The Exchange of Fe\(^{3+}\) between Pyrophosphate and Transferrin

PROBING THE NATURE OF AN INTERMEDIATE COMPLEX WITH STOPPED FLOW KINETICS, RAPID MULTIMIXING, AND ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY*

(Received for publication, July 1, 1985)

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A detailed study of the exchange of Fe\(^{3+}\) between pyrophosphate and human serum transferrin was undertaken to test the hypothesis of a generalized reaction route for exchange of Fe\(^{3+}\) between transferrin and chelators. The initial rate of Fe\(^{3+}\) transfer from pyrophosphate to apotransferrin-CO\(_{3}\) is highly sensitive to the pyrophosphate to iron ratio with a maximal rate being observed at a ratio of 3:1, consistent with the presence of slowly reactive polymeric species at ratios less than 3:1 as revealed by EPR and kinetic measurements. At a ratio of 4:1 the reaction is distinctly biphasic. The rapid first phase results in the formation of an intermediate postulated as a mixed-ligand complex of the type PP\(_{2}\)-Fe\(^{3+}\)-transferrin-CO\(_{3}\)-. The intermediate has a distinct EPR spectrum and an absorption spectrum similar to that of Fe\(^{3+}\)-transferrin-CO\(_{3}\)-, but with a spectral maximum at 450 nm rather than 465 nm. The second phase principally arises from the slow reaction of polymeric iron-pyrophosphate with the apoprotein and has contributions from the breakdown of the intermediate formed in the first phase. The rate of formation of the intermediate shows a hyperbolic dependence on NaHCO\(_{3}\) and apotransferrin concentrations, the latter suggesting a rate-limiting labilization of Fe\(^{3+}\)(PP)\(_{3}\), perhaps to form species of the type Fe\(^{3+}\)(PP)\(_{2}\), prior to attack by apotransferrin-CO\(_{3}\)-. Multimixing stopped flow spectrophotometry was employed to test the chemical reactivity of the Fe\(^{3+}\) to reduction at various times during the first phase. Surprisingly, a diminution of reactivity of 1000-fold was noted after only 2% of the first phase was completed, indicating a fast initial reaction which is not observed by normal rapid flow spectrophotometry. This initial reaction may involve the binding of iron-pyrophosphate to allosteric sites on the protein. The kinetics of iron removal from Fe\(^{3+}\)-transferrin-CO\(_{3}\)- by PP\(_{2}\) are consistent with a rate-limiting conformational change in the protein as proposed earlier.

Iron exchange reactions are a central aspect of the metabolism of iron. From the procurement of iron from the environment by plants and microorganisms to its insertion into proteins of higher organisms, a wide variety of iron exchange reactions takes place. Often disparate species exhibit striking parallels in their management of this element (1). Ferritin in a wide variety of species and transferrin in vertebrates function to store and transport iron, respectively. In addition, a number of low molecular weight chelators and reductants may be utilized intracellularly (2) or exported (3) by the organism in order to accomplish requisite iron procurement and exchange.

Transferrin\(^{1}\) of serum and lactotransferrin of milk and other body fluids play far more comprehensive physiological roles than was realized just a few years ago. Both transferrin and lactotransferrin have been implicated as regulators of cell proliferation (4) and in humoral mediated immunity (5). The release of iron to cells appears to take place in specific vacuoles (6); however, even in this acidic milieu, low molecular weight chelators may play a direct role in iron exchange.

Studies with acetohydroxyacidic acid (7) and other chelators have led to the formulation of a generalized scheme for the exchange of iron between transferrin and chelators (8). The salient features include a conformational change in the Fe\(^{3+}\)-transferrin-CO\(_{3}\)- complex prior to attack by the chelator followed by the formation of a mixed-ligand complex of the type chelator-Fe\(^{3+}\)-transferrin-CO\(_{3}\)- and then iron removal. Further characterization of the exchange reaction and testing of the proposed reaction route is, however, needed at this point. Pyrophosphate, a complexing agent with a possible cellular iron transport role, was chosen for detailed studies...
since it differs markedly in its structure from the hydroxamate and carboxylic acid chelators studied in detail earlier and since it has long been known to be the most effective chelator for the removal of iron from Fe$^{3+}$-transferrin-CO$_2$ (9). The results have provided some surprises and new insights into the chemistry of iron chelate complexes and transferrin.

