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Kinetics of Metal Ion Exchange between Citric Acid and Serum Transferrin

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The exchange of Fe^{3+}, Tb^{3+}, In^{3+}, Ga^{3+}, and Al^{3+} between the C-terminal metal-binding site of the serum iron transport protein transferrin and the low-molecular-mass serum chelating agent citrate has been studied at pH 7.4 and 25 °C. The removal of Ga^{3+}, In^{3+}, and Al^{3+} follows simple saturation kinetics with respect to the citrate concentration. In contrast, removal of both Fe^{3+} and Tb^{3+} shows a combination of saturation and first-order kinetic behavior with respect to the citrate concentration. The saturation component is consistent with a mechanism for metal release in which access to the bound metal is controlled by a rate-limiting conformational change in the protein. The first-order kinetic pathway is very rapid for Tb^{3+}, and this is attributed to a direct attack of the citrate on the Tb^{3+} ion within the closed protein conformation. It is suggested that this pathway is more readily available for Tb^{3+} because of the larger coordination number for this cation and the presence of an aquated coordination site in the Tb^{3+}–CO_3–Tf ternary complex. There is relatively little variation in the k_{max} values for the saturation pathway for Tb^{3+}, Ga^{3+}, Al^{3+}, and In^{3+}, but the k_{max} value for Fe^{3+} is significantly smaller. It is suggested that protein interactions across the interdomain cleft of transferrin largely control the release of the first group of metal ions, while the breaking of stronger metal–protein bonds slows the rate of iron release. The rates of metal binding to apotransferrin are clearly controlled in large part by the hydrolytic tendencies of the free metal ions. For the more amphoteric metal ions Al^{3+} and Ga^{3+}, there is rapid protein binding, and the addition of citrate actually retards this reaction. In contrast, the nonamphoteric In^{3+} ion binds very slowly in the absence of citrate, presumably due to the rapid formation of polymeric In–hydroxo complexes upon addition of the unchelated metal ion to the pH 7.4 protein solution. The addition of citrate to the reaction accelerates the binding of In^{3+} to apoTf, presumably by forming soluble, mononuclear In–citrate complexes.

Introduction

Human serum transferrin is the protein that transports iron though the blood.[1−3] It binds iron tightly enough to prevent hydrolysis and to deny this vital nutrient to potentially harmful bacteria, but in cooperation with its membrane-bound receptor, it is able to deliver iron to tissues efficiently and selectively. The distinguishing characteristic of the transferrins is that the metal binds to the protein only in the form of a ternary complex involving the metal ion, transferrin, and a carbonate anion derived from the buffer. The carbonate anion coordinates to the ferric ion as a bidentate ligand and also hydrogen bonds to charged and polar groups on the protein.[4]

The tertiary structure of transferrin consists of distinct C- and N-terminal lobes.[5,6] Each lobe is further divided by a cleft into two domains, with a single high-affinity iron binding site located within each cleft. There have been numerous studies on the mechanism by which low-molec-

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ular-mass ligands remove iron from native transferrin[7−19] and from a series of recombinant N- and C-lobe half-molecules.[20−26] It is generally believed that iron removal from the native, "closed" form of the protein is regulated at least in part by a rate-limiting conformational change to a more open structure that exposes the iron to attack by the entering ligand.

This conformational change mechanism for iron release, originally proposed by Bates and co-workers,[10,27] predicts saturation kinetics with respect to the ligand concentration. Although iron release by many ligands does follow simple saturation kinetics, several ligands show a more complex ligand dependence. They appear to follow saturation kinetics at lower ligand concentration, but there is an additional first-order component at higher ligand concentration.[8,9,11,17,18,28] The iron release process is strongly influenced by the solution pH, the structure of the ligand, and the binding of anions in the medium to allosteric sites on the protein. However, the details of how these factors all work together to govern the overall rate of iron release are still not clear.

This paper reports studies on a wider variety of metal ions to help delineate which elements of the metal release reaction mechanism are affected by the metal ion. For example, to what extent is the rate of the protein conformational change affected by the metal ion? In addition, transferrin is known to act as the serum transport protein for a number of metal ions, including Al^{3+}, Ga^{3+}, and In^{3+}.[29] Since citrate is one of the most important low-molecular-mass ligands in serum for these metal ions, the results reported here are relevant to their pharmacokinetics.

Experimental Section

Protein Samples. Human serum apotransferrin was purchased from Sigma. A stock solution was prepared by dissolving approximately 200 mg of apoprotein in 2 mL of a pH 7.4 buffer consisting of 0.1 M Hepes and 0.1 M $NaClO_4$. This solution was then flushed several times with the Hepes/perchlorate buffer in a 10 mL Amicon ultrafiltration cell fitted with a PM-30 membrane. The solution was concentrated to about 2 mL and flushed several more times with a 0.1 M Hepes buffer that did not contain any perchlorate. The final solution was passed through a 0.45 μm syringe filter to remove particles that collected during the ultrafiltration. The apotransferrin concentration was determined using a molar extinction coefficient at 278 nm of 93 000 M^{-1} cm^{-1}.

The method for preparing C-terminal monoferric transferrin (Fe_C−Tf) has been reported previously.[13,30] Slightly more than 1 equiv of iron as a pH 4 solution of ferric ion with a 2-fold excess of nitrilotriacetic acid (NTA) was added dropwise to a stirred solution of the apoTf. The NTA selectively delivers iron to the C-terminal binding site.[13,30,31] The Fe_C−Tf solution was purified by ultrafiltration to remove the free NTA.

