electronic configuration differs only by an additional d_π nonbonding electron in high-spin Fe(II), the difference is attributable mainly to the increased nuclear charge on Fe(III). On the basis of Badger's rule,[49] we estimate the decrease in the Fe–OH distance to be 0.124 Å, as expected from the difference in the Fe^{2+} and Fe^{3+} ionic radii (0.135 Å).[50]

When a second OH^- is bound to the high-spin Fe(III) complex, the force constant drops from 2.25 to 2.00 mdyn/Å. (Strictly speaking, the latter value is the sum of the principal force constant and the interaction force constant between the two Fe–OH stretches.) This trans effect is due to the competition between the two OH^- ligands for the Fe(III) bonding orbital, d_{z^2}. The removal of the unpaired electron from this orbital in the low-spin $Fe^{III}(OH)_2$ complex accounts for the increase in the force constant from 2.00 to 2.48 mdyn/Å. Badger's rule gives 0.046 Å for the corresponding decrease in the bond distance, a value that is smaller than the ~0.1 Å difference expected between high- and low-spin Fe(III) complexes.[50] The decrease in the Fe–OH distance brought about by the enhanced σ-interaction is counteracted by the strong π donor properties of the hydroxide ligands. The OH^- π orbitals interact in an antibonding fashion with the partially filled Fe d_π orbitals. This resulting lengthening of the Fe–OH bond might account for the ν_2 core size marker frequency being 14 cm^{-1} higher for low-spin $Fe^{III}(2\text{-}TMPyP)(OH)_2^{3+}(aq)$ than for the low-spin imidazole adduct $Fe^{III}(TPP)(ImH)_2Cl$.[12] The nonbonded repulsions between the pyrrole-N atoms and the axial ligand may be weaker for OH^- than for ImH, allowing the porphyrins to contract.

(49) (a) Badger, R. M. *J. Chem. Phys.* **1934**, *2*, 128. (b) Badger, R. M. *J. Chem. Phys.* **1935**, *3*, 710. (c) Herschbach, D. R.; Laurie, V. W. *J. Chem. Phys.* **1961**, *5*, 458.
(50) Shannon, R. D.; Prewitt, C. T. *Acta Crystallogr.* **1969**, *B25*, 925.
(51) Felton, R. H. In *The Porphyrins*; Dolphin, D., Ed., Academic Press: New York, 1978; Vol. 5, Chapter 3.

Conclusions

The $2\text{-}TMPyP^{2+}$ ligand shows a remarkable ability to stabilize high and low metalloporphyrin oxidation states via control of ligation environment and of molecular charge. We have demonstrated that in the presence of 10^{-2} to 10^{-1} M hydroxide ion the Fe(I), Fe(II), Fe(III), and Fe(IV) complexes of $2\text{-}TMPyP^{2+}$ can be generated via chemical and/or electrochemical means. These complexes are stable at ambient temperature for relatively long periods of time. This stability is remarkable in light of the fact that numerous earlier porphinatoiron(I) and iron(IV) studies have been *plagued* by the presence of trace amounts of water. The Fe(I) stability is based in the ability of the positively charged porphyrin ligand to delocalize electron density from the metal ion via a primarily electrostatic mechanism. In high oxidation states, this same electrostatic influence of the porphyrin ligand results in a high affinity of the metal center for axial ligands. Thus the Fe^{IV}=O complex binds hydroxide and thereby gains thermodynamic and kinetic stability relative to five-coordinate oxoporphinatoiron(IV) complexes. The Fe^{IV}=O stretching frequency has been identified at 763 cm^{-1} and is consistent with axial coordination by a hydroxide ligand trans to the ferryl oxygen.

In summary, the stabilization effects exhibited by this ligand are largely electrostatic in nature and not due to perturbations of the porphine core molecular orbital energies. This has important implications in the design and tailoring of transition metal reagents and catalysts for effecting useful oxidation and reduction chemistry. Moreover, it further illustrates the latitude that nature has at her disposal to "tune" heme reactivity via variations in the electrostatic potential around the prosthetic sites in heme proteins.

Acknowledgment. This work was supported by U.S. Department of Energy Grant DEFG02-88ER13876 (T.G.S.) and the National Science Council of the Republic of China (Y.O.S.). K.R.R. (1 F32 HL08116-01) and R.A.R. (GM12197-02) are recipients of NIH National Research Service Awards.

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Kinetics of Iron Removal from Monoferric and Cobalt-Labeled Monoferric Transferrins by Diethylenetriaminepenta(methylenephosphonic acid) and Diethylenetriaminepentaacetic Acid

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Rate constants for the removal of ferric ion from transferrin by the octadentate chelating agents diethylenetriaminepenta(methylenephosphonic acid) (DTPP) and diethylenetriaminepentaacetic acid (DTPA) have been measured for both N- and C-terminal monoferric transferrin in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ehtanesulfonic acid buffer at pH 7.4 and 25 °C. Iron removal by the amino carboxylate DTPA appears to follow simple first-order kinetics. However, plots of the observed pseudo-first-order rate constant for iron removal versus the concentration of DTPP follow neither simple first-order nor saturation kinetics. The results on DTPP are interpreted in terms of two parallel pathways for iron removal, one which shows saturation kinetics and another which is first order in DTPP. The site preferences for the two ligands are also different. While DTPA preferentially removes iron from the N-terminal site, DTPP preferentially removes ferric ion from the thermodynamically more stable C-terminal binding site. Labeling the vacant binding sites of the monoferric transferrins with kinetically inert cobalt(III) has little effect on the rates of iron removal by DTPP. Equilibrium constants for the binding of the anionic DTPP ligand to the apoprotein have also been determined. The successive macroscopic binding constants are $\log K_1 = 6.25 \pm 0.16$ and $\log K_2 = 4.68 \pm 0.17$.

