conditions. Without additional data this small difference in the rate of decomposition does not allow us to conclude that the oxidized form further stabilizes the Cr(IV) species.

O'Brien and Ozolins[14] noted that at higher pH the dominant Cr(V) complexes decayed to Cr(III) products through a two-term rate law, a first-order process (first order in Cr(V); rate constant = 1.5×10^{-3} s^{-1}) and a second-order process (first order in each Cr(V) and GSH; rate constant = 9.1×10^{-3} M^{-1} s^{-1}). At higher pH it is likely that the Cr(V) species undergoes parallel internal electron transfer and a direct reaction by a GSH molecule to form the Cr(IV) complex. This Cr(IV) complex is then rapidly reduced to Cr(III) products, in accord with our rate data showing that the second-order rate constant for the chromium(IV) decomposition ($k = 0.13$ M^{-1} s^{-1}) in our system is at least 10 times larger than that for ($k = 9.1 \times 10^{-3}$ M^{-1} s^{-1}) the decomposition of Cr(V) complexes.[14]

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Mechanism of Iron Release from Human Serum C-Terminal Monoferric Transferrin to Pyrophosphate: Kinetic Discrimination between Alternative Mechanisms

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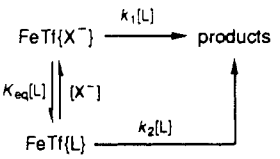
The kinetics of iron release from C-terminal monoferric transferrin (Fe_CTf) to pyrophosphate (PP) under pseudo-first-order conditions show an apparent saturation linear dependence of k_{obs} on [PP]. The variation of k_{obs} with [PP] was studied under conditions of variable temperature and added anion (X) concentration (Cl^-, ClO_4^-, NO_3^-, SO_4^{2-}, HPO_4^{2-}). The results conform globally to a two-path mechanism (mechanism 1) involving iron removal either from a species with added anion bound to a kinetically significant anion binding (KISAB) site on the protein or with PP bound to the KISAB site, these two species being linked by a rapid equilibrium. Microscopic rate and equilibrium constants were evaluated by nonlinear regression of kinetic data to the equation $k_{obs} = (k_1[PP] + k_2K_{eq}'[PP]^2)/(1 + K_{eq}'[PP])$. The conditional constant K_{eq}' ($=K_{eq}/[X]$) obtained in the numerical regression procedure varied with $[Cl^-]$ as required by mechanism 1. Two plausible simple alternative mechanisms were also considered. Both involved a saturation pathway involving attainment of an open conformation of the protein and a nonsaturation pathway involving direct removal of iron from the transferrin. Both mechanisms led to an anion dependence of the microscopic rate and equilibrium constants on $[Cl^-]$ different from that observed experimentally. Furthermore, plots of ln k_1 and ln k_2 vs T^{-1} for mechanism 1 adhere closely to the Arrhenius model, with no curvature or breaks in the plots apparent, as required for true microscopic rate constants, thus arguing further in favor of the correctness of this mechanism.

Introduction

Human serum transferrin is one of a family of iron-binding proteins responsible for transport of iron in the serum and iron sequestering in body fluids, such as milk. It is a single polypeptide chain consisting of 678 amino acid residues arranged into two similar but not identical lobes.[1-5] Each lobe is further organized into two domains, and one Fe(III) ion is bound in the cleft between the two domains along with a (bi)carbonate ion (known as the synergistic anion). The iron-binding ligands have been identified as two tyrosines, a histidine, and an aspartate with the remaining two coordination sites occupied by the synergistic anion, bound in a bidentate manner.[1,2] A number of reviews on the physicochemical behavior of transferrin are available.[3-5]

Considerable interest has focused on the kinetics of iron release from transferrin, both via reduction to weakly bound[6] Fe(II) followed by complexation by a ferrous ion acceptor[7-9] (which provides a significant driving force for the reduction) and by direct chelation of Fe(III). Studies of the latter route using ligands such as acetohydroxamate[10] and *N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (3,4-LICAMS)[11] have shown an apparent saturation dependence on chelator concentration of the observed pseudo-first-order rate constants for iron release (k_{obs}) from the two (N- and C-terminal) sites. On the basis of these (as well as iron uptake) results, Bates and co-workers[10] proposed essentially the following mechanism for iron release: (i) A rate-limiting conformational change of the ferric transferrin to an open conformation; (ii) rapid attack of the chelating agent on the iron to form a so-called quarternary complex (since this follows the rate-determining step, it is not detected during iron release

Scheme I[a]



[a] X^- = an anion such as NO_3^-, SO_4^{2-}, or Cl^-; L = an anionic chelating agent, e.g. citrate or PP; $k_1 > k_2$.

