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# Kinetics of Iron Removal from Human Serum Monoferric Transferrins by Citrate

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The kinetics of release of $Fe^{3+}$ from human serum diferric transferrin ($Fe_2Tf$) and the N- and C-terminal monoferric transferrins ($Fe_NTf$ and $Fe_CTf$) to citrate were studied spectrophotometrically at pH 7.4, 37 °C, and 1.30 M ionic strength. Due to the similarity in rate constants for iron release from the two sites, iron release from $Fe_2Tf$ appears to be a monophasic process. Under the present experimental conditions, the C-terminal site is somewhat more labile than the N-terminal site. The observed rate constants for iron release from $Fe_NTf$ and $Fe_CTf$ show a dependence on citrate concentration (at constant ionic strength) which changes from one apparent linear gradient at low citrate concentration to a second, smaller, apparently linear gradient at higher citrate concentration. This is described by an equation of the form $k_{obs} = \{k_1[Cit][X] + k_2K[Cit]^2\}/\{[X] + K[Cit]\}$, where $k_1$ and $k_2$ are the second-order rate constants for pathways that are dominant at low and high citrate concentration, respectively, and $K$ is an equilibrium constant reflecting the competition by citrate and an anion, X, from solution for anion-binding sites, distinct from the synergistic-anion-binding sites, which control the kinetics of iron release. The experimental data both for $Fe_CTf$ and $Fe_NTf$ are adequately fitted by assuming the existence of one such site. The effect of the anions $HPO_4^{2-}$, $ClO_4^-$, $Cl^-$, and $SO_4^{2-}$ on the kinetics was studied, and it was shown that phosphate, while itself only mobilizing iron very slowly from transferrin, has a marked accelerating effect on metal release to citrate. The present results are compared to the previously reported results for release of $Al^{3+}$ from $Al_2Tf$ to citrate.

## Introduction

Human serum transferrin (Tf) is a single-chain iron-transport protein responsible for carrying two ferric ions per protein molecule between sites of iron absorption, storage, and utilization.[1-5] The iron-binding sites are in two similar but not identical bilobal domains and are respectively termed the N- and C-terminal binding sites. On the basis of the recently reported crystal structures of lactoferrin[6] and rabbit serum transferrin[7] and in view of the common evolutionary ancestry of all Tfs,[2] it can be concluded with a fair degree of certainty that the protein-based ligands for Fe(III) in the human serum protein are His, two Tyrs and Asp, with the other two coordination positions occupied by a (bi)carbonate synergistic anion, itself interacting with the protein via the side chain of an Arg residue and the N-terminus of an $\alpha$-helix.

There has been considerable interest in the mechanism of iron release from diferric transferrin ($Fe_2Tf$) and the monoferric transferrins ($Fe_NTf$ and $Fe_CTf$). Physiologically, $Fe_2Tf$, after binding to a specific receptor on the cell membrane, undergoes endocytosis into a vesicle,[8] which is then acidified to around pH 5.5 by the action of an ATP-driven proton pump.[9] It is probable that a chelating ligand for Fe(III) is required to effect its efficient removal; although $Fe_2Tf$ is unstable in acid solution (dropping the N-terminal Fe around pH 5.7 and the C-terminal Fe around pH 5[10]), in the absence of a chelator the rate of iron release is very slow[11] (of the order of hours) whereas the residence time of $Fe_2Tf$ in cells is only of the order of some minutes.[12,13]

Many studies have been conducted on iron release from $Fe_2Tf$ or the monoferric transferrins either directly to a variety of chelating ligands[14-35] or ultimately to acceptors such as aerobactin[36] and desferrioxamine B[37,38] mediated by small chelating ligands such as pyrophosphate, NTA, and EDTA. These kinetic studies usually, but not always,[17,21] appear to show saturation behavior with ligand concentration and have usually been interpreted by the well-known Bates mechanism,[25] as modified by Chasteen.[5] A feature of this mechanism is that the transition from a "closed" to an "open" protein conformation precedes iron release and is expected to be the rate-determining step. Recent[1] crystallographic evidence appears to provide support for this proposal with the observation of an Fe-bound "closed" conformation and an Fe-free "open" conformation for lactoferrin.

