Magnesium potentiation of iron-transferrin binding in 0.10 M phosphate: kinetic and structural studies

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Prior investigations have shown that phosphate ions destabilize iron-transferrin binding. We have demonstrated previously that iron binding to transferrin (buffered in 0.10 M phosphate) from iron(III) nitrilotriacetate or iron(III) chloride is a magnesium-dependent process. The kinetics of magnesium's effect on iron binding was followed spectrophotometrically at 470 nm, and circular dichroism was used to monitor changes in secondary structure (186–250 nm) induced by this cation. Circular dichroic measurements in the aromatic region (250–350 nm) showed time-dependent activity when iron binding took place in the presence of magnesium. However, similar studies in the intrinsic region (186–250 nm) failed to reveal significant magnesium-induced changes in the secondary structure of transferrin. Kinetic modeling of the data indicate that iron binding to transferrin can be described by a sequence of three reactions, the last of these being dependent on the magnesium concentration. Thus, magnesium may have induced a localized structural change in transferrin, preventing the destabilizing effect of the phosphate anion.

Keywords: transferrin; iron; magnesium; blood transport protein

Introduction

Transferrin is a glycoprotein that is involved in plasma iron-transport. Iron binding to transferrin is synergistically dependent on the presence of carbonate or chemically similar organic anions.[1] Attempts to use inorganic anions such as phosphate in the synergistic role have been unsuccessful.[2] Furthermore, the presence of high phosphate concentration (0.050–0.10 M) destabilizes the iron-transferrin-anion complex.[3,4] Divalent cations may be involved in iron-transferrin binding as Graham and Bates[5] reported that the presence of Ca^{2+} increased the stability of iron bound to transferrin against attack by chelating agents. In a recent report from our laboratory, we demonstrated that iron transfer from either Fe(III)-nitrilotriacetate or freshly prepared ferric chloride to transferrin (in 0.10 M phosphate) is a magnesium-dependent process.[6] This ability of magnesium to influence iron binding to transferrin suggests a new role for this cation.

Since it has been widely reported that iron-transferrin binding is destabilized by the presence of high phosphate concentrations, our earlier reported work merited further studies. Consequently, the present investigation focuses on the possible mechanisms for magnesium's role in potentiating iron binding. We postulate that magnesium may induce an allosteric effect on transferrin that results in a modification of the binding sites to prevent the destabilizing effect of phosphate anions. To investigate this, we have performed kinetic studies of iron binding to transferrin in the absence and presence of varying concentrations of magnesium and structural studies using circular dichroism (CD) to measure changes in the secondary structure of transferrin as affected by magnesium.

Materials and methods

Human apotransferrin (Boehringer Mannheim Biochemicals) was dissolved in a 0.10 M phosphate buffer

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(Na_2HPO_4/NaH_2PO_4, pH = 7.47) to yield final concentrations of 0.031 mg/ml; 1.0 mg/ml; 2.0 mg/ml; and 5.0 mg/ml. Additional apotransferrin solutions (1.0 mg/ml) were prepared in 0.10 M tris-HCl buffer (pH = 7.47). Magnesium nitrate solutions of 0.066 mM; 0.13 mM; and 0.66 mM were prepared in 0.10 M phosphate or 0.10 M tris-HCl. Iron(III)-NTA solutions (pH 2.5–3.0) of 0.032 mM; 0.064 mM; and 0.32 mM were prepared using a 1:2 molar ratio of iron(III) chloride to nitrilotriacetic acid. All solutions were prepared under atmospheric pCO_2. The final pH of all reactions ranged between 7.3–7.4, and all reactions were carried out at room temperature. Kinetic studies were made at 470 nm[7] using a HP8452 Diode Array uv/vis spectrophotometer. CD measurements were made using a JASCO Spectropolorimeter, and the data were analyzed using JASCO Secondary Structure Estimation software version 1.02.[8,9] The kinetic data were analyzed by Chemical Kinetics and Dynamics software.[10] The best set of rate constants was determined by fitting the observed form of the time dependence of concentrations to the software-generated results. The data used to plot *Figures 3* and *4* were analyzed by analysis of variance (ANOVA), and group means within each significant ANOVA were separated by Scheffe's test.[11]

