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Ferric Ion Sequestering Agents. 2. Kinetics and Mechanism of Iron Removal from Transferrin by Enterobactin and Synthetic Tricatechols[1]

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Abstract: The apparent first-order rate constants for iron removal from transferrin by the synthetic sequestering agents 1,5,10-*N,N′,N″*-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane (3,4-LICAMS), 1,3,5-*N,N′,N″*-tris(2,3-dihydroxybenzoyl)aminomethylbenzene (MECAM), and the natural siderophore enterobactin (ent) have been determined at 25 °C. One of these compounds, 3,4-LICAMS, was examined in detail, and the mechanism of its removal of iron from transferrin elucidated. The 3,4-LICAMS ligand is observed to form a complex with transferrin prior to iron removal. The stability constant of this complex and the first-order rate constant for its dissociation to ferric LICAMS and apotransferrin are $K_{eq} = 4.1\ (6) \times 10^2\ M^{-1}$ and $k_2 = 0.066\ (4)\ min^{-1}$, respectively. These results indicate that these catecholate ligands are both kinetically and thermodynamically capable of iron removal from transferrin at physiologically accessible concentrations. In contrast, the hydroxamate-based sequestering agents are kinetically hindered in this reaction. The implications of these results with respect to the possible use of these compounds as drugs in the treatment of iron overload syndromes are discussed.

Introduction

The treatment of certain blood disorders such as sickle cell anemia and β-thalassemia major requires chronic transfusion therapy. This regimen leads to an induced secondary hemochromatosis (buildup of iron in the tissues, particularly the heart, pancreas, and liver) causing fibrotic changes, and eventually failure of vital organs resulting in death.[2] This toxic buildup of iron results from the lack of a physiological mechanism in man for the excretion of this element. Attempts to remove excess iron from the body have led to a search for effective ferric ion chelating agents.[3] The search originally centered on desferriferrioxamine B (Desferal), a hydroxamate siderophore produced by *Streptomyces pilosus*.[4-6] Although Desferal is thermodynamically capable of removing iron from transferrin (the mammalian iron transport protein), its use in iron overload therapy has been only marginally successful. Desferal suffers from a series of drawbacks, among which are a lack of oral effectiveness, moderate toxicity, and, most important, the fact that it has not generally been possible to maintain patients in negative iron balance using this approach without the need for long-term subcutaneous infusion therapy.[7,8] Part of the ineffectiveness of Desferal stems from the fact that despite its high affinity for ferric ion, it is kinetically incapable, both in vivo and in vitro, of removing iron from transferrin.[9,10] This kinetic barrier to iron removal can only be overcome by the addition of other anions to the system,[11] a phenomenon that appears to be shared by other hydroxamate-based compounds.

In contrast, enterobactin, a catechol-containing siderophore, is both thermodynamically and kinetically capable of iron removal from transferrin.[12-14] Use of enterobactin as a drug for chelation therapy is unfortunately precluded by the extreme lability of the molecule itself.[15] Recently we have engaged in the design and synthesis of catechol-based chelating agents modeled on enterobactin (Figure 1).[1,16] We hoped that these molecules would retain the exceedingly high affinity of enterobactin for iron as well as the kinetic ability to remove iron from transferrin, but would prove more resistant to hydrolysis and oxidation. The results of the interaction of several of these model compounds with transferrin are reported herein.