MATERIALS AND METHODS

Transferrin and Reagents—All reagents were of the highest purity commercially available and were not further purified. Iron-free human serum transferrin was obtained from Calbiochem-Behring. Apotransferrin was dialyzed against three changes of 20 mM Hepes$^+$ buffer containing 0.15 M KCl at pH 7.4. Final dialysis was against 20 mM Hepes, pH 7.4. The protein concentration was determined by spectrophotometric titration with Fe$^{2+}$-nitritotriacetate while monitoring the absorbance at 456 nm due to the binding of iron to the protein. Fe$^{3+}$-transferrin-CO$_2$ was prepared by mixing equivalent amounts of apotransferrin and Fe$^{2+}$-nitritotriacetate in 20 mM Hepes containing 20 mM NaHCO$_3$ at pH 7.4. The Fe$^{3+}$-transferrin-CO$_2$ plus nitritotriacetate mixture was then dialyzed against three changes of 20 mM Hepes containing 0.15 M KCl and 10 mM NaHCO$_3$ which removes any nitritotriacetate bound to the protein. The protein was then dialyzed exhaustively against 20 mM Hepes at pH 7.4.

Unless otherwise noted, all reactions were carried out in solutions containing 20 mM Hepes and 20 mM NaHCO$_3$ at pH 7.4. Fe$^{3+}$-nitritotriacetate at a ratio of pyrophosphate to iron of 4:1 was prepared fresh daily by adding Fe(NH$_4$)$_2$(SO$_4$)$_2$ in 0.01 M HCl and rinsed thoroughly in deionized water prior to use. Complete oxidation in 5-10 min was verified by monitoring the amplitude of the relative pyrophosphate concentration or the pyrophosphate to iron ratio was changed to the value obtained at a 3:1 ratio followed by air oxidation of the iron. Addition of bathophenanthroline sulfonate, an Fe$^{3+}$ chromophore, to this solution resulted in a freezing time of approximately 5 s. The fine ice crystals were firmly dewar flask employing a split plastic straw insert in the dewar tip to minimize instrument noise from liquid nitrogen bubbling. EPR tubes were calibrated to one another using an iron pyrophosphate solution. To evaluate EPR intensities, double integrations of first-derivative spectra were calculated between the limits of 55 and 220 milliTesla.

Electrophoresis—Urea polyacrylamide gel electrophoresis (11) was used to determine the distribution of iron between the two sites following 50% saturation of the protein. Samples from the EPR kinetic study were quickly thawed one at a time and immediately diluted in tank buffer/glycerol, pH 8.4, solution and loaded onto the gel. The sample was electrophoresed at 400 V for 5 min until the tracking dye had migrated into the gel after which time the next sample was thawed and loaded. Final electrophoresis was done at 120 V. The Comassie Blue R-250 stained gels were scanned with a Hoeffer Scientific densitometer interfaced to the MINC-23 computer for determination of the area under the peaks.

RESULTS

The Transfer of Fe$^{3+}$ from Fe$^{3+}$-Pyrophosphate to Apotransferrin-CO$_2$^+$

Effect of the Pyrophosphate to Fe$^{3+}$ Ratio—The reaction of Fe$^{3+}$-pyrophosphate with apotransferrin-CO$_2$^+$ at various pyrophosphate to Fe$^{3+}$ ratios was monitored at 456 nm using stopped flow spectrophotometry (for conditions see legend to Fig. 1). At a 1:1 ratio three reaction phases were observed. These phases constituted 11, 61, and 28% of the absorbance change and required approximately 0.1 s, 1 min, and 10 min for completion, respectively. To varying extents these phases were observed as the pyrophosphate to iron ratio was changed from 1:1 to 2.5:1. At ratios of 3:1 and greater the initial absorbance burst disappears, and the reaction becomes biphasic. The first phase at a 3:1 ratio requires about 30 s at room temperature and the second phase approximately 10 min.

The pronounced effect of the pyrophosphate to iron ratio on the initial rate of the reaction is shown in Fig. 1. A 24-fold increase in reaction rate is observed at a ratio of 3:1 compared to the value obtained at a 1:1 ratio. A sharp peak of reactivity is noted at the 3:1 ratio followed by a steep decline as the relative pyrophosphate concentration is further increased.

The simplest explanation of these phenomena lies in the complex equilibria between monomeric and polymeric Fe$^{3+}$-pyrophosphate species. In aqueous solutions at pH 7.4 a stable Fe$^{3+}$ (pyrophosphate), species is present having the formula Fe(P$_2$O$_7$)$_2$(HPO$_4$)$_2$ (12). At pyrophosphate to Fe$^{3+}$ ratios less than 3:1, the decrease in the initial reaction rate is undoubtedly due to the formation of oligomeric or polymeric Fe$^{3+}$-pyrophosphate species (see below).

The initial burst of absorbance change at ratios less than 3:1 mentioned above (but not included in the calculations of the velocity in Fig. 1) probably results from the presence of a small fraction of a highly reactive monomeric Fe$^{3+}$ (pyrophosphate), or Fe$^{3+}$ (pyrophosphate) species having coordination sites available for complexation by the protein.

