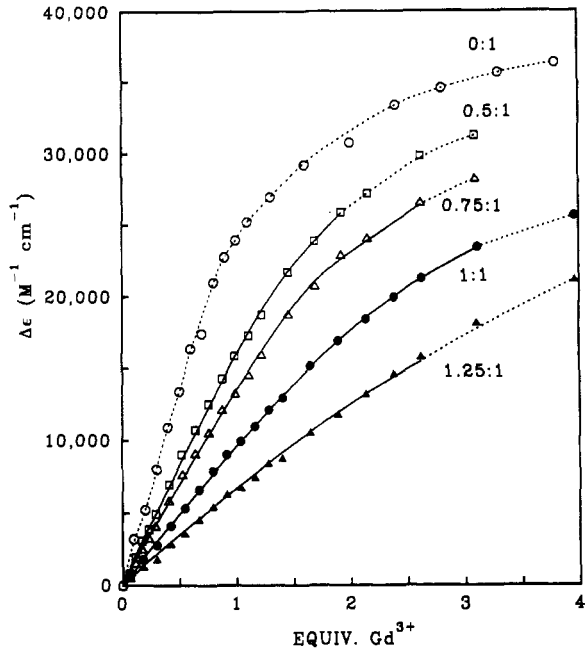

Figure 5. Titrations of approximately 15 μM apoTf in 0.1 M hepes at 25 °C and pH 7.4 with 330 μM solutions of Gd^{3+} that contained varying NTA:Gd ratios. Solid lines represent least-squares fits of the data. Dashed lines show data excluded from the refinements for reasons discussed in the text.

Gd Binding Constants. Successive metal-binding equilibria of transferrin can be described by the following equations:

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

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

The binding of metal ions to apoTf usually releases two or three protons per metal ion.[22] In addition, apoTf is in equilibrium with free bicarbonate and actually exists in solution as a mixture of true apoTf and both 1:1 and 2:1 bicarbonate–Tf species.[23,24] Thus eqs 2 and 3 represent effective binding constants that are valid only under the experimental conditions of pH 7.4 and ambient bicarbonate (0.20 mM).

Titrations with Gd^{3+} were repeated using metal titrant solutions that contained a range of concentrations of either NTA or EDDA. The chelating agents compete with the protein for the metal ions and thus systematically reduce the observed absorptivity. A series of titration curves for Gd–NTA solutions are shown in Figure 5.

The titration data in Figure 5 can be fit using the appropriate mass balance equations for metal ion, transferrin, and competing ligand, a fixed value of the molar absorptivity of Gd–Tf obtained from the initial slope of the apoTf titration curve, and log K_1^* and log K_2^* as the only adjustable parameters. The calculations were restricted to the range of data over which the isosbestic point at 262 nm was maintained. The data were further restricted to points for which the calculated concentration of free Gd was below the upper limit set by the solubility product of $Gd_2(CO_3)_3$. These restrictions limited the Gd–Tf data to $\Delta\epsilon$ values below ~26 000 M^{-1} cm^{-1}. The solid lines in Figure 5 represent the least-squares fits of the restricted data.

The effective binding constants for Gd–Tf are shown in Table III. There is some discrepancy between the log K_1^* values determined with NTA and EDDA. A Student's t-test on the individual means shows that the difference in mean log K_1^* values is statistically significant at $\alpha < 0.005$.[25] There is no statistical significance to the difference in the two mean log K_2^* values.

Table III. Gadolinium–Transferrin Binding Constants

ligand	n_1[a]	log K_1^* ± 2SEM	n_2[a]	log K_2^* ± 2SEM	Δ log K
NTA	13	8.18 ± 0.22	10	6.04 ± 0.28	2.14
EDDA	8	7.61 ± 0.15	3	5.59 ± 0.38	2.02
overall means	21	7.96 ± 0.19	13	5.94 ± 0.26	2.02

[a] Number of replicate titrations used to calculate the corresponding log K value.

The log K values listed in Table III were calculated using a model in which the molar absorptivities of the two transferrin binding sites are equal. Further calculations were conducted for seven of the $Gd(NTA)_x$ titrations at lower NTA ratios for which the extent of binding was sufficiently high to allow one to refine both K_1^* and K_2^*. In these calculations, the molar absorptivity of the weaker N-terminal binding site was varied from 100% to 50% of the value for the stronger C-terminal site. When the N-terminal absorptivity was varied from 100% and 65% of the C-terminal absorptivity, the average value of log K_1^* varied only from 8.18 ± 0.36 to 8.24 ± 0.36. For comparison, a value of log K_1^* of 8.18 ± 0.32 was calculated for five other $Gd(NTA)_x$ titrations in which the degree of binding to Tf was so low that only log K_1^* could be calculated. This value of log K_1^* is independent of any assumptions regarding $\Delta\epsilon_M$ for the weaker binding site. The variation in the N-terminal absorptivity from 100% to 65% also has no significant effect on the overall quality of the least-squares fits for each titration. However, if the absorptivity of the N-terminal site is reduced further to 50% of the absorptivity of the C-terminal site, then the value for log K_1^* begins to increase more sharply and the quality of the fits becomes worse.