An element of the kinetics the Bates mechanism does not explain is the role anions, other than the synergistic anion, play in controlling and modifying the basic kinetic behavior of iron release from the two sites. Iron release kinetics from $Fe_2Tf$ are often at least biphasic[18,23,27,39] and are usually (but not always[17]) interpreted in terms of differences in the kinetic lability of the two sites, with

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the thermodynamically less stable N site usually the more kinetically labile.[24] However, by increasing the ionic strength with anions such as $ClO_4^-$ and $Cl^-$, it is possible to reverse the kinetic lability of the two sites.[40,41]

This study of the kinetics of iron release to citrate was undertaken primarily for two reasons: first, to extend our preliminary studies[21] on the effect of chelating ligand concentration in particular, and anions in general, on the rate of iron release to provide further insight into the intimate mechanism of this process; second, to compare the kinetics of $Fe^{3+}$ release from $Fe_2Tf$ with the rate of $Al^{3+}$ release from $Al_2Tf$ to citrate[42] to establish whether the comparatively labile nature of the latter is attributable to the metalloprotein or whether it is due to citrate being an usually rapid extractor of metal ions from metallotransferrins. The results are presented and discussed in this paper.

## Experimental Section

All glassware used was washed with 30% $HNO_3$ and then exhaustively with water doubly distilled in an all-glass still and further purified by passage through a Millipore MilliQ system (18 MΩ cm). The pH of solutions was measured with a Metrohm 605 pH meter and a Metrohm series 6.0210.100 micro combination glass electrode calibrated against standard buffers. Purified human serum transferrin (Tf) was obtained from Behringwerke. $Fe(NTA)_2$ was prepared by dissolving $FeCl_3 \cdot 6H_2O$ (BDH) and 4 equiv of NTA (Aldrich) in dilute HCl (pH 1) and very slowly raising the pH to 7.4 by dropwise addition of 1 M NaOH. (If the base is added too fast, ferric hydroxide precipitates.) ApoTf (250 mg) and Tris/HCl buffer (1.5 mL, pH 7.4, $\mu$ = 0.05 M) were placed in a glass vial, which was then slowly rotated manually until complete dissolution had been effected (about 10 min).

Diferric transferrin ($Fe_2Tf$) was prepared by adding 2.2 equiv of $NaHCO_3$ and 2.2 equiv of $Fe(NTA)_2$ to apoTf, and the solution was allowed to equilibrate at 5 °C overnight. The protein was then passed through a 1.5 × 20 cm Sephadex G25 column using Tris/HCl (pH 7.4, $\mu$ = 0.05 M) as eluent, at a flow rate of 0.4 mL $min^{-1}$. Fractions with $A_{280}/A_{466}$ and $A_{428}/A_{466}$ ratios near 23 and 0.8, respectively, were pooled and used for the kinetic studies or for the preparation of $Fe_NTf$.

$Fe_CTf$ was prepared by dissolving apoTf (250 mg) in 1.5 mL of 10 mM $NaHCO_3$ (pH 5), adding 0.8 equiv of $Fe(NTA)_2$ (also at pH 5), and incubating at 37 °C for 30 min. The pH was then raised to 7.4 by addition of 1 M $NaHCO_3$, and the solution was allowed to stand at 37 °C for 40 min before being passed through the Sephadex G25 column as described above.

$Fe_NTf$ was prepared[41] by incubating $Fe_2Tf$ with 0.134 M EDTA and 1.6 M $NaClO_4$ at 37 °C for 35 min at pH 7.4 and then rapidly passed through a 2.8 × 35 cm Dowex 1 1X2-400 anion-exchange resin previously stabilized with Tris/HCl buffer (pH 7.4, $\mu$ = 0.05 M).[21] EDTA is readily seen by adding a drop of each fraction to a $Mg^{2+}$/Eriochrome Black T (pH 10) solution. The EDTA-free protein fractions were collected and concentrated by ultrafiltration with an Amicon Model 52 ultrafiltration cell using an XM50 membrane.