Introduction

Transferrin is a serum protein whose primary function is to transport ferric ion among sites of uptake, utilization, and storage.[1-4] The distinguishing characteristic of the transferrins is the requirement of concomitant binding of a synergistic anion to form a stable metal–transferrin complex. Under biological conditions, bicarbonate is the synergistic anion, although a variety of other anions will form weak Fe–L–Tf complexes under bi-

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(1) Harris, D. C.; Aisen, P. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; pp 239-352.
(2) Aisen, P. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; pp 353-372.
(3) Brock, J. H. In *Metalloproteins*; Harrison, P., Ed.; Macmillan: London, 1985; Part II, pp 183-262.
(4) Bates, G. W.; Graybill, G.; Chidambaram, M. V. In *Control of Animal Cell Proliferation*; Boynton, A. L., Leffert, H. L., Eds.; Academic Press: New York, 1987; Vol. II, pp 153-202.

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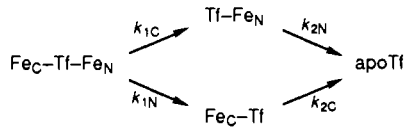
carbonate-free conditions.[5] Recent crystal structures of both transferrin[6] and lactoferrin[7-9] have established that the protein consists of two homologous lobes, designated N-terminal and C-terminal. Each lobe consists of two dissimilar domains, with a single high-affinity metal-binding site located in the cleft between the two domains.

Simultaneous release of iron from the two similar binding sites complicates kinetic studies on iron removal by low molecular weight chelating agents. It is often possible to fit the data on iron release to a simple two-exponential equation, both for serum transferrin[10-15] and for the related protein ovotransferrin.[16,17] However, we have observed that in some cases these two-exponential fits do not give accurate rate constants for the individual transferrin species.[18,19] Thus a more detailed analysis of the microscopic rate constants for iron release from the two binding sites is needed.

There are four microscopic rate constants for the removal of ferric ion from diferric transferrin:



The subscripts to each rate constant indicate the site from which the iron is coming (C-terminal or N-terminal) and whether it is the first or second ferric ion to be lost from the protein.

The two microscopic rate constants for iron release from one binding site when the other site is empty (k_{2C} and k_{2N}) can be easily obtained from studies on pure monoferric transferrins.[14,18-25] We have recently demonstrated that cobalt-labeled monoferric transferrins can be used to obtain reliable estimates for k_{1C} and k_{1N}, the microscopic rate constants for iron removal from one site while the other site remains occupied by ferric ion.[18,19,26] This cobalt-labeling procedure is used in the present study to evaluate cooperativity between the two binding sites during iron removal by diethylenetriaminepenta(methylenephosphonic acid). The results are discussed in terms of previous proposals that the rate of iron removal by exogenous chelating agents is regulated by a protein conformational change.[27,28]

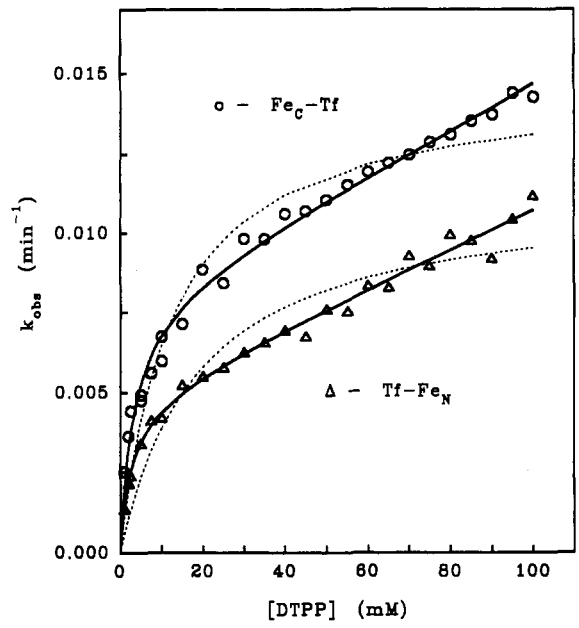


Figure 1. Plots of the apparent first-order rate constants for iron removal from C- and N-terminal monoferric transferrin as a function of the concentration of DTPP in 0.1 M Hepes, pH 7.4, 25 °C. The dashed lines represent the best fit of the data to eq 2. The solid lines represent the best fit of the data to eq 3.

Experimental Section

Materials. Human serum transferrin was purchased from Calbiochem and purified as previously described.[29] Simple monoferric transferrins and cobalt-labeled monoferric transferrins were prepared and purified as described previously.[18,26] A purified sample of diethylenetriaminepenta(methylenephosphonic acid) was a gift from the Monsanto Co. of St. Louis, MO. The ligand gave the expected ^{31}P and ^{1}H NMR spectra and was not purified further.

Methods. Difference ultraviolet spectra were recorded with a Varian 2200 UV-vis spectrophotometer. The concentration of each sample of apoTf was determined from its absorbance at 278 nm on the basis of a molar extinction coefficient of 93 000 M^{-1} cm^{-1}.[30] For difference UV titrations, a baseline of protein versus protein in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.4 was recorded from 310 to 240 nm. Solutions were maintained at 25 °C in a jacketed cell holder by an external constant-temperature bath. Aliquots of DTPP were added to the sample cuvette, while equal volumes of distilled water were added to the reference cuvette. The difference UV spectrum was recorded after each addition. The data were converted to an apparent absorptivity, $\Delta\epsilon$, by dividing the absorbances at the minimum at 245 nm by the analytical concentration of transferrin.