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but has been observed during iron uptake[10,12]); (iii) very fast decay of the quarternary complex to products. The rate of the conformational change (step i above) is an inherent property of the protein, and so this mechanism predicts that all chelating agents should approach the same limiting observed rate constant at high chelator concentration. This is not observed experimentally.[13] In addition, the mechanism does not account for the effect of anions such as Cl^- and ClO_4^-[14,15] on k_{obs}.

In contrast to the above, a number of studies have demonstrated that pyrophosphate (PP),[17-22] certain phosphonate ligands,[16-18,20] and citrate[23] remove the iron in an apparent saturation-linear manner (i.e. k_{obs} saturates to a straight line dependence on chelator concentration, slope > 0). Recent studies on the kinetics of iron release from N-terminal monoferric transferrin (Fe_NTf) to PP[22] and from both monoferric transferrins and diferric transferrin (Fe_2Tf) to citrate has led to the proposal of a new mechanism for iron release from transferrin[23] (Scheme I). It was proposed that the anion binding site ({}) be termed a kinetically significant anion binding (KISAB) site.

Under conditions of constant background anion concentration (X^-), k_{obs} is given by eq 1,[22] where $K_{eq}' = K_{eq}/[X^-]$ (see Results).

$$k_{obs} = (k_1[L] + k_2K_{eq}'[L]^2)/(1 + K_{eq}'[L]) \quad (1)$$

It is in the nature of chemical kinetics that a number of mechanisms of varying complexity can be proposed to account for the observed data. It is usual to choose the simplest mechanism consistent with the observations. Even so, several alternative mechanisms are often available (as is the case here; see Discussion), and in order to provide strong support for any particular mechanism experiments should be specifically designed to discriminate between the alternative mechanisms. We present the results of such a study below.

Methods

Preparation of Transferrin. Human serum transferrin was prepared from serum (separated from discarded blood bank whole blood samples), purified on a Boehringer Mannheim zinc chelate affinity adsorbent column (as has been described for lactoferrin[24]), dialyzed against citrate (pH 5), followed by 0.1 M $NaClO_4$ and 0.1 M KCl, and finally against H_2O, and then lyophilized. The purity of the transferrin was established by SDS-polyacrylamide gel electrophoresis[25] and from the UV-vis spectrum of the iron-saturated transferrin ($A_{280}/A_{466} = 23$ and $A_{428}/A_{466} = 0.8$).[26]

Preparation of Fe_CTf. Fe_CTf was prepared by the method of Baldwin and de Sousa.[15] From the known extinction coefficient ($\epsilon_{465} = 2780\ M^{-1}\ cm^{-1}$ [27]), the final concentration was found to be 17.2 mg mL^{-1}. In the preparation, 0.95 equiv of Fe^{3+} was added to the apotransferrin (Tf) in order to minimize contamination by Fe_2Tf (which leads to biphasic kinetics). The purity of the Fe_CTf was determined by the urea-polyacrylamide gel electrophoresis method of Makey and Seal[28] (except that 1 mM EDTA was used) and was found to be 91% Fe_CTf, 8% Tf, and 1% Fe_2Tf.