N-terminal monoferric transferrin (Tf−Fe_N) was prepared from diferric transferrin (Calbiochem) by a slight modification of the method reported by Baldwin and de Sousa.[13] Approximately 250 μM diferric transferrin was reacted with 100 mM ethylenediaminetetraacetic acid (EDTA) in a pH 7.4 0.1 M Hepes buffer that also contained 2 M $NaClO_4$. The ClO_4^- accelerates iron removal from the C-terminal site while retarding iron removal from the N-terminal site, such that the rate of iron removal from the C-terminal site is about 260-times faster.[13] The reaction was allowed to proceed for about 70 min, at which time the absorbance had leveled off at about 50% of its original value. The sample was ultrafiltered using a Millipore Ultrafree centrifugal filter with a 30 kD nominal molecular mass cutoff to remove the EDTA and stop the iron removal reaction. The purity of the stock solutions of both Fe_C−Tf and Tf−Fe_N was confirmed by urea gel electrophoresis using reported methods.[30]

Metal Stock Solutions. The preparation and standardization of the stock solution of 0.1 M $AlCl_3$ in 0.1 M HCl has been described in detail previously.[32] Essentially the same methods were used to prepare and standardize a 0.1 M stock $GaCl_3$ solution. The preparation of the stock $TbCl_3$ solution has been described previously.[33] The stock $InCl_3$ solution was prepared by weighing a piece of pure indium metal in a 50 mL beaker and adding 10 mL of concentrated HCl. The beaker was loosely covered with a watch glass and gently heated to accelerate the dissolution of the indium metal. More concentrated HCl was added as needed to maintain a vigorous evolution of hydrogen from the metal surface. After all the metal had dissolved, the solution was transferred to a 100 mL volumetric flask and diluted to volume, to give a solution with approximately 0.1 M $InCl_3$ at pH 1. A 0.1 M ferric chloride solution in 0.1 M HCl was prepared and standardized as previously described.[15]

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Metal Ion Removal from Transferrin. The removal of iron from both forms of monoferric transferrin was monitored by the use of visible spectroscopy to follow the decrease in the Fe^{3+}−phenolate charge transfer band at 465 nm. Samples contained approximately 50 μM of the monoferric transferrin in 0.1 M pH 7.4 Hepes buffer along with citrate at a concentration ranging from 40 to 280 mM. Spectra were recorded either on a Cary-14 that had been modernized by OLIS or on a Hewlett-Packard 8452A diode array UV−vis spectrophotometer. The cuvettes were maintained at 25 °C during the equilibration and kinetic phases of the experiment by an external circulating water bath.

The removal of Ga^{3+}, Al^{3+}, and In^{3+} from the C-terminal binding site of transferrin was monitored by difference UV spectroscopy. The transferrin complexes of these metal ions all show a positive peak in the difference UV spectrum near 245 nm.[34−36] Kinetic samples were prepared directly inside a 1 cm quartz cuvette. A 15 μM solution of apoTf was prepared in a buffer consisting of 5 mM bicarbonate and 0.1 M Hepes at pH 7.4. Equal volumes of this solution were added to dry sample and reference cuvettes, and a baseline of apoprotein vs apoprotein was recorded prior to the addition of any metal ion. The addition of 0.8 equiv of metal ion produced a solution of 12 μM M−Tf complex as shown by the peak at 245 nm in the difference UV spectrum. The metal ions were all expected to bind predominantly at the C-terminal binding site of the protein (vide infra).

The equilibration time required for the initial metal−transferrin binding varied. For Ga^{3+} the binding was relatively rapid, and the solutions equilibrated within 10 min. The reaction with Al^{3+} was somewhat slower, and solutions were allowed to equilibrate for 30 min. The binding of In^{3+} to apotransferrin was quite slow, requiring about 3 h. After the metal ion had equilibrated with the apoprotein, the solution was reacted with excess citrate at concentrations ranging from 0.1 to 50 mM. The rate of metal ion removal was followed from the decrease in the 245 nm peak in the difference UV spectrum.

For more rapid reactions, equal volumes of the metal−transferrin complex and citrate were rapidly mixed within the cuvette of a Hi-Tech SFA-11 rapid kinetics accessory using manually operated drive syringes. The syringe drive mechanism was equipped with a switch that initiated data collection by the HP 8452A diode array spectrophotometer.

The release of Tb^{3+} from transferrin was followed by the use of fluorescence spectroscopy. Spectra were recorded with a Perkin-Elmer MPF-44B spectrofluorometer. Samples were prepared in a 1 cm cuvette by adding 0.8 equiv of Tb^{3+} to apoTf in a 0.1 M Hepes buffer with no added bicarbonate. After a few minutes, the complete binding of Tb^{3+} to the protein was verified by recording the fluorescence spectrum of the Tb−Tf complex (λ_{ex} = 278 nm, λ_{em} = 549 nm, corresponding to the $^5D_4 \rightarrow {}^7F_5$ Tb^{3+} f−f band). The appropriate amount of citrate was added to the cuvette, and the removal of Tb^{3+} from transferrin was monitored from the change in the emission intensity at 549 nm. The final transferrin concentration after addition of the citrate was 2 μM. The fluorescence studies on Tb^{3+} were conducted at room temperature.

Metal Ion Donation to Transferrin. A solution of the metal ion with the appropriate concentration of citrate was prepared fresh daily and used within about 1 h. For reactions with In^{3+} and Al^{3+}, 100 μL of the metal citrate complex was added manually to 900 μL of apotransferrin in a narrow cuvette, and the reaction was monitored from the absorbance change near 245 nm. Reactions with gallium citrate were run using the SFA-11 rapid kinetics accessory, which mixed equal volumes of metal−citrate and protein solutions. The concentrations of the metal−citrate solutions were adjusted such that the final samples contained 10 μM concentrations of both protein and metal with citrate concentrations ranging from 0 to 680 μM.

Metal-Binding Site Selectivity. The thermodynamic site selectivity for the binding of Ga^{3+} and Al^{3+} to the C- and N-terminal transferrin binding sites was determined by titrating both forms of the protein with aliquots of the metal aquo ions. The binding of each metal to the protein was monitored from changes in the difference UV absorbance spectrum at 245 nm.

Results

Removal of Iron by Citrate. Iron removal from the monoferric transferrins was followed from the absorbance change at 465 nm. Complete iron removal was obtained only at citrate concentrations above about 140 mM citrate. For reactions that go to completion, the kinetic data were fit to the equation

$$A_t = (A_0 - A_\infty)e^{-kt} + A_\infty \quad (1)$$

where A_0 and A_∞ are initial and final absorbances, A_t is the absorbance at any intermediate time t, and k is the first-order rate constant for iron release.

