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Effect of Ligand Structure on the Pathways for Iron Release from Human Serum Transferrin

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Rate constants for the removal of iron from N-terminal monoferric transferrin have been measured for a series of phosphate and phosphonocarboxylic acids in pH 7.4 0.1 M hepes buffer at 25 °C. The bidentate ligands pyrophosphate and phosphonoacetic acid (PAA) show a combination of saturation and first-order kinetics with respect to the ligand concentration. Similar results are observed following a single substitution at the 2-position of PAA to give 2-benzyl-PAA and phosphonosuccinic acid. In contrast, disubstitution at the 2-position to form 2,2-dibenzyl-PAA leads to a marked reduction in iron removal via the first-order pathway. Rate constants were also measured for tripolyphosphate and phosphonodiacetic acid, which are elongated versions of PP_i and PAA. In both cases, this elongation completely eliminates the first-order component for iron release while having relatively little impact on the saturation pathway. The sensitivity of the first-order component to the structure of the ligand strongly indicates that this pathway involves the binding of the ligand to a specific site on the protein and cannot be attributed to changes in the overall ionic strength of the solution as the ligand concentration increases. It is proposed that this structural sensitivity reflects steric restrictions on the ability of the incoming ligand to substitute for the synergistic carbonate anion to form a relatively unstable Fe–ligand–Tf ternary intermediate, which then dissociates to FeL and apoTf.

Introduction

To meet metabolic needs, the human body must transport 30−40 mg of iron per day through the bloodstream.[1] The bulk of this transport represents the recycling of iron from senescent red blood cells into new reticulocytes for incorporation into hemoglobin. Since free iron is quite toxic, this iron must be carried through blood by a suitable iron-sequestering agent. In humans, this agent is the serum protein transferrin. Serum transferrin is a member of a small class of iron-binding proteins that are distinguished by the requirement of a synergistic anion for effective iron binding.[2−4] In most cases, the physiological synergistic anion is carbonate, which acts as a bidentate ligand to the ferric ion.[4−6] A relatively new class of bacterial iron transport proteins appear to utilize phosphate rather than carbonate as a synergistic anion[7−9] and are often referred to as bacterial transferrins.

Certain genetic disorders such as β-thalassemia require regular blood transfusions.[10,11] Because each unit of whole

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(1) Baker, E.; Morgan, E. H. In *Iron Metabolism in Health & Disease*; Brock, J. H., Halliday, J. W., Pippard, M. J., Powell, L. W., Eds.; W. B. Suanders: London, 1994; pp 63−95.
(2) Aisen, P. In *Metal Ions in Biological Systems*; Sigel, A., Sigel, H., Eds.; Marcel Dekker: New York, 1998; pp 585−631.
(3) Baker, E. N.; Baker, H. M.; Kidd, R. D. *Biochem. Cell Biol.* **2002**, *80*, 27−34.
(4) Baker, E. N. *Adv. Inorg. Chem.* **1994**, *41*, 389−463.
(5) Baker, E. N. In *Perspectives on Bioinorganic Chemistry*; Hay, R. W., Dilworth, J. R., Nolan, K. B., Eds.; JAI Press: London, 1993; pp 161−205.
(6) MacGillivray, R. T. A.; Moore, S. A.; Chen, J.; Anderson, B. F.; Baker, H.; Luo, Y.; Bewley, M.; Smith, C. A.; Murphy, M. E. P.; Wang, Y.; Mason, A. B.; Woodworth, R. C.; Brayer, G. D.; Baker, E. N. *Biochemistry* **1998**, *37*, 7919−7928.
(7) Bruns, C. M.; Nowalk, A. J.; Arvai, A. S.; McTigue, M. A.; Vaughan, K. G.; Mietzner, T. A.; McRee, D. E. *Nat. Struct. Biol.* **1997**, *4*, 919−924.
(8) Guo, M.; Harvey, I. Y. W.; Coghill, L.; Campopiano, D.; Parkinson, J. A.; MacGillivray, R. T. A.; Harris, W. R.; Sadler, P. J. *J. Biol. Chem.* **2003**, *278*, 2490−2502.
(9) Dhungana, S.; Taboy, C. H.; Anderson, D. S.; Vaughan, K. G.; Aisen, P.; Mietzner, T. A.; Crumbliss, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3659−3664.
(10) Hershko, C.; Link, G.; Cabantchik, I. *Ann. N. Y. Acad. Sci.* **1998**, *850*, 191−201.
(11) Pippard, M. J. In *Iron Metabolism in Health & Disease*; Brock, J. H., Halliday, J. W., Pippard, M. J., Powell, L. W., Eds.; W. B. Saunders: London, 1994; pp 271−309.

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blood contains about 200 mg of iron,[11] long-term transfusion therapy leads to lethal iron overload unless accompanied by the administration of a suitable chelating agent to enhance iron excretion.[12,13] The currently approved drug for this purpose is desferrioxamine B (DFO), which is a trihydroxamate microbial iron transport agent.[14] Since transferrin is located in serum, it is a readily accessible target for therapeutic chelating agents. Many hexadentate siderophores, including DFO, as well as a large number of synthetic siderophore analogues, are stronger chelating agents than transferrin.[15−17] However, the rate of iron exchange from transferrin to low-molecular-mass ligands is typically quite slow.[16,18−23] In the case of DFO, the half-life for iron removal from transferrin is approximately 1 day.[24] Thus, we have been investigating the mechanism of iron release from transferrin to low-molecular-mass ligands to determine how to accelerate this reaction so that therapeutic ligands can effectively target transferrin-bound iron for decorporation.

Kinetic experiments on iron removal from transferrin are usually run with a large excess of the chelating agent, so that the reactions follow pseudo-first-order kinetics with respect to the concentration of ferric transferrin. Numerous studies from this and other labs have shown that the order of the iron release reaction with respect to the ligand concentration varies among different ligands. Several ligands follow simple saturation kinetics with respect to the free ligand,[16,18,20,21,25−29] while a few ligands follow simple first-order kinetics.[28,30,31] Many ligands show what appears to be a combination of saturation and first-order behavior.[19,32−35] For these ligands, the reaction appears to follow saturation kinetics at lower ligand concentration, but at high ligand concentrations the rate of iron release continues to increase linearly with increasing ligand concentration instead of reaching a plateau.

