The Mechanism of Iron Transferrin Interactions. Uptake of the Iron Nitrilotriacetic Acid Complex

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The role of the protonation of the transferrin amino acid ligands involved in complex formation with iron in the presence of nitrilotriacetate has been elucidated. The C-terminal site of transferrin binds to Fe(nta) to produce FeH_3T_C; second-order rate constant $k_1 = (7.00 \pm 0.05) \times 10^3$ dm^3 mol^{-1} s^{-1}, stability constant K_1 $(1.00 \pm 0.10) \times 10^{-5}$ mol dm^{-3}. This lowers the deprotonation pK_a of probably the phenolic side-chain of one tyrosine which loses a proton and, thereby, leads to FeH_2T_C; dissociation constant $K_{1a} = (4.50 \pm 0.50) \times 10^{-7}$ mol dm^{-3} and a possible complex stability constant $K_1' \approx 2.3 \times 10^{-9}$ mol dm^{-3}. As for the N-terminal site, it binds to Fe(nta) by a process controlled by a slow proton transfer; second-order rate constant $k_{2a} = (4.50 \pm 0.30) \times 10^6$ dm^3 mol^{-1} s^{-1}, a reverse rate constant $k_{-2} = 0.40 \pm 0.05$ s^{-1}, proton dissociation constant $K_{2a} = (8.5 \pm 1.1) \times 10^{-8}$ mol dm^{-3}. It remains to be shown whether this slow proton transfer controls a change of the conformation of the binding site or if it occurs because of the particular conformations of the binding sites in neutral media.

Transferrin (T), the iron-transport system in vertebrates, is a bilobal glycoprotein of molecular weight *ca.* 80 000.[1-3] Each lobe possesses one iron binding site in which the iron(III) atom is most probably co-ordinated by four protein ligands (Fig. 1).[2,3] Iron binding to T requires the assistance of anions such as carbonate, oxalate or nitrilotriacetate.[4] Naturally, in biological media this binding occurs with carbonate[4,5] with affinity constants of *ca.* 10^{19}–10^{20} dm^3 mol^{-1}.[5] The two binding sites, (*C*) in the C-terminal lobe and (*N*) in the N-terminal lobe, are not kinetically equivalent towards iron removal from the protein.[6] Site *C* has the greatest affinity for iron and retains it in acidic media, while the *N* site is considered more basic and does not retain iron in acidic media.[7] Moreover, in the presence of carbonate, iron binding to the protein is proton dependent and releases three H^+ per bound atom of metal in neutral media.[5] With iron(III) complexes such as those with pyrophosphate or acetohydroxamic acid, iron uptake by T occurs in minutes by the exchange of Fe^{III} between the complex and the protein *via* a mixed T-CO_3^{2-}/HCO_3–Fe-ligand complex [equation (1)].[8,9]

$$\mathrm{FeL} + \mathrm{T} \underset{}{\overset{CO_3^{2-}/HCO_3^-}{\rightleftharpoons}} \mathrm{TFeL} \rightleftharpoons \mathrm{TFe} + \mathrm{L} \quad (1)$$

This process is much faster with iron(III) nitrilotriacetate, Fe(nta), where it occurs in less than ten seconds by biphase kinetics[10] without the assistance of carbonate and without loss of nta.[4] Most of the available data deal with iron depletion by excess of low molecular weight chelating agents and by siderophores.[6,11-16] However, there is a lack of direct kinetic data related to iron release in the absence of competing chelating ligands which is known to be extremely slow.[17] On the other hand, in research dealing with the mechanisms of iron uptake, transport and release by T in living cells and *in vivo*, it was established that T binds to a specific receptor situated in the plasma membrane.[18] The protein and its receptor are internalized in lipid vesicles or endosomes inside the cell by metabolic events known as encocytosis.[19,20] The endosomes are then acidified down to pH 5.5, where the protein loses its iron

