Cooperativity and Heterogeneity between the Two Binding Sites of Diferric Transferrin during Iron Removal by Pyrophosphate

Pawan K. Bali and Wesley R. Harris*

Contribution from the Department of Chemistry, University of Idaho, Moscow, Idaho 83843.
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Abstract: Serum transferrin is a mammalian iron transport protein containing two high-affinity metal binding sites. The vacant binding sites of both C-terminal and N-terminal monoferric transferrin have been labeled with kinetically inert cobalt(III). The rate constants for iron removal by pyrophosphate have been measured in 0.1 M, pH 7.4 *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonate buffer at 25 °C for the following transferrin complexes: Fe_C–Tf–Fe_N, Fe_C–Tf–Co_N, Co_C–Tf–Fe_N, Fe_C–Tf, and Tf–Fe_N, where the C and N subscripts denote the specific metal binding site. The results are discussed in terms of two parallel pathways for iron removal. One is first order in pyrophosphate, while the other shows saturation kinetics with respect to the pyrophosphate concentration. Iron removal from Tf–Fe_N proceeds almost exclusively through the saturation pathway. Labeling of the vacant C-terminal site with cobalt(III) has no significant effect on the rate of iron removal. Iron removal from Fe_C–Tf proceeds through both first-order and saturation pathways, although at concentrations of pyrophosphate above 50 mM the first-order pathway predominates. Labeling the vacant N-terminal site with cobalt(III) accelerates iron removal through both pathways. A very similar degree of cooperativity exists for removal of iron from the C-terminal binding site of diferric transferrin. Thus the cobalt(III)-labeled proteins appear to be good models for the cooperativity between the two transferrin binding sites during iron removal.

The extensive studies on the structure, chemistry, and physiology of the transferrins have recently been reviewed.[1-5] This group of proteins consists of serum transferrin, ovotransferrin, and lactoferrin. The most distinctive feature of the transferrins is the requirement of a synergistic anion for metal binding. Under physiological conditions (bi)carbonate serves this function.

Serum transferrin transports iron(III) through the blood between sites of uptake, utilization, and storage. It is a bilobal protein, with each lobe containing a single high-affinity iron binding site. The two sites are similar, but not equivalent. They differ in their anion requirement for metal binding,[6,7] EPR properties,[8-10] susceptibility to conformational change,[8,11] acid lability,[8,12,13] accessibility to various iron complexes,[8] and metal binding strengths.[8,9,14-17] In addition, anions produce different effects on the kinetics of iron release from the two sites.[10,18-20]

The exchange of iron with chelating agents is one of the most important aspects of the chemistry of the transferrins. The mechanism by which cells remove iron from the very stable transferrin complex has been an intriguing problem, especially when very powerful chelating agents are disappointingly slow at this task. In addition, transferrin is a potential target of chelating drugs for the treatment of iron overload.

The kinetics of iron removal from transferrin by a variety of chelates such as phosphonates,[21-23] catecholates,[24,25] hydroxamates,[26,27] and pyrophosphate (PP_i)[10,21,23,28-30] have recently been investigated. A hyperbolic dependence of the rate of iron removal on the concentration of free ligand has been reported for several ligands. These saturation kinetics had been initially attributed to a preequilibrium between ferric transferrin and the incoming ligand to form a quaternary intermediate as shown in eq 1.[24,27]

$$\text{Fe–HCO}_3\text{–Tf} + \text{L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{L–Fe–HCO}_3\text{–Tf} \xrightarrow{k_2} \text{Fe–L} + \text{Tf} + \text{HCO}_3^- \quad (1)$$

A spectroscopically distinct intermediate, presumably the quarternary complex, has been observed during the donation of iron to transferrin by the ferric chelates of PP_i and acetohydroxamic acid.[26,30] However, such an intermediate has not been observed spectroscopically during iron removal except in the very slow reaction between diferric transferrin and the hydroxamate siderophore aerobactin.[27] Therefore, an alternative mechanism for iron exchange has been proposed that incorporates a conformation

* Address correspondence to this author at the Department of Chemistry, University of Missouri—St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121.