Preparation of Solutions for Kinetic Experiments. All solutions used for the kinetic experiments were made up in double-distilled deionized water and were prepared in 0.05 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonate (HEPES) buffer, pH 7.40. The buffer was prepared by raising the pH of the acid form of HEPES with NaOH. PP stock solutions (0.200 M) were prepared from $Na_4P_2O_7{\cdot}10H_2O$ by addition of the appropriate acid (HCl, HNO_3, $HClO_4$, H_2SO_4, H_3PO_4) to lower the pH to 7.40 and provide the desired background anion (Cl^-, NO_3^-, ClO_4^-, SO_4^{2-}, or HPO_4^{2-}, respectively). From the known pK_as of PP[29] the concentration of background anion (from the monoprotic acids) can be shown to be 0.179 M. In the case of SO_4^{2-} and HPO_4^{2-} the required amounts of acid were added to give final anion concentrations of 0.179 M and the pH was raised to 7.40 with NaOH. For studies in the presence of buffer only, pyrophosphoric acid was prepared by passing $Na_4P_2O_7{\cdot}10H_2O$ down a Dowex 50W-X8 strong acid ion-exchange column (H^+ form). The acid was standardized by pH electrode titration with standardized NaOH. The final PP stock concentration was attained by adding $Na_4P_2O_7{\cdot}H_2O$ to the pyrophosphoric acid, and the pH was raised to 7.40 with NaOH.

Background anion concentrations were kept constant by addition of appropriate volumes of sodium salts of the anions to the solutions used for the kinetic experiments (the phosphate solution was prepared from H_3PO_4 and was pH adjusted with NaOH).

Kinetic Measurements. All kinetic experiments were carried out using a Varian Techtron 635 UV/vis spectrophotometer with thermostated cuvette holder. Data capture was effected by a Hewlett-Packard HP-85 computer using an HP3438 A digital multimeter as an analog to digital interface. Pseudo-first-order rate constants (see Results) were obtained directly by nonlinear least-squares (NLLS) analysis of the absorbance versus time data. A detailed assessment of accuracy, precision, and reproducibility with this experimental system has been reported elsewhere.[30] The dependences of the pseudo-first-order rate constants on PP concentrations were analyzed by NLLS fitting using the Marquardt algorithm.[31] The temperatures at which experiments were performed were measured in situ (in the cuvette) using a calibrated thermocouple and are accurate to ±0.02 °C.

For each kinetic experiment Fe_CTf (50.0 μL of 17.2 mg mL^{-1}) was injected into a deaerated (to prevent bubble formation), temperature-equilibrated (5 min) solution (pH 7.40) containing appropriate volumes of PP, salt, and HEPES solutions. The reaction was monitored by following the decay of the visible absorbance of the Fe_CTf at 465 nm ($\Delta A \simeq 0.015$).

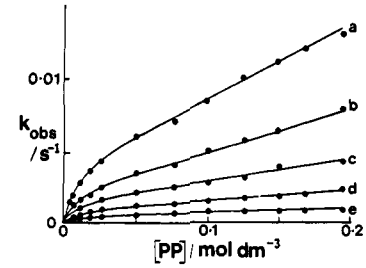

Figure 1. Dependence of k_{obs} on PP concentration at various temperatures. The temperatures for a–e are 37, 33.5, 30, 25, and 20 °C, respectively. The lines drawn through the points are the best fits of eq 1 with rate and equilibrium constants as shown in Table I. Conditions: pH 7.40, 0.05 M HEPES, 0.179 M Cl^-.

Results

The reactions were found to adhere accurately to pseudo-first-order kinetics at [PP] > 0.005 M. Below this concentration the reactions do not reach completion, presumably because PP is thermodynamically unable to compete with the transferrin for Fe^{3+}.

For each set of data collected at a fixed background anion concentration the dependence of the observed pseudo-first-order rate constant (k_{obs}) on PP concentration was fitted by eq 1 (see Introduction). Owing to the fairly large values of K_{eq}' in most cases (i.e. weak competition of anions with PP for the KISAB site) very few points are generally obtained in the region where

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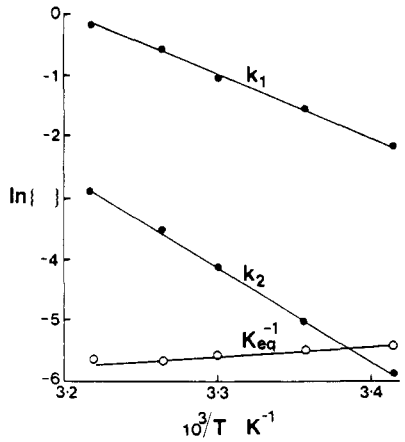

