Lactoferrin: The Role of Conformational Changes in Its Iron Binding and Release

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Abstract: The relative rates of ferric ion removal from human milk lactoferrin and human serum transferrin by the synthetic tricatecholate sequestering agent *N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (3,4-LICAMS) have been compared at pH 7.4 and 37 °C. Hyperbolic plots of the observed pseudo-first-order rate constants with increasing concentrations of 3,4-LICAMS were obtained for both proteins and are consistent with a similar mechanism of iron removal from both proteins by this ligand. However, the average limiting rate of iron removal from milk lactoferrin (k_{max} = 6.5(4) × 10^{-4} min^{-1}) is about 100-fold slower than that found from serum transferrin (k_{max} = 6.3(4) × 10^{-2} min^{-1}) under the same conditions. This accounts for essentially all of the greater thermodynamic stability of lactoferrin. These observations are explained in light of the two conformations, "closed" and "open", seen in protein crystal structures. Lactoferrin's "closed" form is more stable relative to transferrin, leading to a slower rate of iron release. This slower rate of iron removal from lactoferrin is consistent with a passive bacteriostatic function of lactoferrin via iron sequestration. Moreover it demonstrates that the bacteriostasis likely has a kinetic as well as a thermodynamic component.

Introduction

Lactoferrin is an iron-binding protein in the same family as serum transferrin.[1] Its biological,[2,3] iron-binding,[4] and structural[5,6] properties have been recently reviewed. Unlike transferrin, only traces of lactoferrin are found in serum; the highest levels in humans are found in colostrum (about 7 g/L) and milk (about 1 g/L). It also is found in nearly all of the exocrine secretions that bathe the mucosal surfaces of bronchial, nasal, lachrymal, and genitourinary passages of the body. Lactoferrin plays several roles in host defense against infection.[2,3] It has antibacterial, antifungal, and antiviral properties. It also plays an important role in inflammation. However its best characterized role is its ability to maintain iron concentrations below levels required for bacterial growth. The ability of milk to provide bacteriostatic activity against certain species of *Escherichia* is due to the thermodynamic capability of lactoferrin to sequester iron and deprive microorganisms of iron essential for their growth.[7,8] Consistent with this, the bacteriostatic activity of lactoferrin is abolished by saturating the available lactoferrin with ferric ion.

Lactoferrin is phylogenetically (about 44% protein homology[9])- and structurally similar to the well-characterized serum iron transport protein, transferrin. Both proteins are bilobal, oblate spheroids. Each lobe encloses an iron-binding site which contains active site residues consisting of two tyrosines, a histidine, and an aspartate. Both proteins share a concommitant requirement for bidentate carbonate binding upon Fe^{3+} binding.[4,5] However, lactoferrin possesses some crucial differences with respect to iron biochemistry from transferrin. Lactoferrin retains iron down to pH 2, whereas iron is lost quantitatively from transferrin by pH 4.[7,10] The formation constant for the ferric complex of lactoferrin is about 260-fold greater than that of transferrin[7] at physiological pH. This greater stability has been attributed to differences in the relative stability of the favored conformations for the metal complex and free protein.[5,6,11,12]

The iron-complexing ability of lactoferrin is central to its role as a bacteriostatic agent. Despite this there are pathogenic bacteria that are able to extract iron from lactoferrin for their growth. This occurs either through the production of siderophores capable of removing iron from lactoferrin, by absorption of heme iron, or by direct removal of iron from lactoferrin.[13]

The detailed structural data now available for lactoferrin, both as the apoprotein[14] and as the Fe^{3+} complex,[5,6] provide the opportunity for a clearer assessment of the mechanism for iron release from this protein and the high stability of its ferric complex. Of the siderophores or siderophore analogs found to be capable of removing iron from transferrin proteins at significant rates, all have catechol groups as the bidentate ligand subunit. The recently characterized amonabactin siderophores contain two catechol groups and are produced by pathogens that cause human diseases including septicemia, cholera, and enteritis.[15] Several of these organisms are also producers of the tricatecholate siderophore enterobactin (Figure 1). Although enterobactin is not effective in serum as an agent of iron removal from transferrin (because it binds to serum albumin), the more hydrophilic catecholate ligands are effective.