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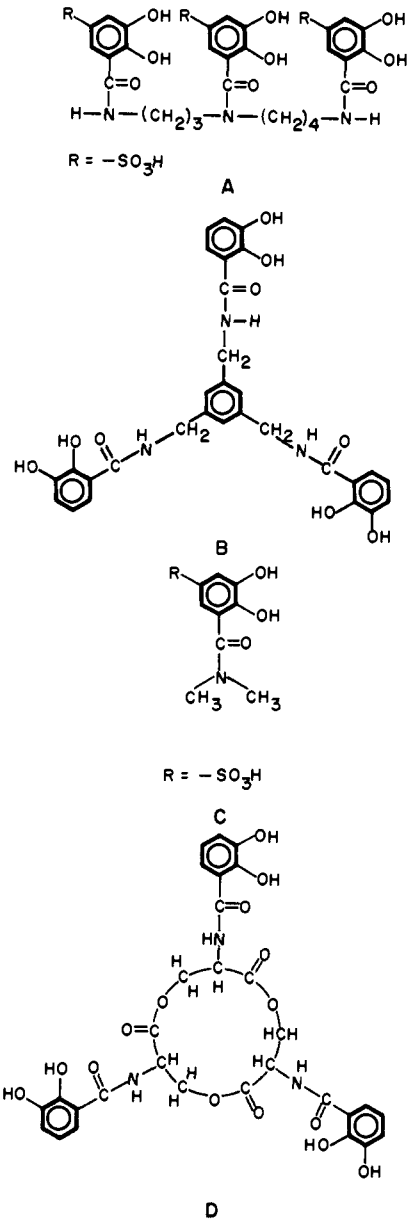

Figure 1. Structural formulas of the synthetic and biogenic tricatecholate ligands: (A) 1,5,10-*N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane (3,4-LICAMS); (B) 1,3,5-*N,N',N''*-tris(2,3-dihydroxybenzoyl)-aminomethylbenzene (MECAM); (C) 5-sulfo-2,3-dihydroxydimethylbenzamide (DMBS); and (D) enterobactin (ent).

Experimental Section

The 1,5,10-*N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane (3,4-LICAMS), 1,3,5-*N,N',N''*-tris(2,3-dihydroxybenzoyl)-aminomethylbenzene (MECAM), and 5-sulfo-2,3-dihydroxydimethylbenzamide (DMBS) were obtained from Dr. F. L. Weitl and prepared as previously described.[1,16] Enterobactin (ent) was isolated and purified by the literature procedures.[15] Apotransferrin (Sigma) was saturated with iron as described by Bates et al.[17] using a 0.02 M solution of freshly prepared ferric nitrilotriacetate (FeNTA) at pH 7.4. The resulting complex was either (a) dialyzed against several changes of 0.1 M $NaClO_4$, followed by dialysis against air-saturated 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4, or (b) gel filtered on Sephadex G-25 (Pharmacia) that had been equilibrated with the same buffers. Only those fractions with $A_{280}/A_{466} \leq 24$ (greater than 95% saturated) were used. The samples were diluted to the desired concentration with 0.1 M Tris buffer at pH 7.4 using an extinction coefficient (per iron) for diferric transferrin of 2500 L/mol·cm.[18]

Kinetic studies were performed in 1-cm quartz cuvettes (maintained at 25 ± 0.1 °C) and monitored at 520 nm by visible spectroscopy using a Cary 118 spectrophotometer equipped with a thermostated cell. Solutions were clarified by membrane filtration (Bio-Rad, Unipore, 0.4 μm) prior to monitoring. The time of mixing was recorded as zero time and the first scan was obtained after 2 min. Pseudo-first-order conditions were maintained by using a large excess of the competing ligand to obtain linear plots of $\ln [(A - A_\infty)/(A_0 - A_\infty)]$ vs. time. The data were corrected for ligand absorption, and values of k_{obsd} (the apparent first-order rate constant) were obtained by linear regression analysis. The k_{obsd} vs. ligand concentration data were analyzed using a nonlinear least-squares computer program, ORGLS.[19]