There is general agreement that the saturation component of iron release is related in some way to a rate-limiting conformational change in the protein.[3,18,26,27,36,37] There is also agreement that the rate of iron release can be affected by the binding of inorganic anions to an allosteric site on the protein.[18,19,37−41] In contrast, there is no universally accepted hypothesis to explain the appearance of a first-order component for iron release. We have previously suggested that the first-order component is associated with a distinct iron release pathway that is available only to ligands that are able to replace the synergistic carbonate anion to form a transient Fe−L−Tf (L, ligand) ternary complex.[20,28,30,31,42] Several carboxylic acids have been evaluated to determine whether they are capable of serving as a synergistic anion to form Fe−anion−Tf complexes under carbonate-free conditions.[43−45] These studies have established a set of structural criteria that a carboxylic acid must meet to function as a synergistic anion. In This Paper, we assess the degree to which these structural criteria correlate with the ability of ligands to remove iron via a first-order kinetic pathway.

Materials and Methods

All UV−vis spectra were recorded by use of a Hewlett-Packard 8452A diode array spectrophotometer. The instrument was equipped with a six-position multicell holder that was maintained at 25 °C by an external water circulating bath.

Diferric human serum transferrin was purchased from Sigma. N-terminal monoferric transferrin (FeNTf) was prepared from diferric transferrin and purified as previously reported.[20] Concentrations of the monoferric transferrin were based on the absorbance at 278 nm and an extinction coefficient of 103 000 $M^{-1}cm^{-1}$. The specific loading of iron into the N-terminal site was verified by polyacrylamide gel electrophoresis using published procedures.[46]

Ligands. Phosphonoacetic acid, pyrophosphoric acid, sodium phosphate, and pentasodium tripolyphosphate were purchased

(12) Hershko, C.; Konijn, A. M.; Link, G. *Br. J. Haematol.* **1998**, *101*, 399−406.
(13) Hoffbrand, A. V.; Wonke, B. *J. Intern. Med.* **1997**, *242*, 37−41.
(14) Matzanke, B. F.; Müller-Matsanke, G.; Raymond, K. N. In *Iron Carrier and Iron Proteins*; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; pp 1−121.
(15) Raymond, K. N.; Carrano, C. J. *Acc. Chem. Res.* **1979**, *12*, 183−190.
(16) Turcot, I.; Stintzi, A.; Xu, J.; Raymond, K. N. *J. Biol. Inorg. Chem.* **2000**, *5*, 634−641.
(17) Pecoraro, V. L.; Weitl, F. W.; Raymond, K. N. *J. Am. Chem. Soc.* **1981**, *103*, 5133−5140.
(18) Li, Y.; Harris, W. R. *Biochim. Biophys. Acta* **1998**, *1387*, 89−102.
(19) Marques, H. M.; Watson, D. L.; Egan, T. J. *Inorg. Chem.* **1991**, *30*, 3758−3762.
(20) Harris, W. R.; Wang, Z.; Brook, C.; Yang, B.; Islam, A. *Inorg. Chem.* **2003**, *42*, 5880−5889.
(21) He, Q.-Y.; Mason, A. B.; Woodworth, R. C.; Tam, B. M.; Wadsworth, T.; MacGillivray, R. T. A. *Biochemistry* **1997**, *36*, 5522−5528.
(22) He, Q.-Y.; Mason, A. B.; Woodworth, R. C. *Biochem. J.* **1997**, *328*, 439−445.
(23) Zak, O.; Aisen, P.; Crawley, J. B.; Joannou, C. L.; Patel, K. J.; Rafiq, M.; Evans, R. W. *Biochemistry* **1995**, *34*, 14428−14434.
(24) Rodgers, S. J.; Raymond, K. N. *J. Med. Chem.* **1983**, *26*, 439−442.
(25) Carrano, C. J.; Raymond, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 5401−5404.
(26) Cowart, R. E.; Kojima, N.; Bates, G. W. *J. Biol. Chem.* **1982**, *257*, 7560−7565.
(27) Kretchmar Nguyen, S. A.; Craig, A.; Raymond, K. N. *J. Am. Chem. Soc.* **1993**, *115*, 6758−6764.
(28) Li, Y.; Harris, W. R.; Maxwell, A.; MacGillivray, R. T. A.; Brown, T. *Biochemistry* **1998**, *37*, 14157−14166.
(29) Kretchmar, S. A.; Raymond, K. N. *J. Am. Chem. Soc.* **1986**, *108*, 6212−6218.
(30) Bali, P. K.; Harris, W. R.; Nesset-Tollefson, D. *Inorg. Chem.* **1991**, *30*, 502−508.
(31) Harris, W. R.; Bali, P. K.; Crowley, M. M. *Inorg. Chem.* **1992**, *31*, 2700−2705.
(32) Bali, P. K.; Harris, W. R. *J. Am. Chem. Soc.* **1989**, *111*, 4457−4461.
(33) Bertini, I.; Hirose, J.; Luchinat, C.; Messori, L.; Piccioli, M.; Scozzafava, A. *Inorg. Chem.* **1988**, *27*, 2405−2409.
(34) Egan, T. J.; Ross, D. C.; Purves, L. R.; Adams, P. A. *Inorg. Chem.* **1992**, *31*, 1994−1998.
(35) Marques, H. M.; Egan, T. J.; Pattrick, G. *S. Afr. J. Sci.* **1990**, *86*, 21−24.
(36) He, Q.-Y.; Mason, A. B. In *Molecular and Cellular Iron Transport*; Templeton, D. M., Ed.; Marcel Dekker: New York, 2002; pp 95−123.
(37) Muralidhara, B. K.; Hirose, M. *J. Biol. Chem.* **2000**, *275*, 12463−12469.
(38) Mizutani, K.; Muralidhara, B. K.; Yamashita, H.; Tabata, S.; Mikami, B.; Hirose, M. *J. Biol. Chem.* **2001**, *276*, 35940−35946.
(39) Kretchmar, S. A.; Raymond, K. N. *Inorg. Chem.* **1988**, *27*, 1436−1441.
(40) Mizutani, K.; Yamashita, H.; Mikami, B.; Hirose, M. *Biochemistry* **2000**, *39*, 3258−3265.
(41) He, Q.-Y.; Mason, A. B.; Tam, B.; MacGillivray, R. T. A.; Woodworth, R. C. *Biochemistry* **1999**, *38*, 9704−9711.
(42) Harris, W. R.; Bao, G. *Polyhedron* **1997**, *16*, 1069−1079.
(43) Harris, D. C.; Aisen, P. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed.; VCH: New York, 1989; pp 239−351.
(44) Schlabach, M. R.; Bates, G. W. *J. Biol. Chem.* **1975**, *250*, 2182−2188.
(45) Dubach, J.; Gaffney, B. J.; More, K.; Eaton, G. R.; Eaton, S. S. *Biophys. J.* **1991**, *59*, 1091−1100.
(46) Bali, P. K.; Harris, W. R. *Arch. Biochem. Biophys.* **1990**, *281*, 251−256.

