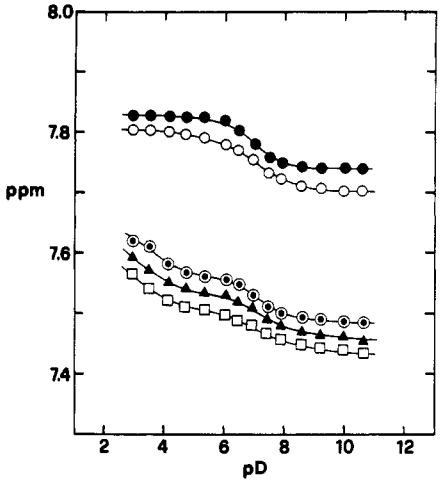

Figure 7. pD dependence of the chemical shift of the multiplet for the equivalent protons 3 and 3′ of 2,2′-bipyridine[19] in the ^{1}H NMR spectrum of the ternary system Cd^{2+}–bpy–bsgly in a 1:1:2 molar ratio. The other signals of 2,2′-bipyridine are either less resolved or overlapped with those of the aromatic ring of bsgly. Analogous patterns are obtained in the presence of the Zn^{2+} ion ($c_{Zn^{2+}} = c_{Cd^{2+}} = 10^{-3}$ M).

protons of bsgly and tsgly as compared to the free ligands. With increasing pH, such a pattern dramatically broadens for both ligands and the signals of 2,2′-bipyridine show a titration pattern with an apparent pK_a of about 7.5 (Figure 7). Such a behavior may indicate the formation of another species, most probably that in which the amino acid ligand acts as a bidentate through the deprotonated sulfonamide nitrogen, in equilibrium with the former:

$$[M(bpy)(LO)]^+ \rightleftharpoons [M(bpy)(LNO)] + H^+$$

The involvement of the deprotonated sulfonamide nitrogen in metal coordination changes the electronic distribution over the complex, so it may well change the shielding of the aromatic protons of both 2,2′-bipyridine and bsgly since, in the latter case, the amino acid moiety is conjugated with the aromatic ring through the SO_2 group.[17] The CH_2 signal of the metal-bound ligand L is, however, not sensitive to sulfonamide nitrogen deprotonation. This is somewhat surprising. An explanation could be that the electronic redistribution due to sulfonamide nitrogen deprotonation in this case also involves the two nitrogens of bpy, so the effect on the CH_2 group may be more complex than that in the above Cd^{2+}–L binary systems and may result in the reciprocal suppression of more effects.

There is a general agreement among the experimental techniques in indicating that for both metal ions the metal-promoted amide deprotonation takes place at lower pH values in the ternary systems as compared to the binary ones. The lowering effect of 2,2′-bipyridine on the pK_{NH} values of this kind of ligands and the corresponding higher stability of the ternary [M(bpy)L] species as compared with the [ML] species were first observed for the Cu^{2+} ion.[4] As for that case, it may be ascribed to a cooperative effect of the π systems of bpy and the amino acid ligands and to the stronger tendency of carboxylate ligands to bind to the $[M(bpy)]^{2+}$ species as compared to the free metal ion[18] (confirmed in this case by the $\Delta E^r_{1/2}$ values of the first step of waves I and II in Figure 4) that strengthens their "anchoring" capability[5] and favors the subsequent closure of the five-membered N,O-chelating ring. The identical $\Delta \log K$ values observed for Cu^{2+} and Cd^{2+} ($\Delta \log K_{Cu} = \Delta \log K_{Cd} = 0.6$) may indeed indicate that the nature of the metal ion plays a secondary role in this effect. The case of the Zn^{2+} ion deserves some comments. Up to now, only one example is known of a deprotonated amide nitrogen coordinated to a Zn^{2+} ion at physiological pH: in the [Zn(Gly-His)] complex the metal is bound to the terminal amino group of the dipeptide, the deprotonated peptide nitrogen, and one imidazole nitrogen of histidine.[7] Also in the present cases, besides the amino acid moiety of L, the Zn^{2+} ion is coordinated to an N-donor aromatic ligand. The presence of such kind of additional ligand could be a necessary requirement for enabling the Zn^{2+} ion to successfully substitute for a proton bound to a peptide or sulfonamide nitrogen.

Acknowledgments. We are grateful to the Centro Grandi Strumenti of the University of Modena for supplying the NMR facilities. Dr. P. Ghedini is acknowledged for the development of a computer program for analysis of the polarographic data.

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Contribution from the Department of Chemistry, University of Missouri—St. Louis, St. Louis, Missouri 63121

Kinetics of Iron Removal from Monoferric and Cobalt-Labeled Monoferric Human Serum Transferrin by Nitrilotris(methylenephosphonic acid) and Nitrilotriacetic Acid

Pawan K. Bali, Wesley R. Harris,* and Diane Nesset-Tollefson

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The kinetics of iron removal from both forms of monoferric transferrin by nitrilotris(methylenephosphonic acid) (NTP) and nitrilotriacetic acid (NTA) have been studied in 0.1 M, pH 7.4 *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonate buffer at 25 °C. The dependence of the observed pseudo-first-order rate constant for removal of iron on the concentration of NTP has been interpreted in terms of two parallel pathways: one that saturates and another that is first order in ligand. For NTP the saturation pathway is more important, while the carboxylate analogue NTA removes iron from both sides entirely by a first-order process. Iron removal from C- and N-terminal monoferric transferrins labeled at the vacant binding site with kinetically inert cobalt(III) has been studied as a model to evaluate cooperativity between the two sites. Cobalt labeling slightly accelerates iron removal by NTP from the C-terminal site and iron removal by NTA from the N-terminal site. The degree of cooperativity is less than that observed previously for iron removal from the C-terminal site by pyrophosphate (Bali, P. K.; Harris, W. R. *J. Am. Chem. Soc.* **1989**, *111*, 4457). Bicarbonate-free Fe–L–Tf ternary complexes are formed with both NTP and pyrophosphate. The pyrophosphate complex is much less stable than the corresponding NTP complex, which may be a factor contributing to the higher first-order rate constant for iron removal by pyrophosphate.