We have earlier explored the kinetics and detailed mechanism by which iron is removed from serum transferrin and bound to several ligands, including 3,4-LICAMS (Figure 1).[1,16–18]

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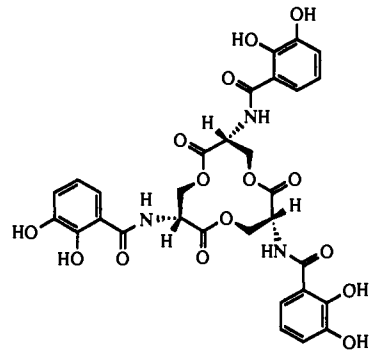
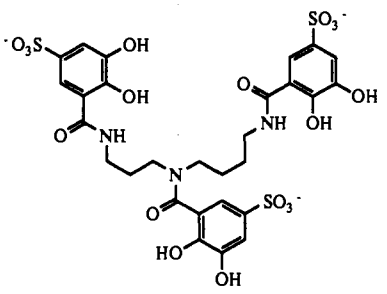


A.



B.

Figure 1. Structural formulas of (A) enterobactin and (B) 3,4-LICAMS (1,5,10-N,N',N''-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane).

In the preceding paper,[1] we have discussed the reversibility of iron binding to transferrin and the mechanism of iron release. Here we contrast the 260-fold greater stability of lactoferrin relative to transferrin and address the question of whether this is largely due to a greater rate of iron binding by the protein or to a slower rate of iron release. The role of the differences in structure in the two proteins and their relative iron-binding properties will be assessed.

Experimental Section

Chemicals. The 1,5,10-*N*,*N'*,*N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)-triazadecane (3,4-LICAMS) was prepared as previously described.[19] Tris-(hydroxymethyl)aminomethane (Tris) and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffers were obtained from Sigma Chemical Co. Acrylamide and gel electrophoresis supplies were obtained from Bio-Rad. Distilled deionized water was used at all times. All stock solutions and buffers were adjusted to pH 7.4 *at 37 °C* with a calibrated Sigma combination electrode (Trizma R.N. E-4878) using a Brinkmann pH 102 meter with temperature compensation. Thereafter, all stock solutions were stored at 5 °C until the actual experiments were performed. All other chemicals were the best commercially available reagent grade materials.

Diferric Transferrin. Lyophilized apotransferrin (>98% pure, Sigma) was saturated according to the procedure of Bates[20] modified by Carrano[21] in 10 mM Tris-HCl (pH 7.4), 0.1 M $NaClO_4$, and 5 mM $KHCO_3$. Only 95% of available sites were saturated (ϵ_{279}(ApoTf) = 9.23×10^4 M^{-1} cm^{-1}) with freshly prepared standardized $Fe(NTA)_2$, pH 4, to insure against nonspecific binding of excess iron. The metal site concentrations were determined from ϵ_{466}(FeTf) = 2500 M^{-1} cm^{-1} (iron concentration) on the basis of the formation of the carbonate complex.[22]

Diferric Lactoferrin. Lyophilized apolactoferrin (70% pure, Sigma) was further purified by chromatography on a Cu^{2+}-affinity gel which was obtained by coupling iminodiacetic acid to epoxy-activated Sepharose 6B (Pharmacia) according to Porath.[23] A modified chromatographic procedure[24] was followed using a gradient from pH 8.2 to 2.8 in 50 mM Tris–acetate buffer. Lactoferrin eluted at ~pH 4. These fractioins were combined, concentrated, and rechromatographed on a Sephadex G-150 (Pharmacia) to remove higher weight molecular weight contaminants[24] such as IgA. Buffer changes were made by dialysis against the desired buffer.