A significant fraction of the kinetics experiments involved iron removal reactions that did not go to completion. For these samples the metal-removal reaction was treated as the reversible reaction

$$\text{M−Tf} + \text{cta} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{apoTf} + \text{M(cta)} \quad (2)$$

which is pseudo-first-order in the forward direction in the presence of a large excess of ligand and second-order on the reverse reaction (cta = citrate). The rate equations for this type of system have been described in detail by Moore and Pearson.[37] The absorbance change at any intermediate time, ΔA_t, is $A_t - A_0$. For reversible reactions, A_∞ is defined as the final absorbance that would have been observed if the reaction had gone to completion and A_e is defined as the actual final absorbance observed at equilibrium. The value of ΔA_t as a function of time is given by the equation

$$\Delta A_t = \frac{\Delta A_0 \Delta A_e (e^{k_f \alpha t} - 1)}{\Delta A_0 - \Delta A_e + \Delta A_0 e^{k_f \alpha t}} \quad (3)$$

where $\Delta A_0 = A_\infty - A_0$, $\Delta A_e = A_e - A_0$, and $\alpha = (2\Delta A_0 - \Delta A_e)/(\Delta A_e)$. To fit the kinetic data for reactions at low citrate concentration, the average value for ΔA_0 for the reactions at high citrate concentration was calculated and used as a fixed constant along with the experimental value for ΔA_e in the calculation of k_f from the nonlinear least-squares fit of ΔA_t versus time to eq 3. This procedure has been used in this laboratory in previous studies on iron removal from trans-

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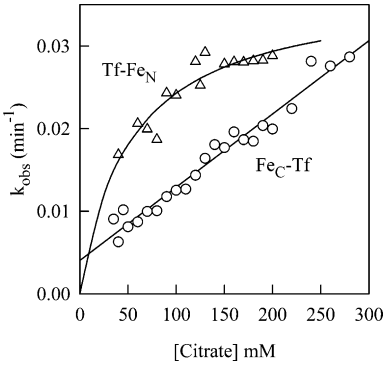

Figure 1. Pseudo-first-order rate constants for iron removal from both forms of monoferric transferrin by citrate as a function of the total citrate concentration: Fe_C-Tf = C-terminal monoferric transferrin; $Tf-Fe_N$ = N-terminal monoferric transferrin.

Table 1. Kinetic Parameters for the Removal of Metal Ions from the C-Terminal Site of Serum Transferrin by Citrate

metal ion	k_{max} (min^{-1})	k_d (mM)	k''' (M^{-1} min^{-1})
Tb^{3+}	2.39 ± 0.14	0.004 ± 0.002	640 ± 40
Ga^{3+}	1.79 ± 0.03	7.2 ± 0.4	
In^{3+}	0.638 ± 0.013	1.02 ± 0.06	
Al^{3+}	0.80 ± 0.04	2.05 ± 0.27	
Fe^{3+}	0.00403 ± 0.00050	nd	0.0887 ± 0.0037
Fe^{3+}(N-terminal)	0.0371 ± 0.0022	53 ± 10	

ferrin.[15] For reactions that go to completion, $\Delta A_0 = \Delta A_e$ and eq 3 reduces to eq 1 with $k = k_f$.

The rate constants for iron removal from each form of monoferric transferrin have been measured as a function of the concentration of citrate. The results are plotted in Figure 1. Iron removal from the N-terminal site follows simple saturation kinetics and can be described by the equation

$$k_{obs} = \frac{k_{max}[cta]}{k_d + [cta]} \quad (4)$$

The solid line in Figure 1 represents the least-squares fit of k_{obs} versus [cta] to eq 4, and the calculated values for k_{max} and k_d are listed in Table 1.

The rate constants for iron removal from the C-terminal site show a linear dependence on the citrate concentration with a nonzero *y*-intercept. Previous studies with several other ligands have shown a combination of a saturation component at low ligand concentration and a linear increase at high ligand concentration.[9,12,18] This type of behavior can be described by eq 5.

$$k_{obs} = \frac{k_{max}[cta]}{k_d + [cta]} + k'''[cta] \quad (5)$$

The results for the C-terminal site are interpreted as indicating that the k_d for the C-terminal site is much less than the minimum citrate concentration of 40 mM. Under conditions where $k_d \ll$ [cta], eq 5 reduces to

$$k_{obs} = k_{max} + k'''[cta] \quad (6)$$

Therefore, the *y*-intercept and slope of the linear plot for the C-terminal site in Figure 1 have been used to determine k_{max} and k''', which are listed in Table 1.

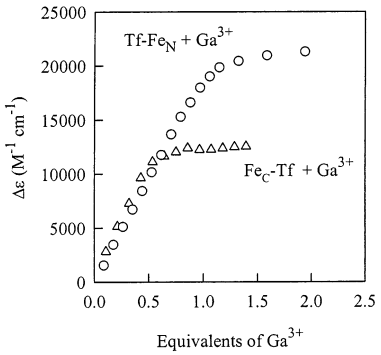

Figure 2. Difference UV titration of both forms of monoferric transferrin with Ga^{3+}.

Site Selectivity for Metal Binding to Apotransferrin. Presently there are no methods for preparing and purifying both the C-terminal and N-terminal transferrin complexes of Tb^{3+}, Ga^{3+}, Al^{3+}, and In^{3+}. Transferrin binding constants for all these metal ions have been reported,[33−36] and the ratio of K_1/K_2 varies from 9 to 3900. Since there does not appear to be significant cooperativity in the binding of metal ions to the two transferrin binding sites,[31] this high ratio of K_1/K_2 indicates that these metal ions bind more strongly to one of the two transferrin binding sites. Thus, the equilibration of ≤ 1 equiv of metal ion with apoTf should result in preferential binding to the more stable site.

The stronger binding site can be easily identified by titrations of two forms of monoferric transferrin. Samples of C-terminal and N-terminal monoferric transferrin (Fe_C-Tf and $Tf-Fe_N$) have been titrated with the Ga^{3+} and Al^{3+} aquo ions. These metal ions do not displace the more tightly bound Fe^{3+} from the monoferric transferrins, so their binding to the vacant binding sites can be monitored using difference UV spectroscopy. The titrations of monoferric transferrins with Ga^{3+} are shown in Figure 2. Both titration curves show an initial linear segment with a slope of approximately 20 000 M^{-1} cm^{-1}, which is the molar extinction coefficient per metal ion for the Ga−Tf complex.[34] This indicates that, in the early stages of the titrations, essentially all the added Ga^{3+} is binding to the protein and that the molar extinction coefficient is the same for each site.