Introduction

The transferrins comprise a family of iron binding proteins that includes serum transferrin, lactoferrin, and ovotransferrin. Several recent reviews of transferrin chemistry are available.[1-6] The characteristic feature of the transferrins is that metal binding

* To whom correspondence should be addressed.

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involves the synergistic binding of a (bi)carbonate anion. Crystallographic studies on human lactoferrin[7-10] and rabbit serum transferrin[11] show that the transferrins are composed of two homologous lobes. Each lobe consists of two dissimilar domains with a single high-affinity iron binding site located within the interdomain cleft.[7,8,11] The metal ligands for both sites are identical, consisting of two tyrosines, one histidine, one aspartate, and the synergistic anion.[8,11] However, the two sites are not chemically equivalent. They differ in their response to pH change and chaotropic agents, thermodynamic stability, ESR and UV-vis spectra, and kinetic lability.[1-6]

One of the most interesting aspects of transferrin chemistry is the exchange of iron between transferrin and low molecular weight chelating agents. With typical iron chelators such as desferrioxamine B, this exchange is quite slow at neutral pH. Since serum transferrin is a potential target for drugs aimed at the treatment of iron overload in patients with certain genetic disorders, an understanding of the kinetics and mechanism of iron removal from this protein is important for the design of new iron chelators for clinical use. In addition, it is not yet completely clear how cells can quickly deplete transferrin of its iron. Intracellular iron release may be accelerated by exposing the transferrin to low pH within an endocytotic vesicle and/or by allosteric effects of the binding of diferric transferrin to its membrane receptor.[5] However, it appears that neither of these factors alone will release the iron and that interaction with an intracellular chelating agent is still required for iron release.

A variety of chelating agents, including ethylenediaminetetraacetic acid,[12-14] pyrophosphate,[15-25] phosphonates,[22,23,26] catecholates,[27-30] hydroxamates,[30-32] and hydroxypyridones,[33,34] have been employed to study the kinetics of iron removal from transferrins. A hyperbolic dependence of the rate of iron removal on the free ligand concentration has been observed with several ligands. This result was originally attributed to a preequilibrium between ferric transferrin and the competing ligand.[27,32] However, the L–Fe–HCO_3–Tf intermediate required by a preequilibrium mechanism did not accumulate to a detectable concentration during iron removal by either PP_i or acetohydroxamic acid.[16,31] These observations led to the proposal of the conformational change mechanism in eqs 1–3, in which the asterisk indicates a

$$\text{Fe–HCO}_3\text{–Tf} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Fe–HCO}_3\text{–Tf*} \quad (1)$$

$$\text{L} + \text{Fe–HCO}_3\text{–Tf*} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{L–Fe–HCO}_3\text{–Tf*} \quad (2)$$

$$\text{L–Fe–HCO}_3\text{–Tf*} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{Fe–L} + \text{Tf} + \text{HCO}_3^- \quad (3)$$

reactive or "open" conformation of the protein.[16,31] This mechanism is further supported by the recent crystallographic data on apolactoferrin, which confirm that there is a significant opening of the interdomain cleft of the N-terminal lobe in the apoprotein.[10]

We have recently reported a new method of evaluating the cooperativity between the two sites of transferrin during iron removal by labeling the vacant site of the monoferric transferrins with kinetically inert cobalt(III).[24] This provides a system in which iron removal from one site can be followed while the other site remains occupied. Using the cobalt-labeled proteins, we demonstrated a degree of cooperativity at the C-terminal site in the removal of iron from transferrin by pyrophosphate.[24] These investigations on cooperativity have now been extended to include nitrilotris(methylenephosphonic acid). Results are also reported for the tripodal carboxylate analogue nitrilotriacetic acid, a ligand for which saturation has not been observed.

Experimental Section

Reagents. All solutions were prepared with deionized water from a four-bowl Milli-Q water system. Reagent grade nitrilotriacetic acid (NTA) was purchased from Eastman and used without further purification. Nitrilotris(methylenephosphonic acid) (NTP) was synthesized by a Mannich reaction of ammonium chloride, formaldehyde, and phosphorous acid as reported previously.[22] Purified human serum transferrin was purchased from Calbiochem. Diferric, C- and N-terminal monoferric, and cobalt-labeled C- and N-terminal monoferric transferrins were prepared and purified as reported earlier.[24]

Electrophoresis. The purity of each preparation of the different protein species was checked by polyacrylamide gel electrophoresis using a variation of the Makey-Seal procedure.[35,36] Gels were 8% by weight of a 19:1 mixture of acrylamide:bis(acrylamide) cast in pH 8.4 tris(hydroxymethyl)aminomethane (Tris) buffer containing 6.1 M urea. Proteins were electrophoresed at a constant current of 30 mA for approximately 14 h. Gels were cooled by circulating water at ~4 °C from an external constant-temperature bath. Bands were visualized by staining with Coomassie Blue. The ferric transferrin species are stable to electrophoresis, whereas cobalt(III) tends to dissociate from the protein, especially from the C-terminal site.

Iron Release Kinetics. The rate of iron removal was measured at 25 °C in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (Hepes) buffer at pH 7.4. The reaction was followed by monitoring the decrease in the absorbance of the iron–phenolate charge transfer band, which appears at 465 nm for both C-terminal (Fe_C–Tf) and N-terminal (Tf–Fe_N) monoferric transferrin and at 440 nm for their cobalt-labeled

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derivatives Fe_C–Tf–Co_N and Co_C–Tf–Fe_N. Spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer equipped with a thermostated multicuvette transport and controlled by an HP 8452 Chemstation computer. There was no detectable loss of cobalt during the iron removal reaction for concentrations of NTA and NTP up to 100 mM.

