Transferrin: The Role of Conformational Changes in Iron Removal by Chelators

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Abstract: Serum transferrin and the other proteins of the transferrin family bind two high-spin Fe^{3+} ions with very high affinity. Strong iron sequestering agents, such as some of the siderophores, are able to remove iron from transferrin, although rates of removal vary dramatically. The thermodynamic and kinetic competition of competing ligands for transferrin iron are strongly connected to issues of iron in medicine. This study explores the release of iron to three prototypical types of ligands: catecholates, hydroxypyridonates, and pyrophosphate. A mechanistic model, consistent with the existing data on transferrin iron removal by chelators, is discussed in light of the detailed structural information that is now available from several crystal structures of the apo- and metalloproteins. In contrast to the catecholates, which show a significant difference in rate of iron removal from the two binding sites of transferrin, the natural product L-mimosine shows monophasic kinetics. Although all anions promote iron removal by binding to the protein and aiding a requisite conformational change (such that the first-order iron removal rate constant for *all* ligands goes to zero as the ionic strength goes to zero), pyrophosphate acts both as an ion mediator and as a ferric ion chelator. The effects of ligand size and charge on iron removal rates from transferrin have been investigated using Tiron (1,5-disulfo-2,3-dihydroxybenzene) and 2,3-dihydroxybenzoate. While the details may differ, all iron removal from transferrin by chelators requires: (1) rapid equilibrium binding of anions, (2) a conformational change of the structurally characterized "closed" form of the protein to the "open" form in which the metal site is exposed, and (3) binding to the metal ion by the attacking ligand and removal of the synergistic carbonate at the iron-binding site. Differences between ligands such as catecholates and hydroxamates or hydroxypyridonates in these steps lead to differences of up to 2 or 3 orders of magnitude in relative rates of iron removal.

Introduction

Serum transferrin is the iron transport agent of mammals.[1-3] Its function and structure are increasingly well understood, particularly because of the relatively recent protein crystal structure information.[2,4-6] Transferrin not only acts as a transport agent but also functions as an iron buffer, maintaining free ferric ion concentrations in the body at a very low but constant value.[3,7] This role of transferrin and our understanding of the mechanisms of iron binding and release by the protein are important in four areas of medical science. First, iron storage and transport are critical to understanding the molecular basis of anemias.[8,9] Iron uptake by the gut involves the eventual complexation by transferrin, but the details of the transport steps between iron in foodstuff and in serum are still being elucidated.[1,3,10,11] Second, iron overload occurs in several disorders, the most common in North America being due to long-term transfusion therapy of β-thalassemia (Cooley's anemia).[10-14] Iron is only regulated by uptake; there is no mechanism for spontaneous removal of iron. Chelation therapy of iron overload has long used desferrioxamine B (Desferal). There is a continuing search for new sequestering agents that would have improved properties, particularly oral activity.[15-17] One issue thus raised is the thermodynamic and kinetic ability of such sequestering agents to remove iron from transferrin.[3,7,17] Third, the release of iron, the reductive generation of Fe^{2+}, and the subsequent free radical generation from reaction with O_2 (Fenton chemistry) are now thought to be the major cause of tissue damage following myocardial infarction.[18,19] The use of iron chelating agents may provide a way to block such damage by keeping free ferric ion at low levels—a function of transferrin in circulating serum that is disrupted by the heart attack.

Finally, the fourth area of medical relevance for transferrin iron binding and release is related to bacterial infection. The role of iron in the pathogenicity of bacterial infections is now well established.[20-22] Iron availability to the invading bacterium is known to be directly connected to the virulence of infections that cause infantile enteritis,[23,24] leprosy,[25] cholera,[26] and tuberculosis,[27]

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(1) de Jong, G.; van Dijk, J. P.; van Eijk, H. G. *Clin. Chim. Acta* **1990**, *190*, 1–46.
(2) Baker, E. N.; Anderson, B. F.; Baker, H. M. *Int. J. Biol. Macromol.* **1991**, *13*, 122–129.
(3) Harris, D. C.; Aisen, P. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; pp 239–352.
(4) Baker, E. N.; Rumball, S. V.; Anderson, B. F. *Trends Biochem. Sci.* **1987**, *12*, 350–353.
(5) Zuccola, H. J. Personal communication (Ph.D. thesis research, Georgia Institute of Technology).
(6) Bailey, S.; Evans, R.; Garratt, R. C.; Gorinsky, B.; Hasnain, S.; Horsburgh, C.; Jhoti, H.; Lindley, P. F.; Mydin, A.; Sarra, R.; Watson, J. L. *Biochemistry* **1988**, *27*, 5804. Sarra, R.; Garratt, R. C.; Gorinsky, B.; Jhoti, H.; Lindley, P. F. *Acta Crystallogr.* **1990**, *B46*, 763.
(7) Kretchmar, S. A.; Reyes, Z. E.; Raymond, K. N. *Biochim. Biophys. Acta* **1988**, *956*, 85–94.
(8) Crosby, W. H. *Arch. Intern. Med.* **1984**, *144*, 471.
(9) Jandl, J. H. In *Blood. Textbook of Hematology*; Little, Brown Publishers: Boston. 1987; pp 192–198.
(10) Bali, P. K.; Zak, O.; Aisen, P. *Biochemistry* **1991**, *30*, 324–328.
(11) Thorstensen, K.; Romslo, I. *Biochem. J.* **1990**, *271*, 1–10.
(12) Kirking, M. H. *Clin. Pharm.* **1991**, *10*, 775–783.
(13) Pippard, M. J. *Acta Haematol.* **1987**, *78*, 206–211.
(14) Borgna-Pignatti, C.; Castriota-Scanderbeg, A. *Haematologica* **1991**, *76*, 409–413.
(15) Martell, A. E.; Motekaitis, R. J.; Murase, I.; Sala, L. F.; Stoldt, R.; Ng, C. Y. *Inorg. Chim. Acta* **1987**, *138*, 215–230.
(16) Olivieri, N. F.; Koren, G.; Louis, P. S.; Freedman, M. H.; McClelland, R. A.; Templeton, D. M. *Semin. Hematol.* **1990**, *27*, 101–104.
(17) Kretchmar, S. A.; Raymond, K. N. *J. Am. Chem. Soc.* **1986**, *108*, 6212–6218.
(18) Wolfe, L.; Olivieri, N.; Sallan, D.; Colan, S.; Rose, V.; Propper, R.; Freedman, M. H.; Nathan, D. G. *New Engl. J. Med.* **1985**, *312*, 1600–1603.
(19) Rolli, R.; Patel, B. S.; Zhu, W.; O'Neill, P. G.; Hartley, C. G.; Charlat, M. L.; Roberts, R. *Am. J. Physiol.* **1987**, *253*, 1372–1380.
(20) Otto, B. R.; Verweij-van Vught, A. M. J. J.; MacLaren, D. M. *Crit. Rev. Microbiol.* **1992**, *18*, 217–233.
(21) Bullen, J. J.; Ward, C. G.; Rogers, H. J. *Eur. J. Clin. Microbiol. Infect. Dis.* **1991**, *10*, 613–617.
(22) Weinberg, E. D. *Drug Metab. Rev.* **1990**, *22*, 531–579.