The apolactoferrin preparation contained about 15% residual iron as estimated from A_{280}/A_{470} = 25–28 and A_{410}/A_{470} = 0.84–0.86 for pure diferric lactoferrin.[25] [A limitation of this experiment is the inability to remove nonspecifically bound iron without affecting the normal properties of the actual metal-binding sites. Under this limitation any real kinetic differences in the N- and C-terminal sites for diferric lactoferrin would be masked.] Apolactoferrin concentration was estimated from ϵ_{280} = 8.85×10^4 M^{-1} cm^{-1}. Diferric lactoferrin was also prepared according to Bates;[20] however, dialysis against or inclusion of 0.1 M $NaClO_4$ in the buffer was avoided as this sometimes resulted in clouding or precipitation of both apolactoferrin and diferric lactoferrin. The metal site concentrations were determined from ϵ_{470}(FeLf) = 2300 M^{-1} cm^{-1} (obtained by titration of purified apolactoferrin with standardized $Fe(NTA)_2$).

The final protein preparations were dialyzed against the desired buffer and concentrated on an Amicon PM-10 ultrafiltration membrane. Protein solutions were stored at 5 °C and clarified by passage through a 0.2-μm filter membrane just prior to each experiment.

The purity of the final protein preparations were ascertained by discontinuous polyacrylamide gel electrophoresis (DISC-PAGE) at pH 8.9 in a 25 mM Tris-HCl buffer according to Davis[26] and also in the presence of sodium dodecyl sulfate by the method of Laemelli[27] in a 7.5% separating gel and 3% stacking gel (2.8% cross-linker). Purities were ascertained to be >97% by densitometry of Coomassie stained gels.[27]

Visible Spectroscopy. Kinetic studies of iron removal from diferric transferrin were performed as described earlier,[18] except using a water-jacketed cell holder maintained at 37 °C with a Brinkmann Lauda K-2/R circulating constant-temperature bath. The absorbance increase at 520 nm was monitored on a Cary 118 spectrophotometer.

Kinetics of iron removal from diferric lactoferrin with 3,4-LICAMS were studied in the same manner as described above for diferric transferrin except longer sampling intervals were taken. Typical reaction cocktails consisted of a 500-μL protein sample (0.2 mM) in 0.1 M Tris-HCl (pH 7.4), 0–500-μL stock 3,4-LICAMS solution, and a balance of buffer to give a final reaction volume of 1.000 mL and a final FeLf concentration of about 0.13 mM. All solutions were equilibrated to 37 °C, and reactions were started by rapid transfer of ligand solution with Eppendorf digital pipets to the protein–buffer solutions followed by several inversions of the capped cell to effect mixing. The first data point was obtained 5 min after mixing, where it was assumed that the temperature had re-equilibrated.

A repetitive scanning accessory was used to obtain absorbance versus time data at constant time intervals. However, some full spectral scans from 200 to 800 nm were obtained as a function of reaction time on a microprocessor-controlled HP 8540A VIS–UV spectrophotometer to allow examination of their derivative spectra ($dA/d\lambda$) and to facilitate data storage and manipulation.

Results and Discussion

Iron Removal from Diferric Transferrin by 3,4-LICAMS at 37 °C. A determination of rates of iron removal from diferric transferrin at 37 °C was desired for direct comparison to previous studies with transferrin at lower temperatures[18,21] and with lactoferrin, which required an elevated temperature for appreciable removal rates. The spectral changes resulting from addition of 3,4-LICAMS to a solution of diferric transferrin at 37 °C are shown in Figure 2. The band maximum shifts smoothly from the 470-nm peak of diferric transferrin to the more intense ~495-nm peak of Fe–3,4-LICAMS. These changes are identical to those seen at 25 °C[17,18] and indicate that transferrin-bound iron is being removed by 3,4-LICAMS. Iron is completely removed as judged from the A_{280}/A_{470} ratio of the recovered apoprotein from a Bio-Rex AG-1-X4 anion-exchange column.