Rate constants for the monoferric and cobalt-labeled monoferric transferrins were calculated by iterative curve fitting of the absorbance versus time data to eq 4, where A_t is the absorbance at time t, A_0 is the

$$A_t = (A_0 - A_\infty)e^{-kt} + A_\infty \quad (4)$$

absorbance at time zero, and A_∞ is the absorbance after infinite time. At concentrations of less than 10 mM NTP or 50 mM NTA, iron was not removed completely, and rate constants were calculated by using only initial data and the value of A_∞ determined from the experiments at higher ligand concentrations. For iron removal reactions that went to completion, data up to 4 or 5 half-lives were considered.

Preparation of Carbonate-Free Complexes. Carbonate-free iron transferrin complexes in which NTP or pyrophosphate was substituted for the synergistic bicarbonate anion were prepared inside a glovebag by using the low-pH purge method.[37,38] Decimolar Hepes buffer (pH 4), stock solutions of ferric nitrate, and weighed solid samples of ligand and apoTf were taken into the glovebag, which was purged for 2 h with CO_2-free N_2. Nitrogen was bubbled through the acidic Hepes and iron solutions to remove dissolved CO_2. A solution of the ferric–ligand complex was prepared in the pH 4 Hepes buffer. The ligand:metal stoichiometry was 1:1 for NTP and 2:1 for pyrophosphate. ApoTf was dissolved in 3.0 mL of the pH 4 buffer, and aliquots of the ferric–ligand solution were added to give varying concentrations of iron. After the reagents had been mixed, the pH of the sample solution was brought to approximately 7.4 by a stream of N_2 that had been passed across the surface of a concentrated ammonia solution. The solution was loaded from within the glovebag into a capped 1-cm cuvette.

After the spectrum of the carbonate-free solution was recorded, 100 μL of 0.1 M sodium bicarbonate was added to the sample cuvette to give a bicarbonate concentration of approximately 3 mM, and a second spectrum was recorded. This large excess of bicarbonate forced the formation of the much more stable Fe–HCO_3–Tf complex. The concentration of transferrin in the cuvette was calculated to be approximately 0.12 mM from the absorbance at 465 nm and an extinction coefficient of 5000 M^{-1} cm^{-1} for diferric transferrin.[39]

Control experiments were run in which a ferric citrate solution containing a 20:1 ratio of citrate:Fe was added to apoTf under carbonate-free conditions. Sufficient ferric citrate was added to give an Fe:Tf ratio of 8:1. Since citrate does not form a ternary complex,[37] any significant absorbance at 465 nm above that of ferric citrate itself was taken as an indication of CO_2 contamination.

Results

Both a preequilibrium mechanism and the conformational change mechanism predict the ligand dependence shown in eq 5 for the apparent first-order rate constant for iron removal.

$$k_{obs} = \frac{k'[L]}{1 + k''[L]} \quad (5)$$

In previous studies with pyrophosphate and aminophosphonic acids,[22,24] it was necessary to modify eq 5 by the addition of a term that was first order in ligand concentration, as shown in eq 6, to

$$k_{obs} = \frac{k'[L]}{1 + k''[L]} + k'''[L] \quad (6)$$

describe the ligand dependence of the observed pseudo-first-order rate constants. These results were interpreted in terms of parallel pathways for iron removal from the protein: one that is first order in ligand and another that saturates.

Iron Removal by NTP. The apparent first-order rate constants for iron removal from C- and N-terminal monoferric transferrin (Fe_C–Tf and Tf–Fe_N) and their cobalt-labeled analogues (Fe_C–Tf–Co_N and Co_C–Tf–Fe_N) have been measured as a function of the concentration of NTP, and the results are shown in Figures 1 and 2. The ligand dependence of the observed pseudo-first-order rate constant for iron removal by NTP from all four transferrin species was evaluated by using both eq 5 and eq 6.

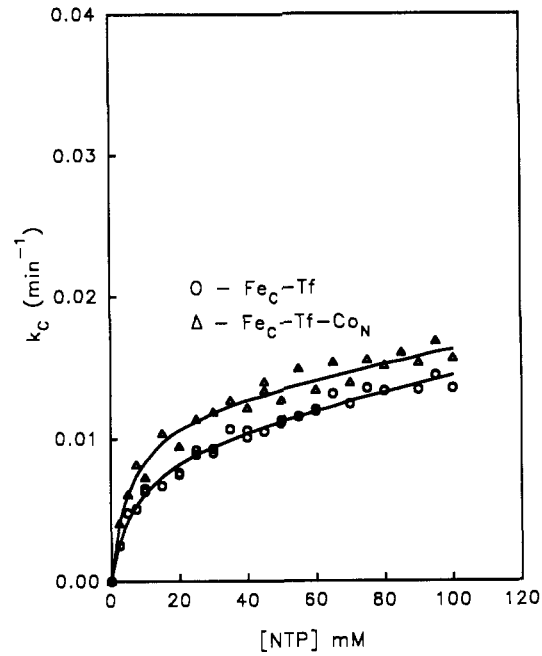

Figure 1. Plots of the pseudo-first-order rate constants for the removal of iron from Fe_C–Tf (O) and from Fe_C–Tf–Co_N (Δ) in 0.1 M Hepes, pH 7.4 at 25 °C, as a function of the concentration of free NTP. The lines are calculated from eq 6 with the parameters listed in Table I.