The decrease in reaction rate beyond a 3:1 ratio can be ascribed to a reaction pathway which probably includes an activation of Fe$^{3+}$ (pyrophosphate), via loss of a ligand to form.
the highly reactive Fe$^{3+}$(pyrophosphate), species. Such processes are well known in transition metal ligand exchange reactions and have been discussed elsewhere in relation to iron delivery to transferrin (8). Excess pyrophosphate at ratios greater than 3:1 then competes with apotransferrin-CO$_3^-$ for such a species and the rate is slowed.

Above a 3:1 ratio, however, EPR data indicates that some polymeric iron species are still present. For example, dilution of an 0.8 mM iron pyrophosphate solution (3.7:1 PP$_3$/Fe) to a concentration of 0.16 mM results in an ~2-fold increase in EPR intensity/iron, suggesting the presence of a polymer = monomer equilibrium. Except where designated, the experiments reported below were carried out at a 4:1 ratio of pyrophosphate to Fe$^{3+}$ in order to minimize Fe$^{3+}$ polymerization products while allowing near-maximal reactivity. Under these conditions the reaction is still heterogeneous, however. The second phase arises in part from slow reaction of polymerized iron with the protein. This phase represents a proportionally smaller fraction of the total reaction (from 50 to 10%) as the concentration of iron pyrophosphate solution is reduced from 0.3 mM to 15 mM.

Reaction Spectra—The family of visible spectra obtained with a Hewlett-Packard 8450A rapid scan spectrophotometer during the reaction of Fe$^{3+}$-pyrophosphate with apotransferrin-CO$_3^-$ is shown in Fig. 2. Spectrum 1 was obtained some 3-5 s after mixing of reagents. Subsequent spectra were obtained at 45-s intervals. Spectrum 1 displays a minimum at 390 nm and a maximum at 450 nm. Fully formed Fe$^{3+}$-transferrin-CO$_3^-$ (the reaction is not complete in the last scan shown) has a spectral minimum at 400 nm and a maximum at 465 nm. The reaction spectra were also constructed from the absorbance changes observed at the end of the first and second phases of the reaction as monitored by stopped flow spectrophotometry. Results similar to that shown for spectrum 1 of Fig. 2 were obtained for the first phase spectrum. Spectrum 1 suggests the rapid formation of an intermediate similar to those observed spectrophotometrically for the reaction of Fe$^{3+}$-acetohydroxamic acid and other Fe$^{3+}$ complexes with apotransferrin-carbonate (7, 8), followed by its subsequent decay to product.

EPR Studies. Correlation with Absorption Spectra—Further evidence for an intermediate is obtained from EPR spectros-
protein obtained by subtracting the spectrum of the reactant treated by
the reaction in which the spectrum of Fe$^{3+}$(pyrophosphate)$_3$
shows the family of difference spectra for the first phase of the reaction
mixed-ligand Complexes of Transferrin
Urea polyacrylamide gels of samples taken throughout the time course of the reaction reveal that the iron is directed to the COOH-terminal site relative to the NH$_2$-terminal iron-binding site in a ratio of 9.5:1. Thus, pyrophosphate is highly site specific in delivering iron to transferrin, and the EPR spectrum is essentially that of the COOH-terminal monoferric site specific in delivering iron to transferrin, and the EPR spectrum of Fe(PPi$_3$) from the experimental spectra (Fig. 3) during the first phase of the reaction. Spectra scaled to the same peak-to-peak amplitude for ease of comparison of lineshapes. The variation in lineshape indicates the presence of three or more EPR active species, i.e. an underlying signal from an intermediate(s) (see "Appendix"). a, 30 s; b, 45 s; c, 60 s; and d, 130 s.

Rapid pathway:

\[
\text{Fe(PPi)$_3$ (monomer) + TRF-CO}_2^- \xrightarrow{k_{2,w}} \text{Fe(PPi)$_3$ (monomer) + TRF-CO}_2^-} \]

Slow pathway:

\[
\text{Fe-PPi (polymer) + TRF-CO}_2^- \xrightarrow{k_{2,p}} \text{Fe-PPi (polymer) + TRF-CO}_2^-} \]

Here $[F_{en}]$ and $[F_{ep}]$ are the concentrations of monomeric and polymeric Fe$^{3+}$ pyrophosphate which react with the transferrin-carbonate complex $T$ according to second-order processes having rate constants $k_{2,w}$ and $k_{2,p}$ respectively, where $k_{2,w} > k_{2,p}$. An intermediate $I$ is formed in both pathways and decays to product $P$ by a first-order process with a rate constant $k_I$. The buildup of intermediate is insignificant by the slow pathway because its formation is slow relative to its decay. The coupled differential equations which describe the kinetics are given by

\[
-d[F]_T dt = k_{2,w}[T][F_{en}] + k_{2,w}[T][F_{en}] \]

\[
d[I] = k_{2,w}[T][F_{en}] + k_{2,w}[T][F_{ep}] - k_I[I] \]

\[
d[P] = k_I[I] \]

\[
d[F_{en}] = k_{2,p}[T][F_{en}] \]

\[
d[F_{ep}] = k_{2,p}[T][F_{en}] \]