The purity of the monoferric transferrins was assessed by measuring the rate of iron release to 0.100 M EDTA at pH 7.4 and 37 °C. The rate constants obtained from the monophasic kinetic traces ($4.6 \times 10^{-4}$ $s^{-1}$ for $Fe_NTf$ in the absence of added salts and $1.1 \times 10^{-3}$ $s^{-1}$ for $Fe_CTf$ in the presence of 0.500 M $NaClO_4$) are in very good agrement with those reported by Baldwin and de Sousa[41] for monoferric transferrins whose purity had been established by electrophoresis.

The kinetics of iron release from the various transferrin species to citrate were studied by following the decrease in the iron–phenolate charge-transfer band at 465 nm as a function of time with a Cary 219 spectrometer fitted with thermostatted cuvette holders connected to an external circulating water bath (37 °C). In all experiments the total buffer concentration was 20 mM, which means that a background of 17.8 mM $Cl^-$ from this source was always present. A stock 1 M citrate solution was prepared by dissolving trisodium citrate (BDH) in water and adjusting the pH to 7.4 with HCl. From the known $pK_a$ values of citrate (3.14, 4.77, and 6.39[43]) it can be shown that at pH 7.4 citrate is present as a mixture of the trianion (91.1%) and the dianion (8.9%); from this the ionic strength contribution of this sequestering ligand was calculated for each experiment. Having taken into account the ionic strength contribution from this and, where appropriate, from added salts ($Na_2SO_4$ (BDH); sodium phosphate (88.8% $Na_2HPO_4$, 11.2% $NaH_2PO_4$), NaCl, and $NaClO_4$ (all Merck)), we adjusted the final ionic strength to 1.30 M by addition of the appropriate volume of 2 M $NaNO_3$ (Merck). In some experiments $Na_2SO_4$ was used instead. The high ionic strength was unavoidable because of the significant contribution to this parameter of the citrate species in solution. The reactions were initiated by adding the protein (final concentration about 40 $\mu$M) to the other reagents in a 1 cm path length cuvette (final volume of solution in the cuvette was 2.00 mL); the reference cuvette contained all reagents except protein.

The reactions were monitored for at least 4 half-times. Between 50 and 60 data points were entered into a nonlinear least-squares computer program employing a Newton–Raphson procedure, and the absorbance–time data were fitted to both a single- and a double-exponential equation. Each reaction was repeated two or three times, and the results were averaged.

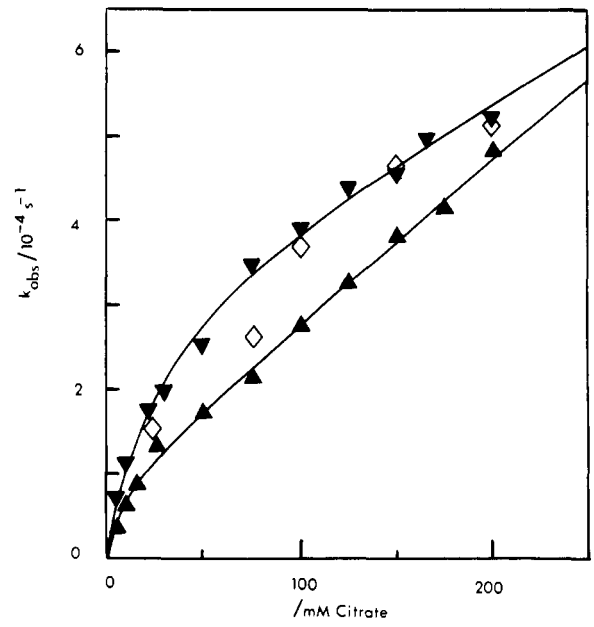


**Figure 1.** Dependence of the observed rate constant, $k_{obs}$, on citrate concentration for iron removal from $Fe_2Tf$ (◇), $Fe_CTf$ (▼), and $Fe_NTf$ (▲) at 37 °C. The ionic strength was maintained constant at 1.30 M for all experiments with $NaNO_3$. The solid lines are fits to eq 3 with the parameter values listed in Table I.