Results

Loading iron into the specific binding sites on transferrin to form an FE(III)-transferrin-anion complex results in an absorption maximum in the 460–470 nm region.[1] In this investigation, similar maxima were obtained using both optical absorption and circular dichroism (CD),[12] indicating that we were measuring specific iron binding to transferrin. The CD wavelength scan of iron-loaded transferrin in the 370–500 nm region showed a negative extremum at 460 nm, and a significant increase in this extremum occurred when magnesium was present during iron binding. These ellipticity measurements parallel our previously reported visible absorption data taken at 470 nm, further demonstrating the ability of magnesium to potentiate this effect.[6]

In the present study, CD changes in the protein's aromatic region (260–290 nm) and in the 300–340 nm region demonstrate time-dependent activity only when iron binding takes place in the presence of magnesium (*Figure 1*). In the 260–290 nm region and the 300–340 nm region, iron binding solutions lacking magnesium (*Figure 2*) did not show discernible time-dependent activity.

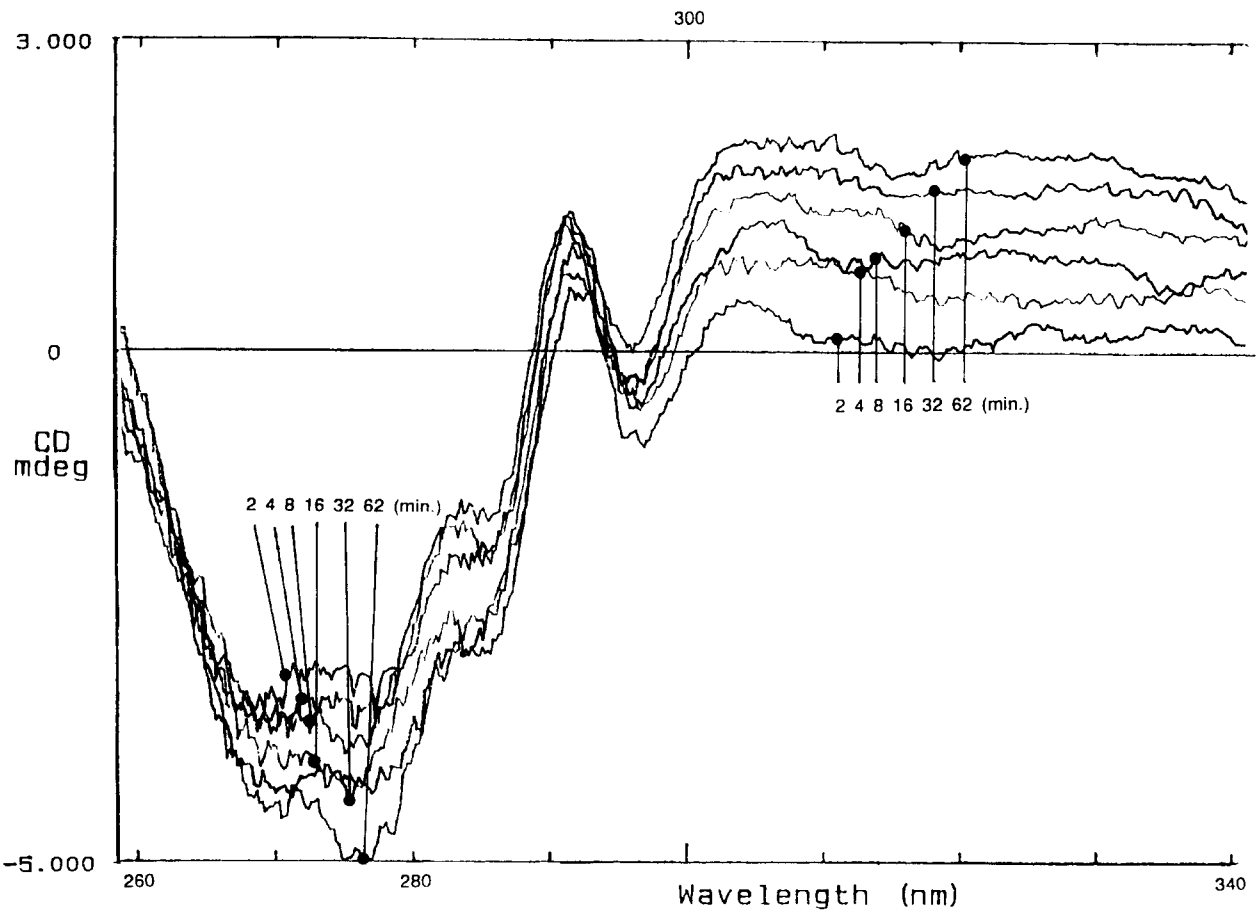

Figure 1 Typical CD scan (260–340 nm) of iron binding to apotransferrin (0.10 M phosphate buffer) in the presence of magnesium. There is time-dependent activity in the 265–285 nm region and in the 300–340 nm region.