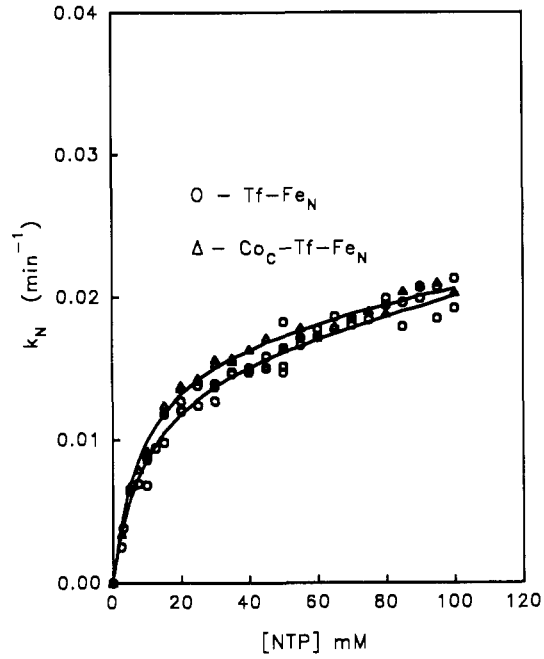

Figure 2. Plots of the pseudo-first-order rate constants for the removal of iron from Tf–Fe_N (O) and from Co_C–Tf–Fe_N (Δ) in 0.1 M Hepes, pH 7.4 at 25 °C, as a function of the concentration of free NTP. The lines are calculated from eq 6 with the parameters listed in Table I.

The addition of the third adjustable parameter in eq 6 should always improve the quality of the least-squares fit. The statistical significance of the observed improvement in the fit of k_{obs} versus [NTP] due to the inclusion of k''' in eq 6 was evaluated by using the R factor ratio test as described previously.[22,24] In all cases, the improvement in fit was highly significant ($\alpha < 0.005$). In Figures 1 and 2 the data points are the observed rate constants, while the lines are calculated from eq 6 by using the constants listed in Table I.

The saturation pathway is best described by the maximum rate constant at saturation, k_{max}, and by k'', which characterizes the approach to saturation as the ligand concentration increases.

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Table I. Parameters Describing the Ligand Dependence of the Rate of Iron Removal from Transferrin

	k_{max}, min^{-1} M^{-1}	k'', M^{-1}	k''', min^{-1} M^{-1}
	NTP		
Fe_C–Tf	0.0098 ± 0.002	131 ± 24	0.053 ± 0.006
Fe_C–Tf–Co_N	0.0125 ± 0.003	171 ± 43	0.004 ± 0.011
Tf–Fe_N	0.0158 ± 0.003	103 ± 19	0.056 ± 0.011
Co_C–Tf–Fe_N	0.0176 ± 0.003	115 ± 19	0.044 ± 0.010
	NTA		
Fe_C–Tf			0.325 ± 0.012
Fe_C–Tf–Co_N			0.335 ± 0.012
Tf–Fe_N			0.212 ± 0.013
Co_C–Tf–Fe_N			0.268 ± 0.014
	PP_i[a]		
Fe_C–Tf	0.0077 ± 0.0016	>1800	0.84 ± 0.03
Fe_C–Tf–Co_N	0.021 ± 0.002	>900	0.99 ± 0.04
Tf–Fe_N	0.051 ± 0.026	59 ± 29	0.25 ± 0.04
Co_C–Tf–Fe_N	0.062 ± 0.031	60 ± 24	0.18 ± 0.12
	LICAMS[b]		
Fe_C–Tf	0.0135 ± 0.005	nd	0.0
Tf–Fe_N	0.039 ± 0.002	nd	0.0
	AHA[c]		
Tf–Fe_N	0.051	32	0.0

[a] Results from ref 24. [b] Constants taken from ref 28. [c] Constants for diferric transferrin taken from ref 31.

Values of k_{max} were calculated as the ratio of k'/k''. Values for k_{max}, k'', and the first-order rate constant k''' are listed in Table I.

The two sites have very similar ligand dependence curves. The faster rate of iron removal from Tf–Fe_N compared to Fe_C–Tf is due entirely to a slightly higher value of k_{max}. The k'' and k''' parameters for Fe_C–Tf and Tf–Fe_N are virtually identical. Loading the N-terminal site with cobalt causes a slight increase in k''. As a result, k_{max} is reached at a lower concentration of NTP, and there is a small acceleration in iron removal from the C-terminal site. Loading the C-terminal site of Tf–Fe_N with Co(III) has essentially no effect on the rate of iron removal.

Iron Removal by NTA. Rate constants for iron removal from Fe_C–Tf, Fe_C–Tf–Co_N, Tf–Fe_N, and Co_C–Tf–Fe_N have also been measured as a function of the concentration of NTA, and the results are shown in Figures 3 and 4. Simple first-order kinetics are observed for both binding sites. The y intercepts of the plots are all within one standard deviation of zero. Thus, it appears that an insignificant amount of iron is removed through the saturation pathway.

Labeling the N-terminal site with Co(III) has no effect on iron removal from the C-terminal site. However, adding Co(III) to the C-terminal site of Tf–Fe_N causes a slight increase in the rate of iron removal from the N-terminal site. The values for the first-order rate constant k''' for NTA with all four transferrin species are listed in Table I. The first-order rate constants for NTA are 3–6 times larger than those for NTP.

Iron Removal from Diferric Transferrin. Iron removal from diferric transferrin is described in terms of four microscopic rate constants (see Scheme I) by eq 7, where ϵ_C and ϵ_N are the molar

$$A_t - A_\infty = (\epsilon_C + \epsilon_N)C_0e^{-(k_{1N}+k_{1C})t} + \left[\frac{\epsilon_C C_0 k_{1N}}{k_{1N} + k_{1C} - k_{2C}}(e^{-k_{2C}t} - e^{-(k_{1N}+k_{1C})t})\right] + \left[\frac{\epsilon_N C_0 k_{1C}}{k_{1N} + k_{1C} - k_{2N}}(e^{-k_{2N}t} - e^{-(k_{1N}+k_{1C})t})\right] \quad (7)$$

extinction coefficients of the transferrin binding sites and C_0 is the initial concentration of diferric transferrin.[12,15]

The rate of iron removal from diferric transferrin was measured for a series of replicate solutions containing approximately 50 mM NTP. Even though values of k_{2C} and k_{2N} for NTP were determined independently from the studies on monoferric transferrins and used as fixed constants in eq 7, it was not possible to refine both k_{1N} and k_{1C} simultaneously from the data on diferric Tf.