commercially. The syntheses for 2-benzyl and 2,2-dibenzylphosphonoacetic acid have been reported previously.[47] Phosphonodiacetic acid was prepared as described in the literature.[48]

To synthesize phosphonosuccinic acid, NaH (60% dispersion, 2.0 g, 50 mmol) was added portionwise to a solution of trimethyl phosphonoacetate (9.1 g, 50 mmol) in dry CH_2Cl_2 (100 mL) at 0 °C under argon. After 5 min, methyl chloroacetate (5.42 g, 50 mmol) was added slowly via a syringe. After the mixture was stirred at room temperature overnight, H_2O (15 mL) was added slowly to quench the reaction. The mixture was extracted with CH_2Cl_2 (3 × 100 mL) and the combined extracts were dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by chromatography (SiO_2, hexane:ETOAc, 1:1) to yield tetramethyl phosphonosuccinate as a colorless oil (8.2 g, 65%). Tetramethyl phosphonosuccinate (4.0 g, 15.7 mmol) was dissolved in 6 N HCl (20 mL). The mixture was heated at reflux for 3 days. The solvent was lyopholyzed to yield phosphonosuccinic acid as a colorless oil (2.8 g, 90% yield). ^{1}H NMR (D_2O) δ 3.27 (1H, ddd, J_{HH} = 11.5, 3.8 Hz, J_{HP} = 15.2 Hz), 2.94 (1H, ddd, J_{HH} = 17.8, 11.4 Hz, J_{HP} = 7.2 Hz), 2.78 (1H, ddd, J_{HH} = 17.6, 3.8 Hz, J_{HP} = 7.9 Hz); ^{13}C NMR (D_2O) δ 175.9 (d, J_{CP} = 18.5 Hz), 174.2 (d, J_{CP} = 5.0 Hz), 43.4 (d, J_{CP} = 125 Hz), 32.2 (d, J_{CP} = 2.0 Hz); ^{31}P NMR: δ 16.4; HRMS (FAB, nba, MH+) calcd. for $C_4H_8O_7P$: 199.008. Found: 199.0008.

Stock solutions of the ligands were prepared by dissolving the solid ligand in 0.1 M hepes buffer and adjusting the pH to 7.4 by the addition of NaOH. Solutions of pyrophosphate were prepared from a concentrated solution of pyrophosphoric acid that was standardized by potentiometric titration with KOH.

Kinetic Methods. Samples were prepared directly in 1-cm quartz cuvettes by mixing approximately 40 μM FeNTf with a large excess (5−200 mM) of the appropriate ligand in 0.1 M hepes buffer at pH 7.4. Samples were maintained at 25 °C, and iron release was followed by monitoring the decrease in the absorbance of the ferric-transferrin charge transfer band at 465 nm. At high ligand concentrations, the iron removal reaction went to completion. At lower ligand concentrations, iron removal was incomplete, and the reaction went to an equilibrium distribution of iron between transferrin and the ligand. Both types of reactions were treated as previously described[18] to give a value of k_{obs}, the pseudo-first-order rate constant for iron removal at each ligand concentration.

Carbonate-Free Tf−Ligand−Fe^{3+} Ternary Complexes. The procedures for preparing carbonate-free transferrin−ligand−Fe^{3+} complexes were based on the methods published by Schlabach and Bates.[44] Two different methods were used. One method involved the displacement of iron from diferric transferrin under an inert atmosphere. A solution of diferric transferrin was adjusted to pH 3 by adding 8.0 M HCl dropwise, at which point a replacement synergistic anion was added. At pH 3, diferric transferrin dissociates to release Fe^{3+} and bicarbonate, which is protonated and forms an equilibrium mixture of H_2CO_3, CO_2(g), and water. The solution was degassed for 1 h by bubbling with N_2(g) to remove the CO_2. After removal of the CO_2, the pH of the protein solution was returned to 7.4 with NH_3(g), which was introduced into the sample by passing the N_2 gas over a solution of ammonium hydroxide prior to bubbling it into the sample. Gaseous ammonia was used as the base to prevent the contamination of the protein solution with the carbonate that accumulates in alkaline solutions. Once the pH reached 7.4, the solution was passed through a 0.2-μ syringe filter to remove any $Fe(OH)_3$ that may have precipitated. The filtered solution was transferred to a cuvette, and the spectrum was recorded.

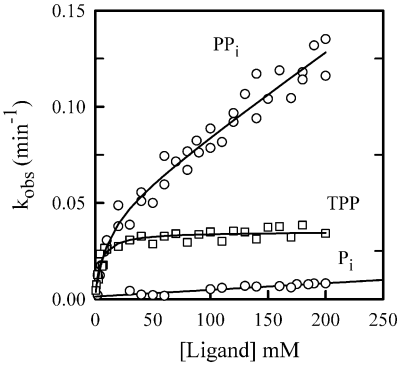


Figure 1. Pseudo-first-order rate constants for iron removal from N-terminal monoferric transferrin by pyrophosphate (PP_i), tripolyphosphate (TPP,), and inorganic phosphate (P_i) in 0.1 M hepes buffer at pH 7.4 and 25 °C.

In a second method, a solution of apoTf was adjusted to pH 4 and degassed to remove CO_2. A solution of Fe^{3+} and the substitute synergistic anion were added, after which the solution was adjusted to pH 7.4 with NH_3(g) and filtered as described above. This method allowed the testing of different ratios of Fe:Tf.

Spectrophotometric Competition. Effective binding constants for PAA and EPAA at pH 6.2 were determined by spectrophotometric competition with the well-characterized iron-chelating agent desferrioxamine B (DFO). Stock solutions of 3 mM DFO, 3 mM Fe-DFO, 0.5 M PAA, and 0.5 M EPAA were prepared in 0.1 M morpholine-*N*-ethanesulfonic acid (MES) at pH 6.2. A stock solution of 5 mM ferric ion was maintained at pH 1.0 in a HCl solution to prevent hydrolysis.

In one series of samples, the preformed Fe−DFO complex was mixed with either PAA or EPAA to give samples with 0.3 mM Fe−DFO and ligand concentrations ranging from 90 to 250 mM. The absorbance at 430 nm because of the Fe−DFO complex was monitored as a function of time. The absorbance decreased to a constant value within about 15 min. In a second series of experiments, ferric ion was allowed to equilibrate with EPAA or PAA for 1 h, after which free DFO was added. In these experiments, the absorbance at 430 nm rapidly increased after the addition of DFO before leveling off after approximately 15 min. For any given concentration of EPAA or PAA, the final absorbance was the same for the two types of competition experiments.