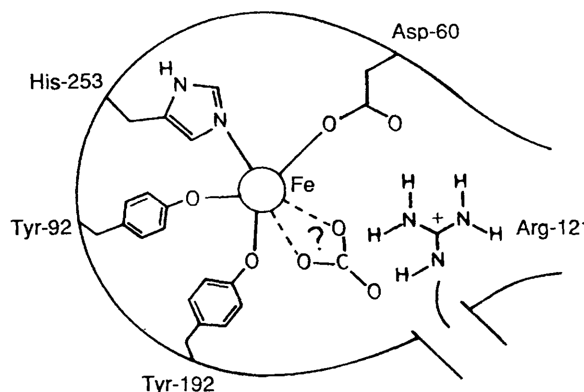


Fig. 1 Schematic view of the iron–lactoferrin complex in the *N*-lobe.[3] The same arrangement is also found in the *C*-lobe[3]

which is then complexed by the chelating agents present in the medium.[19-22] These events reflect the major importance of proton transfers in the processes of iron release and uptake by T which are still poorly understood. The major obstacle for any kinetic analysis dealing with the details of the mechanism of iron uptake and release by T and the influence of proton transfers is the high affinity of the protein for iron in the presence of carbonate.[5] Indeed, if the affinity was as high as 10^{20} dm^3 mol^{-1} a classical physicochemical analysis of iron uptake would be very difficult, simply because the metal will always be complexed with the protein, and the equilibrium is entirely displaced towards the iron–T complex. To overcome this problem, we replaced carbonate by nta and re-examined, in this relaxation kinetic[23-25] and thermodynamic study, Fe(nta) uptake by T. We do not suggest that the behaviour of the protein will be the same as with carbonate, but we believe that this can constitute a first step towards a better understanding of the role played by proton transfers in the processes which govern iron–transferrin interactions.

Experimental

Pure human apotransferrin (>98%, Sigma) (20 mg cm^{-3}) in neutral 0.01 mol dm^{-3} ethylenediamine-*N,N,N',N'*-tetraacetic acid (H_4edta) was dialysed at 4 °C for 20 h against three changes of distilled water and two changes of final buffer. Potassium chloride (Merck Suprapur), H_4edta, NaOH and HCl (Merck Titrisol), $FeCl_3$, hepes [*N'*-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid] (Sigma or Aldrich) and nitrilotriacetic acid (H_3nta) (Aldrich) were used without further purification. Water was distilled twice and boiled about 10 min prior to use. All glassware was cleaned with 5 mol dm^{-3} HCl and abundantly rinsed with distilled water. Moreover, all contact of solutions or reagents with metals was avoided.

Stock Solutions.—All stock solutions were used fresh. They were prepared in the previously boiled distilled water, and were degassed under argon. All final ionic strengths were adjusted to 0.2 mol dm^{-3} with KCl. The hepes concentration in neutral buffers was 50×10^{-3} mol dm^{-3} and final pH values were adjusted to between 6.2 and 8.4 with microquantities of concentrated HCl or NaOH. Transferrin concentrations (c_1) were spectrophotometrically checked at 278 nm after dilution on the basis of a molecular absorption coefficient of 93 000 dm^3 mol^{-1} cm^{-1}.[13] They ranged from 1.5×10^{-5} to 1.3×10^{-4} mol dm^{-3}. The lowest c_1 were used for spectrophotometric measurements, while the highest were used for stopped-flow experiments. Solutions of Fe(nta) (c_2) [(6–7) $\times 10^{-3}$ mol dm^{-3}] were prepared, under argon and at 40 °C, in 10^{-2} mol dm^{-3} HCl with $FeCl_3$ and 1 equivalent of nitrilotriacetic acid. The pH was then gently raised to neutrality with microvolumes of concentrated NaOH.[4] These solutions were further diluted to the required final Fe(nta) concentrations in the buffers.

pH Measurements.—pH Values were measured at 25 ± 0.5 °C, in the sample cell for spectrophotometric measurements and at the end of the mixing for stopped-flow experiments with a Jenco pH meter equipped with an 'Ingold' combined microelectrode. Buffers used for pH standardization were pH 4.00, 7.00 and 10.01 (Beckman).