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as major examples. Increasingly, pathogens that are immune to the usual antibiotics are found to cause disease. This is particularly acute in the re-emergence as a public health problem of tuberculosis, especially among individuals with compromised immune systems.[28] Several siderophores[29,30] of pathogenic microorganisms are capable of removing iron from serum transferrin. The amonabactins,[31] which contain two catechol groups as ligands, are relevant examples of pathogenic bacterial siderophores that are capable of removing iron from transferrin *in vivo*. Hence the mechanism(s) by which iron is released from transferrin to such ligands is of medical, as well as general biochemical, significance.

Early studies by Carrano and Raymond of iron removal from diferric transferrin (Fe_2Tf) by tris-catechol ligand analogs of enterobactin found that at low ligand concentrations the rate was first order in both ferric protein and the ligand. Saturation behavior with respect to the ligand concentration was observed, with the rate eventually becoming independent of ligand concentration as it increased. It was proposed that this was due to formation of a complex between the iron protein and ligand, which gave a mechansim essentially the same as Michaelis–Menton enzyme kinetics.[32] Cowart, Kojima, and Bates (CKB) subsequently found evidence for an intermediate in iron uptake by transferrin from acetohydroxamic acid and interpreted their results as due to a conformation change of the protein.[33] There had long been other evidence for a conformational change as the protein goes from metal-free to metal-bound form. We accordingly subscribed in large part to the CKB mechanism.[34] However, that mechanism also predicts that the limiting rate for iron removal from ferric transferrin to ligands showing saturation behavior should be a constant, characteristic of the rate of conformational change, and hence *independent* of the particular ligand. In general this is not the case.[17] However, recent protein structural information and other kinetic studies provide an explanation for these dichotomies and lead to a mechanism that is essentially a hybrid of those originally proposed by Carrano and Raymond and by Cowart, Kojima, and Bates.

Transferrin is a bilobal protein of molecular weight 78 000 that is apparently the result of the fusion of two units of an ancestral protein.[3] Each of the two lobes contain one metal-binding site that has a very high affinity for high-spin Fe^{3+}. Single crystal structures of rabbit diferric[6] and human monoferric[5] transferrins and lactoferrin[2,4] establish that the iron-binding sites of these closely related proteins are essentially identical, composed of a bidentate carbonate, two phenolate groups (tyrosine), one nitrogen (histidine), and a carboxylate oxygen (aspartate). The stable form of metal-free apotransferrin has an "open" conformation in which the iron-binding site is in an open cleft near the protein surface and is accessible to the surrounding solution. In contrast, the stable form of the iron complex has the cleft closed so that the metal-binding site is buried under the surface of the protein, thus making the metal inaccessible to competing ligands. This then fits the CKB proposal of a required conformation change for iron removal by strong sequestering agents.[33] However, there is also evidence that the protein has anion-binding sites and that anion binding triggers the conformational change.[35-37]

Carbonate binding to transferrin is a prerequisite to iron binding and, in reverse, to iron release.[3] Carbonate and a few other ligands that can take its place have been called "synergistic anions". Certain other compounds (mediators) accelerate the rate of iron removal from transferrin by chelators. These need not be synergistic anions nor effective chelators.[38-40] Egyed found that three "mediator" anions, ATP, ADP, and pyrophosphate (PPi), so acted to promote the removal of iron. Although no rates were calculated, it appears that a 200-fold excess of PPi to diferric transferrin accelerates the half-life of bicarbonate exchange from 20 days to 30 min.[39] Pollack *et al.* showed that PPi has unusually high activity in accelerating iron transfer to the siderophore, desferrioxamine B, and that the ability of a compound to mediate does not correlate with the effectiveness of the anion at removing iron from transferrin.[38] For example, ethylenediaminetetraacetate (EDTA) is twice as fast at iron removal from transferrin as PPi, but it is much slower as a mediator. The rates of mediation were found to be dependent on the concentration of mediator, and combinations of mediators were not additive.

Morgan found biphasic kinetic behavior for iron removal from transferrin by desferrioxamine B when mediated by several phosphate compounds.[40] A linear relationship between proton concentration and rate of iron release was obtained, and it was found that a maximum rate constant was reached as the concentration of mediator was increased for a given pH. However, these experiments were carried out under conditions of high ionic strength and low pH, both of which are known to affect the kinetics.[41-43] Konopka *et al.* also reported a hyperbolic relationship between the observed rate constant and concentration of mediator when PPi was used to mediate the removal of iron from diferric transferrin by the siderophore aerobactin.[44] Recently elegant studies by Harris *et al.* have used substitution of kinetically inert Co(III) for Fe(III) to explore the cooperativity between the two iron sites for Fe(III) removal by aminophosphonate and aminocarboxylate ligands.[45] The equilibrium binding of these anions to Tf was also measured by these authors.