Iron removal reactions were monitored in 0.1 M Hepes buffer at pH 7.4 and 25 °C. The reaction was followed by monitoring the decrease in the iron-phenolate charge-transfer band near 465 nm with a Hewlett-Packard Model 8452A diode array UV-vis spectrophotometer. Pseudo-first-order rate constants were calculated by use of the routines provided by Hewlett-Packard on the HP Chemstation computer to fit the absorbance vs time data to eq 1, where A_0, A_t, and A_∞ are the absorbances at time zero, any intermediate time t, and infinite time, respectively. For reactions that went to completion, A_0, k_{obs}, and A_∞ were treated as adjustable parameters, and data out to 3 or 4 half-lives were included in the calculations. At lower ligand concentrations, where iron removal did not go to completion, the initial data were used to calculate k_{obs} using fixed values of A_0 and A_∞ obtained from the runs at higher concentrations.

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

(5) Schlabach, M. R.; Bates, G. W. *J. Biol. Chem.* **1975**, *250*, 2177-2188.
(6) Bailey, S.; Evans, R. W.; Garrat, R. C.; Gorinsky, B.; Hasnain, H.; Horsburgh, C.; Jhoti, H.; Lindley, P. F.; Mydin, A.; Sarra, R.; Watson, J. L. *Biochemistry* **1988**, *27*, 5804-5812.
(7) Anderson, B. F.; Baker, H. M.; Dodson, E. J.; Norris, G. E.; Rumball, S. V.; Waters, J. M.; Baker, E. M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1769-1773.
(8) Anderson, B. F.; Baker, H. M.; Norris, B. E.; Rumball, S. V.; Baker, E. N. *Nature* **1990**, *344*, 784-787.
(9) Anderson, B. F.; Baker, H. M.; Norris, B. E.; Rice, D. W.; Baker, E. N. *J. Mol. Biol.* **1989**, *209*, 711-734.
(10) Baldwin, D. A. *Biochim. Biophys. Acta* **1980**, *623*, 183-198.
(11) Thompson, C. P.; Grady, J. K.; Chasteen, N. D. *J. Biol. Chem.* **1986**, *261*, 13128-13134.
(12) Bertini, I.; Hirose, J.; Luchinat, C.; Messori, L.; Piccioli, M.; Scozzafara, A. *Inorg. Chem.* **1988**, *27*, 2405-2409.
(13) Harris, W. R. *J. Inorg. Biochem.* **1984**, *21*, 263-276.
(14) Kretchmar, S. A.; Raymond, K. N. *J. Am. Chem. Soc.* **1986**, *108*, 6212-6218.
(15) Kontoghiorghes, G. J. *Biochim. Biophys. Acta* **1986**, *869*, 141-146.
(16) Cheuk, M. S.; Keung, W. M.; Loh, T. T. *J. Inorg. Biochem.* **1987**, *30*, 121-131.
(17) Cheuk, M. S.; Loh, T. T.; Hui, Y. V.; Keung, W. M. *J. Inorg. Biochem.* **1987**, *29*, 301-311.
(18) Bali, P. K.; Harris, W. R. *J. Am. Chem. Soc.* **1989**, *111*, 4457-4461.
(19) Bali, P. K.; Harris, W. R.; Nesset-Tollefson, D. *Inorg. Chem.* **1991**, *30*, 502-508.
(20) Baldwin, D. A.; deSousa, D. M. R. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1101-1107.
(21) Baldwin, D. A.; DeSousa, D. M. R.; von Wandruszka, R. M. A. *Biochim. Biophys. Acta* **1982**, *719*, 140-146.
(22) Kretchmar, S. A.; Raymond, K. N. *Inorg. Chem.* **1988**, *27*, 1436-1441.
(23) Thompson, C. P.; McCarty, B. M.; Chasteen, N. D. *Biochim. Biophys. Acta* **1986**, *870*, 530-537.
(24) Marques, H. M.; Egan, T. J.; Pattrick, G. *S. Afr. J. Sci.* **1990**, *86*, 21-24.
(25) Marques, H. M.; Watson, D. L.; Egan, T. J. *Inorg. Chem.* **1991**, *30*, 3758-3762.
(26) Bali, P. K.; Harris, W. R. *Arch. Biochem. Biophys.* **1990**, *281*, 251-256.
(27) Cowart, R. E.; Swope, S.; Loh, T. T.; Chasteen, N. D.; Bates, G. W. *J. Biol. Chem.* **1986**, *261*, 4607-4614.
(28) Cowart, R. E.; Kojima, N.; Bates, G. W. *J. Biol. Chem.* **1982**, *257*, 7560-7565.
(29) Harris, W. R. *Inorg. Chem.* **1986**, *25*, 2041-2045.
(30) Chasteen, N. D. *Coord. Chem. Rev.* **1977**, *22*, 1-36.

Results

Iron Removal from Monoferric Transferrins. Since it is possible to prepare pure samples of both forms of monoferric transferrin (Fe_C–Tf and Tf–Fe_N), one can directly measure values for k_{2C} and k_{2N}. Apparent first-order rate constants for the removal of iron from both forms of monoferric transferrin have been measured as a function of the concentration of DTPP. The results are shown in Figure 1.