Spectrophotometric Measurements.—Spectrophotometric measurements were performed under nitrogen at 25 ± 0.5 °C on Cary C210 and Varian DMS 200 spectrophotometers equipped with magnetic stirring devices and thermostatted cell carriers. Absorbance spectra of neutral hepes-buffered transferrin solution at fixed pH and c_1 were recorded in the range 400–600 nm at several concentrations c_2 varying from $0.1c_1$ to $5c_1$.

Stopped-flow Measurements.—Kinetic measurements were performed under anaerobic conditions on a High-Tech stopped-flow spectrophotometer equipped with a thermostatted bath at 25 ± 0.5 °C.[25] Output voltage was adjusted to 5 V for zero absorbance and signals were recorded on a Victor PC type computer. Three series of stopped-flow experiments were performed in the neutral hepes buffer. In the first $c_1 < c_2$, in the second $c_1 \geqslant 3c_2$ and in the third series, solutions containing equal concentrations of transferrin and Fe(nta) were mixed with Fe(nta) solutions.

Signal Analysis.—All experimental signals were first analysed by the Padé–Laplace method which gives the number of exponentials contained in a signal and a fair approximation to their exponential constants.[21,26] All the signals recorded under the experimental conditions of this work were either mono- or bi-exponentials. The values from the Padé–Laplace treatment were then used in a more precise biexponential signal analysis by Marquardt[26] which requires prior knowledge of the number of exponentials in the signal and an approximation to their time constants. Monoexponential signals were further analysed by the Guggenheim and phase-plane methods which do not require prior knowledge of asymptotic lines.[24,25]

Results

For reasons of simplicity and lack of knowledge of the state of the charge in the binding sites of T near neutrality,* the charges of the ions involved in the T–iron equilibria are not written. Moreover, since T is bilobal and each lobe possesses a binding site and since iron binding to T in the presence of carbonate releases three H^+ per bound metal,[5] the protein will be represented as $H_3T_CT_NH_3$.

Since experimental signals were pure exponentials, they were dealt with as relaxation modes.[23–25] Moreover, the experimental conditions of this work were chosen so that they were compatible with the use of chemical relaxation formalism.[28] The thermodynamic and kinetic equations are derived in the Appendix to this article.

Iron Uptake in Neutral Media.—When Fe(nta) is added to a neutral solution of T, an absorption band appears in the range 400–600 nm.[4] This absorption is dependent on the concentration of T (c_1), the iron complex concentration (c_2) and the pH. When $c_2 < c_1$, the measured spectrum is that of the first Fe–T complex in the presence of nta (Fig. 2).[4] For $c_1 < c_2$, a new absorption band appears (Fig. 2) corresponding to binding of iron to the *N* site of the $FeT_CT_NH_3$ complex.† Monitoring the protein ligand tyrosine[2,3] absorption band in the UV region is impracticable because in the transferrin concentration range used here the absorption becomes high and the Beer–Lambert law is not obeyed.

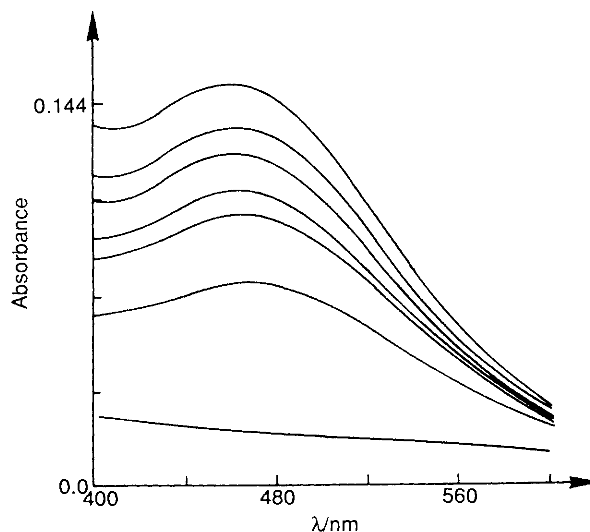


Fig. 2 Absorbance changes at pH 7.64 when a transferrin solution is submitted to several Fe(nta) concentration jumps; with $c_1 = 2.9 \times 10^{-5}$ mol dm^{-3} and c_2 varying from 0 to $5c_1$