In the structure of apolactoferrin, one lobe is found in the open conformation and the other in the closed, and it has been suggested that anion binding helps to stabilize the former.[46] We have shown that the presence of ions is an absolute prerequisite for iron removal from transferrin: the observed rate constant goes to zero as the ionic strength of the solution goes to zero.[43] We proposed a mechanism in which anion binding to ferric transferrin promotes conversion of the closed form of the protein to the open

(23) Lee, L. A.; Gerber, A. R.; Lonsway, D. R.; Smith, J. D.; Carter, G. P.; Puhr, N. D.; Paneish, C. M.; Sikes, R. K.; Fenton, R. J.; Tauxe, R. V. *New Engl. J. Med.* **1990**, *322*, 984–987.
(24) Mofenson, H. C.; Carraccio, T. R.; Sharieff, N. *New Engl. J. Med.* **1987**, *316*, 1092–1093.
(25) Ratledge, C. In *Iron Transport in Microbes, Plants and Animals*; VCH Publishers: New York, 1987; pp 207–221.
(26) Stoebner, J. A.; Payne, S. M. *Infect. Immun.* **1988**, *56*, 2891.
(27) Snider, D. E.; Roper, W. L. *New Engl. J. Med.* **1992**, *326*, 703–705.
(28) Bloom, B. R. *Nature* **1992**, *358*, 538–539.
(29) Matzanke, B. F.; Müller-Matzanke, G.; Raymond, K. N. In *Physical Bioinorganic Chemistry Series, Iron Carriers and Iron Proteins*; VCH Publishers: New York, 1989; pp 1–121.
(30) Neilands, J. B.; Konopka, K.; Schwyn, M. C.; Francis, R. T.; Paw, B. H.; Bagg, A. In *Iron Transport in Microbes, Plants and Animals*; Winkelmann, F., van der Helm, D., Neilands, J. B., Eds.; VCH Publishers: New York, 1987; pp 3–33.
(31) Barghouthi, S.; Young, R.; Olson, M. O. J.; Arceneaux, J. E. L.; Clem, L. W.; Byers, R. *J. Bacteriol.* **1989**, *171*, 1811–1816.
(32) Carrano, C. J.; Raymond, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 5401–5404.
(33) Cowart, R. E.; Kojima, N.; Bates, G. W. *J. Biol. Chem.* **1982**, *257*, 7560–7565.
(34) Raymond, K. N.; Chung, T. D.; Pecoraro, V. L.; Carrano, C. J. In *The Biochemistry and Physiology of Iron*; Saltman, P. S., Hegenauer, J., Eds.; Elsevier: New York, 1982; pp 649–661.
(35) Harrington, J. P. *Int. J. Biochem.* **1992**, *24*, 275–280.
(36) Oe, H.; Takahashi, N.; Doi, E.; Hirose, M. *J. Biochem.* **1989**, *106*, 858–863.
(37) Harris, W. R.; Tollefson, D. N.; Stenback, J. Z.; Mohamed-Hani, N. *J. Inorg. Biochem.* **1990**, *38*, 175–183.
(38) Pollack, S.; Vanderhoff, G.; Lasky, F. *Biochim. Biophys. Acta* **1977**, *497*, 481–484.
(39) Egyed, A. *Biochim. Biophys. Acta* **1975**, *411*, 349–356.
(40) Morgan, E. H. *Biochim. Biophys. Acta* **1979**, *580*, 312–326.
(41) Baldwin, D.; de Sousa, D. M. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1101–1107.
(42) Baldwin, D.; de Sousa, D. M.; Von Wandruszka, R. M. A. *Biochim. Biophys. Acta* **1982**, *719*, 140–146.
(43) Kretchmar, S. A.; Raymond, K. N. *Inorg. Chem.* **1988**, *27*, 1436–1441.
(44) Konopka, K.; Bindereif, A.; Neilands, J. B. *Biochemistry* **1982**, *21*, 6503–6508.
(45) Harris, W. R.; Bali, P. K.; Crowley, M. M. *Inorg. Chem.* **1992**, *31*, 2700–2705.
(46) Anderson, B. F.; Baker, H. M.; Norris, G. E.; Rumball, S. V.; Baker, E. N. *Nature* **1990**, *344*, 784–787.

form—which is then vulnerable to attack by strong sequestering agents. This has subsequently been corroborated Egan *et al.*, who in their investigation of pyrophosphate as a ligand proposed essentially the same mechanism.[47]

It is now becoming clear that the role of "mediator anions" is due to the ability of these ions to promote the protein conformational change. Some strong sequestering agents, such as enterobactin and its analogs, are apparently themselves synergistic anions. Others, such as desferrioxamine B, are not synergistic anions; they are thermodynamically capable of removing iron from transferrin but do not bind to the protein and trigger the conformational change. It is the purpose of this paper to explore this difference in behavior in some prototypical ligand systems and to develop more fully the connection between the structural information about the protein and the kinetic behavior of its iron release to chelators. The kinetic behavior of iron removal from diferric transferrin by the catecholate and hydroxamate chelators, 3,4-LICAMS and desferrioxamine B, have been investigated in the presence of the mediator, PPi. Similar kinetic studies with the monoferric transferrins have been conducted to determine the effects of PPi on the individual sites of transferrin. The effectiveness of catechol as a mediator is not known, and such capabilities might be a factor in contributing to the fast kinetic rates of iron removal from ferric transferrins by catecholate ligands.[32,34,48] Hence 3,4-dihydroxybenzoic acid has been studied because it is a simple monomeric catecholate that is a component of many siderophores and is thermodynamically incapable of removing iron from transferrin by itself in low concentrations, yet is able to accelerate rates of iron removal from the ferric transferrins by desferrioxamine B at these concentrations.

Experimental Section

Distilled deionized water was used at all times unless otherwise indicated. Glassware was washed with phosphate-free RBS-pf (Pierce Chemical Co.) and rinsed thoroughly with tap distilled water. Dialysis tubing (Bethesda Research Laboratories) was boiled in water, stored in a solution containing 0.1% EDTA and 0.02% NaN_3, and washed several times with water prior to use. All solutions were adjusted to pH 7.40 at 25 °C unless otherwise indicated. Urea–polyacrylamide gel electrophoresis was carried out using standard procedures.[49,50]

Diferric Transferrin. Human serum apotransferrin (Sigma Chemical Corp. or Calbiochem-Behring) was dissolved in water (20 mg/mL) and dialyzed at 4 °C for a minimum of 9 h against several changes of water and then against a solution containing 0.050 M *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer and 0.020 M $NaHCO_3$ to remove chelate contaminants.[51,52] The protein was saturated to 100% of total sites by addition of freshly prepared 2.00 mM ferric nitrilotriacetate [$Fe(NTA)_2$].[17] Since some time-dependent hydrolysis of $Fe(NTA)_2$ occurs, the $Fe(NTA)_2$ solutions were prepared just prior to use in the following manner: NTA (Eastman Chemical Corp.) was dissolved in water at pH 4.0, ferric chloride at pH 1.0 was added, and the pH was raised to 4.0–4.5 with dilute NaOH.