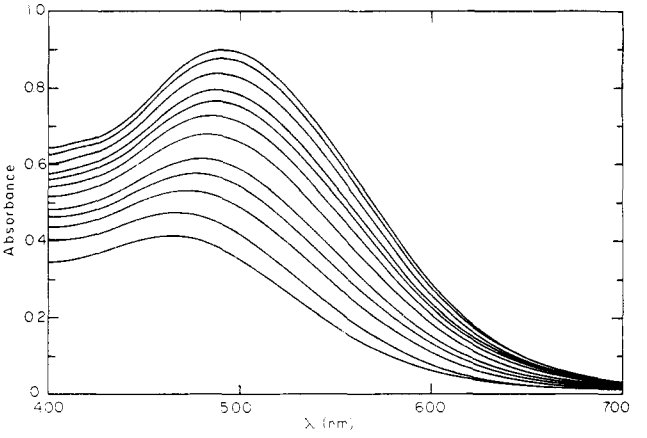

Figure 2. Spectral changes accompanying iron removal from transferrin. The bottom curve represents the unreacted transferrin and the top curve the final product, ferric 3,4-LICAMS.

Separation of the reaction products was carried out on BioRex A6-1 X4 (200–400 mesh) anion exchange resin equilibrated with 0.1 M Tris buffer at pH 7.4. In a typical experiment a ferric transferrin solution (OD_{470} = 0.35) was mixed with an excess of 3,4-LICAMS and allowed to react (24–72 h). The mixture was then chromatographed using Tris buffer as an eluant. The apotransferrin came out in fractions 3, 4, and 5 (2.5 mL/fraction), and the red ferric 3,4-LICAMS remained fixed to the column. Recovery of apotransferrin was 90% of the theoretical value. On addition of a pH 4.0 buffer, the red ferric 3,4-LICAMS turned the blue color characteristic of biscatecholate complexes confirming the iron was bound to the 3,4-LICAMS.

Results

Kinetics of Iron Removal from Transferrin. The addition of 3,4-LICAMS to a solution of differic transferrin results in the spectral changes seen in Figure 2. The band maximum shifts smoothly from the 470-nm maximum of the diferric transferrin to ~495 nm, characteristic of tris(catecholato) iron(III) complexes. This indicates that the iron is being removed from transferrin by the 3,4-LICAMS.

Plots of $\ln [(A - A_\infty)/(A_0 - A_\infty)]$ vs. time gave straight lines over at least 3–4 half-lives for concentrations of 3,4-LICAMS from 0.4 to 6 mM. This represents a 2.5- to 40-fold excess of ligand over transferrin. The results of these experiments are shown in Figure 3. Enterobactin and MECAM are also capable of removing iron from transferrin, but a complete analysis of their solution kinetics was hampered by the insolubility of these ligands in water. Both ent and MECAM were studied (at 0.2 mM concentration) by addition of small aliquots (50–100 μL) of stock solutions in dimethyl sulfoxide (Me_2SO) to solutions of differic transferrin in 0.1 M Tris buffer. Table I shows a comparison of the observed rate constants for the three ligands. It is clear that all three remove iron from transferrin at about the same rate. The percentage of iron removed in 30 min is also compared with Desferal, *which removes less than 5% of the iron (at a 100:1 Desferal:transferrin ratio), while 3,4-LICAMS removes over 50% (at a ratio of only 40:1).*

Mechanism of Iron Removal from Transferrin by 3,4-LICAMS. Previous results have indicated that iron removal from transferrin might involve the formation of a complex

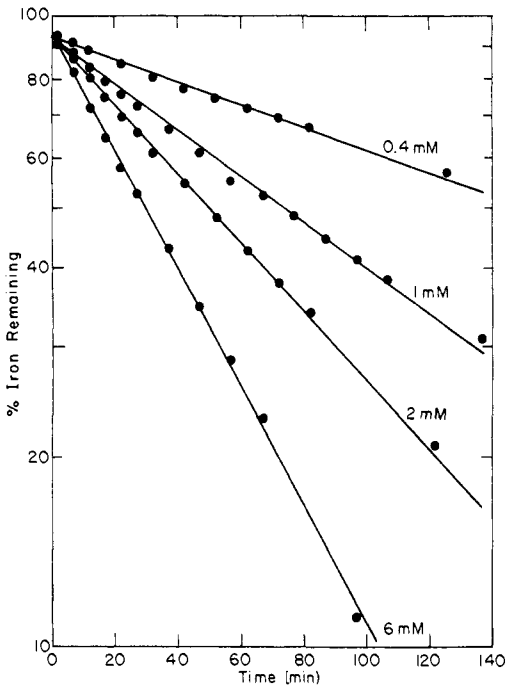

Figure 3. Iron removal from transferrin (~0.2 mM) by various concentrations of 3,4-LICAMS.