Figure 2. Arrhenius and van't Hoff plots of k_1, k_2, and K_{eq}' obtained from the nonlinear least-squares fit of eq 1 to the data in Figure 2. ({} refers to k_1, k_2, or $K_{eq}'^{-1}$ as appropriate). Note that there are no breaks or significant changes in the slope in the Arrhenius plots (i.e. no kinetically significant, temperature-induced conformational changes in the protein between 20 and 37 °C). From the slopes E_a is 93.0 ± 0.7 kJ mol^{-1} (k_1) and 128.6 ± 0.3 kJ mol^{-1} (k_2), and from the intercepts ln A is 36.0 ± 0.3 (k_1) and 47.0 ± 0.1 (k_2). The total change in ln K_{eq} with $1/T$ is smaller than the errors involved; thus, ΔH for the system is not significantly different from zero.

k_1 dominates (i.e. where [PP] is low and $k_{obs} \sim k_1$[PP]) because of the restriction imposed by the non-pseudo-first-order nature of the kinetics below 0.005 M PP. As a result standard deviations are generally large in the case of the parameters k_1 and especially K_{eq}'.

In the case of anions other than Cl$^-$ no values for k_1 and K_{eq}' could be obtained for HPO_4^{2-} and NO_3^- owing to the fact that only the straight line portion of the curve was observed over the entire PP concentration range (see Figure 4 below) apparently due to the very large values of K_{eq}'. On the other hand, in the case of sulfate (where K_{eq}' is exceptionally low) the errors in k_1 and K_{eq}' were only 5% and 10%, respectively. The large errors in the fitted values of k_1 and K_{eq}' (in most cases) are thus unavoidable due to thermodynamic constraints restricting measurements at low PP concentrations.

Effect of Temperature on the Kinetics. In an attempt to further establish the correctness of the proposed mechanism (Introduction, Scheme I), the kinetic experiments were performed at five different temperatures between 20 and 37 °C at a fixed Cl$^-$ concentration of 0.179 M. The dependence of k_{obs} on PP concentration at various temperatures is shown in Figure 1. Figure 2 demonstrates that k_1 and k_2 adhere to the Arrhenius equation. This indicates that they conform to the behavior expected for genuine microscopic rate constants (although products of microscopic rate constants would also exhibit Arrhenius behavior, sums of such constants would produce curved lines (see e.g. ref 32)). The van't Hoff plot of ln K_{eq}' vs $1/T$ is shown in Figure 2. The change in K_{eq}' with temperature is smaller than the errors in K_{eq}' (Table I), indicating that ΔH is not significantly different from zero.

Effect of Chloride Concentration on the Kinetics. Equation 1 is valid for a fixed anion concentration only. The true equilibrium constant K_{eq} is defined by

$$K_{eq} = ([Fe_CTf\{PP\}][Cl^-])/([Fe_CTf\{Cl^-\}][PP]) \quad (2)$$

K_{eq}' and K_{eq} are thus related by

$$K_{eq}/[Cl^-] = [Fe_CTf\{PP\}]/([Fe_CTf\{Cl^-\}][PP]) = K_{eq}' \quad (3)$$

Thus, a plot of K_{eq}' versus $[Cl^-]^{-1}$ should yield a straight line with zero intercept and slope K_{eq}.

The dependence of k_{obs} on PP concentration at four different Cl$^-$ concentrations at 37 °C is shown in Figure 3. A plot of K_{eq}' (obtained by fitting eq 1 to the data) versus $1/[Cl^-]$ is shown in the inset. Within experimental error, the values of K_{eq}' behave as predicted by the proposed mechanism; furthermore, as required by the mechanism, there are no significant changes in k_1 or k_2 with varying Cl$^-$ concentrations (see Table I).