In the titration of the empty C-terminal site of $Tf-Fe_N$, the Ga^{3+} titration curve is linear to approximately 1 equiv, after which it levels off. This is indicative of strong binding of Ga^{3+} to the C-terminal site. In contrast, the titration of the empty N-terminal site of Fe_C-Tf levels off after the addition of only 0.6 equiv of Ga^{3+} at a $\Delta\epsilon$ of about 12 000 M^{-1} cm^{-1}. This indicates weaker binding, such that competition from metal hydrolysis competes with the N-terminal site. This comparison of the titrations of the two monoferric transferrins clearly indicates that Ga^{3+} binds more tightly to the C-terminal site.

Both forms of monoferric transferrin were also titrated with Al^{3+}. The results are essentially the same as those observed for Ga^{3+}, except that the slope of the initial linear segment in each titration curve is about 15 000 M^{-1} cm^{-1}, which reflects the lower molar extinction coefficient of the Al−transferrin complex.[36] The titration curve for Al^{3+} binding

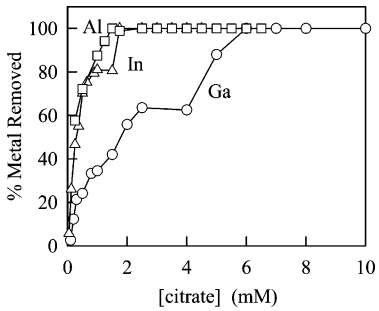

Figure 3. Percentage of metal ion removed at equilibrium as a function of the total citrate concentration for Ga^{3+} (○), In^{3+} (△), and Al^{3+} (□).

to the C-terminal site of Tf−Fe_N is linear to approximately 1.1 equiv, while the plot for the N-terminal site of Fe_C−Tf levels off after the addition of only 0.7 equiv of Al^{3+}. As with Ga^{3+}, the binding of less than 1 equiv of Al^{3+} to the N-terminal site is attributed to competition from hydrolysis.

Similar titrations of monoferric transferrins with Tb^{3+} have shown that Tb^{3+} also binds more strongly to the C-terminal site.[33] Since the binding of Tb^{3+}, Ga^{3+}, and Al^{3+} to transferrin equilibrates relatively rapidly, it is reasonable to infer that the site selectivity for the binding of these metal ions is under thermodynamic control and that addition of ≤1 equiv of metal ion will preferentially load the C-terminal binding site.

No site selectivity has been determined for the binding of In^{3+} because of the very long equilibration time following the addition of the In^{3+} aquo ion to transferrin. It is assumed that In^{3+} mimics Al^{3+} and Ga^{3+} and binds more strongly to the C-terminal site. However, given the slow kinetics of In^{3+} binding, it is less certain that the binding of substoichiometric amounts of In^{3+} will be under thermodynamic control.

Removal of Gallium, Indium, and Aluminum from the C-Terminal Transferrin Binding Site. Samples containing 12 μM concentrations of the metal transferrin complexes with the metal bound preferentially to the C-terminal binding site were prepared as described in the Experimental Section. Citrate was added, and the rate of metal ion removal was followed from changes in the difference UV spectrum. As with iron, this reaction goes to completion only at higher citrate concentrations. The fraction of metal ion removed from transferrin as a function of the citrate concentration is shown in Figure 3. There is relatively little variation among the three metal ions in terms of the competition between citrate and transferrin. The citrate concentration required to remove 50% of the metal is approximately 0.2 mM for Al^{3+}, 0.3 mM for In^{3+}, and 1.8 mM for Ga^{3+}.

The pseudo-first-order rate constants for the removal of Ga^{3+} from serum transferrin are plotted versus the citrate concentration in Figure 4. The data have been fit using both eqs 4 and 5. Use of eq 5 rather than eq 4 resulted in a statistically significant reduction in the overall sum of squares of the residuals in k_{obs} as measured by an *R*-factor ratio test.[38] However, the resulting value of k''' was negative and within two standard deviations of zero. Thus, the data on Ga^{3+} are

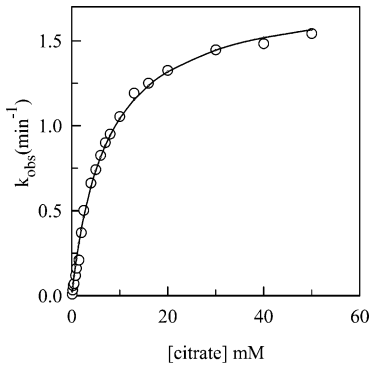

Figure 4. Plot of the observed rate constants for removal of Ga^{3+} from the C-terminal transferrin binding site by citrate. The solid line represents the least-squares fit of the data to eq 4.

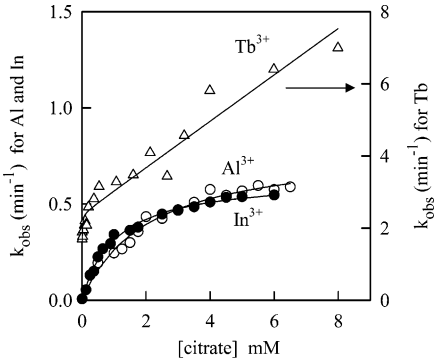

Figure 5. Observed first-order rate constants for the removal of Al^{3+}, In^{3+}, and Tb^{3+} from the C-terminal transferrin binding site by citrate. The solid lines represent least-squares fits to eq 4 for Al^{3+} and In^{3+} or to eq 5 for Tb^{3+}.

best described as simple saturation kinetics by eq 4. The calculated values for k_{max} and k_d for Ga^{3+} are listed in Table 1.

Similar kinetic experiments have been performed for the removal of both Al^{3+} and In^{3+} by citrate, and the results are shown in Figure 5. Both sets of data have been fit to eqs 4 and 5. The addition of the k''' parameter in eq 5 resulted in no statistically significant improvement in the overall quality of the fit for either In^{3+} or Al^{3+}, and the calculated values for k''' were small with relative errors of more than 80%. Thus, both these systems follow simple saturation kinetics. The solid lines in Figure 5 represent the calculated fits to eq 4, and the values of k_{max} and k_d for Al^{3+} and In^{3+} are listed in Table 1.

Removal of Terbium from the C-Terminal Binding Site of Transferrin. The removal of the weakly bound Tb^{3+} ion from the C-terminal binding site of transferrin went to completion at all the citrate concentrations used in this study. The terbium system does not show simple saturation kinetics as shown by the data plotted in Figure 5. There is an obvious first-order component at the higher citrate concentrations, such that the data can only be fit by eq 5. The calculated values for k_{max}, k_d, and k''' are listed in Table 1.