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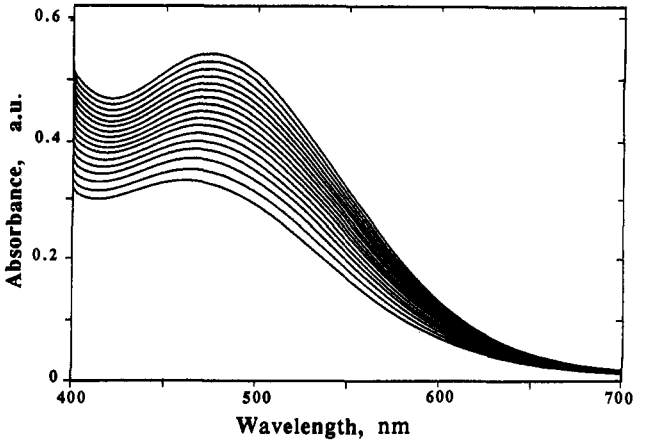

Figure 2. Spectral changes accompanying iron removal from transferrin. The bottom curve represents unreacted transferrin, and the top curve, the final product ferric 3,4-LICAMS. Conditions: 0.1 M Tris-HCl, pH 7.4, 37 °C; [Tf] = 0.062 mM, [LICAMS] = 12.3 mM, ambient CO_2. Each spectrum was taken at successive 2-min intervals after the start of reaction.

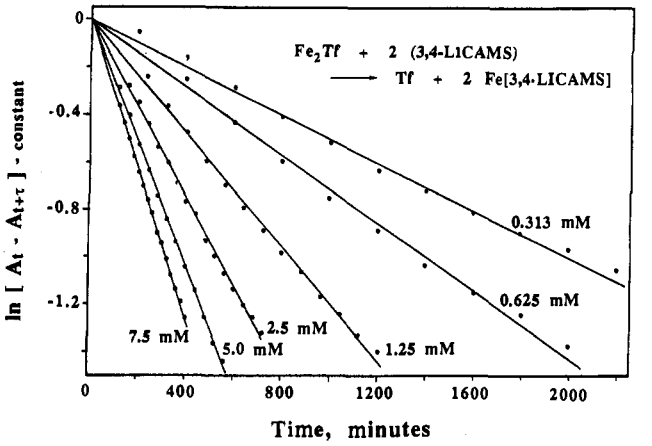

Figure 3. Guggenheim plots for iron removal from diferric transferrin (0.0625 mM) by various concentrations of 3,4-LICAMS at 0.1 M Tris-HCl, pH 7.4, ambient CO_2, and 37 °C.

We have earlier reported that iron removal from diferric transferrin by 3,4-LICAMS is a biphasic process. Baldwin[28,29] found similar biphasic kinetics with EDTA; however, the high concentrations of EDTA necessary to remove iron produce an artificially ionic strength, and ionic strength is known to play a key role in iron removal.[17] Because of experimental limitations imposed by the lactoferrin iron removal, kinetic data of sufficient accuracy to resolve the biphasic rates could not be obtained. Instead we compare the average rates of iron removal from the two proteins. These were obtained by Guggenheim plots[30] of the absorbance versus time. These plots (Figure 3) were nearly linear for 3–4 half-lives for concentrations of 3,4-LICAMS from 0.313 to 7.5 mM, representing a 5–120-fold excess of ligand to protein.[31] The pseudo-first-order rate constants (k_{obs}) were obtained by linear regression analysis of data from the first four half-lives and are compared with those previously obtained at 25 °C in Figure 4.[21] The k_{obs} vs [3,4-LICAMS] plot is hyperbolic in both cases and indicates that no drastic change in mechanism has occurred upon raising the temperature to 37 °C. For the same ionic strength conditions, the average rates of iron removal by 3,4-LICAMS (L) from either transferrin or lactoferrin can be described by the same kind of equation as Michaelis–Menton kinetics:[21]

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(31) Data from the first 10–20 min of these runs were not included, since this is disproportionately due to iron removal from the N-terminal site and direct comparison with the lactoferrin rates were desired.