Results

Iron Release Kinetics. Iron removal from transferrin by PP_i has been studied several times, and it is well-established that the reaction shows a combination of saturation and first-order components.[28,32−35,46,49,50] Figure 1 shows a plot of k_{obs} versus [PP_i] for the removal of iron from N-terminal monoferric transferrin (FeNTf). These results are similar to those previously reported.[28,32,33,35]

The data for PP_i in Figure 1 can be described by eq 1.

$$k_{obs} = \frac{k_{max}[L]}{k_d + [L]} + k'[L] \qquad (1)$$

where k_{max} is the maximum rate constant for the saturation

(47) Harris, W. R.; Brook, C. E.; Spilling, C. D.; Ellepan, S.; Peng, W.; Xin, M.; Van Wyk, J. *J. Inorg. Biochem.* **2004**, *98*, 1824−1836.
(48) Bonadarenko, N. A.; Raytarskaya, M. V.; Rudoming, M. V.; Tsetov, E. N. *Phosphorous, Sulfur, Silicon* **1991**, *56*, 179−182.
(49) Harris, W. R.; Bali, P. K. *Inorg. Chem.* **1988**, *27*, 2687−2691.
(50) Marques, H. M.; Walton, T.; Egan, T. J. *J. Inorg. Biochem.* **1995**, *57*, 11−21.

Table 1. Rate Parameters for Iron Release from N-Terminal Monoferric Transferrin

ligand	$10^3 k_{max}$ (min^{-1})	k_d (mM)	k' (M^{-1} min^{-1})
PP_i[a]	47 ± 6	10 ± 4	0.42 ± 0.03
TPP[b]	35.1 ± 0.8	4.3 ± 0.6	0
P_i			0.035 ± 0.006[c]
PAA[d]	28 ± 2		0.18 ± 0.01
BPAA[d]	10.4 ± 1.7		0.321 ± 0.014
DBPAA[d]	4 ± 1		0.034 ± 0.011
PSA[d]	11 ± 1		0.105 ± 0.008
PDA[d]	8 ± 2		
MPAA[d]	26 ± 1		0.18 ± 0.01
EPAA[d]	20 ± 3		0.19 ± 0.02
AHA[e]	47 ± 2	130 ± 10	0
Deferiprone[f]	44.9 ± 0.7	12.5 ± 0.7	0
LICAMS[g]	39 ± 2		0

[a] Parameters based on least-squares fit to eq 1. [b] Parameters based on least-squares fit to eq 2. [c] For phosphate, this parameter is the slope of eq 3, which is defined as $k_{max}/k_d + k'$. [d] Parameters based on least-squares fit to eq 4. [e] Acetohydroxamic acid, data from ref 37. [f] 1,2-Dimethyl-3-hydroxypyridin-4-one, data from ref 18. [g] 1,5,10-Tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane, data from ref 39.

component, and k_d is a kinetic parameter that reflects the concentration of ligand required to reach half-saturation. The parameter k_d has the units of an equilibrium constant, and one might infer that this is the dissociation constant of a PP_i−Fe−Tf mixed-ligand intermediate. However, Cowart et al.[51] have shown that this intermediate does not accumulate during iron removal, even under saturating concentrations of PP_i. The precise definition of k_d in terms of fundamental rate constants is not well established. The parameter k' is a second-order rate constant that describes the component of iron release that is first-order in both ligand and FeNTf.

Figure 1 also shows kinetic data on iron release by tripolyphosphate (TPP). The observed first-order rate constant for iron release reaches a definite plateau above 50 mM TPP. This type of simple saturation kinetics can be described by the equation

$$k_{obs} = \frac{k_{max}[L]}{k_d + [L]} \quad (2)$$

where k_{max} and k_d are defined as described for eq 1. Table 1 lists the values of k_{max} and k_d for TPP obtained from the least-squares fit to eq 2. The data for TPP were also fit to eq 1, but there was no statistically significant improvement in the quality of the fit because of the addition of k' as a third adjustable parameter. In addition, the value of k' had a relative standard deviation of 75%. In contrast, eq 1 provides a significantly better fit than eq 2 for the PP_i data, with a well-defined value for k'.

Figure 1 also shows the k_{obs} values for iron release by P_i. The plot has a very shallow slope and no discernible curvature. Attempts to fit these data to either eq 1 or eq 2 resulted in large errors in the adjustable parameters. The lack of curvature and the zero *y*-intercept are consistent with either eq 1 or eq 2 if one assumes that $[P_i] \ll k_d$ over the experimental range of P_i concentrations. Under these conditions, eq 1 reduces to

$$k_{obs} = \left(\frac{k_{max}}{k_d} + k'\right)[L] \quad (3)$$

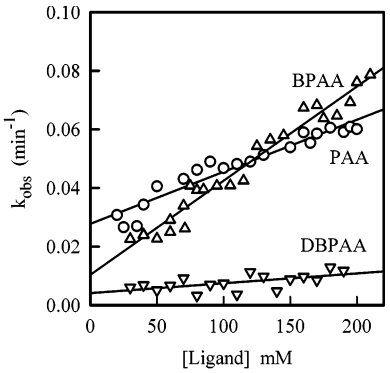
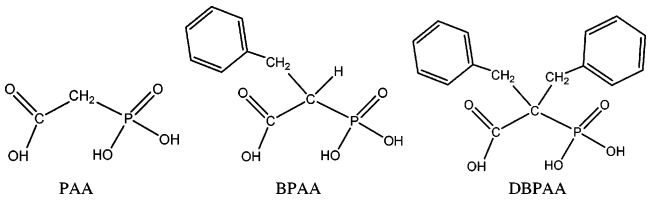


Figure 2. Rate constants for iron release from N-terminal monoferric transferrin by PAA (○), BPAA (△), and DBPAA (▽).

The data in Figure 1 cannot be used to calculate individual values for k_{max}, k_d, and k', and it is possible that $k' = 0$. Table 1 simply lists the slope of the plot of k_{obs} versus $[P_i]$. The data are included primarily to show that iron release by P_i is much slower than iron release by PP_i.

To investigate the steric effect of branching in the ligand structure, rate constants for iron removal from FeNTf have been measured for phosphonoacetic acid (PAA), 2-benzylphosphonoacetic acid (BPAA), and 2,2-dibenzylphosphonoacetic acid (DBPAA).