Iron removal by the catecholate ligand LICAMS[14,31] and the hydroxamate ligands acetohydroxamic acid[28] and aerobactin[32] follows simple saturation kinetics with respect to the free-ligand concentration. In these cases the ligand dependence of k_{obs} is given by

$$k_{obs} = \frac{k'[L]}{1 + k''[L]} \quad (2)$$

The best fits of the k_{obs} values for iron removal by DTPP to eq 2 for both Fe_C–Tf and Tf–Fe_N are shown as the dashed lines in Figure 1. The observed rate constants do not level off at high ligand concentrations, and as a result, the fits are rather poor. The DTPP data can be described more accurately by

$$k_{obs} = \frac{k'[L]}{1 + k''[L]} + k'''[L] \quad (3)$$

The fit of the monoferric rate constants to eq 3 is shown by the solid lines in Figure 1. There is obviously a much better fit to eq 3, which has been used previously to describe the ligand dependence of iron removal by pyrophosphate and other phosphonic acids.[12,18,19,24]

One expects an improvement in the quality of the fit to eq 3 due to the addition of a third adjustable parameter. The R factor ratio test[33] can be used to determine whether the observed improvement in the fit is statistically significant. The R factor associated with the fit of the observed rate constants to eq 2 or 3 is defined as

$$R_x = \frac{\sum(k_{obs} - k_{calc})^2}{\sum k_{obs}^2} \quad (4)$$

where the subscript x denotes the number of adjustable parameters used to fit k_{obs}, k_{obs} represents the observed rate constants at each ligand concentration, and k_{calc} represents the fitted values calculated from either eq 2 or eq 3. The ratio of the R factors for statistically equivalent fits should fall within limits calculated from the F distribution as

$$R_{b,n-p,\alpha} = \frac{R_2}{R_3} = \left(\frac{b}{n-p}F_{b,n-p,\alpha} + 1\right)^{1/2} \quad (5)$$

where p is the maximum number of parameters varied, b is the number of parameters held constant in the more restrictive two-parameter fit to eq 2, and n is the number of observations. On the basis of this test, the improvement in the R factor for the three-parameter fit is significant at $\alpha = 0.005$ for both N- and C-terminal monoferric transferrin. Thus, the rate constants discussed below are all calculated by using eq 3.

The ligand dependences of the monoferric rate constants are interpreted in terms of parallel reaction pathways for iron removal, one which follows saturation kinetics and one which is first order in ligand.[18,19] Three constants are useful for describing the rate constants as a function of the ligand concentration. The parameter k'' from eq 3 describes the approach to saturation. The ratio of k'/k'' is defined as k_{max}, which represents the maximum rate constant for the saturation pathway. The parameter k''' is the rate constant for the first-order pathway. Values of k_{max}, k'', and k''' are listed in Table I.

Table I. Parameters Describing the Ligand Dependence of the Pseudo-First-Order Rate Constants for Iron Removal from Monoferric and Cobalt-Labeled Monoferric Transferrins

	k_{max}, min^{-1}	log (k'', M^{-1})	k''', M^{-1} min^{-1}
	DTPP		
Fe_C–Tf	0.0079 ± 0.0015	2.51 ± 0.08	0.070 ± 0.005
Fe_C–Tf–Co_N	0.0069 ± 0.0022	2.64 ± 0.12	0.078 ± 0.001
Tf–Fe_N	0.0047 ± 0.0013	2.58 ± 0.10	0.061 ± 0.003
Co_C–Tf–Fe_N	0.0052 ± 0.0018	2.84 ± 0.13	0.092 ± 0.004
	DTPA		
Fe_C–Tf	0	0	0.036 ± 0.002
Tf–Fe_N	0	0	0.180 ± 0.008

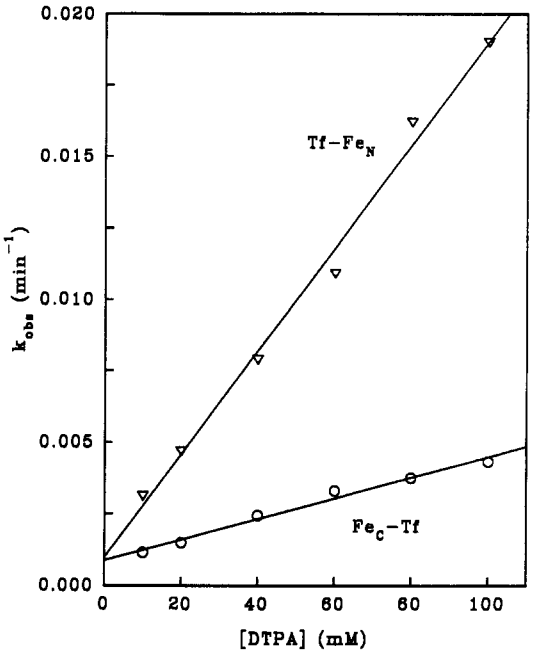


Figure 2. Plots of the apparent first-order rate constants for iron removal from C- and N-terminal monoferric transferrin as a function of the concentration of DTPA in 0.1 M Hepes, pH 7.4, 25 °C.

Iron removal from both forms of monoferric transferrin by DTPA has also been evaluated. Plots of the apparent first-order rate constant vs the concentration of DTPA are shown in Figure 2. In contrast to the case with DTPP, iron removal by DTPA appears to follow first-order kinetics and is significantly faster from the N-terminal binding site. These data have been treated by linear least squares to obtain values of k''' which are listed in Table I.

Iron Removal from Cobalt-Labeled Monoferric Tf. Even when one has accurate values for k_{2C} and k_{2N}, it is very difficult to calculate both k_{1C} and K_{1N} from absorbance vs time data for the removal of iron from diferric transferrin.[18,19] Therefore, we have adopted the practice of labeling the empty sites of monoferric transferrin with kinetically inert cobalt(III). One can then estimate values for k_{1C} and k_{1N} by measuring the rate of iron removal from one site while the other site remains occupied by the trivalent cobalt.