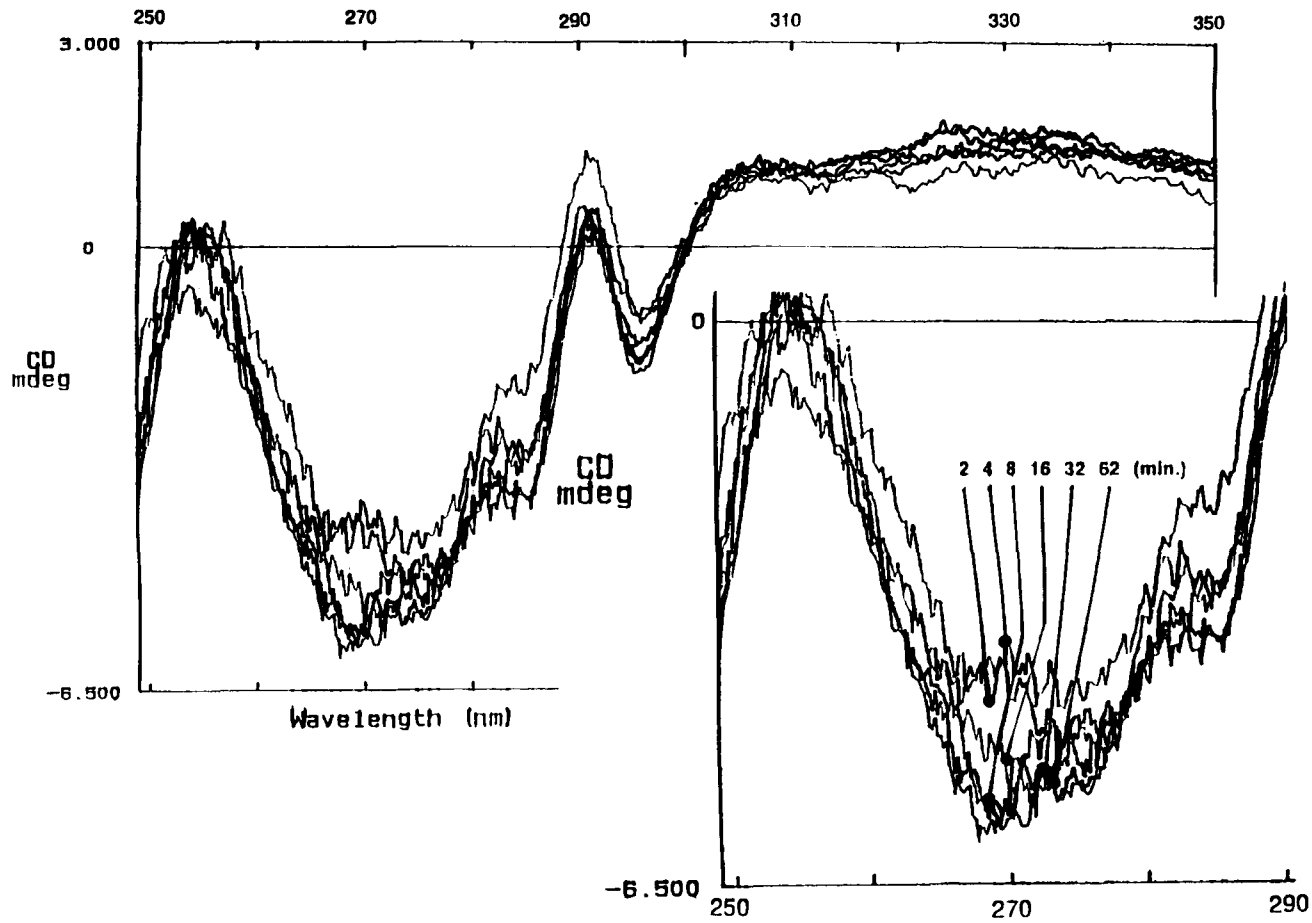

Figure 2 Typical CD scan (260–340 nm) of iron binding to apotransferrin (0.10 M phosphate buffer) in the absence of magnesium. There is no discernible time-dependent activity in either the 265–285 nm region or the 300–340 nm region.

Normal serum values for inorganic phosphate are reported to be 0.001–0.0015 M.[13] Price and Gibson[14] demonstrated that perchlorate ions in excess of 0.10 M induced a conformational change at the metal binding site. Consequently, the use of 0.10 M phosphate concentration in this protocol suggested that a conformational change in transferrin may have occurred, and CD scans in the intrinsic region (186–250 nm) were made subsequently to measure any changes in the secondary structure. However, there was no significant difference in secondary structure when comparing apotransferrin prepared in 0.002 M phosphate to that prepared in 0.10 M phosphate buffer. Additional CD scans (186–250 nm) were made of the following 0.10 M phosphate solutions; apotransferrin plus magnesium, apotransferrin plus Fe(III)-NTA, and apotransferrin plus Fe(III)-NTA and magnesium. There was no significant difference in transferrin's secondary structure when comparing all five of the above solutions.