As one would expect, the value of log K_2^* is sensitive to the molar absorptivity of the N-terminal site. When the N-terminal absorptivity is varied from 100% to 50% of the absorptivity of the C-terminal site, the value of log K_2^* increases from 5.90 to 6.97, while the standard deviation in log K_2^* also increases from 0.3 to 0.5.

Linear Free Energy Relationships. One can compare the transferrin binding constants of different lanthanides using linear free energy relationships (LFER's). Each point in an LFER consists of the stability constant of the sample metal ion as the y-coordinate and the stability constant of a reference metal ion as the x-coordinate. LFER's for Gd^{3+} vs both Nd^{3+} and Sm^{3+} based on approximately 40 metal complexes with low-molecular-weight (LMW) ligands that bind through a combination of nitrogen and oxygen donors are shown in Figure 6. The data for the LMW ligands were taken from Martell and Smith.[21] The LFER's for the LMW ligands are described by the equations

$$\log K_{Gd} = (1.056 \pm 0.009) \log K_{Nd} - (0.15 \pm 0.11) \quad (4)$$

$$\log K_{Gd} = (1.021 \pm 0.007) \log K_{Sm} - (0.14 \pm 0.09) \quad (5)$$

The data points for transferrin are shown as the closed circles for Nd^{3+} and the closed triangles for Sm^{3+}. The Gd–Sm LFER underestimates log K_1^* and log K_2^* by 0.8 and 0.6 log unit, respectively, while the Gd–Nd LFER underestimates the experimental log K^* values by 1.7 and 0.8 log units.

Discussion

Metal–Transferrin Stoichiometry. When metal binding to transferrin is very strong, spectrophotometric titrations show a sharp break at the point of saturation of the two metal-binding

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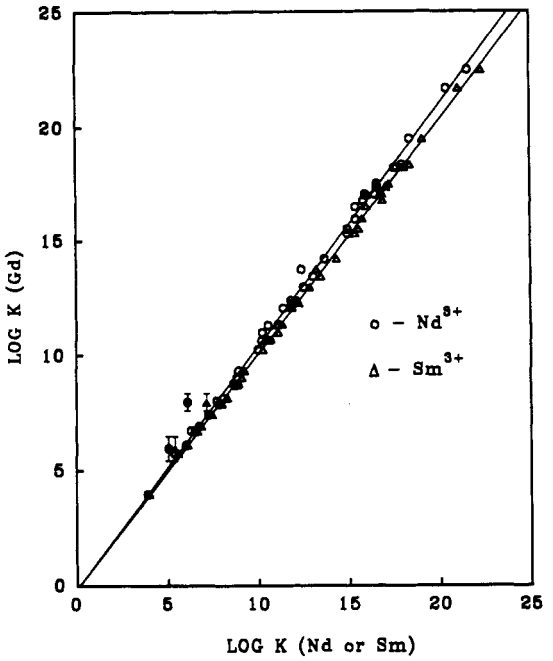

Figure 6. Linear free energy relationships for the complexation of Gd, Nd, and Sm. Each data point represents a different chelating agent. The y-coordinate is the stability constant for Gd^{3+}, while the x-coordinate is the stability constant for either Nd^{3+} or Sm^{3+}. The data points corresponding to the transferrin stability constants with these metals are shown with 95% confidence intervals as the filled circles for Nd and the filled triangles for Sm.

sites. This approach works quite well with the natural substrate, Fe^{3+}, which has an effective binding constant of about $10^{20.7}$.[26,27] However, a distinct end point is seldom observed with other metal ions.[28] In most cases, binding with either hydroxide or bicarbonate will begin to compete with transferrin binding, so that one cannot saturate the two binding sites. This has been observed previously in titrations at pH 7.4 with Ga(III),[21] Zn(II),[29] Cd(II),[30] Nd(III) and Sm(III),[15] and Al(III).[31] Reasonably clear breaks near 2.0 equiv of lanthanide can be observed for titrations at pH >8.5, where the protein binding is stronger.[13,14] However, as a general rule, one cannot rely on the break in the metal titration curve to define the number of available binding sites.