* Although each of the binding sites of T possesses four protein ligands engaged in binding with iron,[2,3] and although the deprotonation p*K* values of their side chains are known for the free amino acids,[27] to our knowledge these values were never reported for T. Furthermore, no information is available on the charge of the carbonate which can be involved in the iron–protein complex either as HCO_3^- or CO_3^{2-}.[2,3]

† The molar absorption coefficient of the Fe(nta)–*C*-site protein complex is about 3100 dm^3 mol^{-1} cm^{-1} at 465 nm. That of the protein in the presence of a very large excess of Fe(nta) ($c_2 > 10c_1$), measured at 465 nm after the subtraction of the spectral contribution of the unbound Fe(nta) ($c_3 - 2c_1$) from the absorption spectrum, is 5200 dm^3 mol^{-1} cm^{-1}

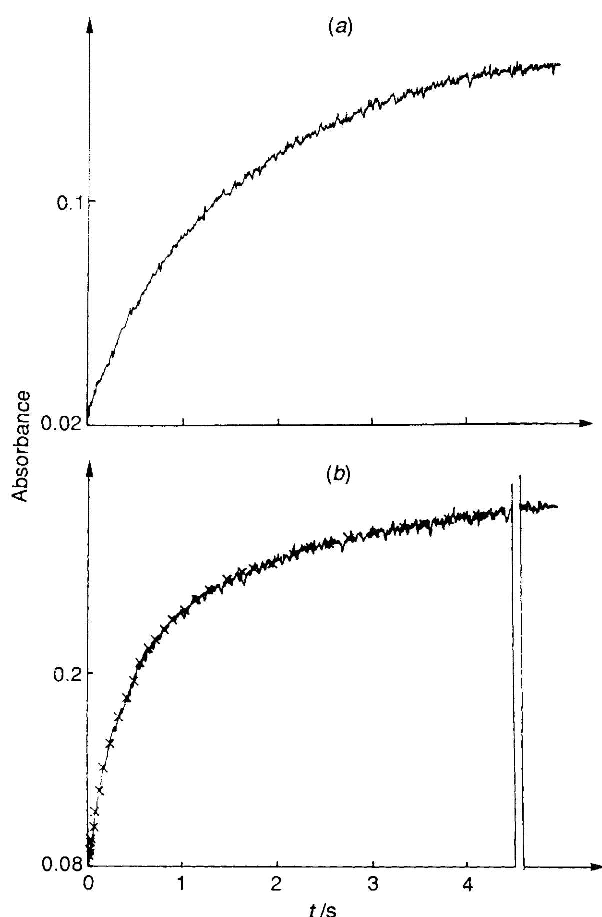


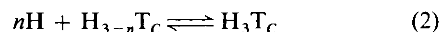
Fig. 3 (*a*) Exponential increase of absorbance at 565 nm when a transferrin solution is submitted to a fast Fe(nta) concentration jump at pH 7.65, 25 ± 0.5 °C and $I = 0.2$ mol dm^{-3}; $c_1 = 4.36 \times 10^{-5}$ mol dm^{-3} and $c_2 = 2.26 \times 10^{-5}$ mol dm^{-3}. (*b*) Biexponential increase of absorbance at 565 nm when a transferrin solution is submitted to a fast Fe(nta) concentration jump at pH 7.44, 25 ± 0.5 °C and $I = 0.2$ mol dm^{-3}; $c_1 = 5.02 \times 10^{-5}$ mol dm^{-3} and $c_2 = 2.77 \times 10^{-4}$ mol dm^{-3}; (× × × ×) represents a biexponential simulation with $\tau_1 = 0.35$ s and $\tau_2 = 1.95$ s

Kinetic observations. When a hepes-buffered neutral solution of T is mixed in a stopped-flow experiment with a neutral solution of Fe(nta), two types of kinetic process are observed in the range 400–500 nm. In the first case, when $c_2 < c_1$, a c_1-, c_2-, and pH-dependent monoexponential increase of absorbance with time is detected [Fig. 3(*a*)]. The second case, when $c_2 \geqslant 5c_1$, is a biexponential increase of absorbance with time [Fig. 3(*b*)]. The amplitudes of both phenomena in Fig. 3(*b*) are independent of c_1, c_2, and pH, and the amplitude of the fast kinetic process is always about 60–65% of the overall experimental amplitude. Moreover, if in the stopped-flow experiment a neutral solution containing equal concentrations of the protein and Fe(nta) is mixed with a neutral solution of Fe(nta), only the second slow kinetic process detected in Fig. 3(*b*) is observed.