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Table I. Fitted Parameters k_1, k_2, and K_{eq}' from Eq 1

anion	anion conc/M	temp/ °C	k_1/M^{-1} s^{-1}	k_2/M^{-1} s^{-1}	K_{eq}'/M^{-1}
Cl$^-$	0.179	37.0	0.86 ± 0.150	0.054 ± 0.001	254 ± 51
Cl$^-$	0.179	33.5	0.57 ± 0.25	0.030 ± 0.001	282 ± 156
Cl$^-$	0.179	30.0	0.35 ± 0.13	0.015 ± 0.001	230 ± 109
Cl$^-$	0.179	25.0	0.21 ± 0.10	0.0067 ± 0.0008	223 ± 37
Cl$^-$	0.179	20.0	0.11 ± 0.03	0.0029 ± 0.0003	209 ± 79
Cl$^-$	0.350	37.0	0.94 ± 0.53	0.054 ± 0.007	172 ± 132
Cl$^-$	0.500	37.0	0.81 ± 0.15	0.050 ± 0.005	93 ± 27
Cl$^-$	0.900	37.0	1.0 ± 0.12	0.035 ± 0.019	52 ± 18
ClO_4^-	0.179	37.0	1.0 ± 0.5	0.057 ± 0.001	122 ± 86
SO_4^{2-}	0.179	37.0	0.66 ± 0.03	0.045 ± 0.002	62 ± 6
NO_3^-	0.179	37.0	*a*	0.049 ± 0.004	*a*
HPO_4^{2-}	0.179	37.0	*a*	0.056 ± 0.002	*a*
		37.0		0.052 ± 0.001	

[a] Parameters numerically unobtainable—see text.

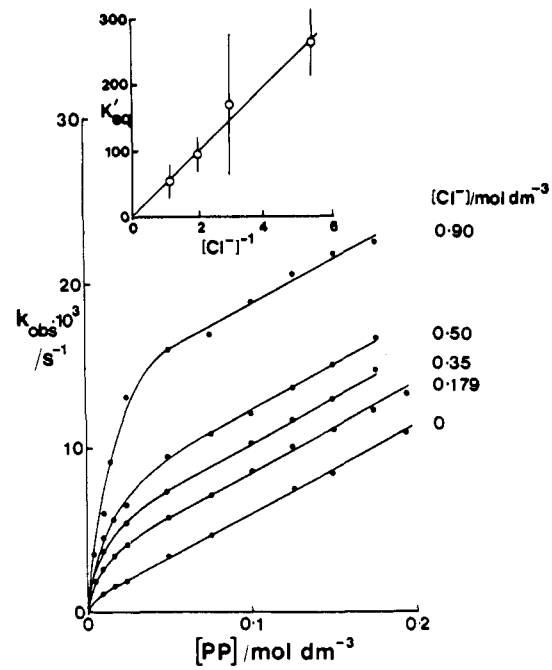


Figure 3. Dependence of k_{obs} on PP concentration at various Cl$^-$ concentrations. The lines drawn through the points are best fits of eq 1. Conditions: pH 7.40, 0.05 M HEPES, 37 °C. Inset: Plot of K_{eq}' versus $1/[Cl^-]$ (units on the inset M^{-1} vs M^{-1}). The line drawn is the least-squares fit to the mean values of K_{eq}'. The line passes through the origin as predicted for Scheme I (2 ± 20). The point at [Cl$^-$] = 0.35 M has an unusually large error associated with it (relative standard deviation more than twice that of the other points; the reasons for relatively large errors in K_{eq}' are discussed in the text even although in this case the error is exceptionally large); nevertheless, the least-squares value of K_{eq}' is in accord with the requirements of mechanistic Scheme I. From the slope of the line K_{eq} (see text) is 45 ± 10.

Effect of Anions Other Than Chloride on the Kinetics. The dependence of k_{obs} on PP concentration in the presence of five different anions at a constant background concentration of 0.179 M as well as in the presence of buffer only (0.05 M HEPES) at 37 °C is shown in Figure 4. The data were fitted by eq 1 except in the case of the buffer curve, which was fitted with a straight line function (between 0.01 and 0.195 M PP) since no significant curvature was apparent even in the lowest region of concentration studied. Clearly the straight line portions of all the curves are parallel; thus, k_2 is the same (within experimental error; see Table I) in all cases. This is to be expected if the proposed mechanism holds true, since k_2 always refers to iron release from the species $Fe_CTf\{PP\}$. Meaningful values of k_1 could not be obtained for NO_3^- and HPO_4^{2-}, but in general the variation in k_1 does not