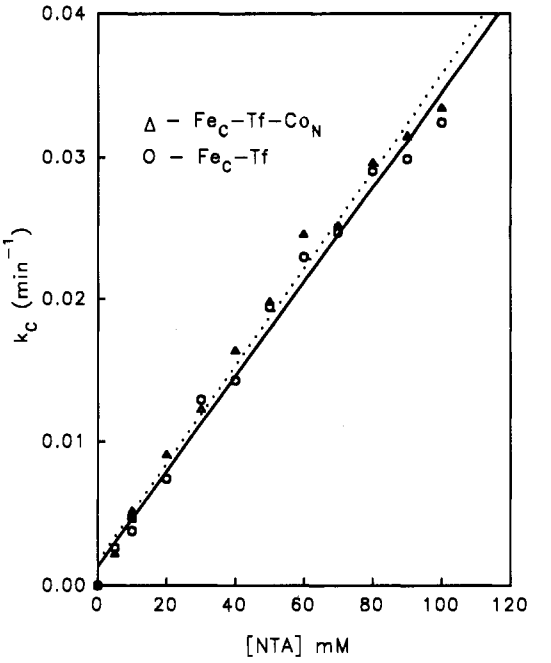


Figure 3. Plots of the pseudo-first-order rate constants for the removal of iron from Fe_C–Tf (O) and from Fe_C–Tf–Co_N (Δ) in 0.1 M Hepes, pH 7.4 at 25 °C, as a function of the concentration of free NTA.

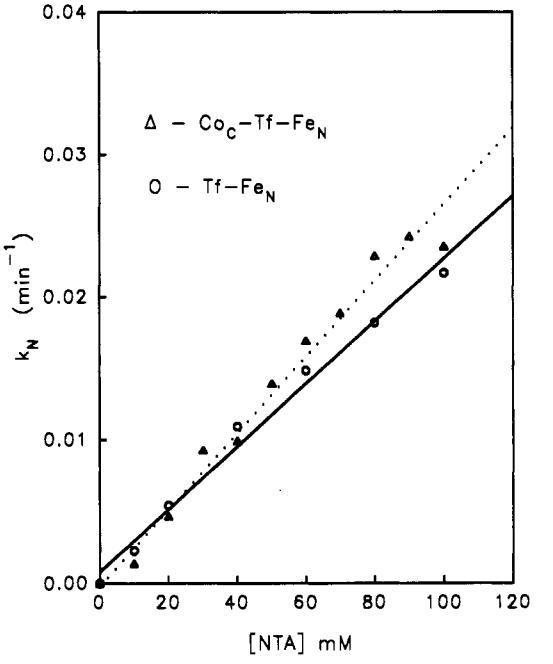


Figure 4. Plots of the pseudo-first-order rate constants for the removal of iron from Tf–Fe_N (O) and from Co_C–Tf–Fe_N (Δ) in 0.1 M Hepes, pH 7.4 at 25 °C, as a function of the concentration of free NTA.

Therefore, the value of k_{1N} was taken as the rate constant for Co_C–Tf–Fe_N, since cobalt labeling has little effect on the N-terminal site. This left k_{1C} as the only adjustable parameter. There was good agreement between the value of k_{1C} calculated for iron removal from diferric transferrin by approximately 50 mM NTP, 0.011 ± 0.001 min^{-1}, and the value of 0.013 ± 0.003 min^{-1} for iron removal from Fe_C–Tf–Co_N by the same concentration of NTP.

If there is no cooperativity at either site, such that $k_{1C} = k_{2C}$ and $k_{1N} = k_{2N}$, then the preexponential coefficients for the $e^{-(k_{1N}+k_{1C})t}$ term sum to zero, and eq 7 reduces to a two-exponential equation. In addition, the microscopic rate constants disappear from the preexponential coefficients. This procedure gives a good fit to the data. However, on the basis of site-specific extinction coefficients of 2150 M^{-1} cm^{-1} for the N-terminal site and 2950

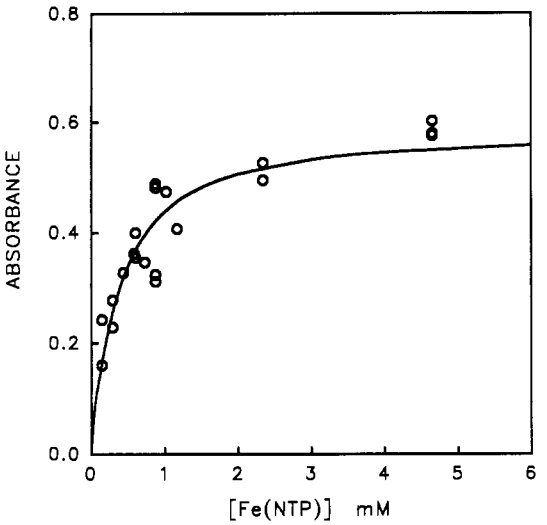


Figure 5. Plots of the absorbance at 465 nm of the Fe–NTP–Tf ternary complex for equilibrium solutions of approximately 1.2×10^{-4} M apoTf and increasing concentrations of the ferric–NTP complex under carbonate-free conditions. The solid line is calculated by using the average log $K_{eq} = 3.5$.

M^{-1} cm^{-1} for the C-terminal site,[39] the calculated rate constants are $k_N = 0.025$ min^{-1} and $k_C = 0.007$ min^{-1}, which do not agree with the measured rate constants for the corresponding monoferric transferrins.