## Results

The kinetic traces for $Fe_2Tf$ and for the two monoferric Tfs could always be fitted by a single-exponential equation (eq 1). Fits to a double-exponential equation (eq 2) were unstable; substantially different values of the rate constants and the contribution of each phase (the values of $A_0$ and $A_1$) were obtained for duplicate runs, which, when fitted by a single exponential, gave good reproducibility (<3% standard deviation). (Bali and Harris[20] have drawn attention to the computational difficulties with extracting two rate constants for iron release from the two sites of $Fe_2Tf$, or even from equimolar mixtures of $Fe_CTf$ and $Fe_NTf$, due to their strong correlation during regression analysis.) It can be concluded that, under the conditions of the experiments reported here, both $Fe_CTf$ and $Fe_NTf$ release $Fe^{3+}$ at very similar rates (iron release from $Fe_2Tf$ is an apparently monophasic process) and that the

$$A = A_0 \exp(-k_{obs}t) + A_\infty \quad (1)$$

$$A = A_0 \exp(-k_{1obs}t) + A_1 \exp(-k_{2obs}t) + A_\infty \quad (2)$$

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**Table I.** Second-Order Rate Constants for Iron Release from Monoferric Tranferrins to Citrate (pH 7.4, 37 °C, $\mu$ = 1.30 M)

| Tf species | ionic strength adjustor | $k_1/10^{-2}$ $M^{-1}$ $s^{-1}$ | $k_2/10^{-3}$ $M^{-1}$ $s^{-1}$ | $K$ | correlation matrix[a] | | | |
|---|---|---|---|---|---|---|---|---|
| $Fe_CTf$ | $NaNO_3$ | 1.3 ± 0.2 | 2.60 ± 0.07 | 50 ± 15 | | $k_1$ | $k_2$ | $K$ |
| | | | | | $k_1$ | 1 | 0.41 | 0.96 |
| | | | | | $k_2$ | | 1 | 0.56 |
| | | | | | $K$ | | | 1 |
| $Fe_CTf$ | $Na_2SO_4$ | 1.8 ± 0.3 | 2.83 ± 0.02 | 56 ± 11 | | $k_1$ | $k_2$ | $K$ |
| | | | | | $k_1$ | 1 | 0.31 | 0.97 |
| | | | | | $k_2$ | | 1 | 0.42 |
| | | | | | $K$ | | | 1 |
| $Fe_NTf$ | $NaNO_3$ | 1.2 ± 0.3 | 2.33 ± 0.03 | 150 ± 65 | | $k_1$ | $k_2$ | $K$ |
| | | | | | $k_1$ | 1 | 0.38 | 0.97 |
| | | | | | $k_2$ | | 1 | 0.49 |
| | | | | | $K$ | | | 1 |

[a] Correlation matrix for nonlinear least-squares fit to eq 3.

quaternary intermediate, $Cit^{3-}Fe^{3+}$–Tf–$CO_3^{2-}$, decays fast (iron release from the monoferric Tfs is also monophasic).

The variation in $k_{obs}$ (eq 1) was studied as a function of citrate concentrations between 5 and 200 mM. At lower concentrations, iron release did not proceed sufficiently far for reliable measurements of the rate constants to be made. The results are shown in Figure 1. Under the conditions of these experiments, the C-terminal site is somewhat more labile than the N-terminal site at all citrate concentrations studied. Further, it is apparent that iron release from $Fe_2Tf$ appears to be a simple exponential process because the rate constants from the two sites are not very different; the apparent rate constant for release of $Fe^{3+}$ from $Fe_2Tf$ is closely bounded by the rate constants for its release from the individual sites.

An examination of the data from the monoferric Tfs, especially in the case of $Fe_NTf$, shows that $k_{obs}$ appears to saturate at low citrate concentrations but is virtually linear in citrate at higher concentrations. The dependence of $k_{obs}$ on citrate concentration is less obvious in the case of the C-site; it could conceivably display the same hyperbolic–linear behavior as the N site, or else be described by a simple hyperbolic curve. However, when nitrate is replaced by sulfate as the background ionic strength adjustor (Figure 2), it is clear that the same hyperbolic–linear behavior is displayed. It is therefore evident that the dependence of $k_{obs}$ on sequestering ligand concentration is contingent upon the ionic milieu.