between diferric transferrin and the competing ligand, followed by iron removal and dissociation of the complex.[20,21] This scheme is outlined in eq 1:

$$\mathrm{FeTr} + \mathrm{L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{LFeTr} \xrightarrow{k_2} \mathrm{FeL} + \mathrm{Tr} \qquad (1)$$

where FeTr is ferric transferrin, L is 3,4-LICAMS, and Tr is apotransferrin. This scheme is qualitatively similar to that of substrate binding by enzymes and should thus follow Michaelis-Menten kinetics. If this mechanism is correct, a hyperbolic relationship should exist between k_{obsd} and the concentration of competing ligand. From Figure 4 we see that this is indeed the case. A replot of these data yields a linear double reciprocal Lineweaver-Burk plot from which an apparent equilibrium constant K_{eq} ($= k_1/k_{-1}$) may be determined. Using the value of K_{eq} thus obtained as a first approximation, the values of K_{eq} and k_2 (for reaction 1) were defined by a nonlinear least-squares fit of k_{obsd} (defined in eq 2) as a function of [L]:[22,23]

$$k_{obsd} = \frac{k_2[\mathrm{L}]K_{eq}}{2.3 + 2.3K_{eq}[\mathrm{L}]} \qquad (2)$$

The values found were $k_2 = 0.066$ (4) min^{-1}, and $K_{eq} = 4.1$ (6) $\times 10^2$ M^{-1}. Under the pseudo-first-order conditions observed here ([L] $\gg$ [Tr]), the initial rate should be directly proportional to Fe_2Tr concentration (Figure 5), while it should be insensitive to the concentration of 3,4-LICAMS. In fact, the initial rate found was 2.87 (5) $\times 10^{-2}$ ΔA_{520}/min over the range of 3,4-LICAMS concentration of 1-6 mM.

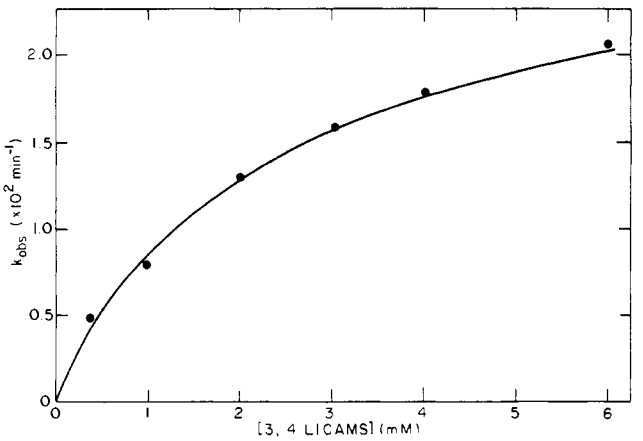

Figure 4. Plot of the observed rate constant for iron removal from transferrin (~0.2 mM) vs. the concentration of 3,4-LICAMS. The points represent the experimental data, the line the computer fit; see text.

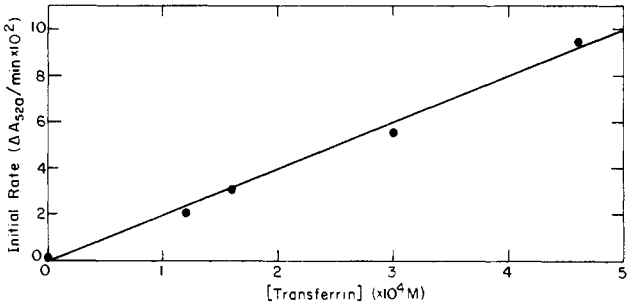

Figure 5. Plot of initial rate of iron removal from transferrin by 3,4-LICAMS (4 mM) as a function of transferrin concentration.