The removal of ferric ion at each concentration of DTPP from both Fe_C–Tf–Co_N and Co_C–Tf–Fe_N follows pseudo-first-order kinetics. Control experiments using dicobalt(III)–Tf show that no significant amount of cobalt(III) is removed over the time period of the iron removal reactions. The rate constants for the removal of ferric ion from both Fe_C–Tf and its cobalt-labeled derivative Fe_C–Tf–Co_N as a function of the concentration of DTPP are shown in Figure 3. It is apparent that the cobalt in the N-terminal site has no effect on iron removal from the C-terminal site. The rate constants for the removal of ferric ion from Tf–Fe_N and Co_C–Tf–Fe_N as a function of the concentration of DTPP are shown in Figure 4. In this case, the cobalt causes a slight ac-

(31) Carrano, C. J.; Raymond, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 5401–5404.
(32) Konopka, K.; Bindereif, A.; Nielands, J. B. *Biochemistry* **1982**, *21*, 6503–6508.
(33) Hamilton, W. C. *Statistics in Physical Science*; Ronald Press: New York, 1964; pp 157–162.

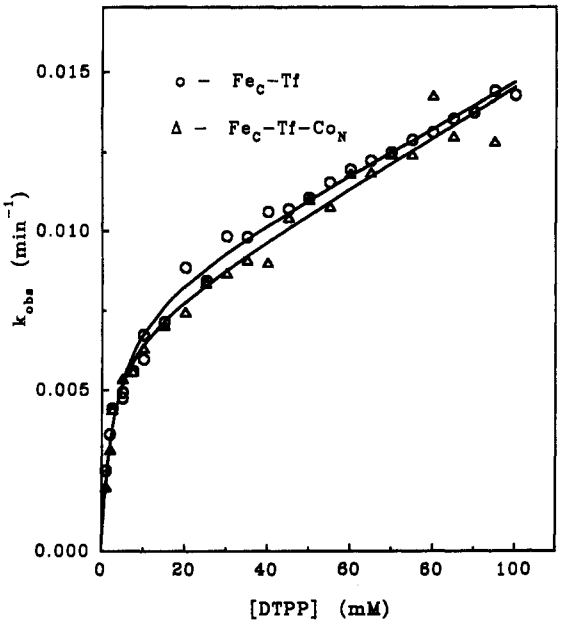


Figure 3. Plots of the apparent first-order rate constants for iron removal from C-terminal monoferric transferrin and C-terminal monoferric transferrin labeled at the N-terminal site with kinetically inert cobalt(III) as a function of the concentration of DTPP in 0.1 M Hepes, pH 7.4, 25 °C.

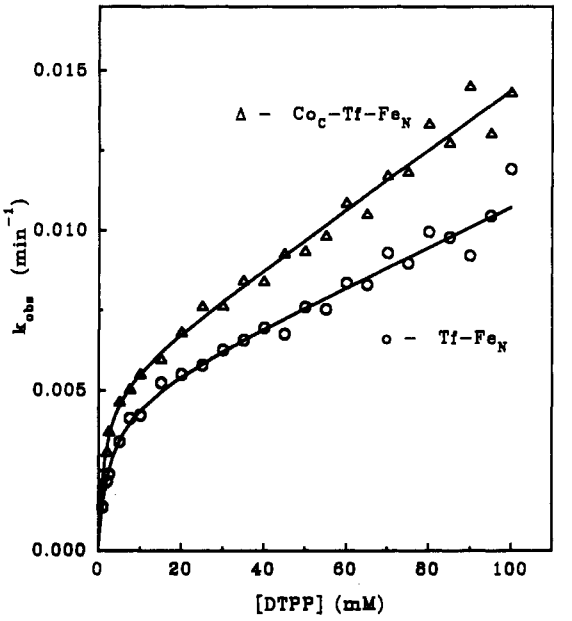


Figure 4. Plots of the apparent first-order rate constants for iron removal from N-terminal monoferric transferrin and N-terminal monoferric transferrin labeled at the C-terminal site with kinetically inert cobalt(III) as a function of the concentration of DTPP in 0.1 M Hepes, pH 7.4, 25 °C.

celeration in the rate of iron removal. The rate constants for the cobalt-labeled samples have also been fit to both eq 2 and eq 3. In both cases, R factor ratio tests indicate that there is a significantly better fit to eq 3. Values of k_{max}, k'', and k''' are listed in Table I.

Anion Binding. The equilibrium constant for the binding of free DTPP to apoTf has been measured by difference UV titrations. The addition of sequential aliquots of DTPP to apoTf produces the family of spectra shown in Figure 5. The spectra are essentially identical to those previously observed for the titration of apoTf with other phosphonates.[34] To correct for dilution effects, the absorbance at 245 nm is converted to an absorptivity

(34) Harris, W. R.; Nesset-Tollefson, D. *Biochemistry* **1991**, *30*, 6930–6936.

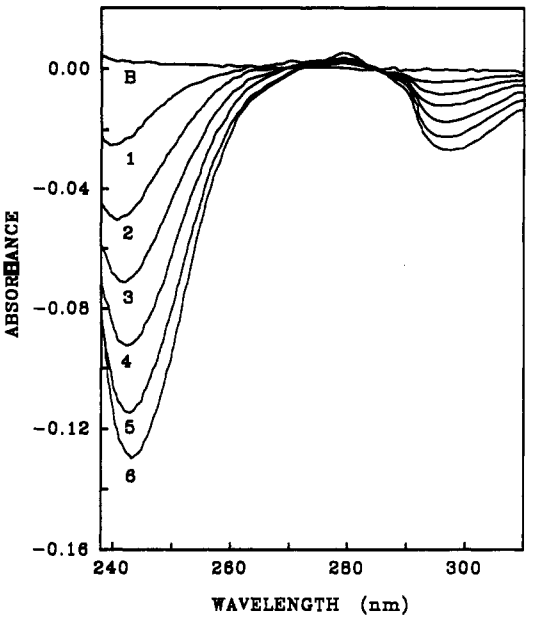


Figure 5. Difference ultraviolet spectra produced by the addition of DTPP to 12.2 μM apoTf in 0.1 M Hepes, pH 7.4, 25 °C. The spectrum marked B is the baseline of apoTf versus apoTf. Volumes of 0.5 mM DTPP added: (1) 10 μL; (2) 30 μL; (3) 50 μL; (4) 80 μL; (5) 125 μL; (6) 250 μL.