Figure 3 depicts the kinetics of iron-transferrin binding as determined by changes in absorbance at 470 nm. This graph appears to represent a biphasic reaction, with the second phase exhibiting magnesium dependence. Further kinetic studies were performed by recording the change in absorbance each second over a 30-second period. There was no significant difference between the magnesium-free and magnesium-containing solutions of Fe(III)-transferrin-NTA during this time period, indicating that the first phase was not magnesium-dependent. During all kinetic studies, the absorbance at 470 nm and 320 nm wavelengths was monitored simultaneously and yielded comparable data demonstrating the effect of magnesium on iron-transferrin binding.

Kinetic studies were run comparing apotransferrin solutions (0.10 M phosphate) to similar phosphate-free solutions buffered by 0.10 M tris-HCl. In magnesium-free solutions, more Fe(III)-transferrin-NTA was formed initially in the tris-buffered solutions (*Figure 4;* curve a) compared to the phosphate-buffered solutions (*Figure 4;* curve d). Although magnesium potentiated iron binding in the presence of either buffer, the concentration-dependence that was found in phosphate-buffered solutions was not evident in the tris-buffered solutions (*Figure 4;* curves b,c,e, and f).

Discussion

Kinetic modeling of the data indicate that iron binding to transferrin can be described by a sequence of three reactions, the last of these being magnesium-dependent in a concentration-dependent manner. The