The kinetic data (with the dependent variable $k_{obs}$ and the independent variables [citrate] and [X] where X = $NO_3^-$ or, in some experiments, $SO_4^{2-}$) for both sites were fitted to eq 3 by

$$k_{obs} = \frac{k_1[Cit][X] + k_2K[Cit]^{n+1}}{[X] + K[Cit]^n} \quad (3)$$

standard nonlinear least-squares methods using a Newton–Raphson procedure and Marquardt's algorithm; the fits were checked by using a program utilizing a simplex optimization procedure. In both cases, the parameters $k_1$, $k_2$, and $K$ were allowed to vary and integral values of $n$ were chosen; good fits were obtained with $n$ = 1. (See Discussion for the significance of this point and of the equation and for the definition of the equilibrium and rate constants.) The best-fitting values, together with the standard errors, are given in Table I.

Virtually the same values of the variables were obtained when very different initial guesses were used to start the minimization procedure or when a simplex method was used for the optimization procedure. As shown by the correlation matrices in Table I, there are weak correlations between $k_1$ and $k_2$ and between $k_2$ and $K$ but a strong correlation between $k_1$ and $K$; this contributes to the greater relative standard errors in $k_1$ and $K$ than in $k_2$.

The effect of various anions on the rate of iron release from the two monoferric Tfs was investigated at a constant citrate concentration of 50 mM, where the first term in [Cit] in eq 3 dominates yet the ionic strength contribution is relatively modest (0.289 M), allowing for ample variation in the contribution to the total ionic strength from the anion studied. In these experiments, too, the total ionic strength was maintained at 1.30 M with the contribution of the anion investigated merely replacing the contribution of nitrate. The results obtained with $SO_4^{2-}$, $Cl^-$, $ClO_4^-$, and $HPO_4^{2-}$ are shown in Figures 3 and 4, respectively. The lines drawn through the points are for clarity only and are not meant to imply a fit to the data.

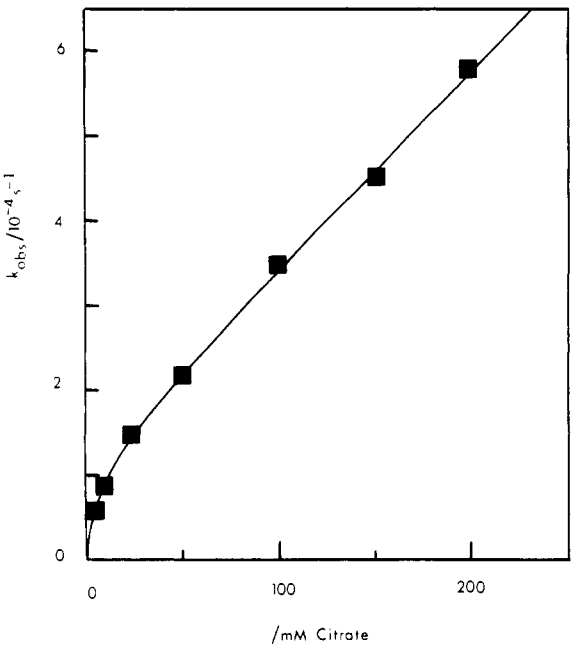


**Figure 2.** Dependence of the observed rate constant, $k_{obs}$, on citrate concentration for iron removal from $Fe_CTf$ with $Na_2SO_4$ as background electrolyte. The ionic strength was maintained constant at 1.30 M in all experiments. The solid line is a fit to eq 3 with the parameter values listed in Table I.

## Discussion

Although most studies on the kinetics of iron release from transferrin have been reported to show saturative (hyperbolic) dependence on sequestering ligand concentration (for example, to ATP,[14] catechols,[26] EDTA,[23,24] acetohydroxamate,[25] and pyrophosphate[36,37]), Harris and co-workers[17] have demonstrated that a plot of the observed rate constants for release of iron from $Fe_2Tf$ to pyrophosphate and a number of tripodal phosphonate ligands curved from an initial slope (with the origin as intercept) at low ligand concentration to reach a second, less steep linear slope at higher ligand concentrations. They have suggested that two pathways exist for iron release and have fitted the curves by an empirical equation (eq 4) incorporating a saturating first term