Plots of k_{obs} versus the concentration of ligand for PAA, BPAA, and DBPAA are shown in Figure 2.

The plots for all three ligands in Figure 2 are linear but with nonzero intercepts. One assumes that these plots would curve toward the origin at lower ligand concentrations. However, the iron-binding constants of phosphonoacetic acid and its derivatives are such that there is a lower limit of about 20 mM on the concentration of ligand that must be used to measure the rate constant for iron release. Below this limit, so little iron is removed from the protein that accurate rate constants cannot be calculated. These results have been interpreted in terms of eq 1. The linearity in the plots and the nonzero intercept suggests that $[L] \gg k_d$ over the available range of ligand concentrations. Under these conditions, eq 1 reduces to eq 4

$$k_{obs} = k_{max} + k'[L] \quad (4)$$

The values of k_{max} and k' determined from the intercepts and slopes, respectively, of the plots in Figure 2 are listed in Table 1.

(51) Cowart, R. E.; Swope, S.; Loh, T. T.; Chasteen, N. D.; Bates, G. W. *J. Biol. Chem.* **1986**, *261*, 4607−4614.

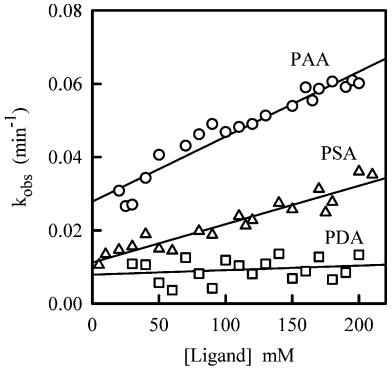


Figure 3. Rate constants for the removal of iron from N-terminal monoferric transferrin by PAA (○), PSA (△), and PDA (□) in 0.1 M hepes at pH 7.4 and 25 °C.

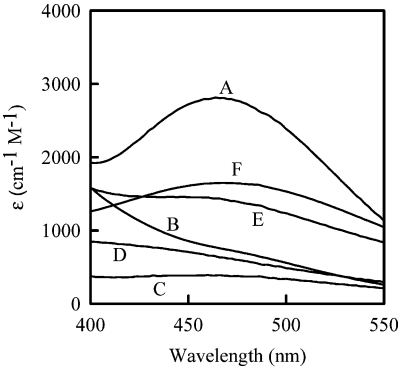
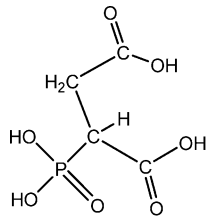
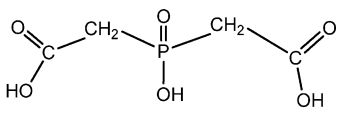


Figure 4. Visible spectra of diferric transferrin and samples of carbonate-free Fe−L−Tf complexes with a series of phosphonate ligands. Curve A, native diferric transferrin with carbonate as the synergistic anion. Curve B, native diferric transferrin after one acid purge cycle in the absence of any anions to substitute for the synergistic carbonate. The other spectra reflect the results of one acid purge cycle in the presence of 1 mM concentrations of PAA (curve C), EPAA (curve D), 3-PPA (curve E), and BPAA (curve F).

Two new ligands have been prepared by adding a carboxymethyl group to PAA. In one case, the carboxymethyl group was added to the central methylene group of PAA to give phosphonosuccinic acid (PSA), while in the other case the carboxymethyl group was added to the phosphonate group to give phosphonodiacetic acid (PDA).



PSA



PDA

The rates of iron release have been measured for both PSA and PDA, and the results are plotted in Figure 3.

The plot for PSA in Figure 3 extends to a lower limit of 5 mM PSA. Over this range, the plot of k_{obs} versus [PSA] is linear with a nonzero *y*-intercept. Below 5 mM PSA, the values of k_{obs} began to curve downward toward the origin, but these constants were not considered to be reliable because so little iron was removed. Therefore, these data have been removed, and the plot of k_{obs} versus [PSA] has been fit to eq 4 to obtain the values for k_{max} and k' listed in Table 1. No value of k_d can be calculated, although this interpretation requires that k_d must be less than 1−2 mM.

The plot of k_{obs} versus [PDA] extends to a lower limit of 35 mM, beyond which too little iron is removed to permit a calculation of k_{obs}. The rate constants for iron removal by PDA are best viewed as conforming to eq 2. Under conditions where [PDA] $\gg k_d$, the *y*-intercept of the plot is equal to k_{max}, which is listed in Table 1.

Carbonate-Free Fe-L-Tf Complexes. Several phosphonocarboxylate ligands were evaluated for their ability to replace the synergistic carbonate anion to form an Fe−L−Tf ternary complex. Since the native carbonate complex is so much more stable, these Fe−L−Tf ternary complexes must be formed and studied under carbonate-free conditions.

The familiar spectrum of the native $(Fe-CO_3)_2$−Tf diferric transferrin complex is shown as curve A in Figure 4. The peak at 465 nm is attributed to a charge-transfer transition between the ferric ion and the coordinated phenolate groups of the two tyrosine residues associated with the transferrin-binding site.[4] To prepare a reaction blank, this diferric transferrin solution was acidified to pH 3.0, purged with nitrogen to remove CO_2, and returned to pH 7.4 in the absence of any replacement synergistic anion. After syringe filtration, the solution gave spectrum B in Figure 4. The tail coming from the UV in this spectrum is attributed to charge-transfer bands of the unchelated, hydrolyzed iron remaining in the solution and possibly to iron nonspecifically bound to the protein. Although spectrum B has an absorbance at 465 nm, it lacks any distinct peak at this wavelength that would indicate binding to the tyrosines of the high-affinity transferrin metal-binding site.

The acidification and purge cycle was repeated in the presence of 1 mM PAA as a potential synergistic anion to give curve C in Figure 4. The presence of PAA reduces the tail from the UV region of the spectrum relative to spectrum B, presumably by chelating the free iron and reducing the degree of hydrolysis or nonspecific protein binding. A similar spectrum is obtained by simply adding ferric ion to excess PAA. There is no distinct peak near 465 nm that one could assign to an Fe^{3+}−phenolate charge-transfer band. Thus, PAA does not appear to promote the formation of a significant concentration of an Fe−PAA−Tf ternary complex.