After 2 h, the resultant red solution was dialyzed at 4 °C for a minimum of 20 h against several changes of a solution containing 0.100 M $NaClO_4$ and 0.050 M HEPES buffer to remove NTA and excess iron and $NaHCO_3$. The desired buffer was used as the final dialysite. The extinction coefficients used were 9.23×10^4 M^{-1} cm^{-1} at λ_{max} 279 nm and 2600 M^{-1} cm^{-1} at λ_{max} 466 nm.[32,51]

C-Terminal Monoferric Transferrin.[52] Apotransferrin was dissolved and dialyzed as described above. Freshly prepared 2.00 mM $Fe(NTA)_2$ was added to the apotransferrin at pH 7.40 to saturate 50% of the sites. The solution immediately turned red and was stirred for 5 min at room temperature. It was dialyzed against 0.100 M $NaClO_4$ and 0.050 M HEPES buffer for 3 h at 25 °C and 12 h at 4 °C, and finally against 0.050 M HEPES buffer adjusted to pH 7.40 at the desired temperature. Urea–polyacrylamide gel electrophoresis was then used to verify the purity of the C-terminal monoferric transferrin.

N-Terminal Monoferric Transferrin.[53] Apotransferrin was dialyzed at 4 °C against several changes of water and finally against a solution of 0.050 M HEPES buffer and 0.020 M $NaHCO_3$, pH 7.80. Freshly prepared 2.00 mM $Fe(NTA)_2$ at pH 4.0 was added to saturate 100% of the sites. The red solution was left overnight at room temperature and then diluted to 0.010 mM transferrin (0.020 mM iron-binding sites); the final concentrations of species were 0.1 M HEPES buffer, 1.00 M $NaClO_4$, and 1.00 mM pyrophosphate, pH 7.5. Desferrioxamine B was added to give a final concentration of 1.00 mM. The solution was left to stand for 4 h at room temperature. Under these conditions, iron was preferentially removed from the C-terminal site as was confirmed by electrophoresis. The solution was concentrated by ultrafiltration (Amicon PM-10 membrane) while stirring under Ar at 4 °C. The N-terminal monoferric transferrin was then dialyzed at 4 °C against several changes of 0.050 M HEPES buffer, which had been filtered on Bio-Rad Chelex 100 (100–200 mesh, Na^+) and adjusted to pH 7.40 at the appropriate temperature.

Chelators and Mediators. Other than 1,5,10-*N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane (3,4-LICAMS),[50] the compounds β-[*N*-(3-hydroxy-4-pyridone)]-α-aminopropionic acid (L-mimosine), tetrasodium pyrophosphate (PPi), 4,5-dihydroxy-*m*-benzenedisulfonic acid (Tiron), and 3,4-dihydroxybenzoic acid (3,4-DHBA) were purchased from commercial sources. Desferal mesylate (desferrioxamine B) was obtained as a gift from Ciba-Geigy Pharmaceutical Co. All compounds were used directly as purchased except for 3,4-DHBA, which was recrystallized from ethyl acetate and cyclohexane.

Visible Spectroscopy. Kinetic studies of iron removal from monoferric and diferric transferrins by 1.00–12.00 mM chelator (L-mimosine, Tiron, 3,4-LICAMS, or desferrioxamine B) in the presence and absence of mediators, PPi and 3,4-DHBA, were performed in 1-cm quartz curvettes maintained at constant temperature and monitored by visible spectroscopy using a Hewlett-Packard 8450A spectrophotometer equipped with a thermostated cell. Solutions containing 0.025–0.050 mM filtered iron transferrin (0.2-μM Gelman Acrodiscs) in the desired buffer were prepared. Potassium chloride was used as the supporting electrolyte for maintaining constant ionic strength. All spectra were measured against a reference solution which contained appropriate amounts of ligand, mediator, buffer, and KCl and subtracted from a background spectrum of the same solution.

The time of mixing protein with ligand was recorded as zero and scans were obtained every 2–15 min depending on the reaction rate (approximately 10 scans per half-life were sought). Absorbance values versus wavelength were collected, baseline connected, and stored in digital format.

Results and Discussion

Kinetics of Iron Removal from Transferrin by L-Mimosine. The addition of L-mimosine (Figure 1) to a solution of diferric transferrin in 0.050 M HEPES buffer resulted in a shift of the band maximum at 466 nm of the diferric transferrin to a band maximum of 450 nm, characteristic of the Fe^{3+}(L-mimosine)$_3$ complex. To calculate the rates of iron removal, the kinetics were monitored at 466 nm. The concentrations of ligand used for the analyses were in 10–100-fold excess over total iron sites in diferric transferrin, so that pseudo-first-order kinetic conditions were maintained. Plots of $\ln[(A - A_\infty)/(A_0 - A_\infty)]$ versus time are linear, as shown in Figure 2.

The simple monophasic first-order kinetic behavior observed for iron removal from diferric transferrin by L-mimosine contrasts with the biphasic behavior earlier observed with 3,4-LICAMS and ascribed to the Baldwin model[42] for iron release from the two different (a and b) sites, as shown in (1),[48,54] which gives the general rate expression of eq 2, where B_0 = concentration of diferric transferrin at initial time; ϵ = molar extinction coefficient per iron; ℓ = cell path length; k_{1a}, k_{1b}, k_{2a}, and k_{2b} are the microscopic rate constants for iron removal from the 'a' and 'b' sites of transferrin; and m_1, m_2, and m_3 are the macroscopic rate

(47) Egan, T. J.; Roos, D. C.; Purves, L. R.; Adams, P. A. *Inorg. Chem.* **1992**, *31*, 1994–1998.
(48) Kretchmar, S. A.; Raymond, K. N. *Biol. Met.* **1989**, *2*, 65–68.
(49) Chasteen, N. D.; Williams, J. *Biochem. J.* **1981**, *193*, 717–727.
(50) Weitl, F. L.; Harris, W. R.; Raymond, K. N. *J. Med. Chem.* **1979**, *22*, 1281–1283.
(51) Bates, G. W.; Schlabach, M. R. *J. Biol. Chem.* **1973**, *248*, 3228–3232.
(52) Bates, G. W.; Wernicke, J. *J. Biol. Chem.* **1971**, *246*, 3679–3685.
(53) Kretchmar, S. A.; Teixeira, M.; Huynh, B.-H.; Raymond, K. N. *Biol. Met.* **1988**, *1*, 26–32.
(54) Baldwin, D. *Biochim. Biophys. Acta* **1980**, *623*, 183–198.