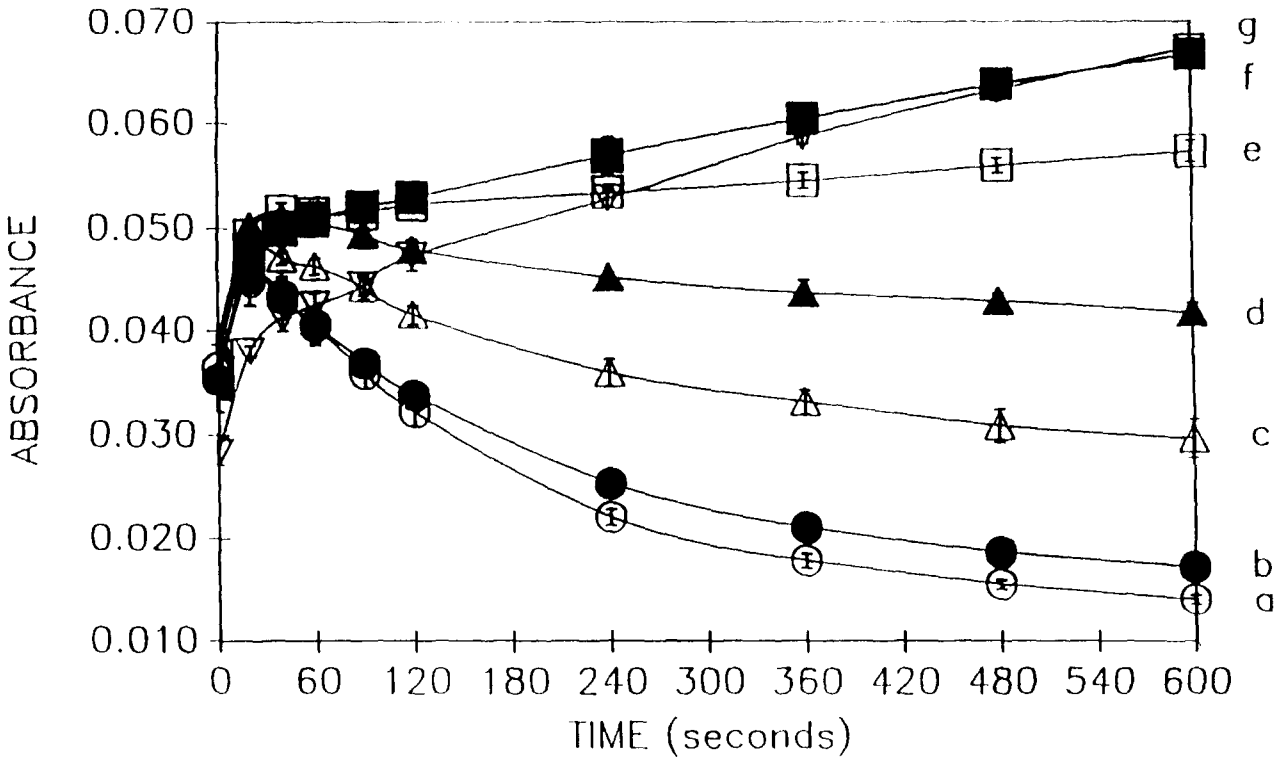

Figure 3 Time-course plot of varying Mg^{2+} concentrations [Mg^{2+}: curves a = 0, b = 0.008 mM, c = 0.032 mM, d = 0.064 mM, e = 0.128 mM, f = 0.256 mM, g = 0.512 mM] in a solution containing apotransferrin (2 mg/ml; Pi = 0.10 M) and iron(III)-NTA (0.065 mM).