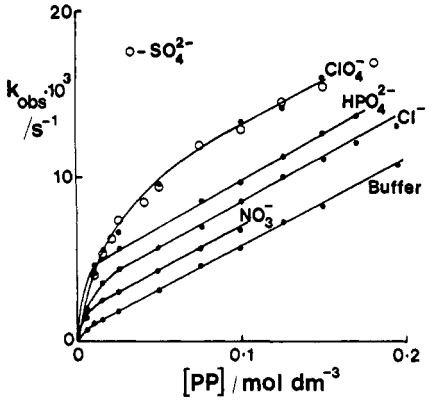
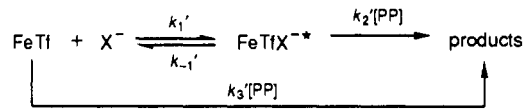


Figure 4. Dependence of k_{obs} on PP concentration both in the presence (0.179 M) and in the absence (buffer) of added anions. Note that the straight line portions of the curves are all parallel. The solid lines are best fits of eq 1 to the data (except for buffer which was fitted by linear least squares). Conditions: pH 7.40, 0.05 M HEPES, 37 °C.

Scheme II



appear to be large; however, the variation in K_{eq}' is significant. Although values of K_{eq}' could not be obtained for NO_3^- and HPO_4^{2-}, they are clearly large (i.e. PP competes very successfully with these anions, implying that their binding to the KISAB site is weak). The strength of binding of anions to the KISAB site on the protein appears to be

$$SO_4^{2-} > ClO_4^- > Cl^- > HPO_4^{2-} > NO_3^-$$

Discussion

The data presented above strongly support the proposed mechanism. The linear Arrhenius behavior of k_1 and k_2 supports the contention that these are genuine microscopic rate constants. Even more significantly, k_1 and k_2 do not vary with Cl^- concentration, while K_{eq}' varies as predicted by the mechanism and k_2 is independent of the nature of the anion used.

Harris and co-workers[16-20] have proposed an empirical equation (eq 4) to describe the dependence of k_{obs} on concentration of PP

$$k_{obs} = k'[L]/(1 + k''[L]) + k'''[L] \quad (4)$$

and several phosphonate ligands. Although these authors have not explicitly proposed a mechanism to account for the behavior of k_{obs}, they have suggested that it results from two parallel pathways, one saturating with increasing ligand concentration and the other exhibiting a linear dependence. The equation does not account for anion effects; however, if one derives an equation (eq 5) on the basis of the suggested saturative/nonsaturative two-path

$$k_{obs} = k_1'k_2'[X^-][PP]/(k_{-1}' + k_2'[PP]) + k_3'[PP] \quad (5)$$

mechanism with anions accelerating the saturative pathway by promoting an open conformation (Scheme II), one finds an anion concentration dependence which differs from that observed experimentally in the study reported here.

In Scheme II the species $FeTfX^{-*}$ is the open conformation analogous to that proposed in the Bates mechanism[10] and is assumed to be present at a very low steady-state concentration. Comparison of eq 5 with eq 1 reveals the following relationships:

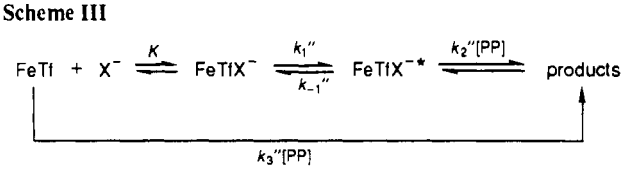
$$k_1 = (k_1'k_2'[X^-]/k_{-1}') + k_3'$$

$$k_2 = k_3'$$

$$K_{eq}' = k_2'/k_{-1}'$$

Thus, if the mechanism in Scheme II were valid, while that in Scheme I (Introduction) were invalid, k_1 would vary in a linear manner with anion concentration (with slope $k_1'k_2'/k_{-1}'$ and intercept k_3') and K_{eq}' would be invariant with respect to $[X^-]$ (k_1 would also be predicted to show non-Arrhenius behavior; however, since k_2 is found to be only about 10% of k_1, the curvature would be experimentally insignificant).