One can also reduce eq 7 to a two-exponential equation by assuming that $k_{2C} = k_{2N}$. If one then defines $m_1 = k_{1C} + k_{1N}$, eq 7 reduces to

$$\frac{A - A_\infty}{A_0 - A_\infty} = \left[1 - \frac{\epsilon_C k_{1N}}{(\epsilon_C + \epsilon_N)(m_1 - k_2)} - \frac{\epsilon_N k_{1C}}{(\epsilon_C + \epsilon_N)(m_1 - k_2)}\right] e^{-m_1 t} + \left[\frac{\epsilon_C k_{1N}}{(\epsilon_C + \epsilon_N)(m_1 - k_2)} + \frac{\epsilon_N k_{1C}}{(\epsilon_C + \epsilon_N)(m_1 - k_2)}\right] e^{-k_2 t} \quad (8)$$

The absorbance versus time data for iron removal from diferric transferrin were fit with three adjustable parameters: m_1, k_2, and c_1, the preexponential coefficient of the $e^{-m_1 t}$ term. The coefficient of the $e^{-k_2 t}$ term is $1 - c_1$ and thus is not an independent parameter. This calculation gives an extraordinarily good fit to the data for values of $m_1 = 0.13 \pm 0.03$ min^{-1}, $k_2 = 0.0115 \pm 0.0008$ min^{-1}, and $c_1 = 0.08 \pm 0.02$.

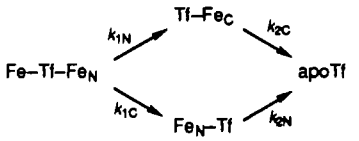
On the basis of the microconstants reported in Table I, c_1 is equal to 0.1, which agrees with the value of 0.08 calculated above. The calculation of m_1 was repeated with c_1 fixed at 0.1 and values of k_2 ranging from 0.011 to 0.016 min^{-1}. When k_{2C} is increased from 0.011 to 0.0137, the average of k_{2C} and k_{2N}, the corresponding value of m_1 decreases by an order of magnitude to only 0.008 min^{-1}. Increasing k_2 further to 0.016 min^{-1}, the value of k_{2N}, causes the value of m_1 to drop to zero (-0.0005 ± 0.002 min^{-1}).

Carbonate-Free Ternary Complexes. ApoTf was reacted with increasing concentrations of a 1:1 ferric–NTP complex under carbonate-free conditions. The resulting Fe–NTP–Tf complex has a broad absorption band ($\lambda_{max} = 470$ nm) that is almost identical with that of the Fe–HCO_3–Tf complex. The absorbance as a function of the concentration of added iron is shown in Figure 5. As a negative control, these experiments were repeated by using 1.8 mM ferric citrate in place of the ferric–NTP complex. Citrate does not form a ternary complex with transferrin,[37] and the measured absorbances were approximately 0.04 AU.

The equilibrium constant for the ternary complex is

$$K_{eq} = \frac{[\text{Fe–NTP–Tf}]}{[\text{Fe(NTP)}][\text{apoTf}]} \quad (9)$$

Scheme I



The N- and C-terminal transferrin binding sites are treated as a homogeneous pool, and the fractional saturation of the binding sites is calculated as

$$\alpha = \frac{A - A_0}{A_{max} - A_0} \quad (10)$$

where A is the absorbance for the equilibrium mixture, A_0 is the background absorbance measured from the solutions containing ferric citrate, and A_{max} is the limiting absorbance observed at high concentrations of iron. For 0.115 mM solutions of apoTf ($A_{max} - A_0$) is 0.548 AU, which corresponds to an extinction coefficient of 2400 M^{-1} cm^{-1} per iron for the Fe–NTP–Tf complex. The value for the equilibrium constant is calculated as

$$K_{eq} = \frac{\alpha}{(1 - \alpha)([\text{Fe}]_{tot} - \alpha[\text{Tf}]_{tot})} \quad (11)$$

An average of log $K_{eq} = 3.5 \pm 0.2$ was calculated from absorbance data on the equilibrium solutions represented in Figure 5 that had less than 3 mM Fe(NTP).

Equilibrium studies under bicarbonate-free conditions were also run with $Fe(PP_i)_2$ as the iron donor. Even with 2.8 mM iron, the absorbance reached only about 0.27 AU at a λ_{max} of 459 nm. Addition of bicarbonate to these solutions resulted in an immediate increase in absorbance to 0.6 AU at 465 nm, which is characteristic of the Fe–HCO_3–Tf complex.

Discussion

To explain fully the kinetics of complete iron removal from a two-sited protein, one needs four microscopic rate constants, as shown in Scheme I. Two of the four microscopic rate constants in Scheme I, k_{2C} and k_{2N}, can be measured directly by following the kinetics of iron removal from the respective monoferric transferrins. It is more difficult to measure k_{1C} and k_{1N} from studies on diferric transferrin, since iron is lost from both sites simultaneously, and these constants appear in the exponential terms of eq 7 only as the sum of $k_{1C} + k_{1N}$. It was not possible to fit the absorbance versus time data to eq 7 by varying k_{1C} and k_{1N} simultaneously.

By loading the vacant site of monoferric transferrin with kinetically inert cobalt(III), one can follow iron removal from Fe_C–Tf–Co_N and Co_C–Tf–Fe_N from the visible spectrum and treat the reaction by simple first-order kinetics.[24] There is no dramatic effect of cobalt labeling on the rate of iron removal by NTP. Loading the C-terminal site of Tf–Fe_N with cobalt(III) does not affect the kinetics of iron release from the N-terminal site by NTP. The same result was observed for iron removal by pyrophosphate.[24] The presence of Co(III) in the N-terminal site does accelerate iron removal from the C-terminal site by NTP by increasing k'' for the saturation pathway. However, the k_{max} values for NTP are very similar for Fe_C–Tf and Fe_C–Tf–Co_N. This contrasts with the pyrophosphate system, where k_{max} increases from 0.0077 to 0.021 min^{-1} when cobalt is loaded into the vacant N-terminal site.

There also appears to be little cooperativity with respect to the first-order pathway. For iron removal by NTA, loading of the N-terminal site has no effect on the kinetics of iron release from the C-terminal site. The rate of iron removal from the N-terminal site is accelerated slightly if the vacant C-terminal site is loaded with Co(III) ion. For iron removal by NTP, cobalt labeling appears to slow down the first-order pathway, although the variation may not be significant due to the relatively high standard deviations of the first-order rate constants.