Discussion

Possible mechanisms of chelate exchange reactions have been described by Basolo and Pearson.[24] The primary step in multidentate ligand exchange is thought to involve the unwrapping of one or more of the ligating groups from the metal ion so that there is a free site of attack for the incoming ligand. A consequence of this reaction scheme is the formation of ternary complexes between the original ligand, the metal ion, and the incoming ligand. Other workers[19-23] have shown that such complexes are formed between ferric transferrin and

Table I. Comparison of Ligand Iron Affinity and Ability to Remove Transferrin-Bound Iron

ligand	log K_f	p [Fe^{3+}] [a]	k_{obsd} ($\times 10^3$ min^{-1}) [b]	% Fe removed (30 min) 40:1 [c]	% Fe removed (30 min) 1:1
enterobactin	51	35.6	2.2		6
MECAM	45.8	29.1	3.4		13
3,4-LICAMS	41.7	27.8	2.2	50	6
DMBS				36	
Desferal	30.6	26.6		5 [d]	
EDTA	25.0	23.1		37 [e]	0
transferrin	24 [f]	23.6			

[a] Free [Fe^{3+}] concentrations in equilibrium with a series of iron complexes where total ligand concentration is 10^{-5} M, total iron concentration is 10^{-6} M, and pH is 7.4. Calculated using the appropriate metal ligand formation constants and ferric hydrolysis constants. See ref 12 and 16. [b] Ligand concentration 0.2 mM. [c] Ratio of ligand to transferrin. [d] 100:1 ratio. [e] 2500:1 ratio. [f] Conditional formation constant, pH 7.4, CO_2-saturated buffer.

competing chelates as well as between many metalloenzymes and competing chelating agents. Previous work has suggested that the exchange in ferric transferrin may begin with the replacement of the carbonate anion of the transferrin complex by a part of the incoming ligand.[25] Steric bulk does not seem to play an important role in the replacement reaction, since we find that the rate of iron removal is the same for both the multidentate chelate, 3,4-LICAMS, and its model monomer, DMBS.

Kochan and co-workers[13] concluded that enterobactin does not actually remove iron from transferrin but merely forms an ent-Fe_2Tr complex. Several lines of evidence in our studies indicate, instead, that enterobactin as well as the synthetic chelates not only form complexes with Fe_2Tr but actually remove the iron. The spectra show changes in λ_{max} from the 470 nm (ϵ 2500 L/mol·cm) of transferrin to the characteristic red color (λ_{max} 495 nm, ϵ 5600 L/mol·cm) of the tris(catecholate) ferric chromophore as the reaction proceeds, indicating that the iron is now coordinated to the catechol moieties and has been removed completely from transferrin.

In addition, ion exchange chromatography of the final reaction mixture at pH 7.4 yields both apotransferrin and ferric enterobactin. Previous reports that the complex was stable resulted from dialysis experiments in which enterobactin did not dialyze from a ferric transferrin solution. We have found that the synthetic chelates tend to bind strongly to dialysis tubing, making any analysis based on this method ambiguous.

Our results for iron removal from transferrin by various chelates suggest that not only the stability constants but also the chemical configuration of the competing ligand play an important role in determining the extent of removal of metal ions from protein binding sites. We have summarized these data in Table I, where we compare the stability constants for the iron complexes of a variety of competing ligands with their rate of iron removal from transferrin.

In summary, the rational design of effective ferric ion chelating agents must take both thermodynamic and kinetic parameters into account. Although the value of these observations to human disease will depend on other factors, including in vivo efficacy, toxicity, and biological half-lives of these complexes, these ligands are capable of removing iron effectively from transferrin in vitro at physiologically accessible concentrations. In this regard, the synthetic chelating agents described here represent an effective combination of both high affinity for ferric ion coupled with the ability to remove this ion from transferrin at a reasonable rate.

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