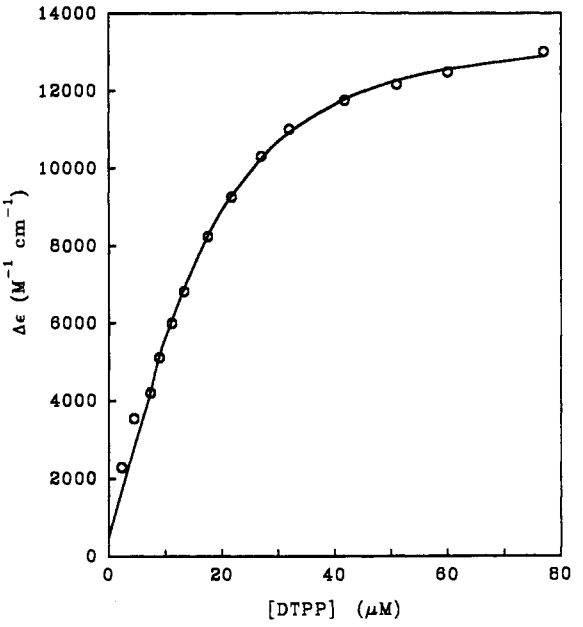


Figure 6. Titration of the apparent absorptivity of the DTPP–Tf complex versus the concentration of total DTPP. [apoTf] = 12.2 μM in 0.1 M Hepes, pH 7.4, 25 °C.

by dividing by the analytical concentration of transferrin at each point in the titration. A titration curve of absorptivity versus concentration of DTPP is shown in Figure 6.

The binding of anions to apoTf is described by two macroscopic binding constants defined as

$$K_1 = \frac{[\text{A–Tf}]}{[\text{A}][\text{apoTf}]} \tag{6}$$

$$K_2 = \frac{[\text{A–Tf–A}]}{[\text{A}][\text{A–Tf}]} \tag{7}$$

where A represents the anion. The absorptivity at any point in the titration can be calculated by first using initial guesses of K_1 and K_2 to solve the appropriate mass balance equations for [apoTf] and [DTPP] and then using these values in eq 8, where $\Delta\epsilon_M$ is the molar absorptivity of the DTPP–Tf complex per anion-binding

$$\Delta\epsilon = \frac{(\Delta\epsilon_M)K_1[\mathrm{DTPP}][\mathrm{apoTf}] + 2(\Delta\epsilon_M)K_1K_2[\mathrm{DTPP}]^2[\mathrm{apoTf}]}{[\mathrm{apoTf}]_{tot}} \quad (8)$$

site.[35] Previous studies on simple inorganic anions and on phosphonic acids have shown that there is no detectable difference in the molar absorptivities for the two anion-binding sites.[34,36] Initial values of $\Delta\epsilon_M$ are determined from the plateau in the titration curves at high DTPP concentrations. This leaves K_1 and K_2 as the only unknowns needed to calculate $\Delta\epsilon$. Values for these two constants have been calculated from nonlinear least-squares fits of $\Delta\epsilon_{obs}$. During these refinements, values of $\Delta\epsilon_M$ were allowed to vary within ±10% of the initial value. The final values are $\Delta\epsilon_M = 6300\ M^{-1}\ cm^{-1}$, $\log K_1 = 6.27 \pm 0.23$, and $\log K_2 = 4.88 \pm 0.11$.

The anion binding data shown in Figure 6 are fit quite well by a simple two-binding-site model. This model is supported by several studies which indicate direct competition between ferric ion and those anions which bind to apoTf to produce the characteristic difference UV spectrum.[36-38] Although the anion-binding data could have certainly been fit to more complex models, there is currently no justification for use of a more complicated model for anion binding.

Discussion

Anion Binding. Simple anions such as phosphate and sulfate as well as aminophosphonic acids bind electrostatically to apoTf.[34-36] Divalent anions such as HPO_4^{2-} and SO_4^{2-} have binding constants of $\sim 10^4$.[35] The $\log K_1$ for DTPP is 6.27, which is essentially identical to the binding constants for PP_i, the triphosphonate nitrilotris(methylenephosphonic acid) (NTP), and the diphosphonate di(phosphonomethyl)glycine (DPG).[33] Thus there appears to be a cap of $\log K \sim 6.3$ for the binding of phosphonates which is reached whenever there are two phosphonate groups in the ligand.

The pattern in the anion-binding constants for a series of aminophosphonic acids has been attributed to a ditopic anion-binding site.[34] It is proposed that there is a primary binding locus, which corresponds to the binding location of the synergistic bicarbonate anion, and a secondary locus, which consists of cationic residues that extend into the interdomain cleft near the metal-binding site. Simple anions appear to bind at the primary locus, which contains a tyrosine residue and thus produces the characteristic difference UV spectrum. Anions which have two phosphonate groups can bind at both loci, increasing the binding constant from 10^4 to 10^6. However, the additional phosphonate groups of NTP and DTPP do not contribute further to anion binding and presumably extend into the cleft between the two domains of each lobe of the protein.