These equations cannot be solved in closed form. Accordingly, they were integrated numerically in increments of 0.1 s using a computer program to give the concentrations of all species as a function of time for a given set of rate constants and initial concentrations. The input parameters $k_{2,w}$, $k_I$, and $k_{2,p}$.
were varied iteratively to give a least-squares fit to the 0 °C absorbance data (Fig. 4). The other parameters were held fixed. Their values were determined as described in the legend of Fig. 4. The model reproduces the data very well (Fig. 4).

The rapid pathway principally gives rise to the first phase and the slow pathway to the second phase under the conditions in Fig. 3. The molar extinction of the intermediate is comparable to that of the product at 465 nm (Fig. 4, title) and at 245 nm.

EPR spectra of Fig. 3 were then deconvoluted to obtain the spectrum of the intermediate. By using molar fractions calculated from the curve-fitting parameters (Fig. 4) as a guide, spectra of the EPR active reactant Fe₃⁺ and product P were appropriately weighted and subtracted from the spectrum of the reaction mixture. The spectrum of the intermediate obtained in this way for various times is shown in Fig. 6. The spectra all have the same principal features, giving us confidence that the analysis is correct. Moreover, the spectrum of the putative intermediate is different than that of either the reactant or product (cf. Figs. 3 and 6). The half-life of the intermediate as estimated from the EPR data is approximately 1.5 min at 0 °C.

Detailed Kinetics of Intermediate Complex Formation and Breakdown—The dependence of the velocity of the reaction on the concentration of reactants can provide insight into the reaction pathways involved in mixed ligand complex formation. At a constant apotransferrin-CO₂⁻ concentration (5 x 10⁻⁵ eq liter⁻¹) the initial velocity exhibits a linear dependence on the Fe³⁺ concentration over the range 0.1 x 10⁻⁵ to 9 x 10⁻⁵ M when the pyrophosphate concentration is maintained constant (4 x 10⁻⁴ M). A slope of 0.38 s⁻¹ was obtained for the line.

The initial velocity of intermediate formation is a hyperbolic function of the apotransferrin-CO₂⁻ concentration as shown in Fig. 7. The hyperbolic plot indicates a zero order dependence on apotransferrin-CO₂⁻ at high concentrations. The inset double reciprocal plot gives an extrapolated maximal velocity of 2.7 x 10⁻⁵ eq liter⁻¹ s⁻¹. From that and the Fe³⁺ concentration (5 x 10⁻⁵ M), a first-order rate constant of 0.54 s⁻¹ corresponding to infinite apotransferrin concentration is calculated. The concentration of apotransferrin giving half-maximal velocity is 3.0 x 10⁻⁵ eq liter⁻¹.

It is possible to correct the observed rate constant obtained from the slope of the plot of initial velocity versus Fe³⁺ concentration (0.38 s⁻¹) to a value anticipated for infinite apotransferrin concentration (7). This value is 0.61 s⁻¹ (k₂ = 1.14 s⁻¹) in good agreement with the value of 0.54 s⁻¹ obtained from the extrapolated maximal velocity value of Fig. 7.

The initial velocity of intermediate complex formation shows a hyperbolic dependence on NaHCO₃ concentration as has been observed in other metal ion-binding reactions of apotransferrin (7, 8, 14). Half-maximal velocity was obtained at a NaHCO₃ concentration of 12 mM.

A linear Arrhenius plot giving an activation energy of 55 kJ mol⁻¹ (13.1 kcal mol⁻¹) was obtained for the first phase of the reaction.

Probing the Reactivity of Fe³⁺ Intermediate Complexes with Multimixing Stopped Flow Spectrophotometry—Multimixing stopped flow spectrophotometry is a technique that, among other things, allows one to examine the chemical reactivity of intermediates by the automated sequential mixing of three solutions containing reactants. In our studies we mixed Fe³⁺-pyrophosphate with apotransferrin-CO₂⁻ in the first mixing event followed at various time intervals by the addition of a solution containing thioglycolate and bathophenanthroline sulfonate. This reductant/chromogen combination reacts with the variety of Fe³⁺ populations that may be present. The rate of formation of Fe³⁺-bathophenanthroline sulfonate, monitored at 535 nm, thus serves as an index of the chemical reactivity of the iron.

The results of such a study are presented in Fig. 8. The rate of Fe³⁺-bathophenanthroline sulfonate formation following addition of the reductant/chromogen combination is plotted as a function of the age of the initial reaction mixture (Fe³⁺-pyrophosphate plus apotransferrin-CO₂⁻) at the time of the second mixing event. For comparison, the rate of reaction of the reductant/chromogen with Fe³⁺-pyrophosphate is shown as point A. This would be the value obtained at age zero of the initial reaction mixture.