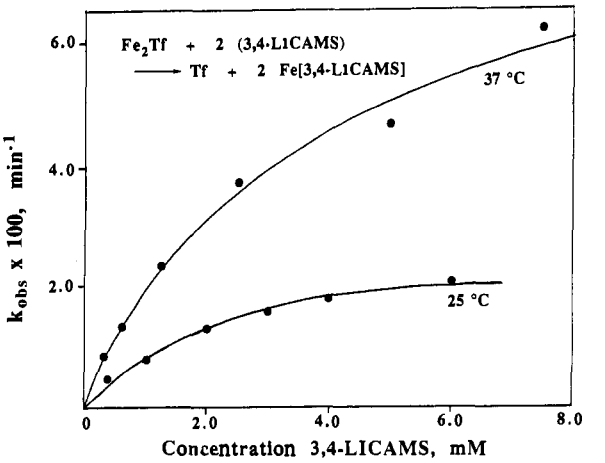

Figure 4. Plots of the observed rate constants for iron removal from transferrin (0.0625 mM) vs the concentration of 3,4-LICAMS at 37 and 25 °C.

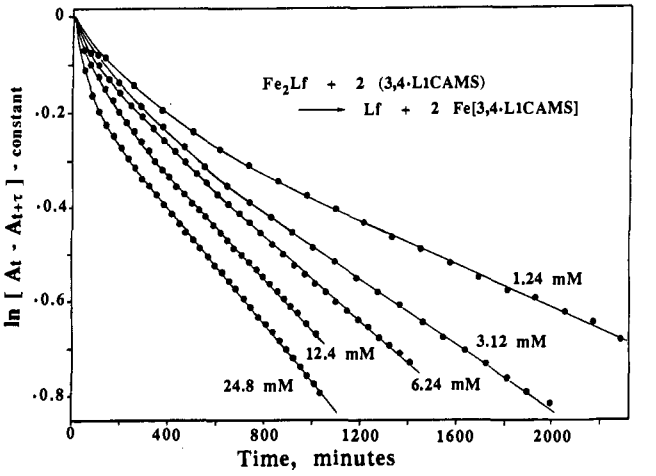

Figure 5. Infinite-time plots for iron removal from diferric lactoferrin (0.0625 mM) by various concentrations of 3,4-LICAMS at 0.1 M Tris-HCl, pH 7.4, and 37 °C.

$$k_{obs} = \frac{k_{max}K[L]}{1 + K[L]} \quad (1)$$

The underlying mechanism behind this resultant behavior will momentarily be ignored while comparing the quantitative differences in average rates of Fe^{3+} removal from transferrin versus lactoferrin. The change in k_{obs} versus [L] for both proteins is amenable to double-reciprocal plots analysis to give values of $k_{max} = 0.063(4)$ min^{-1} and $K = 4.9(3) \times 10^2$ M^{-1} for transferrin at 37 °C, whereas $k_{max} = 0.029(2)$ min^{-1} and $K = 4.1(6) \times 10^2$ M^{-1} at 25 °C.[21]

Iron Removal from Diferric Lactoferrin with 3,4-LICAMS. Addition of 3,4-LICAMS to a solution of diferric lactoferrin yields a series of spectra identical to that obtained with diferric transferrin (Figure 2) except that the absorbance increases occur at a much slower rate. Initial data analysis gave Guggenheim plots with pronounced curvature, consistent with the biphasic kinetics described for transferrin.[1] However, it was difficult to obtain accurate, stable infinite time values due to the long half-lives ($7t_{1/2}$ = 200 h) required for 99% completion and resultant development of turbidity in the solutions. Therefore final absorbance values were calculated from $\epsilon_{520} = 4960$ M^{-1} cm^{-1} for Fe–3,4-LICAMS assuming complete iron removal, and rate constants for the average rate of Fe^{3+} removal were obtained from the linear portions of the $(A - A_\infty)$ plots by linear regression solutions. At very long times, A_{obs} exceeded A_{calc} due to the increased scattering for turbid solutions. These plots of [ln$(A - A_\infty)$ + constant] versus time (Figure 5) are curved, indicative of a slow side reaction or decomposition accompanying the faster