The degree to which cobalt(III) mimics iron(III) in terms of its effects on the kinetics of iron release has been evaluated by fitting data on iron release from diferric transferrin by 50 mM NTP to eq 7 with k_{1C} as the only adjustable parameter. The

observed rate constant is in good agreement with the k_{1C} value calculated for iron release by the same concentration of NTP from Fe_C–Tf–Co_N. Similar studies on iron removal by pyrophosphate showed good agreement between the data on diferric transferrin and Fe_C–Tf–Co_N over the entire range of pyrophosphate concentrations.[24] In addition, iron removal from diferric transferrin by 100 mM concentrations of both NTP and PP_i has been followed by electrophoresis,[39] and the calculated values for k_{1C} and k_{1N} are in good agreement with the values from cobalt-substituted transferrins. Thus, there is growing evidence that cobalt-loaded monoferric transferrins provide very good models to evaluate cooperative interactions between the two sites during iron removal from transferrin.

Kretchmar and Raymond[28] have reported a slight positive cooperativity in the kinetics of iron removal from the N-terminal site by the catecholate ligand LICAMS. No cooperativity could be detected at either of the sites in iron removal by EDTA.[12,13] Thus, cooperativity between the two sites appears to be sensitive to the identity and concentration of the competing ligand. In general the cooperativity effects are rather small.

Biphasic kinetics of iron removal from diferric transferrin have often been reported.[12,13,15-19,25,26,31] Usually, the absorbance versus time data are fit to simple two-exponential equations. One rationalization for a two-exponential fit is that one of the k_1 terms is much greater than the other three rate constants. However, this is ruled out for iron removal by NTP by the electrophoresis study.[39] A two-exponential fit could also reflect intrinsic heterogeneity between noninteracting sites, in which case the $e^{-(k_{1C}+k_{1N})t}$ term in eq 7 disappears and the two calculated rate constants for diferric transferrin should correspond to the rate constants of the monoferric transferrins. Although this type of calculation gives a good fit to the data, the calculated rate constants do not match the observed values for the monoferric transferrins. The same result was observed for iron removal by pyrophosphate.[24]

It has also been proposed to obtain two-parameter fits by assuming that $k_{1C} + k_{1N} \gg k_{2N} \approx k_{2C}$,[12,28] which leads to eq 8. In this case, the preexponential terms are not restricted to any value and may be treated as a third adjustable parameter. The values of k_{2C} and k_{2N} are similar, and the approximation that $k_{2C} = k_{2N}$ would appear to be reasonable. However, the small value of $c_1 \approx 0.1$ makes the calculation of m_1 extraordinarily sensitive to the values of k_2, such that the approximation of $k_{2C} = k_{2N}$ is not appropriate in this system. The low value of c_1 results primarily from the similarity between k_{1C} and k_{2C} and between k_{1N} and k_{2N}. In systems where there is a larger degree of cooperativity between the sites and the value of c_1 is greater, the two-parameter fits might be justified.

On the basis of the conformational change mechanism and the assumption of steady-state concentrations for Fe–HCO_3–Tf* and L–Fe–HCO_3–Tf, the following equation for the observed first-order rate constant as a function of ligand concentration can be derived:[26]

$$k_{obs} = \frac{k_1 k_2 k_3[L]}{k_{-1}(k_{-2} + k_2) + k_2 k_3[L]} \qquad (12)$$

Under saturation conditions, the first term in the denominator of eq 12 can be ignored, and the equation reduces to $k_{obs} = k_1$. Thus, the maximal rate constant for iron removal under saturating conditions (k_{max}) will be equal to the forward rate constant for the conformation change, which is an intrinsic property of the protein.

Site-specific rate constants have been measured for a limited number of systems. Table I gives a partial list of k_{max}, k'', and k''' for iron removal by NTP, NTA, PP_i, acetohydroxamic acid (AHA), and 1,5,10-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (LICAMS). The ligand dependence of iron removal from the N-terminal site by PP_i can be fit equally well by either eq 5 or eq 6,[24] and the parameters for eq 6 are listed in Table I. For the catecholate ligand LICAMS, values of k_{max} are taken as the observed rate constants at 12 mM LiCAMS,[28] since ligand dependence curves for diferric transferrin indicate that the reaction is approaching saturation at this concentration of ligand.[27] There is no indication of a first-order pathway for LICAMS, so k''' for this ligand is set to zero. Values for acetohydroxamic acid are rough estimates from data on iron removal from diferric transferrin.[31] Since this system goes to equilibrium with only 50% of the iron removed, the observed constants are assigned to the thermodynamically weaker N-terminal site.

The interpretation of these site-specific rate constants rests primarily on previous reports on the donation and removal of iron by PP_i[16] and AHA.[31] In both cases, spectroscopically distinct L–Fe–HCO_3–Tf intermediates were detected during the donation of ferric ion to transferrin. However, in neither case did detectable concentrations of these intermediates accumulate during iron removal by these ligands. In addition, no intermediate was observed during iron removal by LICAMS.[27,28] It has been proposed that the saturation process for these ligands reflects a rate-limiting conformational change in the protein.[16,31]

The k_{max} values for iron removal from Fe_C–Tf by NTP, PP_i, and LICAMS cluster near an average of 0.010 ± 0.003 min^{-1}. Thus, it appears that iron removal from the C-terminal site operates at or very near the limit set by the forward rate constant of the conformational change for all these ligands. For N-terminal monoferric transferrin there is a wider range of k_{max} values. For iron removal by LICAMS, acetohydroxamate, and PP_i, the k_{max} value is approximately 0.04–0.05 min^{-1}. This is consistent with the hypothesis that iron removal by these three ligands is limited by the rate of the conformational change. However, the rate constant for NTP of only 0.016 min^{-1} is clearly lower than this limiting value. Saturation during iron removal from the N-terminal site is attributed to the preequilibrium formation of an NTP–Fe–HCO_3–Tf quaternary intermediate.