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

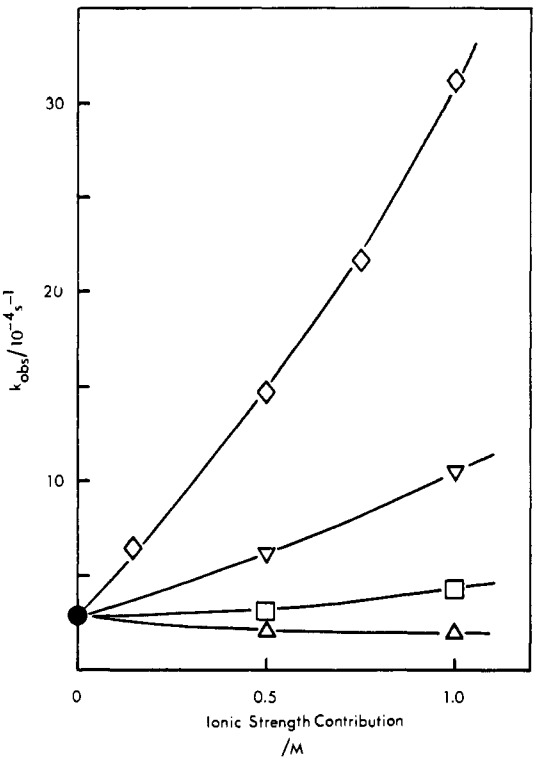


**Figure 3.** Effect of anions on the rate of iron release from $Fe_CTf$ to citrate: $Na_2HPO_4$ (◇); $NaClO_4$ (▽); NaCl (□); $Na_2SO_4$ (△).

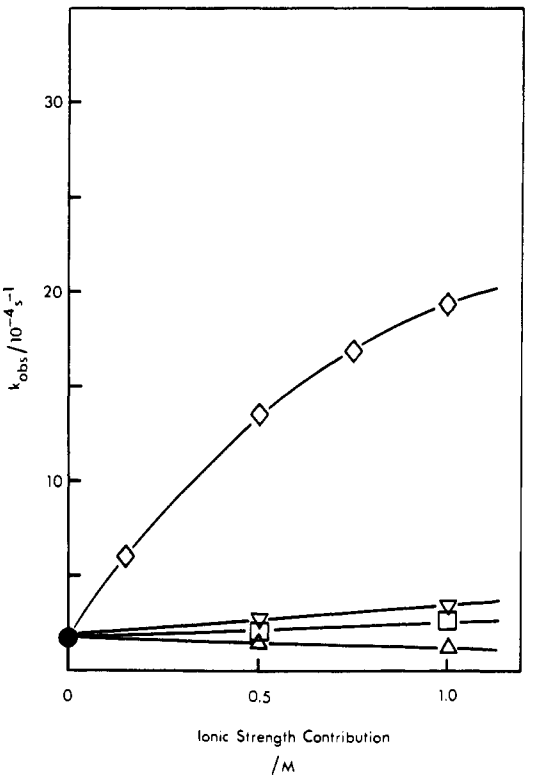


**Figure 4.** Effect of anions on the rate of iron release from $Fe_NTf$ to citrate: $Na_2HPO_4$ (◇); $NaClO_4$ (▽); NaCl (□); $Na_2SO_4$ (△).

and a linear second term; however, no suggestions appear to have been advanced as to the nature of the events at the molecular level which account for this behavior. A similar behavior was demonstrated for $Fe^{3+}$ release to pyrophosphate from $Fe_2Tf$,[18] $Fe_cTf$,[20] $Fe_NTf$,[20,21] and transferrins containing $Fe^{3+}$ in one site and kinetically inert $Co^{3+}$ in the other.[20]

The present results for iron release to citrate, and especially the results for $Fe_CTf$ with nitrate and sulfate, respectively, as ionic strength adjustors, suggest that hyperbolic behavior may be a special case of a general pattern of kinetic behavior where at low and high ligand concentrations the rate constants appear to saturate and to increase linearly, respectively. The linear portion of such a curve may fall outside the ligand concentration range investigated and not be obvious. Further, the degree of curvature, which is dependent on the ionic milieu, may be so gradual that an apparently purely hyperbolic curve is described. For example, we have found (results not shown) that the previously reported results for iron release to EDTA[23] can be adequately fitted by equations such as (3) and (4).