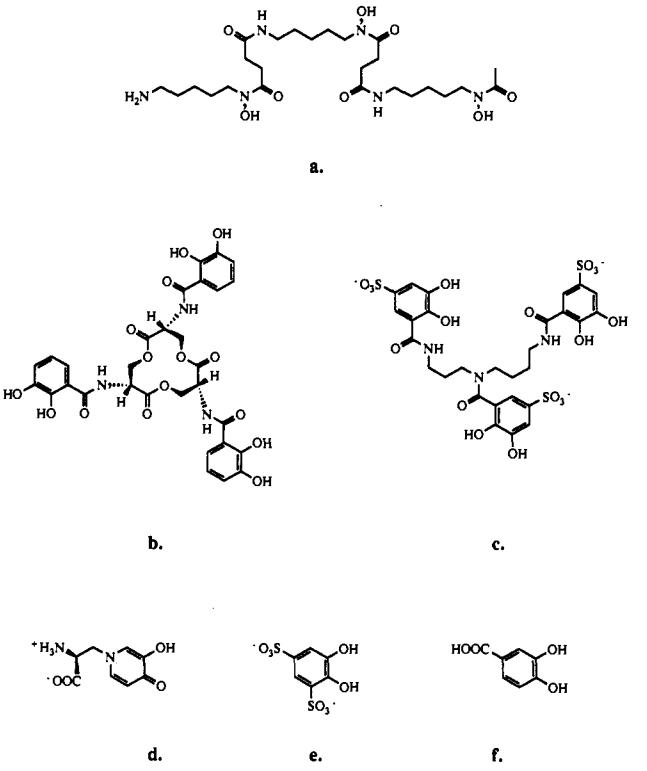


Figure 1. Structures of ligands discussed in this paper: (a) desferrioxamine B; (b) enterobactin; (c) 3,4-LICAMS; (d) L-mimosine; (e) Tiron; (f) 3,4-dihydroxybenzoic acid (DHBA).

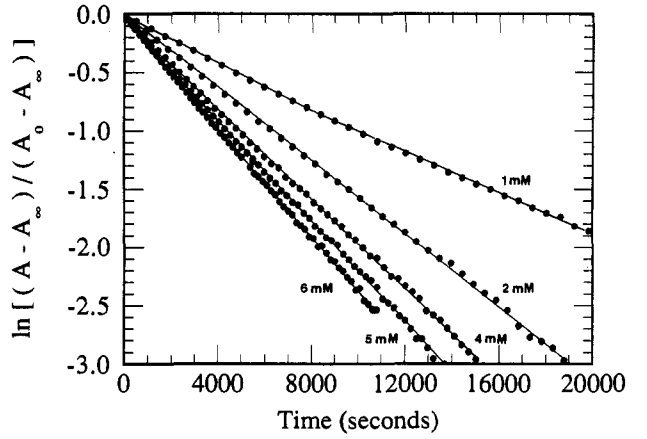
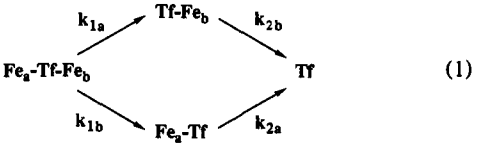


Figure 2. Iron removal from 0.100 mM diferric transferrin by various concentrations of L-mimosine (0.050 M HEPES buffer, pH 7.4, 25 °C, μ = 0.026).



(1)

$$A - A_\infty = B_o \varepsilon \ell \left[\left(2 - \frac{k_{1a}}{(m_1 - m_2)} - \frac{k_{1b}}{(m_1 - m_3)} \right) e^{-m_1 t} + \left(\frac{k_{1a}}{(m_1 - m_2)} \right) e^{-m_2 t} + \left(\frac{k_{1b}}{(m_1 - m_2)} \right) e^{-m_3 t} \right] \quad (2)$$

constants which are related to the microscopic rate constants by the following expressions: $m_1 = k_{1a} + k_{1b}$, $m_2 = k_{2b}$, and $m_3 = k_{2a}$.[54,17]

We have shown by direct measurement of m_2 and m_3 for 3,5 LICAMS[17,48] that their values are similar, and smaller than m_1, which allows for eq 2 to be further simplified:

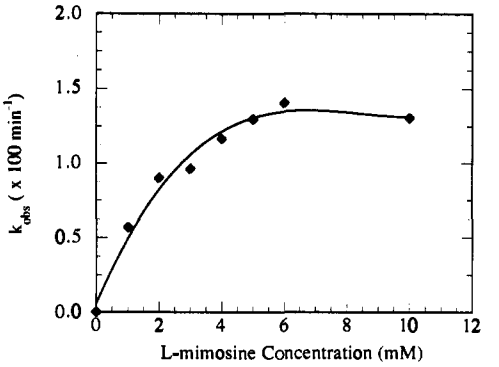


Figure 3. Plot of observed rate constants as a function of increasing concentrations of L-mimosine as determined by linear-least-squares analyses for iron removal from 0.100 mM diferric transferrin (0.050 M HEPES buffer, pH 7.4, 25 °C, μ = 0.026).

$$A - A_\infty = B_o \varepsilon \ell \left[\left(2 - \frac{m_1}{(m_1 - m_2)} \right) e^{-m_1 t} + \left(\frac{m_1}{(m_1 - m_2)} \right) e^{-m_2 t} \right] \quad (3)$$

Finally, for the case in which iron is removed from the two sites at nearly identical rates, eq 2 collapses to the single-term exponential equation for a first-order rate process:

$$A - A_\infty = 2B_o \varepsilon e^{-kt} \quad (4)$$

Attempts to fit the data for iron removal from diferric transferrin by L-mimosine to eqs 2 and 3 using nonlinear-least-squares regression[55] failed because of the number of variables. However, the data were successfully fit to eq 4. Thus the rates of iron removal from the two sites are essentially identical (the analysis cannot discriminate rate constants which differ by less than about 30%). The observed rate constants are plotted against concentration of ligand in Figure 3. In accordance with the mechanism described earlier in this paper, saturation of the observed rate occurs with respect to increasing ligand concentration.