The first multimixing point is obtained at an initial reaction mixture age of 56 ms. At this point the rate is one thousandth that obtained for the reaction of the reductant/chromogen with Fe³⁺-pyrophosphate. The Fe³⁺-pyrophosphate has interacted with the apotransferrin-CO₂⁻ in such a way that at least 99.9% of the Fe³⁺ has become more resistant to reduction. A minimum second order rate constant of 3 x
Mixed-ligand Complexes of Transferrin

**Kinetic Parameters and Concentration Effects**—The rate of removal of Fe$^{3+}$ from Fe$^{3+}$-transferrin-CO$_3^-$ exhibits a hyperbolic or saturation dependence on pyrophosphate in the concentration range 1–100 mM. A linear double reciprocal plot of 1/velocity versus 1/[PPi] indicates a maximal velocity at 3.6 × 10$^{-5}$ M s$^{-1}$ (not shown). One half-maximal velocity is obtained at a pyrophosphate concentration of 38 mM. Division of the maximal velocity by the concentration of Fe$^{3+}$-transferrin-CO$_3^-$ yields an apparent first order rate constant of 3.6 × 10$^{-3}$ s$^{-1}$ ($t_{1/2} = 3.2$ min).

The reaction exhibits a linear dependence on Fe$^{3+}$-transferrin-CO$_3^-$ concentration at a constant 0.1 M level of pyrophosphate. The slope of the plot gives a first-order rate constant of 2.7 × 10$^{-3}$ s$^{-1}$ ($t_{1/2} = 4.3$ min). The velocity at 0.1 M pyrophosphate is 72% of maximal velocity. Thus a corrected slope value gives an apparent first-order rate constant of 3.7 × 10$^{-3}$ s$^{-1}$ ($t_{1/2} = 3.1$ min) in excellent agreement with the value of 3.2 min above.

The time course of the reaction at 0.1 M pyrophosphate (pseudo first-order conditions) exhibits curvature when plotted as a first-order function, consistent with two sites that differ by a factor of 5.1 in reactivity toward iron removal. The rate constant obtained from the initial slope of a first order plot is 2.6 × 10$^{-3}$ s$^{-1}$. When corrected to “infinite” pyrophosphate concentration for comparison with the value obtained from maximal velocity one obtains a value of 3.6 × 10$^{-3}$ s$^{-1}$ ($t_{1/2} = 3.2$ min). Thus by three independent measures we have obtained the same value for the maximal first-order rate constant. Ref. 7 may be consulted for a more detailed description of these calculations.

**The Effect of pH on the Rate of Removal of Fe$^{3+}$ from Fe$^{3+}$-Transferrin-CO$_3^-$**—The removal of Fe$^{3+}$ from Fe$^{3+}$-transferrin-CO$_3^-$ by pyrophosphate was examined between pH 5.6 and 8.0. A marked increase in the rate is observed as the pH is decreased. A plot of the log of the velocity as a function of the pH exhibits a slope of 0.65 in the pH 5.6–6.6 range and 0.5 in the pH 7–8 range. These values are considerably lower than the slope of 2.0 observed for the reductive release of iron from Fe$^{3+}$-transferrin-CO$_3^-$ (14). Protonation destabilizes Fe$^{3+}$ binding by transferrin, but also decreases reactivity of chelators as well.

**DISCUSSION**

This study of the exchange of Fe$^{3+}$ between transferrin and pyrophosphate is instructive in that it supports a previously enunciated hypothesis and points toward heretofore unsuspected aspects of transferrin chemistry. Specifically, this work is consistent with the general form of a reaction route proposed for iron exchange between transferrin and nonreducing chelators. The study gives further insight into the properties of the mixed-ligand intermediate, chelate-Fe$^{3+}$-transferrin-CO$_3^-$, and it suggests the possible involvement of allosteric sites in both binding and release of Fe$^{3+}$ by the protein.

The absorption and EPR spectra (Figs. 2 and 6) clearly reveal the presence of an intermediate which we propose to be a mixed-ligand complex. In the case of iron delivery to transferrin by acetohydroxamic acid, formation of such a complex has been demonstrated spectrophotometrically (7). Formation of mixed-ligand complexes is probably a common feature of metal ion exchange reactions between transferrin and chelators. However, we cannot exclude the possibility that pyrophosphate is not bound to the iron in the intermediate since other interpretations of the spectral data are also possible (see below). Conceivably more than one intermediate is formed in the reaction pathway, each contributing to the observed spectrum.