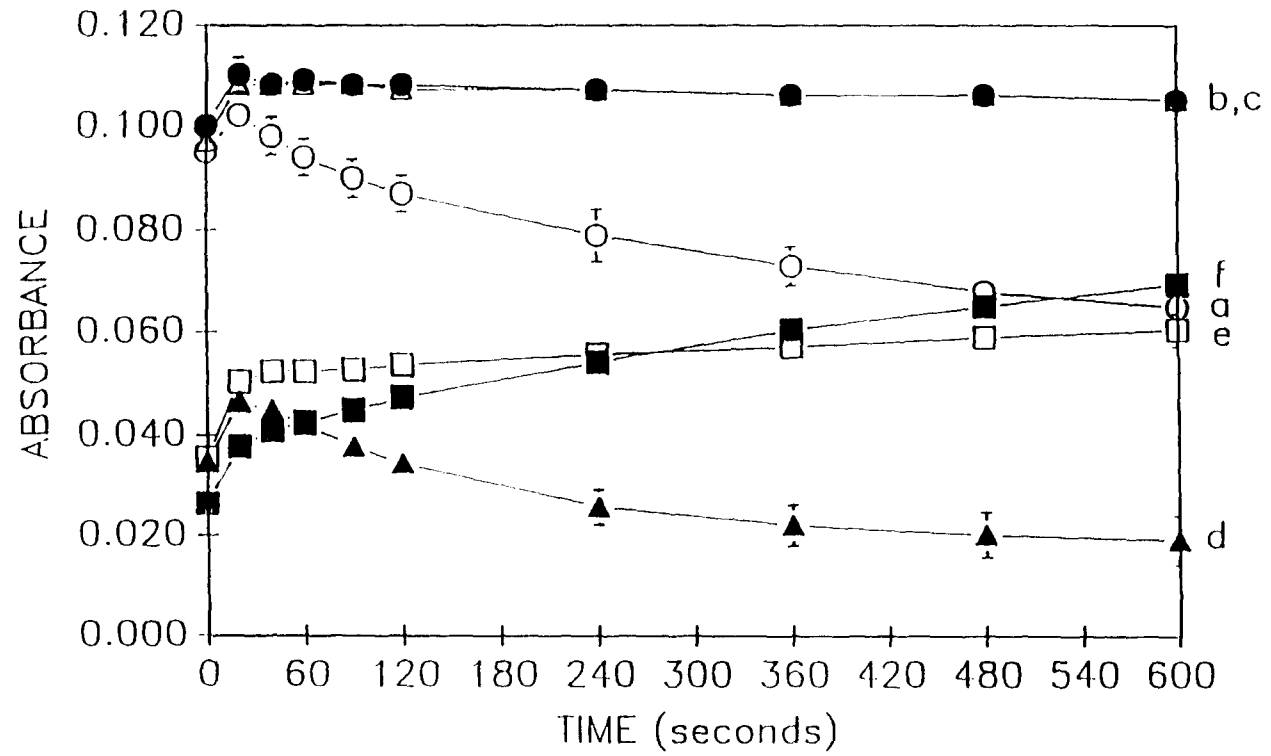

Figure 4 Time-course plot comparing solutions of Fe-NTA binding to transferrin (1 mg/ml) buffered in phosphate (0.10 M; pH = 7.47; curves d,e, and f) to tris-HCl (0.10 M; pH = 7.47; curves a,b, and c) buffered solutions. (Mg^{2+}: curves a,d = 0; curves b,e = 0.13 mM; curves c,f = 0.53 mM).

first phase of the reaction (0–30 seconds) depicted in *Figure 3* is the delivery of iron to form a ternary complex with transferrin.[15] The series of slopes formed from 30–600 seconds clearly demonstrates a concentration-dependent effect of magnesium on iron binding. The following reaction sequence appears to account for the effect of magnesium on binding and is consistent with the data.

Apotransferrin +

$$\text{Fe(III)NTA} \underset{k_{-1} = .05\ s^{-1}}{\overset{k_1 = 35\ Lm^{-1}s^{-1}}{\Leftrightarrow}} \text{Fe(III)-transferrin-NTA} \quad (1)$$

Fe(III)-transferrin-NTA +

$$\text{Pi} \underset{k_{-2} = .0001\ L^2m^{-2}s^{-1}}{\overset{k_2 = .2\ s^{-1}\ \text{(pseudo first order)}}{\Leftrightarrow}} \text{transferrin-Pi} + \text{Fe(III)} + \text{NTA} \quad (2)$$

Fe(III)-transferrin-NTA +

$$\text{Pi} + \text{Mg} \underset{k_{-3} = .0001\ s^{-1}}{\overset{k_3 = 40\ Lm^{-1}s^{-1}\ \text{(pseudo second order)}}{\Leftrightarrow}} \text{Fe(III)-transferrin-NTA-Pi-Mg} \quad (3)$$

This model indicates that there are at least two equilibria involved in iron binding in the absence of magnesium, and a third that occurs only in the presence of magnesium. The first equation accounts for the iron-transferrin-anion signal recorded at 470 nm using both spectrophotometric and circular dichroic (CD) measurements. The second equation accounts for the loss of the signal over time in the absence of magnesium, most probably due to phosphate destabilization of the iron-transferrin-anion complex. The third equation accounts for the ability of magnesium to potentiate iron-transferrin binding in the presence of high phosphate concentrations. Although the exact nature of the third reaction has not been elucidated, we suggest that magnesium may effect a localized structural change to stabilize iron binding against phosphate anion attack. The rate constants used in the rate expressions allowed us to replicate the form of our kinetic data, but they did not yield a direct fit. As a consequence, they should be viewed as relative rather than absolute. However, at low (0.0081 mM to 0.26 mM) magnesium concentrations, a quantitative fit between experimental and calculated concentrations of Fe(III)-transferrin-anion can be obtained by use of a constant multiplier of 42.2 ± 3.93.