The kinetic equivalence of iron removal from the two sites of transferrin by L-mimosine was further tested by studying the rates of iron removal from the two monoferric transferrins. First-order kinetic behavior was observed for iron removal from the C-terminal and N-terminal monoferric transferrins by 6.00 mM L-mimosine; the average observed rates from three trials were $0.94(1) \times 10^{-2}$ and $1.42(2) \times 10^{-2}$ min^{-1}, respectively.[56] Similar results were found when the concentration of L-mimosine was 3.60 mM. The rates of iron removal from the N-terminal monoferric transferrin are identical to those of the diferric transferrin. However, the measurably slower rate for iron removal from the C-terminal monoferric transferrin suggests that some difference does exist between the rate of iron removal from the C-terminal sites of the monoferric and diferric transferrins.

From the crystal structures of rabbit serotransferrin, human monoferric transferrin, and the related protein, human lactoferrin, it is apparent that each lobe of the transferrin contains a channel leading to the ferric ions.[4-6] One model to test is that release of the Fe^{3+} may involve the access of small molecules to the Fe^{3+} sites through these channels. Although the N-terminal and C-terminal domains are structurally equivalent, the location of the channels leading to the Fe^{3+}-binding sites are not equivalent; the channel leading to the C-terminal Fe^{3+}-binding site is located close to the interface between the two lobes and is restricted by the protein, while that leading to the N-terminal site is far from the interface and is open to the surrounding solution. Also, the extra disulfide bridges in the C-terminal lobe appear to reduce

(55) Schreiner, W.; Kramer, M.; Krisher, S.; Langsam, Y. *Pers. Tech. Comput. J.* **1985**, *3*, 170–199.

(56) The estimated standard deviation is presented in parantheses (in the least significant digits) following the corresponding parameter.

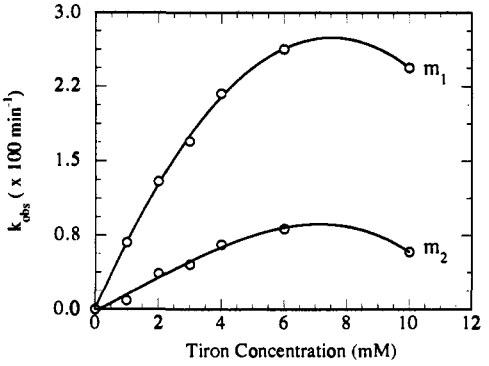


Figure 4. Plot of observed rate constants as a function of increasing concentrations of Tiron as determined by nonlinear-least-squares analyses for iron removal from 0.100 mM diferric transferrin (0.050 M HEPES buffer, pH 7.4, 25 °C, μ = 0.166). The macroscopic rate constants m_1 and m_2 give rise to the upper and lower curves, respectively.

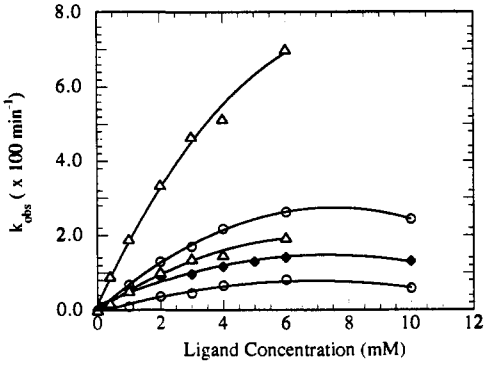


Figure 5. Plot of observed rate constants as a function of increasing concentrations of 3,4-LICAMS (△), L-mimosine, (◆), and Tiron (○) for iron removal from 0.100 mM diferric transferrin.

access to the Fe^{3+}-binding site. These differences have been used to account for the greater stability of the C-terminal lobe toward temperature-induced conformational change. It seemed this might also explain the difference in rate of removal of Fe^{3+} by the hexadentate ligand 3,4-LICAMS. For the smaller and lower charge L-mimosine molecule, these protein access constraints should be less important. To probe the role of ligand size and charge on Fe^{3+} removal from transferrin, a study was carried out with Tiron, a monomeric catecholate (Figure 1).

Kinetics of Iron Removal from Transferrin by Tiron. The addition of Tiron to a solution of diferric transferrin in 0.050 M HEPES buffer resulted in a shift of the band maximum at 466 nm of the diferric transferrin to a band maximum of 480 nm, characteristic of the $Fe(Tiron)_3$ complex. To calculate the rates of iron removal, the kinetics were monitored at 460 nm. The concentrations of ligand used for the analyses were in 10–100-fold excess over total iron sites in diferric transferrin. Plots of $\ln[(A - A_\infty)/(A_0 - A_\infty)]$ versus time (not shown) were *not* linear and were similar to those seen when 3,4-LICAMS was used as the competing ligand.[48] Consequently, it is not simply the size of the 3,4-LICAMS molecule that determines its kinetic behavior. [We note that other highly charged species, such as EDTA and desferrioxamine B, and hydroxypyridinones (1,2-dimethyl-3-hydroxypyrid-4-one, 1-methyl-3-hydroxypyrid-2-one, and 2-methyl-3-hydroxypyrid-4-one) also exhibit biphasic kinetic behavior.][4,33,54] The data were fit to eq 3.[55] As shown in Figure 4, saturation of the observed macroscopic rate constant occurs with respect to increasing ligand concentration.

Mechanisms of Iron Removal from Transferrin by Chelators. The observed rates are plotted against ligand concentration for iron removal from diferric transferrin by 3,4-LICAMS, L-mimosine, and Tiron in Figure 5. The saturation levels are dependent upon the ligand type, consistent with the requirement for anion binding in the conformational change of the protein as earlier described.