An interesting feature of the Tb^{3+} data is that the saturation component reaches its maximum rate at a very low citrate concentration, as shown by a k_d value of only 4 μM, compared to mM concentrations for the group 13 metal ions. Although the terbium data extend to a citrate concentration

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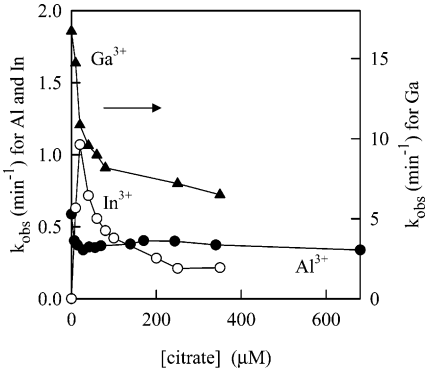

Figure 6. First-order rate constants for the binding of metal ions to apotransferrin in the presence of citrate.

of 6 μM, only the upper portion of the saturation curve is defined by the experimental data. As a result of this limited coverage of the saturation curve, the standard deviation for k_d is high, and this constant should be handled cautiously. In contrast, the values for k_{max} and k''' are well defined by the data.

Donation of Metal Ions to Transferrin. A 1 equiv amount of metal ion in solution with varying concentrations of citrate was reacted with 10 μM apoTf. Metal binding was monitored from the increase in absorbance at 245 nm. Equation 1 was converted to its logarithmic form, and the rate constants for metal binding were calculated from linear least-squares fits of the equation

$$\ln(A_\infty - A_t) = -kt + \ln(A_\infty - A_0) \qquad (7)$$

In some cases deviation from first-order kinetics led to curvature in the plot of eq 7, and values of k were calculated only on the basis of the initial, linear portions of the curves.

Rate constants for the binding of Ga^{3+} as a function of the citrate concentration are shown in Figure 6. The rate of Ga^{3+} binding is most rapid when there is no citrate present in the solution. There is a rapid decrease in the rate of metal binding as the citrate concentration increases from 0 to about 20 μM. Above 20 μM, the rate continues to decrease, but the rate of decline is much less.

The apparent first-order rate constants for the binding of Al^{3+} to apotransferrin are also shown in Figure 6. Just as observed for Ga^{3+}, the binding of Al^{3+} to transferrin is most rapid when there is no citrate present. There is a decrease in the observed rate constant as the citrate concentration increases to about 20 μM. There is essentially no change in the rate constant as the citrate concentration increases from 20 to 680 μM.

The apparent first-order rate constants for the donation of In^{3+} from citrate to apoTf are plotted as a function of the total citrate concentration in Figure 6. Unlike the Ga^{3+} and In^{3+} systems, the reaction of In^{3+} with apoTf in the absence of citrate is extremely slow ($k_{obs} \sim 10^{-4}$ min^{-1}). The addition of citrate up to 20 μM leads to a sharp increase in the rate of binding to apoTf. The addition of citrate beyond 20 μM leads to a decrease in the rate of binding as was observed for both Ga^{3+} and Al^{3+}.

The rate of In^{3+} donation as a function of the citrate concentration has also been measured for both forms of monoferric transferrin. The results are virtually identical for all three forms of the protein (apoTf, Fe_C-Tf, and $Tf-Fe_N$), with a maximum rate constant of $\sim$1 min^{-1} when the ratio of citrate:In is 2:1. Indium may bind slightly more rapidly to the vacant N-terminal site of Fe_C-Tf, but the site selectivity, if there is any, is small. For a citrate:In ratio of 2:1, $k_{obs} = 1.3$ min^{-1} for the N-terminal site compared with 0.94 min^{-1} for the C-terminal site.

Discussion

Metal Ion Donation. It was established in the early transferrin literature that adding unchelated ferric ion was a very slow and inefficient method for loading iron into Tf, despite the very high binding affinity of Tf for the Fe^{3+} ion.[39] Rapid hydrolysis of the free ferric ion leads to polymeric complexes that react very slowly with apoTf. In contrast, the addition of iron complexes with ligands such as NTA, citric acid, or pyrophosphate leads to complete binding within a few minutes.[27,40,41]

The rates of ferric ion donation from pyrophosphate and citrate have been studied in detail.[27,41] The presence of relatively low ratios of ligand:iron leads to a rapid increase in the rate of iron binding to apotransferrin. As the ratio of ligand:iron increases, the rate of binding reaches a maximum and then declines. The explanation for this trend is that low concentrations of the ligand prevent the formation of inert, polymeric iron−hydroxo species by forming coordinatively unsaturated mononuclear complexes that can rapidly donate iron to transferrin. Higher ligand concentrations lead to fully chelated bis and tris iron complexes, and the rate of iron donation to transferrin slows down as it becomes dependent on the loss of a ligand to expose coordination sites on the metal.

The reaction of In^{3+} with transferrin follows the pattern previously observed for Fe^{3+}. The addition of unchelated In^{3+} to a neutral solution of apoTf leads leads to very slow protein binding, presumably due to the rapid to the hydrolysis of the In^{3+} ion. At these dilute concentrations, $In(OH)_3$ does not actually precipitate. Instead, it is expected to form colloids that react very slowly with the apoTf. It appears that the addition of low concentrations of citrate forms soluble mononuclear In(cta) complexes which react much more rapidly with the protein. The addition of higher concentrations of citrate leads to a reduction in the rate of reaction, presumably due to the conversion of the In^{3+} from a 1:1 complex to a less reactive $In(cta)_2$ complex.

The kinetics of transferrin binding for Al^{3+} and Ga^{3+} are quite different from those of In^{3+} and Fe^{3+}. The rate of binding is highest when no ligand has been added. This is attributed to the amphoteric nature of the Al^{3+} and Ga^{3+} ions. At pH 7.4 a substantial fraction of both Ga^{3+} and Al^{3+} is

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present as the soluble, mononuclear $M(OH)_4^-$ anion.[42] These hydroxo complexes react relatively rapidly with apoTf. The addition of citrate leads to the formation of 1:1 and 2:1 citrate complexes which react more slowly.