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**FIG. 8.** The rate of reduction of Fe$^{3+}$ as a function of age of the reaction following the mixing of Fe$^{3+}$-pyrophosphate with apotransferrin. The reaction mixture was allowed to age for various intervals following which a solution containing thioglycolate and bathophenanthroline sulfonate was added. The initial velocity of the reduction of Fe$^{3+}$ was monitored by formation of Fe$^{3+}$-bathophenanthroline sulfonate at 536 nm. Concentrations of reactants were as follows: Fe$^{3+}$, 5 × 10$^{-5}$ M; pyrophosphate, 2 × 10$^{-3}$ M; apotransferrin, 7 × 10$^{-5}$ eq liter$^{-1}$. The reductant-indicator solution consisted of 10 mM thioglycolate and 0.25 mM bathophenanthroline sulfonate. Other standard conditions are stipulated in the legend to Fig. 1. The experiments were carried out in a multimixing stopped flow spectrophotometer.

It should be noted that under these conditions about 2% of the Fe$^{3+}$-pyrophosphate has reacted with apotransferrin-CO$_3^-$ at 50 ms to form the intermediate complex as judged by the rate of the first phase of the reaction. Thus, we are left with the conclusion that a very rapid interaction occurs between Fe$^{3+}$-pyrophosphate and the protein preceding the formation of the mixed ligand complex observed spectrophotometrically.

From the maximal velocity obtained at “infinite” apotransferrin-CO$_3^-$ concentration (Fig. 7) a first order rate constant for the apparent conversion of Fe$^{3+}$ (pyrophosphate)$_2$ to the putative Fe$^{3+}$ (pyrophosphate)$_3$ of 0.61 s$^{-1}$ (see above) is calculated. Thus, at 50 ms only about 3% of the Fe$^{3+}$ (pyrophosphate)$_3$ has undergone labilization via loss of a ligand molecule. The rapid quenching of Fe$^{3+}$ reduction evidently is the result of an interaction of apotransferrin-CO$_3^-$ with Fe$^{3+}$ (pyrophosphate)$_3$, and not with the putative Fe$^{3+}$ (pyrophosphate)$_2$.

After the rapid dip in Fe$^{3+}$ reactivity, a transient rise by a factor of 5 is seen with a peak near 0.4 s (Fig. 8). Then a diminution in rate is observed reaching an apparent plateau at 10 s. It is during this latter time period in which the intermediate complexes becomes fully formed at room temperature. The peak at 0.4 s may result from a complex formed from the initial association of Fe$^{3+}$ (pyrophosphate)$_2$ with apotransferrin-CO$_3^-$, ultimately leading to the spectrophotometrically observable intermediate.

The Removal of Fe$^{3+}$ from Fe$^{3+}$-Transferrin-CO$_3^-$ by Pyrophosphate

**Spectral Family**—The family of spectra obtained during the time course of the reaction in which the Fe$^{3+}$ is removed from Fe$^{3+}$-transferrin-CO$_3^-$ by pyrophosphate gives no evidence for a mixed ligand intermediate complex. A smooth monotonic decrease in absorbance is observed with no apparent shift in the 465-nm peak toward shorter wavelengths. This is in contrast to the shifts observed in the spectra of Fig. 2.
The effect of the reactant concentrations on the initial velocity of the "forward" reaction (the reaction in which pyrophosphate delivers Fe$^{3+}$ to transferrin) is hyperbolic for apotransferrin and NaHCO$_3$, linear for Fe$^{3+}$, and inhibitory for pyrophosphate when the pyrophosphate concentration exceeds three times the Fe$^{3+}$ concentration. The "reverse" reaction shows a linear dependence on Fe$^{3+}$-transferrin-CO$_2$- and a hyperbolic dependence on pyrophosphate. These data and the spectroscopic evidence for an intermediate complex are consistent with the following reaction route.

1. $\text{TRF} + \text{HCO}_3^- \rightarrow \text{TRF-CO}_2^- + \text{H}^+$
2. Fe$^{3+}(\text{PPi})_3 \rightarrow \text{Fe}^{3+}(\text{PPi})_2 + \text{PPi}$
3. Fe$^{3+}(\text{PPi})_2 + \text{TRF-CO}_2^- \rightarrow \text{PPiFe}^{3+}.\text{TRF-CO}_2^- + \text{PPi}$
4. PPi.Fe$^{3+}.\text{TRF-CO}_2^- \rightarrow \text{Fe}^{3+}.\text{TRF-CO}_2^- + \text{PPi}$
5. Fe$^{3+}.\text{TRF-CO}_2^- \rightarrow \text{Fe}^{3+}.\text{TRF}$

Here PPi and TRF are pyrophosphate and apotransferrin, respectively. A double dagger symbol ($\ddagger$) is used to designate an "open" or "apotransferrin-like" protein formation.