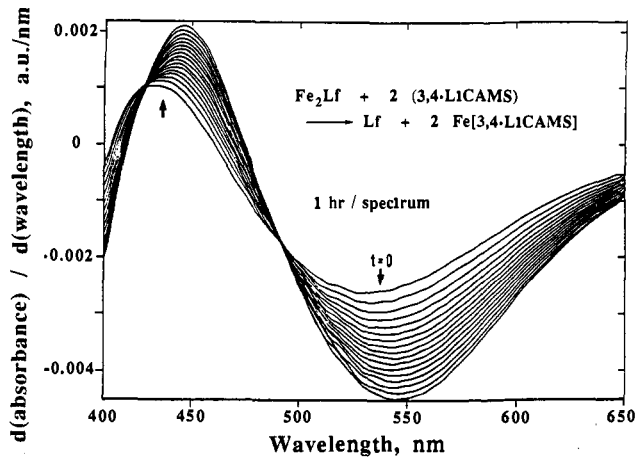

Figure 6. Derivative spectra obtained during iron removal from diferric lactoferrin by 3,4-LICAMS. Derivative spectral changes increase with time; each spectrum was taken at successive 1-h intervals after the start of the reaction.

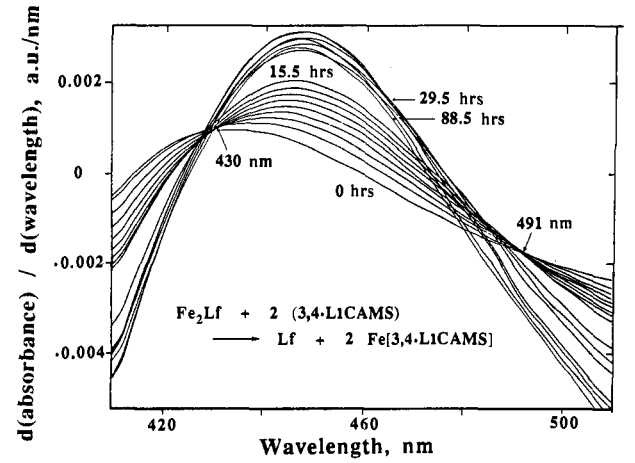

Figure 7. Derivative spectra at very early and long times for iron removal from diferric lactoferrin by 3,4-LICAMS.

iron-removal reaction or the incomplete averaging of the rates of iron removal from the two Fe^{3+} sites.

The presence of isosbestic points in a series of spectra is good evidence of a clean conversion between only two absorbing species with no buildup of intermediates. No isosbestic points are seen in the iron-removal reaction with 3,4-LICAMS since the spectra (ϵ vs λ) of the absorbing species do not cross at any point. However isosbestic points in the derivative spectra ($dA/d\lambda$) can be similarly interpreted, and the greater curvature of the derivative spectrum leads to a crossover in ($d\epsilon/d\lambda$) and hence an isosbestic point. The derivative spectra obtained during the course of the iron-removal reaction from diferric lactoferrin with 3,4-LICAMS are shown in Figures 6 and 7. The derivative spectra in Figure 6 present isosbestic points at 430 and 491 nm over most of the reaction time from 3 to 18 h. These isosbestic points are missed at times earlier than 3 h, while spectra at >29.5 h show an increasing deviation. These results are consistent with a fast process (nonspecifically bound iron) which gave derivative spectra that miss the isosbestic point, followed by a slower process (isosbestic behavior) which is iron removal from the specific iron-binding sites. Incipient turbidity due to protein precipitation and absorbance increrases due to ligand oxidation are seen at times longer than 25 h and lead to increasing absorbance due to scattering. This is consistent with the more steeply deviating scans (480–520 nm) seen at later times. In contrast, derivative spectra for transferrin gave similar isosbestic points through to reaction completion.