Scheme III



A further mechanism which would predict the observed dependence of k_{obs} on PP concentration is presented in Scheme III. This leads to

$$k_{obs} = \frac{\dfrac{k_{-1}''k_3'' + k_1''k_2''K[X^-]}{k_{-1}'' + k_{-1}''K[X^-]}[L] + \dfrac{k_2''k_3''}{k_{-1}'' + k_{-1}''K[X^-]}[L]^2}{1 + \dfrac{k_2''}{k_{-1}''}[L]} \quad (6)$$

Comparison with eq 1 reveals that k_1 would again vary with anion concentration, as also would k_2 (with the consequence that the linear portions of the curves in Figure 3 would not be parallel); K_{eq}' would again be independent of anion concentration.

In spite of the relatively large errors in K_{eq}', it is clearly possible to discriminate between these three mechanisms on the basis of the data presented in this paper. It would thus seem that the mechanism presented in Scheme I is the only simple mechanism which accounts for the observed kinetics. The 12-parameter equation proposed by Bertini et al.[21] was not used in the analysis of our data.

Implicit in our proposed mechanism is the requirement that an anion be bound to the KISAB site for iron release to occur (whether the anion is a simple ion such as Cl^- or a chelating agent such as PP). Binding of the anion to the KISAB site results in a conformation which is more open to the aqueous medium than that which is present at zero ionic strength (but this does not imply a completely open conformation such as that observed in apotransferrin). This is in accord with the observation of Kretchmar and Raymond[33] that the observed rate constant for iron release at fixed 3,4-LICAMS concentration extrapolates to zero at zero ionic strength. Under the conditions of ionic strength at which kinetic studies on transferrin have been conducted the KISAB site would almost certainly be occupied.

It is significant that our proposed mechanism explains the kinetics of iron release not only from Fe_CTf to PP but also from Fe_NTf to PP,[22] as well as from both monoferric transferrins to various phosphonate ligands[16-18,20] and citrate[23] and from Fe_2Tf to EDTA[14] (see in ref 23). Furthermore, if K_{eq}' is sufficiently low (i.e. the strength of binding of the chelating agent to the KISAB site is similar to that of the background anion(s)) or the experiments are conducted up to a fairly low chelator concentration, the observed kinetics will be experimentally indistinguishable from saturation kinetics (see Figure 5). It is thus possible that other chelating agents studied in the literature which seem to display saturation kinetics (e.g. acetohydroxamate[10] and 3,4-LICAMS[11]) may also follow this mechanism. This would account for the fact that the predicted saturation rate constants for various ligands are not the same;[13] this is a point of disagreement with the prediction of the Bates mechanism.

Scheme I shows attack of chelating agents on the $FeTf\{X^-\}$ and $FeTf\{L\}$ species leading directly to products. However, it is apparent that the reaction would proceed via at least one intermediate which probably contains chelator, amino acid side chains, and carbonate as ligands of the iron (the so-called quarternary complex). Because the breakup of this complex is very rapid in the iron release reaction, it results in no significant curvature in the linear portion of the k_{obs} versus chelator concentration plots, and

(33) Kretchmar, S. A.; Raymond, K. N. *Inorg. Chem.* **1988**, *27*, 1436-1441.

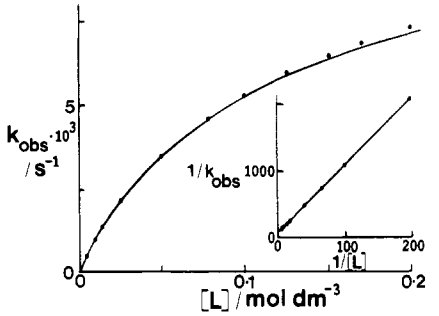


Figure 5. Hypothetical points calculated from eq 1 with k_1, k_2, and K_{eq}' arbitrarily set at 0.010 M^{-1} s^{-1}, 0.005 M^{-1} s^{-1}, and 10.0 M^{-1}, respectively. The solid line is a hyperbolic function. Note that the hypothetical kinetics would be experimentally indistinguishable from simple saturation kinetics under these conditions. Inset: Double reciprocal plot of the hypothetical data (units s vs dm^3 mol^{-1}).

no direct evidence for this complex is observed; i.e., attack of chelating agent on the iron is thus the rate-determining step. In the iron uptake reaction,[10,12] however, evidence for this intermediate has been obtained implying that the reverse of the k_1 step is the rate-determining step (the reverse of the k_2 step would not be significant in iron uptake studies since the concentration of chelator would necessarily be very low). The mechanism of the reverse reaction would thus be formally similar to the Bates mechanism, i.e., the present mechanism is thus also consistent with the reported iron uptake studies.