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change from an unreactive "closed" to a reactive "open" form of ferric transferrin as the rate-determining step in iron removal,[26,30] as shown in eq 2–4. The asterisk indicates the open conformation of ferric transferrin.

$$Fe\text{–}HCO_3\text{–}Tf \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe\text{–}HCO_3\text{–}Tf^* \quad (2)$$

$$L + Fe\text{–}HCO_3\text{–}Tf^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} L\text{–}Fe\text{–}HCO_3\text{–}Tf^* \quad (3)$$

$$L\text{–}Fe\text{–}HCO_3\text{–}Tf^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Fe\text{–}L + Tf \quad (4)$$

Both the conformational change and the preequilibrium mechanisms predict the same ligand dependence for the apparent first-order rate constant for iron removal:

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

The definitions for k' and k'' will differ for the preequilibrium and conformational change mechanisms.

We have previously reported on the removal of iron by PP_i and tripodal phosphonic acid analogues of nitrilotriacetic acid (NTA).[21] To describe adequately the ligand dependence of the pseudo-first-order rate constant for iron removal, eq 5 had to be modified by inclusion of a first-order term to give eq 6. This two-term

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

equation was interpreted in terms of parallel pathways for the removal of ferric ion from the protein, one which is first order in ligand and another which shows saturation kinetics.

This paper describes the kinetics of iron removal from diferric transferrin, both forms of monoferric transferrin, and monoferric transferrins which have been labeled at the vacant binding site with cobalt(III). The kinetically inert cobalt(III) allows one to follow iron release from one site while the other site is occupied by a metal ion. These systems are used as models for the release of iron from each site of differic transferrin. This approach provides new evidence for positive cooperativity for iron removal at the C-terminal site.

Experimental Section

Reagents. Reagent-grade tetrasodium pyrophosphate (PP_i) was purchased and used without further purification. Purified human serum transferrin was purchased from Calbiochem and further purified by published procedures.[16] Diferric and C-terminal monoferric transferrin were prepared by addition of 2 and 1 equiv of bis(nitrilotriacetato)-ferrate(III), respectively. The N-terminal monoferric transferrin was prepared from diferric transferrin by the method of Baldwin and de Sousa,[31] except that 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) was substituted for tris(hydroxymethyl)aminomethane (Tris) as the buffer.

Dicobalt transferrin was prepared by addition of slightly in excess of 2 equiv of cobalt(II) to a pH 7.4 solution of apotransferrin at ambient bicarbonate concentration, followed by addition of a 5-fold excess of 0.015% hydrogen peroxide.[32] The same procedure was used to load cobalt into the vacant binding sites of monoferric transferrins.

All transferrin species were purified by elution through a 1.5 × 30 cm Sephadex G-25 column with 0.1 M, pH 7.4 Hepes. They were then washed with 3–4 volumes of buffer and concentrated on an Amicon ultrafiltration cell with an XM-50 membrane. The overall metal content of each transferrin sample was determined by UV/vis spectroscopy. The spectra of the cobalt transferrin species were compared to those previously reported.[33]

The distribution of different protein species was checked by polyacrylamide gel electrophoresis according to published procedures.[10,29,34]