Equilibrium of first iron binding to transferrin. In Fig. 3(*a*), a single exponential kinetic process is observed when an Fe(nta) solution is mixed with a solution of T with $c_2 < c_1$. At the end of this phenomenon, the absorption spectrum is that of first Fe(nta) binding to T (Fig. 2). When, in the vicinity of neutrality, c_2 exceeds c_1, a new exponential kinetic phenomenon is detected [Fig. 3(*b*)]. At the end of this phenomenon (final state of equilibrium), the absorption spectrum is that of binding of a second iron to the protein (Fig. 2). Moreover, in the vicinity of neutrality, for $c_2 \geqslant 5c_1$ and at $\lambda = 465$ nm, the ratio of the absorbance related to Fe_2–protein complex to that of Fe–protein is about 1.6–1.7, which is also the value found for the ratio of the overall amplitude to that of the slow kinetic process [Fig. 3(*b*)]. Therefore, the single kinetic process observed in Fig. 3(*a*) is related to the binding of one iron complex to the protein, whereas the two kinetic processes of Fig. 3(*b*) are related to the binding of the first and second iron to the protein.

The complex moiety Fe(nta) binds first to the binding site of the C-terminal lobe.[7,11,12] Furthermore, when c_2 is lower or equal to c_1, it is known that only site *C* is involved in the binding.[11,12] Therefore, to a first approximation, we will consider that Fe(nta) binds to the protein according to either mechanism A [reactions (2) and (3)] or mechanism B [reactions (4) and (5)], where Fe represents the Fe(nta) complex.

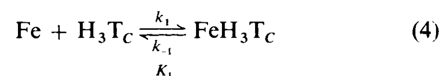
Mechanism A; $K_{1a}' = [H]^n[H_{3-n}T_C]/[H_3T_C]$, $K_1' = [Fe][H_{3-n}T_C]/[FeH_{3-n}T_C]$



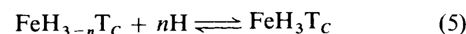
$$nH + H_{3-n}T_C \rightleftharpoons H_3T_C \qquad (2)$$

$$H_{3-n}T_C + Fe \rightleftharpoons FeH_{3-n}T_C \qquad (3)$$

Mechanism B; $K_{1a} = [H]^n[FeH_{3-n}T_C]/[FeH_3T_C]$, $K_1 = [Fe][H_3T_C]/[FeH_3T_C]$



$$Fe + H_3T_C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FeH_3T_C \quad (K_1) \qquad (4)$$



$$FeH_{3-n}T_C + nH \rightleftharpoons FeH_3T_C \qquad (5)$$

In the case of mechanism A, for an optical path length of 1 cm a Benesi–Hildebrandt type relationship is derived [equation (6)] in which $\Delta A = A - A_0$ and $\Delta\varepsilon' = \varepsilon_1 - (\varepsilon_2 + \varepsilon_2')$, where

$$\Delta\varepsilon'/\Delta A = 1/c_1 + K_{obs}/c_1[c_2 - (\Delta\varepsilon'/\Delta A)] \qquad (6)$$

A is the absorbance, A_0 is the initial absorbance in the absence of Fe, and ε_1, ε_2 and ε_2' are the molar absorption coefficients of species $FeH_{3-n}T_C$, H_3T_C and $H_{3-n}T_C$, respectively. The expression for K_{obs} is then given by equation (7). At

$$K_{obs} = K_1' + [H]^nK_1'/K_{1a}' \qquad (7)$$

constant pH, K_{obs} is constant. Therefore, six series of experiments were performed in hepes-buffered media from pH 6.3 to 7.31. At each of the six pH values a linear least-squares regression of the experimental data against equation (6) gave one K_{obs} value (Table 1). However, attempts linearly to correlate K_{obs} against [H], $[H]^2$ or $[H]^3$ were unsuccessful.