The synthesis of a carbonate-free transferrin complex was repeated in the presence of ethylphosphonoacetic acid (EPAA), benzylphosphonoacetic acid (BPAA), and 3-phosphonopropionic acid (3-PPA). In the presence of EPAA, there is a slight increase in the visible absorbance compared to the PAA sample (curve D), although there is still no discernible peak near 465 nm. Both 3-PPA (curve E) and BPAA (curve F) give a significant absorbance near 475 nm, indicating the formation of ternary Fe−L−Tf species. On the basis of the relative absorbance intensities at 475 nm, the stabilities of the Fe−L−Tf ternary complexes appear to fall in the order PAA < EPAA < 3-PPA ∼ BPAA.

Binding Constants for Fe−Phosphonocarboxylate Complexes. During the kinetic studies on iron release as a

function of the ligand concentration, it appeared that PAA bound free ferric ion more strongly than either EPAA or BPAA. The amount of ligand required to remove 50% of the iron from FeNTf was about 25 mM for PAA as compared to approximately 50 mM for EPAA and BPAA. If one assumes that these ligands all form tris(bidentate) complexes, this difference in ligand concentration would correspond to a difference of about one log unit in the β_3 formation constants of the FeL_3 complexes.

To obtain a more accurate measure of the iron-binding affinity of PAA and EPAA, spectrophotometric competition studies were conducted between these ligands and the well-known ferric ion sequestering agent DFO. Stock solutions of free ligand and the ferric−DFO complex were mixed to give solutions with a final concentration of 0.3 mM Fe−DFO and from 90 to 250 mM of the phosphonate ligand. The transfer of iron from the Fe−DFO complex to the phosphonate ligand, which was measured by the decrease in the Fe−DFO charge-transfer band at 430 nm, reached equilibrium within approximately 15 min.

A second set of samples was prepared in which free DFO was added to the preformed iron−phosphonate complex. Equilibration between DFO and the phosphonates was complete within a few minutes, and there were no significant difference in the final spectra because of variations in the order of mixing.

The metal exchange equilibrium can be described as

$$\mathrm{Fe(PAA}n + \mathrm{DFO} \rightleftarrows \mathrm{Fe{-}DFO} + n\mathrm{PAA} \qquad (5)$$

This equilibrium is described by the exchange constant K_x, defined as

$$K_x = \frac{[\mathrm{Fe{-}DFO}][\mathrm{PAA}]^n}{[\mathrm{Fe(PAA})_n][\mathrm{DFO}]} \qquad (6)$$

Equation 6 was rearranged to the logarithmic form

$$\log \frac{[\mathrm{Fe{-}DFO}]}{[\mathrm{Fe(PAA})n][\mathrm{DFO}]} = \log K_x - n \log[\mathrm{PAA}] \qquad (7)$$

Competition experiments were run with a series of ligand concentrations, and the value of n was determined by plotting the left-hand side of eq 7 versus log [PAA]. The resulting plot was linear ($r^2 = 0.9957$), with $n = 2.9 \pm 0.1$ and log $K_x = 2.36 \pm 0.09$. This confirms the 3:1 ligand:metal stoichiometry of the ferric−PAA complex.

A similar set of competition experiments were conducted using EPAA. As with PAA, the plot of log[Fe(DFO)]/[Fe(EPAA)$_n$][DFO] versus log[EPAA] was linear, although the scatter was higher ($r^2 = 0.899$). The plot produced values of $n = 3.1 \pm 0.4$ and log $K_x = 3.2 \pm 0.3$. The higher scatter can be attributed to the fact that EPAA removed less iron from DFO and that the experimental errors become more difficult to control when less than 10% of the iron is removed.

The K_x values represent the ratio of the effective binding constants for Fe−DFO and the ferric phosphonate complexes at the experimental pH of 6.2. The effective binding constant for Fe−DFO was calculated to be $10^{22.08}$ on the basis of the literature values for the Fe−DFO stability constant and the DFO ligand protonation constants.[52] This leads to values of log β_3* of 19.7 for PAA and 18.9 for EPAA. These values are consistent with the previous estimate that the binding constant for PAA was about one log unit larger than that of EPAA. It must be stressed that these are effective binding constants for pH 6.2. They have not been corrected for the protonation of the phosphonate ligand, and the FeL_3 complexes formed at this pH may also be protonated. The results are presented here to verify that PAA forms a more stable ferric complex than EPAA.

Discussion

Saturation Pathway for Iron Release. Many ligands follow simple saturation kinetics for iron removal from transferrin. Given the high binding affinity of transferrin, it is not plausible that the iron exchange reaction could proceed at measurable rates from the spontaneous dissociation of free ferric ion, followed by chelation by the competing ligand.[53] Although it is virtually certain that the exchange reaction must proceed through some sort of ligand−Fe−protein mixed-ligand intermediate, no such intermediate accumulates to detectable concentrations during iron release by AHA or PP_i, even under saturating concentrations of the ligand.[26,51] These observations led to the Bates mechanism for iron removal, in which there is a rate-limiting conformational change in the protein.[26] Crystal structures have confirmed that removal of the iron does trigger a significant conformational change in the protein, which takes the form of a widening of the cleft in the protein in which the ferric ion binding site resides. The importance of a conformational change in the kinetics of iron release is broadly accepted.[3,18,26,27,36,37] One modification to the original mechanism is that there appears to be an allosteric anion-binding site that affects iron release.[33−35,39,42,54]

In the original Bates mechanism, the rate constant under saturating conditions, k_{max}, corresponds to the rate constant for the conformational change from the native "closed" form to a more reactive "open" conformation. If the incoming ligand were not involved in this step, then this rate constant would be characteristic of the protein, and k_{max} would be the same for all ligands. The data in Table 1 illustrate that this is not the case. Among the ligands listed, k_{max} varies by about a factor of 10.

There have been several suggestions that the binding of an anion from solution influences the protein conformational change that leads to iron release.[36−39] It is suggested that anionic ligands such as PAA can also bind to this allosteric anion-binding site and that this is responsible for the variation in k_{max} values seen in Table 1. Neutral ligands such as deferiprone and AHA have k_{max} values of about 0.04 min^{-1},[18]

(52) Evers, A.; Hancock, R. D.; Martell, A. E.; Motekaitis, R. J. *Inorg. Chem.* **1989**, *28*, 2189−2195.
(53) Pollack, S.; Aisen, P.; Lasky, F.; Vanderhoff, G. *Br. J. Haematol.* **1976**, *34*, 231−235.
(54) Egan, T. J.; Ross, D. C.; Purves, L. R. *S. Afr. J. Sci.* **1994**, *90*, 539−543.

which are larger than the k_{max} values for any of the PAA derivatives. Thus, the binding of phosphonocarboxylate ligands to the allosteric site appears to slow the rate of iron release from the N-terminal site.