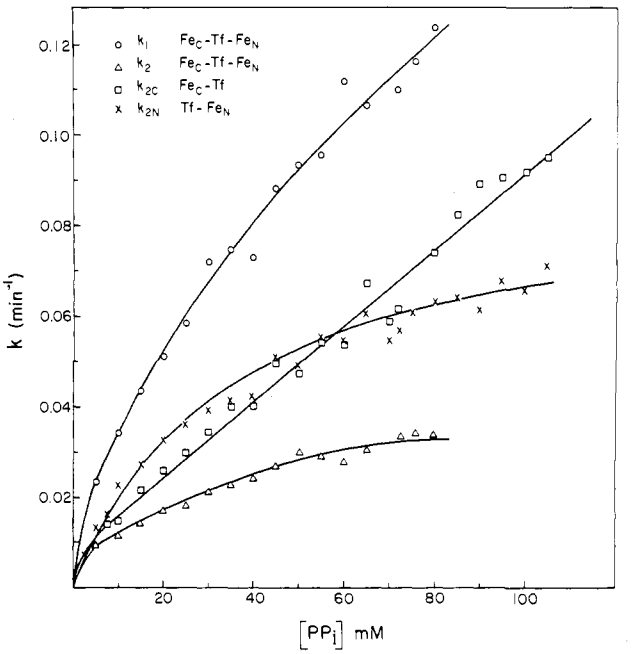

Figure 1. Plots of pseudo-first-order rate constants for removal of iron from transferrin as a function of the [PP_i] in 0.1 M Hepes, pH 7.4 at 25 °C. k_1 (○) and k_2 (△) are from a two-exponential fit of data on iron removal from diferric transferrin. k_{2C} (□) and k_{2N} (×) are from one-exponential fits of data on iron removal from Fe_C–Tf and Tf–Fe_N, respectively.

Gels were 7% by weight of a 19:1 mixture of acrylamide–bis(acrylamide) containing 6.5 M urea in a pH 8.4 Tris buffer. From 4 to 12 μg of protein was loaded and run at a constant 120 V for 15–20 h. Bands were visualized by staining with Coomassie Blue. The ferric transferrin species were stable to electrophoresis, whereas cobalt(III) tended to dissociate from the protein, especially from the C-terminal site.

Methods. The rate of iron removal was measured at 25 °C in 0.1 M Hepes buffer by monitoring the decrease in the iron–phenolate charge-transfer band at 465 nm. Rate constants were calculated by nonlinear least-squares fits of absorbance vs time to either a two-exponential function (for diferric transferrin) or a one-exponential function (for monoferric and cobalt-labeled transferrins) with the nonlinear least-squares program ORGLES.[35] In most calculations, the final absorbance value (A_∞) was treated as an adjustable parameter as long as its value stayed within experimentally reasonable boundaries.

Results

Monoferric Transferrins. The first-order rate constants for iron removal from both forms of monoferric transferrin have been measured as a function of the free PP_i concentration, and the results are shown in Figure 1. The two sites release iron at similar rates but have much different ligand-dependence curves. The ligand dependence of the N-terminal site has been fit to eq 5 and 6. The quality of each fit is given by its R factor, where R is the square root of the sum of the squares of the residuals divided by the sum of the squares of the observations.[36]

Inclusion of the third parameter (k''') is expected to decrease the R factor in all cases. The statistical significance of this decrease is evaluated by the R factor ratio test,[36] in which the experimental ratio of the two R factors is compared with the test statistic:

$$R_{b,n-p,\alpha} = \left(\frac{b}{n-p}F_{b,n-p,\alpha} + 1\right)^{1/2} \quad (7)$$

where n is the number of observations, p is the maximum number of adjustable parameters, b is the number of parameters held fixed in the two-parameter fit, and F is the appropriate element from

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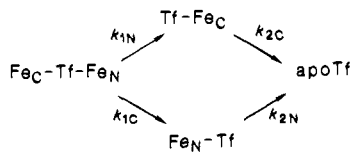
Scheme I



the F distribution table. If the observed ratio of R factors exceeds $R_{b,n-p,\alpha}$, then one can reject the hypothesis that the two sets of parameters give equally good fits of the experimental data.

For the N-terminal site, the addition of the k''' parameter produces a decrease in R that is significant at $\alpha = 0.005$. However, this calculation gives a value of $k'' = 59 \pm 29$ M^{-1}. Since the standard deviation is almost 50% of the value of this parameter, the two-parameter fit from eq 5 has been used. The resulting parameters are $k' = 2.44 \pm 0.16$ min^{-1} M^{-1} and $k'' = 27 \pm 3$ M^{-1}. The parameter k_{max}, which corresponds to the ratio of k'/k'', is the limiting pseudo-first-order rate constant for the saturation process. For the N-terminal site, $k_{max} = 0.091 \pm 0.011$ min^{-1} M^{-1}.