In the case of mechanism B, for an optical path length, of 1 cm the Benesi–Hildebrandt type relationship is given by equation (8) in which $\Delta\varepsilon = (\varepsilon_1 + \varepsilon_1') - \varepsilon_2$, where ε_1' is the molar

$$\Delta\varepsilon/\Delta A = 1/c_1 + K_{obs}/c_1[c_2 - (\Delta\varepsilon/\Delta A)] \qquad (8)$$

absorption coefficient of species FeH_3T_C. The expressions for K_{obs} and K_{obs}^{-1} are given by equations (9) and (10).

$$K_{obs} = K_1[H]/(K_{1a} + [H]) \qquad (9)$$

$$K_{obs}^{-1} = 1/K_1 + K_{1a}/K_1[H]^n \qquad (10)$$

Equation (8) is comparable to equation (6). Therefore, the K_{obs} values are those reported in Table 1. The only possible linear least-squares regression of K_{obs}^{-1} against equation (10) is achieved for $n = 1$ (Fig. 4). This clearly indicates that between pH 6.3 and 7.31 only a single proton exchange is involved in Fe(nta) binding to the protein, which probably occurs according to mechanism B. Values of K_1 and K_{1a} are determined from

Table 1 Values of K_{obs} measured at differing pH values at 25 ± 0.5 °C and I = 0.2 mol dm^{-3}

pH	$10^6 K_{obs}$/mol dm^{-3}
6.34	5.06
6.50	4.61
6.71	2.87
6.84	2.62
7.07	1.6
7.31	1.00

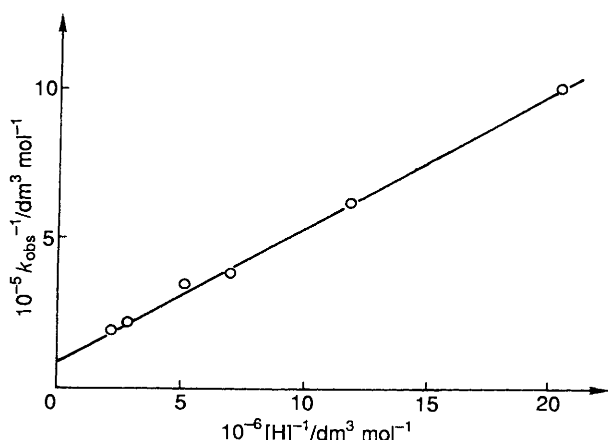


Fig. 4 Plot of K_{obs}^{-1} against [H]$^{-1}$ (Table 1) at 25 ± 0.5 °C and I = 0.2 mol dm^{-3}. Intercept, (1.00 ± 0.08) × 10^5 dm^3 mol^{-1}; slope, (4.45 ± 0.10) × 10^{-2}, r = 0.998 09

the slope and the intercept of the experimental line of Fig. 4: K_1 = (1.0 ± 0.1) × 10^{-5} mol dm^{-3}, K_{1a} = (4.5 ± 0.5) × 10^{-7} mol dm^{-3}.

Attempts to measure the equilibrium constants involved in the binding of the second iron to T were fruitless. They were performed near neutrality (pH ⩾ 7.4) by adding Fe(nta) to solutions containing equal amounts of iron complex and protein.

First relaxation process. In neutral media, the preceding thermodynamic study allowed us to ascribe the single relaxation process observed for $c_2 < c_1$ [Fig. 3(*a*)] and the first fast relaxation observed for $c_2 \geqslant 5c_1$ [Fig. 3(*b*)] to the binding of Fe to the *C* site of the protein *via* Mechanism B, with n = 1.