The saturation pathway for iron removal by phosphate appears to reach saturation at a much higher ligand concentration compared with either PP_i or TPP. This is consistent with the previous observation that the value of k_d is inversely related to the iron-binding affinity of the ligand.[18] The sharp difference between P_i and both PP_i and TPP suggests that the transient ternary species associated with the Bates model for the saturation pathway involves bidentate coordination of the incoming ligand to the ferric ion. The similarity between the k_d values for PP_i and TPP suggests that there is little steric hindrance to the formation of this ternary species for the open Fe−Tf conformation.

First-Order Component for Iron Release. There is no generally accepted explanation for the appearance of a first-order component for iron release by certain ligands. Our working hypothesis is that this component is associated with a separate reaction pathway in which the incoming ligand avoids the rate-limiting conformational change in the protein by replacing the synergistic carbonate anion in the closed ferric transferrin conformation to form an Fe−L−Tf ternary intermediate.

Schlabach and Bates tested the formation of carbonate-free Fe−L−Tf complexes with a series of carboxylic acids as potential synergistic anions.[44] They established three criteria for a potential replacement anion for the synergistic carbonate in the Fe−CO_3−Tf ternary complex. (1) The anion must have a Lewis base functional group in addition to the carboxylate group. The Lewis base groups studied by Schlabach and Bates included carboxylate, alcohol, ketone, aldehyde, amine, and thiol moieties. (2) Increasing the separation between the Lewis base and the carboxylate group results in a rapid loss of function as a synergistic anion. For example, oxalate is a very effective synergistic anion, malonate is less effective, and succinate is a nonsynergistic anion. (3) Anions can be monosubstituted α to the carboxylate group, but disubstitution at this carbon destroys the ability to serve as a synergistic anion. For example, 2-hydroxypropanoic acid is an effective synergistic anion while 2-hydroxy-2-methylpropanoic acid is not.

Subsequent EPR studies showed that both the carboxylate group and the Lewis base of the synergistic anion were coordinated to the ferric ion.[45] More recently, several crystal structures of transferrin and lactoferrin have shown that oxalate as the synergistic anion coordinates as a 1,2-bidentate ligand to the ferric ion.[55−57] Given this bidentate model for the synergistic anion, one can now view the length restriction on the anion reported by Schlabach and Bates[44] as a chelate ring effect. The synergistic anions oxalate and malonate form five- and six-membered chelate rings, respectively. The nonsynergistic anion succinate would have to form a much less stable seven-membered chelate ring.

Since pyrophosphate and the phosphonoacetic acid derivatives also form six-membered chelate rings, we would expect them to serve as synergistic anions, and both of these ligands show a clear first-order component for iron release. According to the Bates criteria for a synergistic anion, one should be able to add one substituent to the 2-position of PAA and retain function as a synergistic anion, but disubstitution at the 2-position should produce a nonsynergistic anion. Our hypothesis would predict that disubstitution would abolish the first-order pathway for iron release. For PAA and the three monoalkyl PAA derivatives in Table 1, the average value of k' is 0.22 ± 0.07. The k' for the disubstituted ligand DBPAA is only 0.034 M^{-1} min^{-1}, well outside the range of the monalkyl derivatives.

As shown in Figure 1, pyrophosphate has a clear first-order component for iron release, with $k' = 0.427$, whereas $k' = 0$ for TPP. Both PP_i and TPP are capable of forming a six-membered chelate ring. Thus, the lack of a first-order component for TPP is attributed to a steric effect from the additional phosphate group of TPP. Similar results are observed when comparing the kinetic profiles for PSA and PDA. In PSA, the additional carboxylate group is attached to the central methylene carbon. This type of branching is allowed under the Bates model and, as expected, PSA shows a significant first-order term for iron removal, with $k' = 0.105 \pm 0.008$. With the ligand PDA, the carboxymethyl substituent is attached to the phosphonate group of PAA, mimicking the elongation of the ligand seen in the comparison of PP_i and TPP. As observed for the TPP, this elongation results in $k' = 0$ for PDA. This steric effect associated with elongation of the anion has not been previously reported.

Phosphonocarboxylates as Synergistic Anions. Since we have proposed that iron release through the first-order pathway proceeds via an Fe−L−Tf intermediate, we attempted to prepare these complexes under carbonate-free conditions. No ternary complex was observed with PAA, whereas the ternary complex with BPAA was easily observed. The formation of the ternary transferrin complex in the presence of an excess of a chelating synergistic anion can be represented by the equilibrium

$$FeL_3 + \text{apoTf} \rightleftarrows \text{Fe−L−Tf} + 2L \qquad (8)$$

There is little reason to believe that bulky substituents on PAA directly stabilize the Fe−L−Tf complex. Instead, they appear to destabilize the competing FeL_3 species, as indicated by the effective binding constants of PAA and EPAA. Bulky substituents appear to shift the equilibrium in eq 8 to the right, leading to the formation of detectable concentrations of the Fe−L−Tf ternary complex.

The observation of a ternary complex with 3-PPA is consistent with this competition model. The larger seven-membered chelate ring size for this ligand will drastically reduce the stability of the FeL_3 complex on the left side of eq 8, shifting the equilibrium in favor of the Fe−L−Tf

(55) Shongwe, M. S.; Smith, C. A.; Ainscough, E. W.; Baker, H. M.; Brodie, A. M.; Baker, E. N. *Biochemistry* **1992**, *31*, 4451−4458.
(56) Halbrooks, P. J.; Mason, A. B.; Adams, T. E.; Briggs, S. K.; Everse, S. J. *J. Mol. Biol.* **2004**, *339*, 217−226.
(57) Baker, H. M.; Anderson, B. F.; Brodie, A. M.; Shongwe, M. S.; Smith, C. A.; Baker, E. N. *Biochemistry* **1996**, *35*, 9007−9013.

species. It is not clear why this longer phosphonocarboxylate can form the Fe−L−Tf complex when no such complex forms with the analogous dicarboxylate, succinic acid. One can speculate that a more favorable electrostatic interaction between the phosphonate group and the positively charged side chain of Arg 124 may permit the formation of a stable ternary species in which only the carboxylate group of 3-PPA is coordinated to the ferric ion.

The tendency to form an Fe−L−Tf ternary complex depends on a rather delicate balance between the relative stability of the ternary complex and the low-molecular-mass FeL_3 chelate. In the case of PAA, the equilibrium in eq 8 appears to be shifted too far to the left to allow for the formation of detectable concentrations of the Fe−PAA−Tf ternary complex. This does not preclude the formation of this ternary complex as a short-lived kinetic intermediate during iron removal by PAA.