There is no spectroscopic evidence that would support direct ligation of NTP to the ferric ion in the formation of the proposed NTP–Fe–HCO_3–Tf intermediate. However, several anionic ligands, including both NTP and PP_i, bind quite strongly to apoTf.[40,41] Therefore, it is proposed that the quaternary intermediate involves the binding of NTP to ferric transferrin as an anion to cationic protein side groups that extend into the interdomain cleft of each lobe of transferrin.[11] The lack of a saturation pathway for iron removal by NTA can be attributed to its very weak binding to the protein as an anion.[42]

The variation in kinetic behavior of the two transferrin binding sites is consistent with the recent crystallographic studies on lactoferrin,[7,8] and apolactoferrin.[10] The N-terminal site, which has the higher k_{max}, undergoes a substantial conformational change between apo- and diferriclactoferrin, indicating a flexible protein structure. Conversely, for the C-terminal site, which behaves kinetically as a more rigid system, there is relatively little change in the conformations of apo and diferric forms of the protein. It is noteworthy that there are three extra disulfide bonds in the C-terminal lobe of transferrin, one of which bridges the interdomain cleft.[10,11]

It has been proposed that the first-order pathway is associated with the displacement of the synergistic carbonate anion followed by rapid dissociation of the ferric chelate.[22] Therefore, we have looked for some correlation between the rate constant for this pathway and the tendency of the ligand to form a bicarbonate-free ternary Fe–L–Tf complex. The equilibrium constant of the ternary complex with NTP is $\log K = 3.5 \pm 0.2$. On the basis of data from Schlabach and Bates,[37] we estimate the binding constant for NTA to be 5–10 times that for EDTA,[43] or $\log K \approx 3.5$.

It has been reported that PP_i does not form a ternary complex.[44] Although we do detect a ternary complex with PP_i, it is so weak that we are unable to measure a value for its equilibrium constant. Formation of the PP_i ternary complex may be complicated by the fact that the free ligand binds to apoTf with an equilibrium

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constant of 10^6,[41] so that any free PP_i will compete with ferric ion for the binding site.

There is no clear correlation between the stability constants of the ternary complexes and the first-order rate constants for iron removal. However, the unique combination of a weak ternary complex combined with a very stable binary apoTf–anion complex may be the basis for the unusually high first-order rate constant for iron removal from the C-terminal site by PP_i.

Bertini et al.[17] have proposed that the first-order term for iron removal by pyrophosphate arises not from an independent first-order pathway but from PP_i binding to allosteric sites on the protein and increasing the rate constants of the saturation pathway. The presence of anion binding sites on diferric transferrin has been implicated by chemical modification studies[35] and ESR spectroscopy.[45]

We have previously studied iron removal from diferric transferrin by a series of phosphonates in the presence of 200 mM concentrations of several anions.[23] The perchlorate anion has the strongest effect on the kinetics of iron removal and consistently increases the initial rates of iron removal by all ligands. On the basis of the perchlorate binding constants reported by Folajtar and Chasteen,[45] these allosteric sites will be saturated by 200 mM perchlorate. If the Bertini model were correct, one would expect simple saturation kinetics as a function of the ligand concentration in the presence of 200 mM perchlorate, with the k_{max} and k'' values associated with the modified form of the protein. The first-order term would disappear, since the change in concentration of NTP would not produce any change in the populations of modified and unmodified transferrins. This is not observed. The addition of 200 mM perchlorate actually increases the first-order term for both NTP and PP_i.[23]

The ionic strength of the Hepes buffer is only 0.041 M. Thus, the change in ligand concentration up to 100 mM causes a substantial change in ionic strength. The nonzero values for k''' could be due in part to the change in ionic strength. This is very difficult to evaluate because specific anion effects appear to be much more important than simple changes in ionic strength.[13,23] Increasing KCl concentrations increase the rate of iron removal for both sites by the catecholate LICAMS.[29] For iron removal by EDTA, NaCl accelerates iron removal from the C-terminal site but has no effect on the N-terminal site.[13] For iron removal by NTP, NaCl accelerates iron removal from Fe_C–Tf but decreases the rate of iron removal from Tf–Fe_N.[23] It is not clear that any anion can be used to adjust ionic strength without serious concern over specific anion effects.

Although one cannot rule out an ionic strength effect on the magnitude of k''', several factors argue in favor of the interpretation of k''' as representing an independent pathway for iron release. The magnitude of k''' for PP_i, NTA, and NTP does not correlate with the magnitude of the change in ionic strength. On the basis of the Bronsted equation, one would not predict a linear increase in rate constant with increasing ionic strength.[46] Lastly, NTA removes iron strictly by the proposed first-order pathway, and it would be difficult to account for a pathway that dominates for NTA yet is completely inaccessible to the structurally analogous ligand NTP. In fact, iron removal by NTP becomes strictly first order in the presence of several anions due to a decrease in k_{max} to ~ 0.[23]

Conclusions

Iron removal from ferric transferrins can proceed by two parallel pathways: one that is first order in ligand and another that shows saturation kinetics. The relative importance of the two pathways is a function of the identity and concentration of the competing ligand and the site of bound iron. Rapid saturation and low k_{max} values for the C-terminal site appear to indicate a more sluggish conformation change for the C-terminal lobe, which is consistent with structural features of the protein. Cobalt-labeled monoferric transferrins provide very good models for evaluating cooperativity between the two binding sites during iron removal. A slight positive cooperativity is observed at the C-terminal site during iron removal by PP_i and NTP and at the N-terminal site during iron removal by NTA. The cooperativity effect at the C-terminal site appears to be associated with the saturation pathway. The uniquely high first-order rate constant for iron removal by PP_i may be related to the combination of very strong binding of free PP_i to apoTf and a relatively unstable Fe–PP_i–Tf ternary complex.

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Registry No. NTA, 139-13-9; NTP, 6419-19-8; Fe, 7439-89-6; Fe^{3+}–NTP, 122681-17-8.

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