Site Selectivity of Metal Binding. The metal removal reactions for Al^{3+}, Ga^{3+}, In^{3+}, and Tb^{3+} were all conducted on protein samples that contained only 0.8 equiv of bound metal ion. This substoichiometric amount of metal was used so that the kinetics would not be complicated by the removal of metal ion from two different metal binding sites. The site preference for the binding of Ga^{3+} and Al^{3+} was determined by difference UV titrations of the monoferric transferrins. In the case of Al^{3+}, the monoferric titrations confirm a previous report of stronger binding to the N-terminal binding site at pH 7.4.[36] The preferential binding of Tb^{3+} to the C-terminal site has also been reported previously.[33]

A thermodynamic preference for binding at the C-terminal site has also been reported for Bi^{3+},[43,44] Fe^{3+},[31] Nd^{3+},[45] Sm^{3+},[45] Lu^{3+},[46] Gd^{3+},[46] Zn^{2+},[47] Mn^{2+},[48] Cu^{2+},[49] and Cd^{2+}.[50] Only the Ni^{2+} ion has shown a small preference for binding at the N-terminal site, and it has been proposed that the binding of this ion is strongly influenced by losses in ligand field stabilization energy associated with the distortions from octahedral symmetry at the transferrin binding site.[51]

Difference UV titrations of apotransferrin with Ga^{3+} have been reported previously.[34] The titration curve was linear through the addition of about 1.5 equiv of Ga^{3+} with a slope of 20 000 M^{-1} cm^{-1}. The curve leveled off at a $\Delta\epsilon$ value of about 33 000 M^{-1} cm^{-1}. The partial saturation of the weaker binding site was attributed to competition from hydrolysis of the Ga^{3+}, but no titrations of monoferric transferrins were conducted to identify the stronger and weaker binding site. The data on Ga^{3+} binding to monoferric transferrins shown in Figure 2 are completely consistent with this previous study on apotransferrin. The titration of apotransferrin reflects the saturation of the stronger C-terminal site in the initial phase of the titration, followed by the partial saturation of the weaker N-terminal site. There is no indication that occupancy of one site by Fe^{3+} has a significant impact on the binding of Ga^{3+} at the other site. This is consistent with a quantitative analysis of site specific binding constants for Fe^{3+} [31] and indicates that the titrations of the monoferric transferrins give a reliable indication of the site selectivity for metal binding to apoTf.

Bates Mechanism of Metal Release from Transferrin. The saturation component of iron release is consistent with the mechanism initially proposed by Bates and co-workers for iron removal by acetohydroxamic acid and pyrophosphate:[10,27]

$$Fe{-}CO_3{-}Tf \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe{-}CO_3{-}Tf^* \quad (8)$$

$$Fe{-}CO_3{-}Tf^* + L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} L{-}Fe{-}CO_3{-}Tf^* \quad (9)$$

$$L{-}Fe{-}CO_3{-}Tf^* \xrightarrow{k_3} FeL + HCO_3^- + apoTf \quad (10)$$

Equation 8 shows a conformational change in the ferric transferrin complex from its native "closed" conformation to a reactive, "open" conformation (designated with an asterisk) from which iron is rapidly removed by the incoming ligand. This mechanism was proposed to explain the appearance of saturation kinetics without the accumulation of a significant concentration of the mixed-ligand $L{-}Fe{-}CO_3{-}Tf^*$ intermediate.

It is well established that the rates of iron release are altered in the presence of inorganic anions such as Cl^- and ClO_4^-.[7,12,13,15,19,28,52−54] This is generally attributed to the binding of the anion to an allosteric site on the protein, although no specific anion binding site has been identified. Raymond and co-workers have proposed that this allosteric site must be occupied by a suitable anion before iron can be released from the protein.[7] Anion binding is presumed to alter the rate constants for the conformational change in eq 8. Its impact on eqs 9 and 10 is not known.

It has been suggested several times that an anionic chelating agent might bind to the allosteric anion-binding site and alter its own rate of iron removal.[8,15−17] Because of its 3− charge, one might have expected citrate to act in this way. However, the k_{max} values for citrate are very similar to those of the neutral ligand deferiprone,[15] so allosteric binding of citrate does not appear to be a major factor.

Effect of the Metal Ion on k_{max}. Iron removal by catechols, hydroxamates, and hydroxypyridinones tend to follow simple saturation kinetics as described by eq 4,[10,14,15,55] while aminophosphonic acids and pyrophosphate tend to show a combination of saturation and first-order kinetics as described by eq 5.[8,9,11,17] Both mathematical descriptions of the ligand dependence of iron removal result in the calculation of k_{max}, the maximum rate of metal release via the saturation pathway shown by eqs 8−10. According to this mechanism, k_{max} corresponds to k_1, the forward rate constant for the protein conformational change.

One objective of this study was to determine how the rate of this protein conformational change varies for different metal ions. The results in Table 1 show that the k_{max} values for Tb^{3+}, Ga^{3+}, Al^{3+}, and Ga^{3+} vary by only a factor of 3. In contrast, the water exchange rates for the aquo ions of

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these metals vary by $10^{8.7}$ [56] and the metal transferrin binding constants vary by $10^{8.6}$. Thus, the rate of the protein conformational change appears to be relatively independent of the intrinsic lability of the bound metal ion and the strength of the metal−transferrin binding.

Ferric transferrin appears to be the exception, in that k_{max} for Fe^{3+} is much smaller than that for any of the other metal ions. It is not clear why the release of iron is so unusually slow. Our current hypothesis is that the overall activation energy for the protein conformational change can be divided into two components. One component represents various factors that are characteristic of the protein and independent of the metal ion. This would include factors such as the requisite breaking of electrostatic and hydrogen-bonding interactions across the interdomain cleft, solvation effects, and conformational changes in the protein backbone. It appears that, for the removal of Al^{3+}, Ga^{3+}, In^{3+}, and Tb^{3+}, these protein interactions dominate the overall activation energy.

These metal ions have transferrin binding affinities ranging from $10^{11.2}$ for Tb^{3+} to $10^{19.75}$ for Ga^{3+}.[33,35] The binding of Fe^{3+} is stronger, with a binding constant of $10^{21.44}$.[35] Even though there is not a large gap between the binding of Ga^{3+} and Fe^{3+}, it is possible that the increase in the stability of the iron complex is such that the disruption of metal−protein interactions now makes a meaningful contribution to the overall activation energy, so that one observes a reduction in the rate constant for the protein conformational change. This model is still speculative, and it should be noted that there is no overall correlation between k_{max} and the metal-transferrin binding constants.