Step 1 depicts the binding of bicarbonate to apotransferrin to activate the nucleophile properties of the protein (7, 8, 15). The conversion of Fe$^{3+}$ (pyrophosphate)$_3$ to a labile species via loss of a chelator group is depicted in step 2. This is consistent with the hyperbolic dependence of rate on apotransferrin concentration, the linear dependence on Fe$^{3+}$ concentration, and the decrease in rate as the pyrophosphate concentration is increased beyond the 3:1 pyrophosphate to Fe$^{3+}$ ratio. Data support the formation of similar labile species in the donation of iron to apotransferrin by citrate (8) and by acetohydroxamic acid (7).

The attack of apotransferrin-CO$_2^-$ on the putative Fe$^{3+}$ (pyrophosphate)$_2$ species to form the mixed-ligand intermediate complex gives rise to what is observed as the first phase of the reaction. This is shown as step 3. The kinetic, spectral, and EPR studies support this suggestion; however, the exact points of pyrophosphate release are indefinite. The decay of the intermediate is ascribed to steps 4 and 5, the loss of a chelator, and folding of the protein to give a stable Fe$^{3+}$-transferrin-CO$_2^-$ conformation. A detailed account of this type of reaction route including correlations of observed kinetics with the five steps and supporting citations from the literature is given in Refs. 7 and 8 and will not be repeated here.

The distinctive 3-component $g' = 4.3$ EPR signal of the high spin Fe$^{3+}$-transferrin-CO$_2^-$ complex (Fig. 3, bottom) arises from transitions within the middle Kramers doublet of the $S = 5/2$ manifold of spin states (16). At present the relationship between the lineshape of $g' = 4.3$ EPR signals and metal site structure is not well understood (17). However, the EPR spectrum of the intermediate (Fig. 5) lacks the 3-component feature of the fully developed Fe$^{3+}$-transferrin-CO$_2^-$ complex, indicating a difference in coordination environment of the Fe$^{3+}$. Fe$^{3+}$-transferrin-anion complexes in which carbonate has been replaced by other anions are known to exhibit markedly different EPR spectra (18). Thus it appears that ligand substitution in only one coordination position can substantially alter the EPR spectrum. Loss of structure in the EPR signal of the C-terminal site has also been observed upon binding of lyotropic anions such as perchlorate to sites on the protein (19-21). Based on the EPR data alone, it is, therefore, not possible to say how pyrophosphate is bound in the intermediate complex. Both allosteric sites and direct coordination to the iron may be involved.

The dependence of the initial rate on CO$_2$ concentration implies that CO$_2$ is bound to the iron in the intermediate and that substitution of pyrophosphate for carbonate has not occurred. The fact that the molar extinction coefficients at 245 and 465 nm for the intermediate are comparable to those of Fe$^{3+}$-transferrin-CO$_2^-$ suggests that the coordination sphere of the iron in the intermediate also contains two tyrosine ligands (22).

It is instructive to compare the rate constants and spectral transitions of the various steps as observed with various chelators. In the forward direction the most important factor in determining rates is the chemical properties of the Fe$^{3+}$ complex. A fundamental characteristic of Fe$^{3+}$-chelate complexes in aqueous solutions is their propensity to form condensation products via oxo and hydroxo groups which act to bridge iron centers. Thus, Fe$^{3+}$-chelate complexes may exist as monomers, dimers, oligomers, or polymers depending primarily on the pH, the nature, and concentration of the chelator and the concentration of the iron. A competition exists between the oxo bridges with their drive toward condensation of metal centers and chelators which isolate iron through ligand groups. Since the various products that form vary in their ligand exchange characteristics, we can anticipate, and have previously observed (7, 23), marked variation in the reaction of apotransferrin-CO$_2^-$ with Fe$^{3+}$-chelate complexes as a function of the chelator to Fe$^{3+}$ ratio. In our studies Fe$^{3+}$-pyrophosphate was observed to exhibit a competition between a polymeric and monomeric species that is dependent upon solution conditions. Fe$^{3+}$-pyrophosphate at a 1:1 pyrophosphate to Fe$^{3+}$ ratio near neutral pH is composed primarily of a polymeric species. In dilute solution at ratios of 3:1 and greater an Fe$^{3+}$ (pyrophosphate)$_3$ complex predominates. The maximal reactivity observed here for Fe$^{3+}$pyrophosphate occurs at a 3:1 ratio. The maximally reactive chelator to iron ratios are approximately 5:1 for acetohydroxamic acid and 20:1 for citrate. Weaker chelators require greater chelator:iron ratios to suppress polymerization. Nitritotriacetate which is a strong tetradentate chelator is fully reactive at a 1:1 ratio (10). The Fe$^{3+}$ chelator labilization of step 2 obtained from Figs. 7 and 8 (vide supra) is 0.6 s$^{-1}$ for pyrophosphate and 2.2 s$^{-1}$ for acetohydroxamate (7). Both chelators exhibit half-maximal velocity at an apotransferrin concentration of 3 x 10$^{-4}$ eq liter$^{-1}$.