For the C-terminal site, there is a simple first-order dependence of k_{obs} on [PP$_i$] at concentrations of PP$_i$ above 5 mM. The thermodynamic stability of the C-terminal binding site precludes rate measurements below 5 mM PP$_i$. The lack of curvature indicates that $k''[L] \gg 1$, such that eq 6 reduces to

$$k_{obs} = k_{max} + k'''[L] \tag{8}$$

It is impossible to fit the data to eq 6 to calculate values of k' and k''. Instead, the data on Fe$_C$–Tf are fit to eq 8 to give values of $k''' = 0.84 \pm 0.03$ M^{-1} min^{-1} and $k_{max} = 0.0077 \pm 0.0016$ min^{-1}. One can estimate a lower limit of k'' by assuming that linearity requires that $k''[L] \geq 10$. On the basis of the lowest ligand concentration which falls on the linear plot, one estimates a value of $k'' > 1800$ M^{-1} for Fe$_C$–Tf.

Diferric Transferrin. Iron removal from diferric transferrin can be represented by Scheme I. The coupled differential equations which describe the kinetics, on the basis of Scheme I, have previously been presented in integrated form by Baldwin[18] and Thompson et al.[29] The concentrations of the different transferrin species in terms of site-specific rate constants are given by

$$[Fe_C\text{–Tf–}Fe_N] = C_0 e^{-(k_{1N}+k_{1C})t} \tag{9}$$

$$[Fe_C\text{–Tf}] = \frac{C_0 k_{1N}}{k_{1N} + k_{1C} - k_{2C}}(e^{-k_{2C}t} - e^{-(k_{1N}+k_{1C})t}) \tag{10}$$

$$[Tf\text{–}Fe_N] = \frac{C_0 k_{1C}}{k_{1N} + k_{1C} - k_{2N}}(e^{-k_{2N}t} - e^{-(k_{1N}+k_{1C})t}) \tag{11}$$

where C_0 is the concentration of diferric transferrin at time zero. The absorbance at any time during the iron removal reaction is given by the sum of the absorbances of the three species of transferrin absorbing at 465 nm:

$$A_t - A_\infty = (\epsilon_C + \epsilon_N)[Fe_C\text{–Tf–}Fe_N] + \epsilon_C[Fe_C\text{–Tf}] + \epsilon_N[Tf\text{–}Fe_N] \tag{12}$$

where A_T is the absorbance at any time t, A_∞ is the absorbance at infinite time, ϵ_C is the molar extinction coefficient of the C-terminal site, and ϵ_N is the molar extinction coefficient of the N-terminal binding site. Substitution for the concentrations in eq 12 in terms of eq 9, 10, and 11 yields

$$A_t - A_\infty = (\epsilon_C + \epsilon_N)C_0 e^{-(k_{1N}+k_{1C})t} + \frac{\epsilon_C C_0 k_{1N}}{k_{1N} + k_{1C} - k_{2C}}(e^{-k_{2C}t} - e^{-(k_{1N}+k_{1C})t}) + \frac{\epsilon_N C_0 k_{1C}}{k_{1N} + k_{1C} - k_{2N}}(e^{-k_{2N}t} - e^{-(k_{1N}+k_{1C})t}) \tag{13}$$

The total absorbance change is given by

$$A_0 - A_\infty = (\epsilon_N + \epsilon_C)C_0 \tag{14}$$

Dividing eq 13 by eq 14 gives

$$\frac{A_t - A_\infty}{A_0 - A_\infty} = 1e^{-(k_{1N}+k_{1C})t} + \frac{\epsilon_C}{\epsilon_C + \epsilon_N}\frac{k_{1N}}{k_{1N} + k_{1C} - k_{2C}}(e^{-k_{2C}t} - e^{-(k_{1N}+k_{1C})t}) + \frac{\epsilon_N}{\epsilon_C + \epsilon_N}\frac{k_{1C}}{k_{1N} + k_{1C} - k_{2N}}(e^{-k_{2N}t} - e^{-(k_{1N}+k_{1C})t}) \tag{15}$$

The term on the left-hand side of eq 15, usually denoted by R_t, is the normalized reaction coordinate which goes from 1 to 0 as the iron removal reaction proceeds to completion. We define noncooperativity to mean that the binding of iron at one site does not alter the rate constant for iron removal at the alternate site, i.e., $k_{1N} = k_{2N} = k_N$ and $k_{1C} = k_{2C} = k_C$. Assuming that the two sites have the same extinction coefficient, eq 15 reduces to

$$\frac{A_t - A_\infty}{A_0 - A_\infty} = 0.5e^{-k_C t} + 0.5e^{-k_N t} \tag{16}$$