Effect of the Metal Ion on k_d. During the removal of the metal ion via the Bates mechanism, the competing ligand reacts with the open form of the metal−transferrin complex to form a L−M−Tf mixed-ligand intermediate. Saturation is achieved when there is enough ligand in solution to ensure that every conformational transition from the closed to the open form results in the loss of the metal; i.e., there is no significant back-reaction to the closed form of ferric transferrin.

The kinetic parameter k_d corresponds to the ligand concentration required to reach half-saturation. If one assumes steady-state concentrations of Fe−CO_3−Tf* and L−Fe−CO_3−Tf*, then k_d is defined as

$$k_d = \frac{k_{-1}(k_{-2} + k_3)}{k_2 k_3} \qquad (11)$$

Although this parameter has the units of an equilibrium constant, it is not the dissociation constant of the mixed-ligand intermediate. In the Bates mechanism, saturation is not associated with the complete conversion of Fe−Tf into the L−Fe−Tf* mixed-ligand intermediate, and k_{max} does not correspond to the rate at which this intermediate dissociates into products.

It is reasonable to expect that a higher ligand concentration will be required to remove a more tightly bound metal ion from the protein, and this is indeed the case. There is a relatively clear correlation between the value of k_d and the metal−transferrin binding constants. A plot of log k_d vs log K_M has a linear correlation coefficient of $r = 0.87$. It has previously been observed that the k_d values for the removal of iron by a series of ligands decrease as the metal−ligand binding affinity increases.[15] This was reflected in a plot of log($1/k_d$) vs pM, where pM is −log [Fe^{3+}] calculated for 10^{-4} M total iron, 6×10^{-4} M total ligand, and pH 7.4. The k_d value reported here for the removal of iron from the N-terminal binding site by citrate fits this previous correlation with an overall correlation coefficient for four ligands of $r = 0.97$. Thus it now clear that k_d is directly related to the competitive metal-binding affinities between transferrin and the incoming ligand.

Effect of the Metal Ion on k'''. The k''' rate parameter is associated with the first-order component for metal ion removal that is observed at higher concentrations of some, but not all, ligands. This first-order term cannot be explained by the Bates mechanism. Previous studies on iron removal have shown that the value of k''' is highly dependent on the identity of the ligand. One hypothesis is that k''' is related to the ability of the ligand to displace the carbonate anion from the closed form of ferric transferrin, thus providing a reaction mechanism that avoids the rate-limiting conformational change in the protein.[9,11,12,15]

The formation of carbonate-free ternary Fe−ligand−transferrin complexes has been experimentally confirmed for a number of ligands.[9,57−59] Bates and co-workers have proposed structural criteria by which one can predict whether a ligand will be able to serve as a synergistic anion.[57,58] Table 2 lists the k''' values for iron removal by a series of ligands, along with an indication of the likelihood that each ligand can serve as a synergistic anion.

There is an obvious correlation between the ability of a ligand to serve as a synergistic anion and the appearance of a first-order kinetic pathway for iron release. Ligands such as acetohydroxamic acid and deferiprone, which do not fit the structural requirements for a synergistic anion, have values of $k''' = 0$. In contrast, nonzero k''' values are observed for ligands that are able to serve as a synergistic anion.

Citrate does not meet the usual criteria for a synergistic anion.[57] Thus one would predict that there would be no iron removal via the first-order pathway, and this is observed for the N-terminal binding site. However, the C-terminal site unexpectedly does show a first-order pathway with a k''' value of 0.089 M^{-1} min^{-1}. The reasons for this exceptional behavior are not clear. It appears that the Bates structural model for a synergistic anion is a useful but not perfect predictor of whether a ligand will have a nonzero value for k'''.

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Table 2. Rate Constants (k''') for Iron Removal via the First-Order Pathway

ligand	status as a synergistic anion	k''' ($M^{-1}\,min^{-1}$) Fe_C-Tf	k''' ($M^{-1}\,min^{-1}$) $Tf-Fe_N$	ref
pyrophosphate	confirmed[9]	0.84 ± 0.03	0.25 ± 0.04	18
nitrilotriacetic acid	confirmed[58]	0.33 ± 0.01	0.21 ± 0.01	9
ethylenedinitrilotetracetic acid	confirmed[58]	0.11	0.03	12
NTP[a]	confirmed[9]	0.053 ± 0.06	0.056 ± 0.011	9
DTPP[b]	expected	0.070 ± 0.005	0.061 ± 0.003	11
DTPA[c]	expected	0.036 ± 0.002	0.180 ± 0.008	11
citrate	not expected	0.089 ± 0.04	0	this work
acetohydroxamic acid	not expected	0	0	15
Deferiprone[d]	not expected	0	0	15
1H2P[e]	not expected	0	0	15

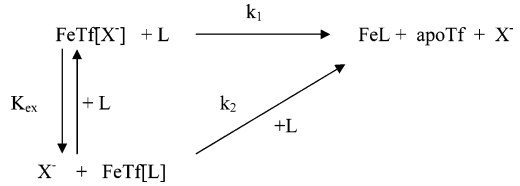
[a] Nitrilotris(methylenephosphonic acid). [b] Diethylenetriaminepentakis(methylenephosphonic acid). [c] Diethylenetriaminepentaacetic acid. [d] 1,2-Dimethyl-3-hydroxypyridin-4-one. [e] 1-Hydroxypyridin-2-one.

On the basis of the results on the removal of iron, there is no obvious reason Ga^{3+}, Al^{3+}, and In^{3+} should not also show a first-order pathway for metal removal from the Fe_C-Tf by citrate, but the data in Figures 4 and 5 do not indicate a significant first-order pathway for these metal ions. Given that Ga^{3+}, Al^{3+}, and In^{3+} have much larger values of k_{max} and much smaller values for k_d compared with Fe^{3+}, it is possible that a small k''' similar to that of iron would simply be undetectable. If the group 13 metal ions had a k''' value identical to that of Fe^{3+}, only 0.1−0.2% of the metal ion would be removed via the first-order pathway, even at the highest citrate concentrations used in this study.