Transferrin binds iron with a high affinity in non-phosphate buffered systems (stability constant $K = 10^{20}$).[16] In comparison, our data demonstrate a relatively slow development of iron binding as measured by optical absorbance. This slow signal development may be the result of a significantly larger concentration of destabilizing phosphate anion (100 mM) when compared to the concentrations of transferrin's binding sites (0.025 mM), iron (0.032 mM), and magnesium (0.066 mM). There is also a difference in the rate of signal development when comparing the CD data (*Figure 1*) with the kinetic data (*Figure 3*). This may be due in part to the differences in concentrations of iron, transferrin, and magnesium used in the two protocols. The following concentrations were used in the CD studies; $[Fe^{3+}] = 0.032$ mM; apotransferrin = 1 mg/ml; $[Mg^{2+}] = 0.66$ mM; and [Pi] = 23 mM, whereas the following concentrations were used in the kinetic studies; $[Fe^{3+}] = 0.065$ mM; apotransferrin = 2.0 mg/ml; $[Mg^{2+}]$ = (0 to 0.512 mM); and [Pi] = 100 mM. The time-dependent activity in the 300–320 nm region (*Figure 1*) reflects a change in the dihedral angle of disulfide bonds.[17] Thus, time-dependent activity in the presence of magnesium suggests localized structural changes are being made. Since the magnesium concentration is significantly lower than the phosphate concentration, it is unlikely that the potentiation of iron binding is simply the result of magnesium bonding directly with phosphate to prevent its attack on the binding site.

Inorganic anions such as phosphate appear not to exhibit a synergistic role in iron binding,[2] and they destabilize the iron-transferrin-anion complex.[3,4] This type of observation has led to the hypothesis that inorganic anions may play a role in cellular ion release from transferrin.[4] Although the initial Fe(III)-NTA concentrations were sufficient to saturate transferrin's binding sites (*Figures 2* and *3*), our absorbance measurements at 470 nm did not confirm that saturation had occurred. This is to be expected when iron-transferrin binding takes place in the presence of high phosphate concentration and in the absence of added carbonate.

It has been reported that transferrin binds transition metals but does not bind Mg^{2+}.[17,18] Unpublished atomic absorption studies by this laboratory using previously reported techniques[6] to separate solution Mg^{2+} from protein bound Mg^{2+} concur with this finding. Calculations using the Mg^{2+} concentrations of this protocol and the solubility product of $Mg(HPO_4)_2$, $K_{sp} = 10^{-5}$,[19] do not indicate that Mg^{2+} will precipitate out of solution. Rogers and coworkers[3] have demonstrated previously that the iron-transferrin complex formed in 50 mM phosphate under atmospheric conditions of CO_2 is unstable. When they added 50 mM carbonate to the iron-transferrin solutions containing 50 mM phosphate, the complex achieved stability for 24 hr. Our iron-transferrin complexes buffered in 100 mM phosphate were similarly unstable under atmospheric conditions of CO_2 with no additional carbonate added.

In these studies, the apotransferrin was used as received from the supplier without further purification. This may raise the question as to whether any impurity introduced during commercial purification may be acting at the iron-transferrin binding sight. If such an impurity were influencing iron binding, then it must be recognized that in our protocol such an effect only occurred in the presence of magnesium.

Our typical CD wavelength scans took approximately two minutes to complete, so there are no recorded ellipticity measurements for the zero time-point. The CD activity at the 275 nm region suggested that tyrosine participated in the formation of the chromophore,[17] but time-dependent activity was only evident when magnesium was present (*Figure 1*). Addi-

tionally, there was noticeable time-dependent activity in the 300–340 nm region in the presence of magnesium (*Figure 1*), but a lack of same in the absence of magnesium (*Figure 2*). In this protocol, the apparent lack of significant change in secondary structure, as measured by CD scans in the intrinsic region (186–250 nm), is consistent with previous findings of Mazzurier and coworkers[20] and Mason and Brown.[21]

In conclusion, our data suggest that magnesium's presence may have stabilized the binding site or a nearby modifier site[22] from attack by the phosphate anion. To accomplish this, magnesium may be involved in some localized structural preparation to facilitate iron binding in the presence of high phosphate or high tris-HCl concentration. These findings suggest that the hypothesis calling for anion displacement as a mechanism in Fe^{3+} release from transferrin[4] should also account for Mg^{2+} interactions with this protein.

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