To include A_∞ as an adjustable parameter, eq 16 has been rearranged to

$$A_t = \frac{A_0 - A_\infty}{2}(e^{-k_C t} + e^{-k_N t}) + A_\infty \tag{17}$$

For calculations of rate constants for iron removal from simple monoferric and cobalt-labeled monoferric transferrins from absorbance vs time data, eq 17 reduces to

$$A_t = (A_0 - A_\infty)e^{-kt} + A_\infty \tag{18}$$

Iron removal from diferric transferrin has been studied as a function of the free PP$_i$ concentration. Equation 17 does not fit the data when k_C and k_N are restricted to the values for the respective monoferric transferrins. If the two rate constants are allowed to vary, one obtains reasonably good fits. However, these calculations invariably give one rate constant that is higher than either monoferric rate constant and a second rate constant that is lower than either monoferric rate constant, as shown in Figure 1.

The rate of iron release from a simple, equimolar mixture of C-terminal and N-terminal monoferric transferrins has also been measured. Data on this simple mixture give rate constants similar to those calculated for diferric transferrin, which again bracket the actual monoferric rate constants.

Dicobalt Transferrin. Dicobalt(III) transferrin was prepared as described under Experimental Section. The absorbance maximum at 400 nm and extinction coefficient of 8900 M^{-1} cm^{-1} agree with values previously reported.[33] The rate of cobalt(III) removal by PP$_i$ was determined for 80 mM PP$_i$. The apparent first-order rate constant is 0.001 27 min^{-1}, which is almost 2 orders of magnitude lower than the rate constant for iron removal. This indicates that one can use the kinetically inert cobalt(III) ion to occupy one of the transferrin binding sites and treat iron removal from the other site as a simple first-order process.

Mixed-Metal Cobalt–Iron Transferrins. The vacant sites of both forms of monoferric transferrin were saturated with cobalt(III). The spectroscopic parameters are $\lambda_{max} = 440$ nm and $\epsilon = 5400$ M^{-1} cm^{-1} for Fe$_C$–Tf–Co$_N$ and $\lambda_{max} = 440$ nm and $\epsilon = 3600$ M^{-1} cm^{-1} for Co$_C$–Tf–Fe$_N$. The addition of PP$_i$ leads to removal of the ferric ion, with a shift in λ_{max} to 404 nm, which is characteristic of the intact monocobalt–Tf complex. The ligand dependence of iron removal was measured for each of these cobalt-labeled proteins. The results for Tf–Fe$_N$ and Co$_C$–Tf–Fe$_N$ are plotted in Figure 2. The addition of cobalt to the C-terminal site has virtually no effect on iron removal from the N-terminal site. The R factor ratio test shows that the data for Co$_C$–Tf–Fe$_N$ can be fit to eq 5. Adding k''' as an adjustable parameter produces no signficant improvement in the fit. The values of k', k'', and k_{max} are listed in Table I.

The ligand dependence of the rate constants for Fe$_C$–Tf and Fe$_C$–Tf–Co$_N$ are shown in Figure 3. The addition of cobalt to the vacant N-terminal site does affect iron removal from the C-terminal site. The ligand dependence is still linear, but both the slope and intercept of the curve are increased from the values measured for simple C-terminal monoferric transferrin. The values of k_{max} and k''' are listed in Table I.

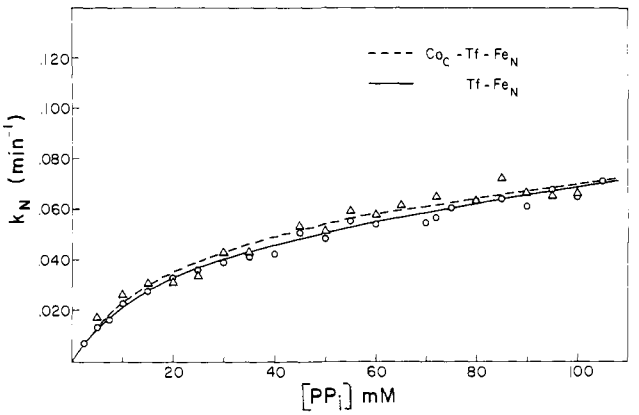


Figure 2. Plots of pseudo-first-order rate constants for the removal of ferric ion from Tf–Fe$_N$ (O) and from Co$_C$–Tf–Fe$_N$ (Δ) as a function of the [PP$_i$] in 0.1 M Hepes, pH 7.4 at 25 °C. The symbols represent experimental data points. The lines are calculated from eq 5 with the parameters listed in Table 1.