The k''' value for Tb^{3+} is 640 $M^{-1}\,min^{-1}$, which is 4 orders of magnitude larger than the k''' for Fe^{3+} with citrate. The key difference between Tb^{3+} and the other metal ions included in this study is the larger coordination number for Tb^{3+}. The Tb^{3+} aquo ion is 8-coordinate,[60] while the other metal ions tend to form 6-coordinate complexes. Fluorescence lifetime studies have shown that at least one coordinated water molecule is retained in the $Tb^{3+}-Tf$ complex.[61] This suggests that the very high k''' for Tb^{3+} is associated with direct citrate attack at the aquated coordination site.

Marques−Egan Model for Metal Release from Transferrin. Kinetic studies have been previously reported for the removal of both Fe^{3+} and Al^{3+} by citrate.[28,62] Compared with the work reported here, these studies used significantly different experimental conditions. They were conducted at 37 °C instead of 25 °C, and the sample solutions contained high concentrations of a salt, either Na_2SO_4 or $NaNO_3$. In addition, the study on the removal of Al^{3+} used Al_2Tf and attempted to calculate site specific rate constants by fitting the data to a complex function with six adjustable parameters.[62] The computational difficulties associated with obtaining site specific rate constants from data on diferric transferrin have been previously reported.[9,18,28] While the overall rates reported here are generally similar to those in the two previous studies on citrate,[28,62] these differences in experimental conditions preclude a detailed comparison of rate constants.

Scheme 1 . Marques−Egan Mechanism for Iron Removal from Transferrin



The previous studies show a more significant first-order term at higher citrate concentration for the removal of both Al^{3+} and Fe^{3+}.[28,62] This may be related at least in part to the difference in the solution temperatures between this paper and the previous studies. Egan et al.[17] have shown that the first-order term for iron removal by pyrophosphate increases 7-fold as the temperature is increased from 25 to 37 °C.

Marques and Egan have proposed a different mechanism for iron release and thus use a different mathematical model for fitting the rate constants.[17,19,62] The Marques−Egan mechanism for iron release is shown in Scheme 1. For simplicity, the scheme includes only one of the two metal binding sites of transferrin, and the synergistic carbonate anion, which is always present when iron is bound, is omitted.

The brackets in Scheme 1 denote the allosteric anion binding site, often referred to as the KISAB site. It is presumed that this site is initially occupied by an anion from the buffer, designated as X^-. The Marques−Egan mechanism consists of two iron removal reactions, each of which is first-order with respect to the ligand concentration (k_1 and k_2), that are coupled by an anion exchange equilibrium in which the ligand displaces the buffer anion (K_{ex}). On the basis of this mechanism, the ligand dependence of the observed rate constant for iron removal is given by eq 12.[17]

$$k_{obs} = \frac{k_1[L] + k_2K_{ex}[L]^2}{1 + K_{ex}[L]} \qquad (12)$$

Equation 12 is essentially an algebraic rearrangement of eq 5, and we have previously tested both functions on common data sets and established that one obtains the same quality least-squares fit using either equation.[15] We have confirmed this by analyzing the data on gallium removal in Figure 4 using both eqs 5 and 12.

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Since the quality of the least-squares fits to eqs 5 and 12 is virtually identical, one must use different criteria for comparing the two-pathway model proposed by us with the Marques−Egan mechanism. As noted above, there is a very clear correlation between the parameter k_d and the relative strength of metal binding of the ligand and transferrin. This does not prove the validity of the definition of k_d shown in eq 11, but it is consistent with this equation. In the Marques−Egan mechanism, k_d is related to the binding affinity of the ligand for the allosteric anion-binding site. A comparison of the k_d values for the simple ligands PP_i,[9] deferiprone,[15] 1-hydroxypyridin-2-one,[15] citric acid, and acetohydroxamic acid[15] shows that there is no correlation at all between k_d and the anionic charge on the ligand at pH 7.4. The neutral ligand deferiprone would appear to bind more tightly to the allosteric site than the trianionic citrate molecule. Furthermore, the previous study on iron release by deferiprone showed that the addition of 0.4 M chloride and perchlorate resulted in saturation at lower ligand concentrations for iron removal from the N-terminal site,[15] which is opposite of what one would predict on the basis of Scheme 1.

The second factor is that we have a simple structural model that explains why some ligands show a first-order component for iron release and others do not, as shown by Table 2. To account for simple saturation kinetics in the Marques−Egan mechanism, one must presume that the ligand binds as an anion to the allosteric site and produces a value of $k_2 = 0$ in Scheme 1. While this is possible, there is no clear rationale for explaining why some ligands result in $k_2 = 0$ and others do not.

In Vivo Metal Binding. The data in Figure 3 show that a relatively large excess of citrate is required to remove 50% of the group 13 metal ions from transferrin. The higher carbonate concentration in serum would further favor transferrin binding. Since the ratio of citrate:transferrin in serum is only about 3:1, it is not surprising that, in serum, transferrin binds about 90% of aluminum[63−66] and almost 100% of both gallium[67−69] and indium.[68,70,71] The kinetic results reported here indicate that this binding should be complete within a few minutes of the metal entering the blood. This is consistent with reports that both Ga^{3+} and In^{3+} bind rapidly to transferrin in vivo.[67,72−74]

Transferrin is able to facilitate the cellular uptake of both gallium[69,75−78] and aluminum[78−82] via the normal transferrin receptor-mediated mechanism for iron uptake. In contrast, transferrin cannot facilitate the cellular uptake of In(III),[72,83,84] even though spectroscopic studies indicate that In^{3+} and Fe^{3+} induce the same change from open to closed protein conformations[85] and that In−Tf and Fe−Tf bind with similar affinity to the transferrin receptor.[83] Since intracellular metal release from transferrin takes place within an endosome at a pH of about 5.6,[3] the rate constants reported here cannot be directly related to the rates of receptor-mediated uptake. Nevertheless, these results do not indicate any unusual kinetic inertness on the part of the In−Tf complex that would account for the lack of receptor-mediated uptake of In^{3+}.

We have previously reported effective transferrin binding constants for Ga^{3+}, In^{3+}, and Al^{3+} that take into account competition from hydrolysis of the metal ions.[35] Although Ga^{3+} has the larger formal transferrin binding constant, the In^{3+} ion suffers the least competition from hydrolysis. The net effect is that at pH 7.4 the In−Tf complex is about 400 times more stable than Ga−Tf and about 1000 times more stable than Al−Tf with respect to the loss of the metal ion to hydrolysis. The lack of receptor-mediated uptake of In^{3+} might be related to this difference in thermodynamic stability rather than to differences in rates of metal release.

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