In addition to the synergistic anion, transferrins have many other anion-binding sites some of which are likely to play a crucial role in regulating iron release kinetics. The existence of these anion-binding sites has been deduced from electrophoretic mobility and isoionic pH studies,[44] spin-echo NMR methods,[45] ESR difference spectroscopy,[15] and the effect of anions on kinetics.[11,21,28,46]

Bertini and co-workers[18] have proposed a comprehensive scheme for iron release from $Fe_2Tf$ to pyrophosphate (PP) in the presence and absence of anions. Their proposed mechanism proceeds via two Bates-type conformations, one with bound anion (either chelating anion or nonchelating lyotropic anion) and the other without. The rates of attainment of both conformers is an intrinsic property of the protein or protein–anion complex; both paths should therefore be saturable. While this mechanism cannot be discounted, (i) release via the anion-free open conformation is unlikely in view of the observation of Kretchmar and Raymond[28] that iron release drops to zero as ionic strength drops to zero and (ii) the behavior of the system can be adequately described by changes in $K$ in eq 3, as observed in this work, which has the advantage of greater simplicity.

It is an inescapable conclusion that anion-binding sites are an important feature of transferrin chemistry. There is no reason to suppose that all these sites control the kinetic behavior of the protein. We suggest that it is the occupation of some of them, which we shall term kinetically significant anion-binding (KISAB) sites, by different anions which is responsible for controlling and modifying the kinetic behavior demonstrated in this and other studies.

A vacant KISAB site will be denoted as {·}, and {X}FeTf and {L}FeTf will denote iron transferrin species with KISAB sites occupied by an anion X from solution and by the sequestering ligand itself, respectively. In solutions of high ionic strength as used in this and most other studies, it is reasonable to assume that the concentration of {·}FeTf is insignificant. The release of Fe from FeTf can therefore proceed by attack of a sequestering ligand, L (in this study, citrate), on either {X}FeTf or {L}FeTf. $K$ is an equilibrium constant for competition for the KISAB site between X and L (eq 5). If $k_1$ and $k_2$, respectively, represent the sec-

$$\{X\}FeTf + L \rightleftharpoons \{L\}FeTf + X \quad (5)$$

ond-order rate constants for iron release to L from {X}FeTf and {L}FeTf, then the overall kinetic expression is given by eq 6. Upon substitution of [{X}FeTf] and [{L}FeTf] by $[FeTf]_{tot}$, the concentration of all ferric transferrin species in solution, eq 7 is obtained. Under pseudo-first-order conditions, $k_{obs}$ is then given

$$\frac{d[products]}{dt} = k_1[L][\{X\}FeTf] + k_2[L][\{L\}FeTf] \quad (6)$$

$$\frac{d[products]}{dt} = \frac{k_1[L][X][FeTf]_{tot}}{[X] + K[L]} + \frac{k_2K[L]^2[FeTf]_{tot}}{[X] + K[L]} \quad (7)$$

by eq 3 (L = citrate) where, for a single KISAB site, $n = 1$. Hence, as X is replaced by L in the KISAB site, the reaction pathways changes from one where iron release is from {X}FeTf to one where release is from {L}FeTf. As $[L] \rightarrow 0$, $k_{obs} \rightarrow k_1[L]$,

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and as [L] → ∞, $k_{obs} \rightarrow k_1[X]/K + k_2[L]$. If $k_1 > k_2$, then, at low [L], $k_{obs}$ varies linearly with [L] with a slope of $k_1$; at higher [L], the plot becomes nearly (but not quite, since [X] varies) linear with slope approximating $k_2$. The apparent linear portions of curves such as shown in Figures 1 and 2 are therefore controlled by the magnitude of $k_1$ and $k_2$, and the extent of curvature is controlled by $K$.

The kinetic data were fitted to eq 3 quite adequately with $n = 1$ (i.e., assuming a single KISAB site per monoferric Tf); the curves generated with $n > 1$ (not shown) did not fit as well. It may be possible to generate fits by invoking multiple KISAB sites and iron release from a series of transferrin species each with KISAB sites satured to a different extent, but this seems unnecessarily complicated. It may be concluded that a single KISAB site is the *minimum* requirement for an explanation of the data.