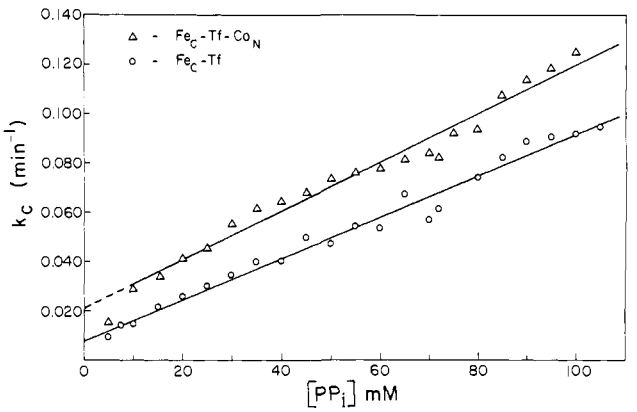


Figure 3. Plots of pseudo-first-order rate constants for removal of ferric ion from Fe$_C$–Tf (O) and Fe$_C$–Tf–Co$_N$ (Δ) as a function of the [PP$_i$] in 0.1 M Hepes, pH 7.4 at 25 °C. The symbols represent the experimental data points. The lines are calculated from eq 8 with the parameters listed in Table I.

Table I. Parameters Describing the Ligand Dependence of the Rate of Iron Removal from Transferrin by PP$_i$

protein	k' (min^{-1} M^{-1})	k'' (M^{-1})	k_{max} (min^{-1})	k''' (min^{-1} M^{-1})
Fe$_C$–Tf		>1800	0.0077 ± 0.0016	0.84 ± 0.03
Fe$_C$–Tf–Co$_N$		>900	0.021 ± 0.002	0.99 ± 0.04
Tf–Fe$_N$	2.44 ± 0.16	27 ± 3	0.090 ± 0.010	
Co$_C$–Tf–Fe$_N$	2.75 ± 0.26	30 ± 5	0.092 ± 0.014	

One must ask whether cobalt(III) will induce the same cooperativity effects as iron(III). The removal of iron from diferric transferrin is described by four microscopic constants, as shown in Scheme I. Two of these constants, k_{2C} and k_{2N}, can be measured directly from the appropriate monoferric transferrins. It is not possible to calculate k_{1C} and k_{1N} from the diferric data because of the very high correlation between these two parameters. Since there is very little effect from adding cobalt(III) to the empty C-terminal site, we make the approximation that the rate constant for the Co$_C$–Tf–Fe$_N$ protein gives a fairly good estimate of k_{1N} of diferric transferrin. This leaves k_{1C} as the only adjustable parameter needed to fit the data on iron release from the diferric protein to eq 15.

Values of k_{1C} calculated by this procedure are shown in Figure 4, along with values of the first-order rate constant for iron removal from Fe$_C$–Tf–Co$_N$. There is reasonably good agreement between these two parameters. The intercepts are within 1 standard deviation of each other. The slope for the Fe$_C$–Tf–Co$_N$ species is slightly greater than that for diferric transferrin (0.99 ± 0.04 vs 0.87 ± 0.05). In addition, for concentrations of PP$_i$ below 80 mM, the fit of the absorbance vs time data obtained from the four