Table I. Maximum Observed Rates for Iron Removal from Transferrin by Various Ligands at 25 °C and pH 7.4[a]

ligand	$k_{max} \times 10^2$, min^{-1}	estimated μ,[b] M	buffer
3,4-LICAMS	7.0(1)	0.151	50 mM HEPES
EDTP[c]	3.0(1)	0.371	100 mM TRIS[k]
Tiron	2.6(3)	0.166	50 mM HEPES
PPi[d]	2.0(8)	0.064	100 mM HEPES
HDEP[e]	1.9(7)	0.271	100 mM TRIS
L-mimosine	1.4(1)	0.026	50 mM HEPES
NTP[f]	1.0(3)	0.104	100 mM HEPES
DPG[g]	1.0(2)	0.095	100 mM HEPES
PIDA[h]	0.7(3)	0.106	100 mM HEPES
aerobactin[i]	0.3		10 mM HEPES
AHA[k]	0.0004	0.028	20 mM HEPES/20 mM HCO_3^-

[a] For the cases in which the kinetic behavior is biphasic; the observed rate for the faster phase is reported. See ref 56 regarding esd's. [b] Calculated for the concentration of ligand at which saturation occurred. Also included are the contributions from the buffer medium. [c] Ethylenediamine-*N,N,N',N'*-tetrakis(methylenephosphonic acid); ref 57. [d] Reference 58. [e] 1-Hydroxyethane-1,1-diphosphonic acid; ref 57. [f] Nitrilotris(methylenephosphonic acid); ref 58. [g] *N,N*-Bis(phosphonomethyl)glycine; ref 58. [h] *N*-(Phosphonomethyl)iminodiacetic acid; ref 58. [i] Reference 44. [k] Acetohydroxamic acid; ref 33.

The maximum observed rates for Fe^{3+} removal from transferrin by several different ligands are listed in Table I.[57,58] (For the cases in which the kinetic behavior is biphasic, the observed rate for the faster phase is reported.) Even accounting for the varying experimental conditions, the observed rate values are significantly different between 3,4-LICAMS and the other ligands. An even greater variance in the maximum observed rates would be obtained by inclusion of those rates of removal for ligands which are very slow to remove iron from transferrin, such as EDTA and desferrioxamine B, where high concentrations and temperatures are needed to achieve significant rates. The maximum observed rate in Fe^{3+} removal from transferrin is dependent upon the ligand type; the ligand is involved in the rate-limiting step.

Iron Removal from Transferrin as Mediated by Pyrophosphate. The kinetics of Fe^{3+} removal from diferric and the monoferric transferrins by 1.00 and 3.00 mM 3,4-LICAMS in the presence of 1–40 mM PPi have been investigated by UV/VIS spectroscopy. The spectral changes were identical to those reported previously for the unmediated reaction of Fe^{3+} removal from transferrin by 3,4-LICAMS, in which the band maximum shifted from 466 to 490 nm.[17,32] The kinetics were monitored at 520 nm. Plots of $\ln[(A - A_\infty)/(A_0 - A_\infty)]$ versus time showed biphasic kinetic behavior. Nonlinear-least-squares refinements of the data using eqs 2 and 3 were statistically indistinguishable.[55] First-order kinetic behavior was observed for mediated Fe^{3+} removal from monoferric transferrins, and the rate constants were obtained from the slopes of the first-order kinetic plots of $\ln[(A - A_\infty)/(A_0 - A_\infty)]$ versus time. The observed rate constants for mediated Fe^{3+} removal by 1.00 mM 3,4-LICAMS are plotted against mediator concentration in Figure 6. (The macroscopic rate constants for Fe^{3+} removal from diferric transferrin were determined from eq 2.) The results were identical for Fe^{3+} removal by 3.00 mM 3,4-LICAMS (data not shown). The rates of Fe^{3+} removal for the monoferric and diferric transferrins increase by a factor of ca. 3 before saturation of the observed rate occurs at 10 mM PPi. The rates of Fe^{3+} removal from the C-terminal monoferric transferrin are enhanced to a greater extent than those of the N-terminal monoferric transferrin. As noted in the Introduction, this agrees with other observations on the effects of anions on the transferrin Fe^{3+} sites.

An analogous study, in which PPi was used to mediate Fe^{3+} removal from diferric transferrin by 1.00 and 20.00 mM

(57) Harris, W. R. *J. Inorg. Biochem.* **1984**, 263–276.
(58) Harris, W. R. *Inorg. Chem.* **1987**, *26*, 2711–2716.

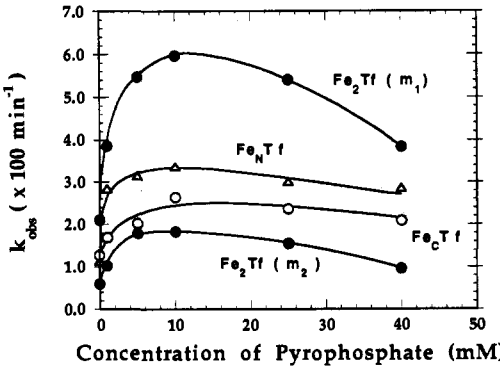


Figure 6. Plot of observed rate constants as a function of increasing concentrations of PPi for iron removal from 0.100 mM diferric (●), C-terminal monofrric (○), and N-terminal monoferric (△) transferrins by 1.00 mM 3,4-LICAMS (0.050 M HEPES buffer, pH 7.4, 25 °C, μ = 0.292). For diferric transferrin, the macroscopic rate constants m_1 and m_2 give rise to the upper and lower curves, respectively. The solid lines are drawn only for the purpose of distinguishing among the four data sets.