Since acid–base reactions [reaction (5)] are usually diffusion controlled,[23–25] it is assumed that binding of Fe to protein is rate-limiting [reaction (4)]. Chemical relaxation methodology can be applied in buffered media if $c_2 \geqslant 3c_1$ or $c_2 < c_1$.[28] In this case the reciprocal relaxation time equation associated with reaction (4) is derived by the substitution method according to equation (11) which can be modified to equation (12). At the

$$\tau_1^{-1} = k_1([FeH_3T_C] + [Fe])([H] + K_{1a})/K_{1a} + k_{-1}[H]/K_{1a} \quad (11)$$

$$(\tau_1)^{-1}/[H] = k_1([H_3T_C] + [Fe])(K_{1a} + [H])/K_{1a}[H] + k_{-1}/K_{1a} \quad (12)$$

end of Fe binding to the *C* site, and before binding to the *N* site, [H_3T_C] and [Fe] can be determined from equations (13) and (14) where β = ([H] + K_{1a})/K_1[H].

$$[Fe] = (c_1 - [H_3T_C])/\beta[H_3T_C] \quad (13)$$

$$[H_3T_C] = \{\beta c_1 - \beta c_2 - 1 + [(\beta c_1 - \beta c_2 - 1)^2 + 4\beta c_1]^{\frac{1}{2}}\}/2\beta \quad (14)$$

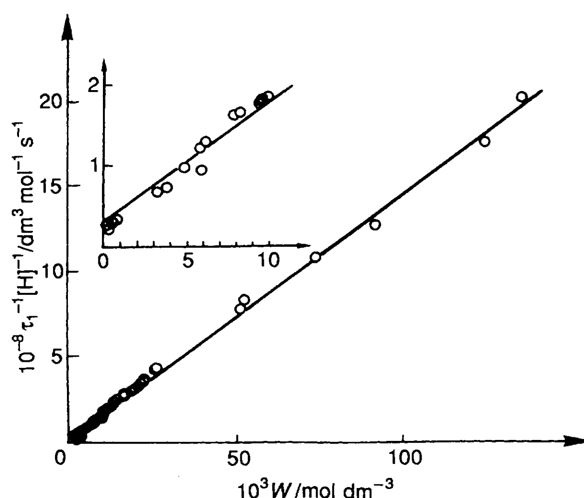


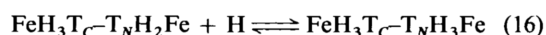
Fig. 5 Plot of τ_1^{-1}/[H] against W = ([H_3T_C] + [Fe])(K_{1a} + [H])/[H] at 25 ± 0.5 °C and I = 0.2 mol dm^{-3}. Intercept, (4 ± 6) × 10^7 dm^3 mol^{-1} s^{-1}; slope, (1.45 ± 0.05) × 10^{10} dm^6 mol^{-2} s^{-1}, r = 0.996 37. The scale is expanded 5 times for the first 17 points

A good linear least-squares regression of the data against equation (12) is obtained for c_1 in the range (3.5–6) × 10^{-5} mol dm^{-3}, c_2 from 2 × 10^{-5} to 1 × 10^{-3} mol dm^{-3} and pH from 7.13 to 8.40 (Fig. 5). The value of rate constant k_1 = (7.00 ± 0.05) × 10^3 dm^3 mol^{-1} s^{-1} is determined from the slope of regression line.

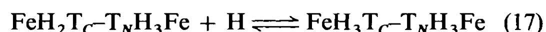
Neutral media, second kinetic process. In neutral media, the amplitude of the slow kinetic phenomenon in Fig. 3(*b*) and that of the single kinetic process observed when the iron–transferrin monocomplex is mixed with Fe(nta) is that of the transformation of the Fe–(T_C–T_N) complex to the Fe_2–(T_C–T_N) complex. Furthermore, since sites *C* and *N* have similar structures towards metal binding,[2,3] we will presume that binding to *N* occurs by a mechanism similar to that to *C* [mechanism C, reactions (15)–(19)].