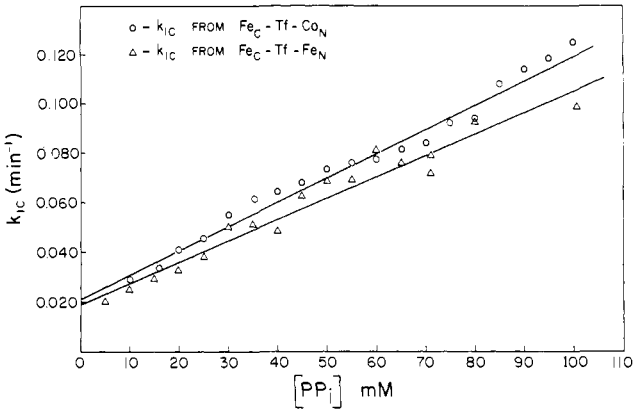


Figure 4. Plots of the pseudo-first-order rate constant for removal of ferric ion from Fe$_C$–Tf–Co$_N$ (O) and the value of k_{1C} calculated from data on iron release from diferric transferrin (Δ). All data were collected in 0.1 M Hepes buffer, pH 7.4 and 25 °C.

microconstants with eq 15 is significantly better than that obtained from a simple two-exponential fit of eq 17, even though k_{1C} is the only microconstant allowed to vary. These results confirm that the cobalt-labeled proteins provide reasonable estimates for the rates of iron release from each site of diferric transferrin.

Iron removal from diferric transferrin was also followed by polyacrylamide gel electrophoresis. This technique allows one to follow the changes in concentration of all four transferrin species: Fe$_C$–Tf–Fe$_N$, Fe$_C$–Tf, Tf–Fe$_N$, and apoTf. At this time the results are only semiquantitative. Both forms of monoferric transferrin are detected early in the iron removal reaction, but the N-terminal species accumulates to higher levels. In addition, the C-terminal monoferric transferrin disappears faster than the N-terminal monoferric transferrin. This indicates that the C-terminal site is reacting faster. A more thorough investigation of iron removal by electrophoresis is in progress.

Discussion

It is obvious that the two rate constants calculated for the removal of iron from diferric transferrin with eq 17 do not correspond to k_C and k_N determined from the monoferric transferrins. One could explain the increase in the faster rate constant as arising from cooperativity. However, the second iron comes from a simple monoferric species. Thus cooperativity cannot account for a rate constant slower than either of the monoferric rate constants.

It now appears that the least-squares fit of the PP$_i$ data to eq 17 produces incorrect results. The rate of iron removal was measured for a mixture of monoferric transferrins. This mixture provides two pools of iron, but with no chance of cooperativity. Thus the system should be described by the two-exponential fit with rate constants k_{2C} and k_{2N}. Nonetheless, the computer fit to eq 17 does not give the known monoferric rate constants. Instead, one again obtains one rate constant that is higher than either monoferric constant, along with a second rate constant that is significantly lower than either of the monoferric values.

These computational problems are presumably due in part to the similarity in magnitude of the two rate constants. One assumes that the results would be more accurate if the constants were much different in magnitude. Two-exponential fits have been used to fit iron removal data for several ligands.[18,20,25,29] Although we do not wish to suggest that a similar problem exists in all cases, we would suggest that caution be used in future calculations.

The ligand dependence of the observed first-order rate constants for iron removal from monoferric and cobalt-labeled monoferric transferrins is explained in terms of two parallel pathways for iron removal, one which is first order in ligand and one which shows saturation behavior.[21,23] There are three kinetic parameters that describe the ligand dependence: k'', which describes the approach to saturation; k_{max}, which gives the maximal rate constant for the saturation process ($k_{max} = k'/k''$); and k''', which is the rate constant for the first-order process. These parameters are listed in Table I.

The data for iron removal from C-terminal transferrin can be fit only to eq 8. Although saturation of the C-terminal site occurs at very low [PP_i], the k_{max} for this site is small. Thus at concentrations of PP_i at or above 50 mM, most iron is removed via the first-order pathway.

The interpretation of iron removal from the N-terminal site is more difficult. For simple Tf–Fe_N the three-parameter fit to eq 6 is significantly better than the two-parameter fit to eq 5 and gives $k_{max} = 0.051$ min^{-1} and $k''' = 0.25 \pm 0.04$ min^{-1} M^{-1}. However, this procedure gives a very poorly defined value of $k'' = 59 \pm 29$ M^{-1}. In addition, the data for iron removal from Co_C–Tf–Fe_N are fit equally well by either eq 6 or eq 5. Therefore, we have adopted eq 5 as the best description of the ligand dependence of iron removal from the N-terminal site. Even if k''' is not zero for the N-terminal site, it is clear that at concentrations of PP_i below 50 mM the predominant pathway for iron removal is the saturation process.