The proposed mechanism is relatively simple (compared, for example, with that proposed by Bertini[18]). It (i) explains why the limiting rate constants predicted from the Bates mechanism differ for different chelating agents (the curves are not truly hyperbolic), (ii) is consistent with the finding of Kretchmar and Raymond[28] that the extrapolated rate constant for iron removal is zero at zero ionic strength (iron release is not possible unless the KISAB site is occupied), and (iii) provides an explanation of how anions affect the rate of iron release.

The position of the KISAB site(s) is as yet unknown, and the precise series of events on the molecular level can only be speculated upon. It seems reasonable to imagine that interdomain forces may hold the protein clamped in a closed conformation at very low ionic strength and the release of iron is impossible. Binding of an anion to a KISAB site disrupts this interaction to some degree and allows attack of a chelating agent on the metal. Some anions such as $ClO_4^-$ and $HPO_4^-$ significantly accelerate iron release, while others such as $NO_3^-$ and $SO_4^{2-}$ are less effective. The factors governing their effectiveness at promoting iron release are not yet clear. Phosphate appears to be particularly efficient in this regard and this may be physiologically significant. Larger anions such as citrate and pyrophosphate may sterically impede the approach of a sequestering ligand to the metal ion, and this would explain why, when these species occupy the KISAB site, the rate constant for iron release decreases (i.e., $k_2 < k_1$ and {X}FeTf is more labile than {L}FeTf).

This study is not a good model for the physiological process of iron release in acidic endocytotic vesicles, since both the ionic strength (1.30 M) and the pH (7.4) are too high. Nevertheless the mechanism of iron release to citrate at this high ionic strength is essentially the same as that found for release to pyrophosphate at variable ionic strength (at least for $Fe_NTf$).[21] It seems reasonable to expect that anions could have a similar marked effect under physiological conditions, and experiments with pyrophosphate as chelating agent are in progress.

Citrate mobilizes $Al^{3+}$ from the two sites of $Al_2Tf$ with second-order rate constants of 0.60 and 0.38 $M^{-1}$ $s^{-1}$, respectively, and from the resulting AlTf with rate constants of 0.27 and 0.12 $M^{-1}$ $s^{-1}$, respectively.[42] There is a question about the reliability of rate constants obtained by nonlinear regression analysis of kinetic traces of metal ion release from dimetalated transferrins (vide supra). Further, the study on $Al_2Tf$ and the present study were conducted at different ionic strengths (0.70 and 1.30 M, respectively). Nevertheless, it is probably valid to conclude that the fast release of $Al^{3+}$ to citrate is not due to any special ability of this ligand to mobilize metal ions from transferrin, since the rates reported here for mobilization of $Fe^{3+}$ (Table I) are of the same order as for other ligands previously studied such as EDTA[14,23,24] and pyrophosphate.[14-22,31] The lability of $Al_2Tf$ appears to be related to the lower thermodynamic stability of $Al_2Tf$ ($\log \beta_2 = 31$) compared to $Fe_2Tf$ ($\log \beta_2 = 40$),[47] which may be a consequence of differences in ionic radii of the two ions (0.675 Å for $Al^{3+}$; 0.785 Å for high-spin $Fe^{3+}$[48]). The displacement of $Al^{3+}$ by $Fe^{3+}$ would presumably require release of the former prior to binding of the latter; the results obtained indicate that such a process is both thermodynamically favored and kinetically facile. Thus, if $Al^{3+}$ is transferrin-bound in the serum, as has been suggested,[49,50] any excess of $Fe^{3+}$ is likely to displace $Al^{3+}$, resulting in its deposition. In this respect, it is interesting to note that abnormally high $Fe^{3+}$ saturation of transferrin has been observed in the brain tissue of persons suffering from Alzheimer's disease (59% versus 39% in normal persons.)[51]

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**Registry No.** Fe, 7439-89-6; $HPO_4^-$, 14066-19-4; $ClO_4$, 14797-73-0; $Cl^-$, 16887-00-6; $SO_4^-$, 14808-79-8; citrate, 77-92-9.

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