This is certainly not the first proposal that external anionic ligands can exchange with the synergistic carbonate anion. Aisen et al.[58] measured the rate of exchange between the synergistic carbonate and free carbonate and showed that both citrate and NTA accelerated the rate of carbonate exchange. They proposed that the acceleration in carbonate exchange occurred by the mechanism

$$\mathrm{Fe-H^{*}CO_3^-} - \mathrm{Tf} + \mathrm{A^-} \rightarrow \mathrm{Fe-A^-} - \mathrm{Tf} + \mathrm{H^{*}CO_3^-} \quad (9)$$

$$\mathrm{Fe-A^-} - \mathrm{Tf} + \mathrm{HCO_3^-} \rightarrow \mathrm{Fe-HCO_3^-} - \mathrm{Tf} + \mathrm{A^-} \quad (10)$$

Pollack et al.[59] proposed that the ability of small ligands to accelerate the transfer of iron from transferrin to DFO involved this same carbonate substitution reaction to give a labile intermediate. Morgan[60] also considered eqs 9 and 10 as a mechanism by which small ligands could accelerate carbonate exchange, although they also noted that the ligand could remove both the iron and the carbonate, rather than simply substituting for the synergistic carbonate.

Ionic Strength Effects. The comparisons of PP_i versus TPP, PSA versus PDA, and BPAA versus DBPAA were intended to probe steric effects on the mechanism of iron removal. However, they serve to address another issue. The kinetics experiments were run in a 100 mM hepes buffer. Given the relatively high ligand concentrations used and the charges on the ligands, the ionic strength of the solutions varies as one goes from low to high ligand concentrations. This raises the possibility that the apparent first-order component for iron release could be an artifact associated with changing ionic strength. In this model, the system initially reaches saturation as described by the parameters k_d and k_{max}. As more ligand is added, the increase in the ionic strength gradually increases the numerical value of k_{max}. Thus, the values of k_{obs} continue to increase with increasing ligand concentration, even though the reaction is following a simple saturation pathway.

The new data reported here provide an excellent basis to test this ionic strength hypothesis. The phosphonoacetate ligands PAA, BPAA, and DBPAA all have a net charge of approximately −2 at pH 7.4, and thus they all make essentially identical contributions to the ionic strength. The ligands PSA and PDA both exist as trianions at pH 7.4 and will make a larger contribution to the ionic strength. If the apparent first-order component were due to changes in the ionic strength, then one should observe the same value of k' for PAA, BPAA, and DBPAA, as well as a larger k' for both PDA and PSA. Figures 2 and 3 show clearly that this is not the case.

At neutral pH, PP_i caries an average charge of approximately −3, while TPP has an even more negative charge of approximately −4. Despite its higher charge, TPP shows no detectable first-order component for iron removal. The ligand comparisons shown in Figures 1−3 clearly show that one cannot attribute the appearance of a first-order component for iron removal to increases in ionic strength. The first-order component depends much more on the structure of the ligand rather than on its charge. This is consistent with the interaction of the ligand with a specific binding site.

Other Kinetic Models. Egan and Marques have proposed a different kinetic model consisting of two first-order pathways for iron removal linked by an anion-binding equilibrium.[19,34,35,54] This mechanism would also be consistent with specific structural requirements for ligands that show a first-order component at high ligand concentrations. As we have noted previously,[18,20] the Egan−Marques mechanism leads to a relationship between k_{obs} and the ligand concentration that is an algebraic rearrangement of eq 1. Thus, one cannot distinguish between the two mechanisms on the basis of the quality of the least-squares fits of k_{obs} versus [ligand] for ligands that follow complex kinetics. The primary difference is how the two mechanisms account for simple saturation kinetics. The proposed mechanism associates simple saturation kinetics with ligands whose structure prevents them from replacing the synergistic anion, leading to a k' of zero. This includes neutral ligands such as acetohydroxamic acid and deferiprone,[18] as well as sterically hindered anionic ligands such as TPP and DBPAA. To obtain a plateau at high ligand concentrations in the Marques−Egan mechanism, the ligand must bind at an anion-binding site with mM k_d values, and this binding must result in a rate constant of zero for iron removal from the ligand−Fe−protein species. This is highly unlikely, particularly for neutral ligands such as deferiprone.

Hirose and co-workers have also proposed kinetic models for both simple saturation and complex kinetics. The mechanism for simple saturation involves a preequilibrium formation of an Fe−L−Tf ternary complex.[38] The model for complex kinetics involves iron release from two different conformational states of transferrin.[37] We have tested these models on our data for iron release by both TPP and PP_i. While we can obtain least-squares fits of k_{obs} versus [L] that have the same standard error in k_{obs} as those associated with

(58) Aisen, P.; Leibman, A.; Pinkowitz, R. A.; Pollack, S. *Biochemistry* **1973**, *12*, 3679−3684.
(59) Pollack, S.; Vanderhoff, G.; Lasky, F. *Biochim. Biophys. Acta* **1977**, 481−487.
(60) Morgan, E. H. *Biochim. Biophys. Acta* **1977**, *499*, 169−177.

eqs 1 and 2, the more complex Hirose models lead to very large errors for some of the adjustable parameters.

Bertini et al.[33] also reported complex kinetics for iron removal from transferrin by PP_i. They proposed a mechanism that consists of two saturation pathways linked by an anion-binding preequilibrium. This mechanism gives a very complex function relating k_{obs} to the ligand concentration, but these authors note that this function reduces to eq 1 with only a few assumptions. The primary requirement is that the anion-binding preequilibrium must be far from saturation to account for the linear increase in k_{obs}. The new data reported here would be consistent with this mechanism, and the concept of an allosteric anion-binding site is mentioned above in the context of variations in k_{max}. However, the relationship between the structural criteria for a synergistic anion and the observation of the first-order pathway for iron release makes it more likely that the first-order component is due to binding of the ligand as a synergistic anion.

Conclusions

For a series of polyphosphate and phosphonocarboxylate ligands, the relative importance of the saturation and first-order components for iron release from N-terminal monoferric transferrin varies sharply with changes in the structure of the ligand but shows no correlation with the contribution of the ligand to the ionic strength of the solution. The sensitivity of the reaction mechanism to structure, rather than ionic strength, suggests that the first-order component for iron release involves an interaction between the ligand and a specific binding site on the protein. It is proposed that this ligand−protein interaction is the replacement of the synergistic carbonate anion by the incoming ligand to give a transient intermediate that quickly dissociates to apotransferrin and the ferric−ligand complex.

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