The cooperativity associated with iron release from the C-terminal site is stronger for k_{max} than for k'''. Thus the cooperativity factor k_{1C}/k_{2C} starts out quite high at low [PP_i] and gets smaller as the concentration of PP_i increases. It has been proposed that for PP_i saturation is associated with a rate-determining conformational change in the protein, while the first-order pathway is associated with substitution of PP_i for the synergistic bicarbonate anion.[21] If these assignments are correct, it appears that metal binding at the N-terminal site accelerates the rate of the conformational change in the C-terminal domain but that this change has relatively little direct effect on the rate of anion substitution at the C-terminal site.

The mixed-metal Fe–Co proteins provide reasonably accurate estimates of k_{1C} and k_{1N}. This is indicated by the agreement between k_{1C} calculated from studies on Fe_C–Tf–Co_N and the value obtained from the four-parameter fit of the data on diferric transferrin (Figure 4). The four-parameter model also provides a more accurate fit of the diferric data over most of the PP_i concentration range.

The distance between the two sites has been estimated to be 35 Å,[37] which is obviously too far for any direct interaction between the two ferric ions. Nevertheless, cooperativity between the two metal binding sites of transferrin is of interest with regard to both the thermodynamics of metal binding and the kinetics of iron removal. Cooperativity in the thermodynamics of metal binding appears to be no more than 0.2–0.3 log unit,[8,9,34] which is comparable to the experimental uncertainties in the equilibrium constants.

Cooperativity in the kinetics of iron removal has been evaluated several times.[13,25,29] Thompson et al.[29] have used gel electrophoresis to study cooperativity in iron removal by 3.0 mM PP_i at pH 6.9 and 37 °C. Desferrioxamine B was used as a thermodynamic sink for the iron. They have reported a slight negative cooperativity for the N-terminal site and a substantial positive cooperativity for the C-terminal site: $k_{1N}/k_{2N} = 0.7$ and $k_{1C}/k_{2C} = 3.0$. At 25 °C, pH 7.4, and 10 mM PP_i, we observe noncooperativity at the N-terminal site and a positive cooperativity factor of 1.9 for the C-terminal site. We cannot accurately extrapolate our C-terminal data to 3 mM PP_i for a more detailed comparison with the results of Thompson et al.[29] Nevertheless, it appears that these two studies agree reasonably well on the extent of cooperativity in iron removal by PP_i.

Kretchmar and Raymond[25] report a slight positive cooperativity in the kinetics of iron removal from the N-terminal binding site by the catecholate ligand 1,5,10-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane at pH 7.4. Conversely, iron removal by EDTA at pH 7.4 shows no cooperativity.[13] Thus it appears that cooperativity effects are sensitive to the identity of the competitive ligand, the pH, and perhaps other factors such as temperature and salt concentration.

Although the cooperativity effects for PP_i are similar at pH 6.9 and at pH 7.4, Thompson et al.[29] report that iron is removed faster from the N-terminal site at pH 6.9, while at pH 7.4 iron is removed more quickly from the C-terminal site. This shift is consistent with the observation that the ratio k_N/k_C for iron removal from the monoferric transferrins by EDTA increases with decreasing pH.[13] A faster reaction of the C-terminal site with PP_i at pH 7.4 has also been noted by Bertini et al.[38]

Summary

Iron removal from monoferric C-terminal transferrin by PP_i proceeds predominantly through a first-order pathway, whereas iron removal from the N-terminal site follows a saturation pathway. Labeling of the vacant site of each monoferric transferrin with cobalt(III) allows one to measure directly cooperativity for iron release. The addition of cobalt to the C-terminal site has no effect on iron release from the N-terminal site. However, addition of cobalt to the N-terminal site significantly accelerates iron release from the C-terminal site. This acceleration is due primarily to an increase in the k_{max} for the saturation process. Comparison of rate constants for diferric transferrin with those for the cobalt-labeled monoferric transferrins indicates that Co(III) mimics the cooperativity effects of iron(III).

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