The separation between K_1 and K_2 for DTPP binding to apoTf is 1.4 log units. This value is comparable to that observed for other polyphosphonates[34] and is substantially greater than the value of 0.6 log unit, which is the statistical factor for the separation between the macroscopic binding constants for two identical binding sites. Thus, there is a substantial preference for DTPP binding to one of the two transferrin binding sites.

Simple inorganic anions show only a very slight preference for binding to the N-terminal site.[36] Thus the selectivity for polyphosphonates is associated primarily with the secondary cleft binding locus. Examination of the crystal structure of diferric transferrin suggests that the cleft site is likely to consist of two lysines for the N-terminal lobe and one lysine and one arginine for the C-terminal lobe.[34] Studies on simple model systems indicate that protonated amino groups form more stable anion complexes than guanidinium groups.[39] Thus, we tentatively assign the higher binding constant to the N-terminal site.

Ligand Dependence of Iron Removal. It is clear that iron removal by DTPP follows neither saturation nor first-order kinetics. It is our working hypothesis that there are two parallel pathways for iron removal, one which is first-order in ligand and another which follows simple saturation kinetics. This hypothesis is based in part on the variation in kinetics among different ligands. Iron removal by LICAMS[14,31] and acetohydroxamic acid[28] follows simple saturation kinetics as described by eq 2. Conversely, iron removal by NTA follows simple first-order kinetics,[19] and iron removal by the analogous triphosphonate NTP changes to a simple first-order process in the presence of 200 mM concentrations of several anions.[40]

It now appears that iron removal by DTPA also follows first-order kinetics with respect to the ligand concentration. However, DTPA shows a significant selectivity for iron removal from the N-terminal site, while NTA showed a slight selectivity for iron removal from the C-terminal site. It is difficult to attribute this change in selectivity to the greater size of DTPA, since the first-order process for DTPP is essentially the same for both sites and is faster for the larger pentaphosphonate DTPP than for the smaller triphosphonate NTP.

Neither of the lines shown in Figure 2 extrapolates back to zero, as expected for a simple first-order process. However, the intercept for Fe_N–Tf is within two standard deviations of zero. The data for Fe_C–Tf are inherently less reliable at low DTPA concentrations because the stronger binding of iron to the C-terminal site of the protein results in a relatively small amount of iron being removed from transferrin during the reaction. Finally, the intercepts are quite small, $\sim 1 \times 10^{-3}\ min^{-1}$. Thus, at this time, we do not ascribe any chemical significance to the non-zero intercepts.

It has been proposed[27,28] that iron release from transferrin is regulated by a conformational change in the protein as described in eqs 9–11, where the asterisk denotes an "open", reactive form

$$\mathrm{Fe\text{–}HCO_3\text{–}Tf} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Fe\text{–}HCO_3\text{–}Tf^*} \quad (9)$$

$$\mathrm{Fe\text{–}HCO_3\text{–}Tf^* + L} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{L\text{–}Fe\text{–}HCO_3\text{–}Tf^*} \quad (10)$$

$$\mathrm{L\text{–}Fe\text{–}HCO_3\text{–}Tf^*} \overset{k_3}{\rightleftharpoons} \mathrm{Fe\text{–}L + apoTf + HCO_3} \quad (11)$$

of ferric transferrin. This mechanism accounts for the observation of saturation kinetics during the removal of iron by ligands such as acetohydroxamic acid[28] and LICAMS[31] without the accumulation of detectable concentrations of any mixed-ligand intermediates.

This simple model predicts that all ligands should reach the same k_{max}, which would be equal to k_1, the forward rate constant for the conformational change. If iron release kinetics for the phosphonates are treated by eq 2, there is no consistency in the k_{max} values. However, if eq 3 is used, the k_{max} values of the C-terminal site for the four ligands studied thus far cluster around $0.010 \pm 0.003\ min^{-1}$.[14,18,19] Thus, the data are consistent with the hypothesis that a relatively slow conformational change in the C-terminal lobe is rate limiting in all cases.

The situation is more complicated for the N-terminal site. The k_{max} of $0.016\ min^{-1}$ for NTP[19] is significantly lower than the value of $0.051\ min^{-1}$ for PP_i[18] or $0.039\ min^{-1}$ for LICAMS.[14] The k_{max} reported here for DTPP is only $0.0047\ min^{-1}$, a full order of magnitude less than the PP_i constant. Thus, this study confirms that a ligand-independent conformational change in the protein cannot be the common rate-limiting step for all ligands at the N-terminal site.

One possibility is that the interactions of the phosphonates with transferrin involve the binding of these highly charged ligands as anions to the protein, rather than direct coordination to the metal. This could account for the lack of any detectable mixed-ligand intermediates, even in cases where the conformational change is not rate-limiting and such an intermediate would be expected. We have previously estimated the anion-binding affinity

(35) Harris, W. R. *Biochemistry* **1985**, *24*, 7412–7418.
(36) Harris, W. R.; Nesset-Tollefson, D.; Stenback, J. Z.; Mohamed-Hani, N. *J. Inorg. Biochem.* **1990**, *38*, 175–183.
(37) Foley, A. A.; Bates, G. W. *Biochim. Biophys. Acta* **1988**, *965*, 154–162.
(38) Rogers, T. B.; Feeney, R. E.; Meares, C. F. *J. Biol. Chem.* **1977**, *252*, 8108–8112.
(39) Dietrich, B.; Fyles, D. L.; Fyles, T. M.; Lehn, J. M. *Helv. Chim. Acta* **1979**, *62*, 2763–2787.
(40) Harris, W. R.; Bali, P. K. *Inorg. Chem.* **1988**, *27*, 2687–2691.

of the cleft binding locus by comparing the apoTf binding constants for the polyphosphonates with that of (aminomethyl)phosphonic acid.[34] Such an analysis predicts that the cleft locus would bind DTPP with an equilibrium constant of $10^{3.8}$. This is more than large enough to account for the value of $10^{2.6}$ for k'', which describes the approach to saturation for Tf–Fe_N.