The removal of Fe$^{3+}$ from Fe$^{3+}$-transferrin-CO$_2^-$ by chelators has been examined by several groups. Often, variations in conditions such as pH, ionic strength, the type of transferrin, or the presence of an iron acceptor such as desferrioxamine B compromises comparisons of rate constants. We are, however, able to make an interesting comparison between the rate-limiting step of iron removal for those cases in which the rate is a hyperbolic function of the chelator concentration as is shown above. The half-life for iron removal from Fe$^{3+}$-transferrin-CO$_2^-$ at "infinite" chelator concentration is 3.2 min for pyrophosphate (vide supra), 14 min for acetohydroxamic acid (7), and 21 min for 1,5,10-N,N',N''-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane (24).

Harris (25) has pointed out that the dependence of the rate constant for the rate-limiting step on the nature of the chelator is not inconsistent with the basic reaction route shown above in which the first step of the iron release reaction (reverse of step 5) is shown as a monomolecular conformational transition dependent on Fe$^{3+}$-transferrin-CO$_2^-$. Both the concentration of mixed-ligand intermediate and its rate of breakdown to products can be rate-determining factors (25).
It is also possible that interactions between the chelator and Fe$^{3+}$-transferrin-CO$^2-$ affect a rate-limiting transition by the iron-protein complex. The binding of a chelator to Fe$^{3+}$-transferrin-CO$^2-$ might have any (or all) of several forms, including bonding to allosteric sites (19). Recent models of the proposed metal-binding region of transferrin based on amino acid conservation, chemical modification, spectroscopy, and other approaches indicate clusters of positive charges near the metal and anion binding center (19, 26-29). Binding and/or bridging of positive clusters could alter the energy of activation for a conformational transition by Fe$^{3+}$-transferrin-CO$^2-$.

It is probable that transferrin possesses structural attributes that facilitate iron exchange with agents in its environment in such a way that the apoprotein is a highly effective nucleophile in seeking adventitious iron and Fe$^{3+}$-transferrin-CO$^2-$ readily uploads iron in acidic subcellular compartments. In understanding these properties we may well need to look beyond the coordinating residues of what we have called the specific site.

Acknowledgments—Heather Read (R. A. Welch Foundation Undergraduate Fellow at Texas A&M University) provided technical assistance in the exploratory phases of this research.

APPENDIX

EPR Difference Spectral Lineshapes of Mixtures

EPR difference spectra are useful in the analyzing reaction mixtures for the presence of more than two EPR-active components. Consider the following simple system consisting of pure reactant and product, R and P, which exhibit EPR spectra $S_R$ and $S_P$, respectively. The observed spectrum $S_{obs}$ of the mixture is the sum of the individual spectra $S_R$ and $S_P$, weighted by the molar fractions $X_R$ and $X_P$ (Equation 1). Substitution of $X_R = 1 - X_P$ leads to Equation 2 for the difference spectrum $S_{diff}$

$$ S_{obs} = X_R S_R + X_P S_P $$

(1)

$$ S_{diff} = S_{obs} - S_P = X_R (S_R - S_P) $$

(2)

The spectrum $S_{diff}$ is obtained by subtracting the spectrum $S_P$ of the product from the observed spectrum $S_{obs}$ of the mixture. The lineshape of the difference spectrum is constant and given by the difference spectrum of the pure components, i.e. $S_R - S_P$, and its amplitude is proportional to the molar fraction $X_R$ of reactant. Similarly, an expression for the difference spectrum obtained by subtracting the spectrum of pure reactant $R$ from that of the mixture is given by Equation 3.

$$ S_{diff} = S_{obs} - S_R = X_P (S_P - S_R) $$

(3)

Note that the lineshapes of the difference spectra obtained in these two ways are simply the negative of one another. For a simple interconversion $R \rightarrow P$, the lineshape $S_R - S_P$ or $S_P - S_R$ of either difference spectrum (Equations 2 or 3) will be constant throughout the time course of reaction, but the amplitude will change in proportion to the molar fraction $X_R$ or $X_P$, respectively. For a process $R \rightarrow I \rightarrow P$ in which an intermediate $I$ is present, the appropriate equation is given by

$$ S_{diff} = S_{obs} - S_I = X_I (S_I - S_P) + X_R (S_R - S_P) $$

(4)

and contains a term $X_I (S_I - S_P)$ for the intermediate. In this case neither the lineshape nor amplitude of the difference spectrum will be constant throughout the course of the reaction. Thus, lineshape variations in difference spectra enable the presence of three or more EPR-active species in a reaction mixture to be readily detected.

REFERENCES


Mixed-ligand Complexes of Transferrin