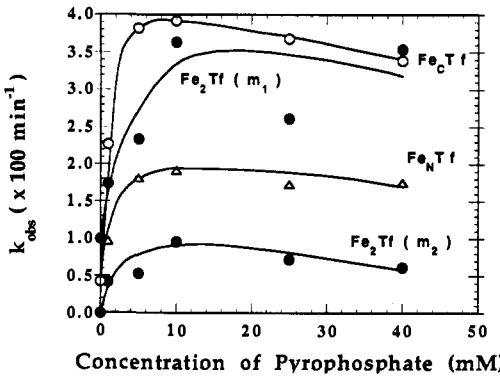


Figure 7. Plot of observed rate constants as a function of increasing concentrations of PPi for iron removal from 0.100 mM diferric (●), C-terminal monofrric (○), and N-terminal monoferric (△) transferrins by 20.00 mM desferrioxamine B (0.050 M HEPES buffer, pH 7.4, 25 °C, μ = 0.292). For diferric transferrin, the macroscopic rate constants m_1 and m_2 give rise to the upper and lower curves, respectively. The solid lines are drawn only for the purpose of distinguishing among the four data sets and are not representative of calculated values.

desferrioxamine B, gave spectral changes which were identical to those of the unmediated reactions (in which the band maximum shifted from 466 to 420 nm), and biphasic kinetic behavior was observed. The data for Fe^{3+} removal from diferric transferrin by 20.00 mM desferrioxamine B were fit to eq 3. First-order kinetic behavior was observed for Fe^{3+} removal from the monoferric transferrins. These observed rates are plotted against mediator concentration in Figure 7. Identical results were obtained for mediated Fe^{3+} removal by 1.00 mM desferrioxamine B (data not shown). The rates of Fe^{3+} removal from diferric and N-terminal monoferric transferrins increase by a factor of ca. 3, while those of the C-terminal monoferric transferrin increase by a factor of ca. 8 before saturation of the rate occurs at 10 mM PPi. Thus the effects of PPi are essentially independent of the type and concentration of the chelator and give rates of Fe^{3+} removal greater than the sum of individual PPi chelator rates.

The saturation behavior observed for the PPi-mediated experiments agrees with previous studies for this ion.[30,40,47] This behavior differs from that observed for Fe^{3+} removal from transferrin by chelators in the presence of simple salts,[41,42] which promote Fe^{3+} release by binding to ion-binding sites of the protein—often at multiple sites. Because PPi is effective as an Fe^{3+} chelator, accelerates the exchange of the synergistic bicarbonate, and can itself behave as a synergistic anion, we infer that it forms a complex with FeTf prior to the ligand attack

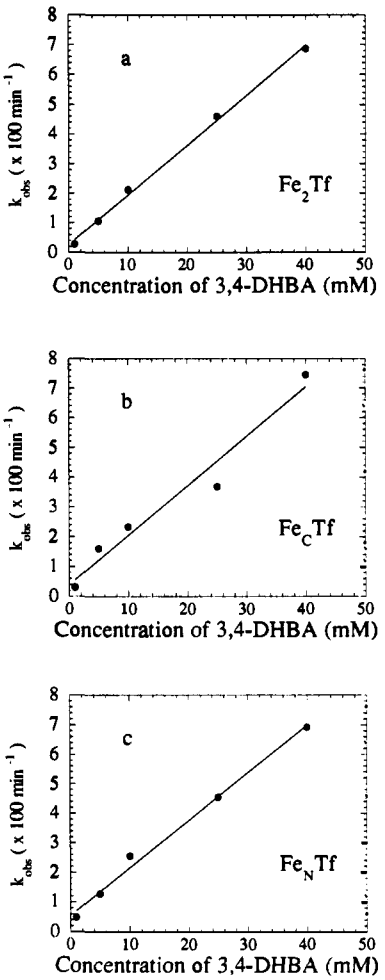


Figure 8. Plots of observed rate constants as a function of increasing concentrations of 3,4-DHBA for iron removal from 0.100 mM (a) diferric, (b) C-terminal monoferric, and (c) N-terminal monoferric transferrins by 1.00 mM desferrioxamine B (0.050 M HEPES buffer, pH 7.4, 25 °C, μ = 0.025).

of the Fe^{3+} site—the same process that occurs for the most effective chelators, which act both as mediator and as Fe^{3+} removal agent, as described in the Introduction. Evidence for a FeTf(PPi) intermediate has been observed by electron paramagnetic resonance in the exchange of Fe^{3+} from PPi to transferrin.[59] When the concentration of PPi is high enough (10 mM) to bind all of the apotransferrin, the rates of Fe^{3+} removal from transferrin by chelators are not longer accelerated. Indeed, the rates begin to decrease slightly.

Iron Removal from Transferrin as Mediated by 3,4-DHBA. UV/VIS spectroscopy was employed to investigate the kinetics of iron removal from diferric and the monoferric transferrins by 1.00 mM desferrioxamine B in the presence of 1–40 mM 3,4-DHBA. The spectral changes were identical to those observed for the unmediated reaction. In contrast to the biphasic kinetic behavior observed when PPi was used to mediate iron removal from diferric transferrin, simple first-order kinetics were obtained when 3,4-DHBA was used as a mediator. Plots of the observed rate constants for iron removal from monoferric and diferric transferrins by 1 mM desferrioxamine B in the presence of 1–40 mM 3,4-DHBA are shown in Figure 8. The observed rates are linearly dependent on concentration of 3,4-DHBA, and iron is removed from the different iron sites of transferrin at identical rates. Saturation rates could not be reached at higher concentrations of 3,4-DHBA because at concentrations above 40 mM

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

the UV/VIS spectral changes are no longer characteristic of the mediation reaction. These spectral changes did not indicate formation of an Fe^{3+} complex of either desferrioxamine B or 3,4-DHBA. Finally, from Figure 8 it can be seen that 3,4-DHBA achieves a greater than 7-fold enhancement of the rates of iron removal from the ferric transferrin complexes by desferrioxamine B.

Conclusions

To further elucidate the mechanisms by which iron is removed from transferrin by chelators and to correlate this information with the protein structural data now available, kinetic studies of iron removal by three different types of ligands have been carried out under identical reaction conditions. In contrast to the biphasic behavior observed with 3,4-LICAMS and Tiron, no resolution of the two different rates was seen when L-mimosine was used to remove iron from diferric transferrin. The effective charge (rather than size) of the ligand most strongly affects the kinetic behavior. Saturation of the observed rate with increasing ligand concentration was seen for the three ligands, as predicted by a mechanism in which protein conformational change, required for access to the iron center, is promoted by anion binding. That mechanism, which we described in an earlier study,[43] predicts that the first-order rate constant for iron removal from transferrin by any ligand will go to zero as the ionic strength goes to zero. This has been found for every ligand system described to date. This model fits well what is now known about the dramatic differences in conformation seen in the metal-bound versus metal-free forms of the transferrin proteins and the effect of anion binding on the relative stability of these conformations.

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