Another important finding of this study is the very significant difference in the kinetics when various background anions are used (Figure 4). Although it has long been known that anions have a considerable influence on the observed rate constants for iron release, it does not appear to have been appreciated that the anions can dramatically change the apparent nature of the dependence of k_{obs} on chelator concentration. This therefore highlights the importance of using the same buffers for comparative studies as well as the same acids for all pH adjustments and the same salts for all adjustments of ionic strength.

It is not possible at this stage to account for the strength of binding of the various anions to the KISAB site. The order (see Results) clearly does not follow the lyotropic series and is not related to anionic charge. It is also unclear why k_2 is always observed to be smaller than k_1; however, it is possible that this is a steric effect, due to the much larger size of the chelating agents compared with the background anions.

At present there is no direct evidence permitting the location of the KISAB site on the transferrin molecule. Nevertheless, fluorescence and kinetic studies on $Ru^{III}(NH_3)_5$-modified transferrin[34] have indicated two histidines as possible sites affecting the kinetics. These are His-207 (535) and His-242 (577) (human serum transferrin numbering, N- (C-) terminal lobes). One is located in the cleft between the two domains, and the other is located close to one of the two β-strands linking the two domains of each lobe. Interactions of anions with sites in these regions could alter the strength of interaction between the domains, promoting or retarding opening of the cleft to the iron-binding site. The physiological significance of the KISAB site is unknown, since iron release in vivo occurs with the transferrin bound to its receptor[35,36] on the cell membrane and occurs at low pH (5.5) in an endocytotic vesicle[37] (although anions have been shown to increase rates of iron release at low pH[38]). Recent studies have shown that the binding transferrin to the transferrin receptor affects the rate of iron release both to PP[39] and as a function of pH.[40] At pH 7.4 the receptor decreases the rate of iron release to PP,[39] and it is an intriguing possibility that the KISAB site may in fact be part of the receptor binding site on transferrin and that the observed effects of anions in vitro are somewhat artifactual, reflecting electrostatic alterations at this site.

Conclusions

The results of this study are consistent with the simple mechanism for iron release from transferrin proposed in Scheme I. The Arrhenius plots for the microscopic rate constants k_1 and k_2 are straight lines, the conditional equilibrium constant K_{eq}' varies with chloride concentration as predicted, and the value of k_2 is independent of the nature of the background anion. The generality of the mechanism is attested to by its ability to explain apparent saturation-linear kinetics found with other chelating agents (and for iron release from Fe_NTf) as well as the inconsistent limiting rate constants found for various chelating agents which display apparent saturation kinetics. The mechanism is also not inconsistent with iron uptake studies.

For iron release from Fe_CTf to pyrophosphate the average value of k_2 at 37 °C is 0.050 ± 0.007 M^{-1} s^{-1}, those for k_1 in the presence of Cl^-, ClO_4^-, and SO_4^{2-} are 0.91 ± 0.10 M^{-1} s^{-1}, 1.0 ± 0.5 M^{-1} s^{-1}, and 0.66 ± 0.04 M^{-1} s^{-1}, respectively. The values of K_{eq} (K_{eq}'[anion]) are 45 ± 10 (Cl^-), 22 ± 15 (ClO_4^-), and 11 ± 1 (SO_4^{2-}).

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Registry No. PP, 14000-31-8; Fe, 7439-89-6; Cl^-, 16887-00-6; ClO_4^-, 14797-73-0; NO_3^-, 14797-55-8; SO_4^{2-}, 14808-79-8; HPO_4^{2-}, 29505-79-1.

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