The first-order pathway is most effective for ligands such as NTA and PP_i that are able to substitute for the synergistic bicarbonate anion. Thus, this pathway has been attributed[18,19] to the process

$$Fe\text{–}HCO_3\text{–}Tf + L \xrightarrow{k'''} Fe\text{–}L\text{–}Tf + HCO_3 \quad (12)$$

$$Fe\text{–}L\text{–}Tf \xrightarrow{fast} Fe\text{–}L + apoTf \quad (13)$$

A variety of ligands can substitute for the synergistic bicarbonate complex to form Fe–L–Tf.[5] These complexes are usually very unstable and are easily dissociated by the addition of bicarbonate or divalent anions such as HPO_4^{2-} and SO_4^{2-}.[19,37,38] However, preliminary studies indicate that neither DTPP nor DTPA is capable of forming carbonate-free Fe–L–Tf complexes.[41]

The addition of ligand up to 100 mM causes a substantial change in ionic strength. The precise impact of this change on the magnitude of the rate constants is difficult to evaluate. However, it is fairly certain that the hyperbolic-linear shape of the ligand dependence curves is not simply an artifact of changes in ionic strength. The ligand dependence for PP_i, the triphosphonate NTP, and the diphosphonate DPG has been measured in the presence of 200 mM concentrations of several anions.[40] Not only did this increase in the background ionic strength fail to dampen the linear component of the ligand dependence curves, but it actually increased the value of k''' compared to Hepes buffer alone for all three ligands. More recently, Marques et al.[25] have observed the same type of hyperbolic-linear behavior for iron removal by citrate even when the ionic strength was held constant at 1.3 M. In addition, if one considers the net ionic charge at pH 7.4 for the ligands PP_i, LICAMS, NTP, DPG, and DTPP, one observes that the magnitude of k''' does not correlate with the magnitude of the change in ionic strength associated with the ligand. Lastly, iron removal by the carboxylates NTA and DTPA follows simple first-order kinetics, and it seems very unlikely that this dominant reaction pathway would be completely inaccessible to the phosphonic acid analogs of these ligands.

Kinetic data on iron removal by PP_i that conform to the mathematical form of eq 3 have been observed in two other laboratories.[12,24,25] However, there is no agreement on a mechanism to explain these results. Bertini et al.[12] ascribe the unusual ligand dependence for PP_i to binding of the anionic ligand to allosteric modifier sites, so that there are two, parallel saturation pathways for iron removal: one for the native protein and one for the modified protein. This model is suggested by the observation that a variety of anions bind to differic transferrin and perturb the EPR signal.[42] It has been further proposed[12] that these allosteric sites could also be involved in the variety of anion effects observed during iron removal.[10,12,20,22-25,40] However, this complex kinetic model can be reduced to eq 3 only by assuming that the modified protein has a much higher k_{max} and that allosteric binding is so weak that only a small fraction of the protein is converted to the modified form, so that the concentration of the modified protein is linearly dependent on the free PP_i concentration. In addition, saturation with respect to perchlorate and chloride is not observed over the concentration range required by the anion-binding constants of diferric transferrin,[10,12,20,22,40] which rules out the binding sites detected by the EPR experiments of Folajtar and Chasteen.[42]

Marques et al.[24,25] also propose an allosteric model, in which both the native and modified protein react in pseudo-first-order processes with PP_i[24] or citrate[25] to form a common, reactive mixed-ligand intermediate. It is proposed that the modified protein reacts more slowly, so that the observed ligand dependence represents the transition from a more rapid first-order reaction with the native protein to a slower first-order reaction with the modified protein.

Cooperativity in Iron Removal by DTPP. The use of cobalt-labeled protein allows one to estimate values of k_{1C} and k_{1N}, the rate constants for iron removal from one site when the other site is still occupied by a metal ion. Iron removal from cobalt-labeled transferrins has been previously studied for both PP_i and NTP.[18,19] In addition, iron removal from diferric transferrin by these ligands has been followed by electrophoresis, which allows one to calculate k_{1C} and k_{1N} directly.[26] For both ligands the cobalt-labeled protein is a very good model for the diferric protein.

There is relatively little cooperativity between the two sites for iron removal by DTPP. Addition of cobalt to the C-terminal site results in a small but measurable increase in the rate of iron removal from the N-terminal site. This increase is due primarily to a 50% increase in k''' for Co_C–Tf–Fe_N compared to Tf–Fe_N. There is almost no change in k_{max} for the N-terminal site. Addition of cobalt to the N-terminal site has virtually no effect on iron removal from the C-terminal site.

No consistent pattern has emerged in the cooperativity for iron release by different ligands. Cobalt labeling accelerates removal of iron from the N-terminal site via the first-order pathway for both DTPP and NTA.[19] It also appears that $k_{1N} > k_{2N}$ for the saturation pathway for LICAMS.[14] However, for both PP_i and NTP, cobalt labeling enhances the rate of the saturation pathway at the C-terminal site. Since the cooperativity in general is rather small, there may be no dominant factor controlling cooperativity, and this type of scatter may continue to be observed as new ligands are studied.

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(41) Harris, W. R. Unpublished results.
(42) Folajtar, D. A.; Chasteen, N. D. *J. Am. Chem. Soc.* **1982**, *104*, 5775–5779.