The difference UV titrations of apoTf as well as N- and C-terminal monoferric transferrins demonstrate unequivocally that all the lanthanides with the possible exception of Pr^{3+} bind in substantial amounts to both Tf binding sites at pH 7.4. The final absorptivity values in the titrations of apoTf are at least 1.5 times larger than the molar absorptivities, which requires binding to both sites. In addition, significant absorbances are observed upon titration of both forms of monoferric transferrin. The C-terminal monoferric transferrin has no detectable N-terminal monoferric transferrin and only about 4% apoTf. The measured absorbances are much too large to be attributed to the apoTf impurity and therefore must be due to binding at the weaker N-terminal site.

These difference UV results are consistent with the Eu–Tf fluorescence spectra, which show distinct peaks for each binding site.[9] However, these results conflict with Gd EPR titrations, which failed to detect any binding of Gd^{3+} to the N-terminal binding site at pH 7.4.[16] Although we clearly detect binding at both sites, the degree of site selectivity for Gd^{3+} does appear to be unusually large. Typically, the two sites differ by a factor of 10–20 in their binding affinities for a given metal ion,[28] while the Gd constants differ by a factor of 100.

Differences in solution conditions may account for the variation between difference UV and EPR results at pH 7.4. The EPR titrations of monoferric transferrins were performed with solutions that are over 30 times more concentrated than our difference UV samples. As mentioned earlier, we are operating near the limit of saturation with respect to lanthanide–carbonate complexes. In addition, the EPR spectra were recorded at 77 K.

The steady decrease in n_{max} as a function of ionic radius probably reflects two factors. One would expect the lanthanide–Tf binding constant to decrease as the charge:radius ratio decreases. In addition, the solubility product for the lanthanide carbonate complexes decreases by 2 orders of magnitude with increasing ionic radius from 10^{-31} for Yb^{3+} to 10^{-33} for Nd^{3+}.[21] Both these factors work to restrict Tf binding as the ionic radius increases. There is no visible precipitation of insoluble lanthanide–carbonate complexes. However, isosbestic points for the larger lanthanides are lost at higher metal:Tf ratios. The loss of the isosbestic points and the small increase in absorbance during the titration of both diferric transferrin and hepes buffer alone are attributed to light scattering from polymeric lanthanide–carbonate complexes.

It is important to note that the n_{max} values are not reached until excess lanthanide is added. Spectroscopic studies are often conducted by adding stoichiometric amounts of the lanthanide. If one simply adds 2 equiv of lanthanide to apoTf, only 1.3–1.7 equiv will bind to the protein at pH 7.4 and ambient bicarbonate concentration.

Molar Absorptivities. Previous difference UV studies on lanthanide–transferrin complexes assumed that two metal ions were bound and focused on the maximum absorptivity at saturation, rather than measuring molar absorptivities from the initial slopes of the titration curves.[5,13,14,16] As a result, the molar absorptivities reported in this study are slightly higher than most of the literature values. On the basis of the spectra of the model compound ethylenebis((*o*-hydroxyphenyl)glyine), Pecoraro et al.[13] calculated a molar absorptivity of 8300 M^{-1} cm^{-1} in the difference UV spectrum for each tyrosine coordinated to a lanthanide. The observed molar absorptivities of the lanthanide–transferrin complexes correspond to about 2.5 tyrosines/metal ion. It has been suggested that a third tyrosine might be included in an expanded coordination shell of the lanthanides.[9,16]

The crystal structure of rabbit serum transferrin shows noncoordinating tyrosines near the metal binding sites.[20] In the C-terminal lobe, the nearest uncoordinated tyrosine is Tyr-412 (residue numbers correspond to the rabbit serum transferrin sequence from ref 32), which is located in the cleft between the two domains and is only 6.28 Å from the iron. However, it appears to be hydrogen bonded to Arg-632 and partially blocked from the iron by the coordinated histidine ligand. Tyr-85 occupies a similar position in the N-terminal lobe at a distance of 6.30 Å from the iron. Luminescence lifetime studies have indicated that Tb^{3+} in the transferrin binding site probably retains one coordinated water molecule.[5,7] It may be that one or both of these tyrosines are hydrogen bonded to coordinated waters, rather than coordinated directly to the metal ion, which could contribute to the larger $\Delta\epsilon_M$ for the lanthanides.

Gadolinium–Transferrin Binding Constants. The first lanthanide–transferrin binding constants were reported by Harris for Nd^{3+} and Sm^{3+}.[15] The stronger C-terminal binding site had constants of only $10^{6.09}$ and $10^{7.13}$ for Nd^{3+} and Sm^{3+}, respectively. Since one expects increasing log K values with decreasing ionic radius, the Gd log K_1* value of 7.96 is qualitatively consistent with the Nd and Sm values. The much weaker binding of the lanthanides compared to Fe^{3+} is consistent with the smaller charge:

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radius ratio of the lanthanides and the weaker covalent bonding of the 4f orbitals versus the 3d orbitals of the first-row transition metals.[33]

The n_{max} values from the titrations of C- and N-terminal monoferric transferrins indicate that binding to the C-terminal site is stronger. A preference for the C-terminal site has been observed in all cases evaluated thus far, with the exception of Ni^{2+}.[28] Thus the larger K_1^* value is associated primarily with the C-terminal site.