The plot of the observed rate constants versus ligand concentration in Figure 4 gives a hyperbolic profile with $k_{max} = 6.5(4) \times 10^{-4}$ min^{-1} and $K = 3.3(1) \times 10^{2}$ M^{-1}, when interpreted according

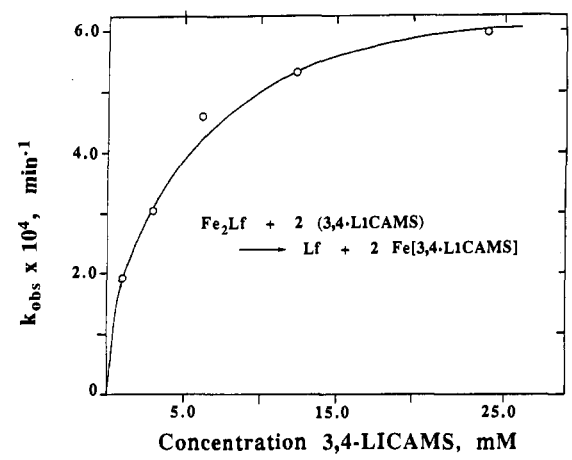

Figure 8. Plot of the observed rate constants for iron removal from lactoferrin (0.0625 mM) versus the concentration of 3,4-LICAMS at 37 °C.

to the kinetic model (eq 1).[16] This result indicates that iron removal from lactoferrin proceeds by a mechanism essentially the same as that for the removal of iron from transferrin, and is consistent with the crystal structures that show the iron-binding sites in lactoferrin and transferrin are essentially the same.[5,6] However there is a larger kinetic barrier to iron removal in lactoferrin: the k_{max} is slower by a factor of 100! The values of the apparent equilibrium constants indicate that there is only a slightly lower affinity between the ligand and diferric lactoferrin relative to diferric transferrin.

The recent structural information shows that there are two conformations for the iron-binding sites in the two different lobes of the transferrins.[5,6,12] The iron complex favors the "closed" conformation in which the iron is forced well below the protein surface and is inaccessible to attacking ligands. The apoprotein favors an "open" conformation in which the iron-binding site is near the protein surface and exposed to the surrounding solution. The apolactoferrin structure shows several features that explain observations indicating that the "closed" conformation is more stable relative to "open" as compared with transferrin. Indeed, one lobe of the lactoferrin apoprotein, even though there is no bound Fe^{3+}, was found in the "closed", normally metal-bound, conformation.[12] Anion binding to the protein is found in the structure and is suggested as essential for stabilization of the "open" form of the protein. Such anion binding has been suggested as the key step in promoting the conformational change as presented in the mechanism for iron release from transferrin to all sequestering agents.[17] Baker *et al.* have supported this view,[5] speculating that the closed C-lobe of the apolactoferrin structure was due to the low ionic strength of the crystallization conditions.

In summary, the kinetics of iron removal from lactoferrin to the catecholate ligand 3,4-LICAMS are found to follow the same behavior as that of transferrin. However, under comparable conditions, the rate of iron removal from lactoferrin is 100 times slower than from transferrin. This explains nearly all of the greater thermodynamic stability of the lactoferrin complex relative to transferrin. The rates of iron complexation are therefore essentially the same. The slower iron-removal rate correlates well with what is now known about the relative stabilities of the "closed" versus "open" structures of these two closely related proteins. Lactoferrin, with a bacteriostatic role dependent on its ability to deny iron to an invading organism, seems extraordinarily well suited for this role. Not only does it have a very high iron affinity, but its rate of change to a conformation in which the iron site is exposed is very slow: it locks up iron and throws away the key.

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