A smaller Gd^{3+}–transferrin binding constant of log $K_1^* = 6.83$ based on equilibrium dialysis studies has been reported.[16] However, these samples covered Gd:Tf ratios from 0.9 to 16. Our results indicate that the Gd–Tf equilibrium is significantly perturbed by the formation of carbonates at higher Gd:Tf ratios. The calculations from difference UV spectra reported here are based primarily on data where the Gd:Tf ratio is between 0 and 2.

The behavior of the lanthanide–Tf solutions after the disappearance of the isosbestic points at high Gd:Tf ratios is difficult to describe. There is no doubt that we exceed the K_{sp} of gadolinium during the titrations, yet no precipitate is observed. This suggests that the solutions at high metal:Tf ratios are not at equilibrium but are supersaturated with respect to free gadolinium. This would account for the continuing increase in $\Delta\epsilon_{obs}$ under conditions where K_{sp} would predict no further increase in transferrin binding. We would stress, however, that the uncertainty in the nature of the solution at high metal concentrations does not affect the credibility of the reported binding constants, which are calculated from solutions in which the isosbestic point is retained and the K_{sp} of gadolinium carbonate is not exceeded.

Binding constants have been calculated using a model in which the transferrin binding sites have equal molar absorptivities. The titrations of monoferric transferrin with Lu^{3+} indicate that the sites have *comparable* molar absorptivities, but the data are not sufficiently accurate to establish *equal* molar absorptivities. The value of log K_1^* and the overall quality of the fits are insensitive to changes in the N-terminal absorptivity over the range of 100%–65% of the value of the C-terminal site.

At higher NTA:Gd ratios, the binding is restricted to less than 1 equiv of metal ion, and one can calculate values of K_1^* that are independent of any assumptions regarding the value for the molar absorptivity of the weaker binding site. The results for these independent calculations agree with the results in which the N-terminal site molar absorptivity is 100% of the C-terminal site molar absorptivity. In addition, the standard deviation in log K_2^* is at a minimum when the N-terminal molar absorptivity is 100% of the C-terminal molar absorptivity. Any decrease in $\Delta\epsilon_M$ for the N-terminal site would increase the n_{max} values for both apoTf and Fe_C–Tf. Since the n_{max} values for Lu^{3+}, Er^{3+}, and Ho^{3+} are in the ranges 1.8–1.9 for apoTf and 0.7–0.9 for Fe_C–Tf, one could not decrease the N-terminal molar absorptivity by more than about 20% without pushing n_{max} above 1.0 for the N-terminal site. These data do not prove that the molar absorptivities of the two sites are identical. However, there is no compelling reason to set the N-terminal molar absorptivity at less than 100% of the C-terminal absorptivity, and any decrease in $\Delta\epsilon_M$ for the N-terminal site must be less than about 20%.

Since log K_2^* is sensitive to the value of the absorptivity for the N-terminal site, the real uncertainty in log K_2^* is larger than that indicated by the standard deviation given in Table III. A 20% decrease in $\Delta\epsilon_M$ for the N-terminal site would shift log K_2^* upward by about 0.3 log unit and decrease the apparent site selectivity for Gd^{3+} by a factor of 2.

The stability constants for LWM lanthanide complexes form unusually good LFER's as shown by the low standard deviations for both the slopes and intercepts in eqs 4 and 5. However, these LFER's consistently underestimate the Gd–Tf binding constants. It appears that the transferrin binding constants decrease with increasing ionic radius at a faster rate than do the stability constants of the LWM ligands. The transferrin binding constants of Zn^{2+} and Cd^{2+} ($r = 0.95$ Å) are quite consistent with the LFER between these metal ions. Thus large size alone does not lead to unusual binding affinity.

It is possible that changes in coordination number among the lanthanides affect the binding constants of the lanthanide–transferrin complexes. The assignment of coordination numbers for the lanthanide aquo ions has been a controversial issue.[33] However, it now appears that the aquo ions have a coordination number of 8 for the ions from Tb^{3+} to Lu^{3+}, a coordination number of 9 for the ions La^{3+} to Nd^{3+}, and intermediate coordination numbers for Sm^{3+}, Eu^{3+}, and probably Gd^{3+}.[34-37] Thus it is possible that variations in desolvation energies associated with complexation by Tf may affect the relative stabilities of the lanthanide–Tf complexes.

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