Mechanism C

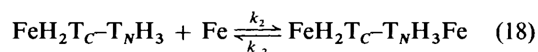
$$FeH_3T_C\text{–}T_NH_3 + Fe \rightleftharpoons FeH_3T_C\text{–}T_NH_3Fe \quad (15)$$



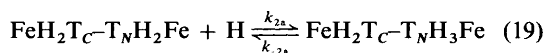
$$FeH_3T_C\text{–}T_NH_2Fe + H \rightleftharpoons FeH_3T_C\text{–}T_NH_3Fe \quad (16)$$



$$FeH_2T_C\text{–}T_NH_3Fe + H \rightleftharpoons FeH_3T_C\text{–}T_NH_3Fe \quad (17)$$



$$FeH_2T_C\text{–}T_NH_3 + Fe \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} FeH_2T_C\text{–}T_NH_3Fe \quad (18)$$



$$FeH_2T_C\text{–}T_NH_2Fe + H \underset{k_{-2a}}{\overset{k_{2a}}{\rightleftharpoons}} FeH_2T_C\text{–}T_NH_3Fe \quad (19)$$

However, under our experimental conditions (pH ⩾ 7 and $c_2 \geqslant 5c_1$), at the end of the fast first iron binding to the protein, the iron protein complex is mostly in the FeH_2T_C form. Therefore, the slow relaxation mode observed for second iron binding [Fig. 3(*b*)] would involve reaction (18) or (19). If reaction (18) is rate-limiting, its reciprocal relaxation time will be given by equation (20), with K_2 = [Fe][FeH_2T_C–T_NH_3]/

$$\tau_2'^{-1} = k_2([Fe] + [FeH_2T_C\text{–}T_NH_3])(K_{2a} + [H])/(K_{1a}'' + [H]) + k_{-2}[H]/K_{2a} \quad (20)$$

[FeH_2T_C–T_NH_3Fe], K_{2a} = [FeH_2T_C–T_NH_2Fe][H]/[FeH_2-T_C–T_NH_3Fe] and K_{1a}'' = [FeH_2T_C–T_NH_3Fe][H]/[FeH_3T_C–T_NH_3Fe].

Under our experimental conditions, $c_2 \geqslant 5c_1$ leads to

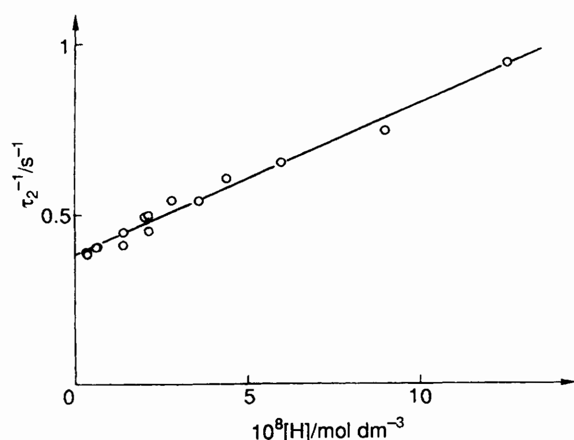


Fig. 6 Plot of $\tau_2'^{-1}$ against [H] at 25 ± 0.5 °C and I = 0.2 mol dm^{-3}. Intercept, 0.40 ± 0.05 s^{-1}; slope, (4.50 ± 0.30) × 10^6 dm^3 mol^{-1} s^{-1}, r = 0.992 41. Each experimental point represents the average value measured at least four different concentrations c_2

$[FeH_2T_C–T_NH_2Fe] < [Fe]$. Consequently, at constant pH $\tau_2'^{-1}$ should depend on [Fe]. However, the reciprocal relaxation times measured at constant pH are independent of c_2 when c_2 ranges from 2.5 × 10^{-4} to 1 × 10^{-3} mol dm^{-3}. This kinetic process may then describe the slow deprotonation of the second transferrin–iron complex [reaction (19), reciprocal relaxation equation (21)].

$$\tau_2^{-1} = k_{2a}[H] + k_{-2a} \quad (21)$$

Linear least-squares regression of the data against equation (21) (Fig. 6) gave k_2 = (4.50 ± 0.30) × 10^6 dm^3 mol^{-1} s^{-1} and k_{-2} = 0.40 ± 0.05 s^{-1}. The ratio $k_{-2}/k_2 = K_{2a}$ is (8.5 ± 1.1) × 10^{-8} mol dm^{-3} which leads to pK_{2a} = 7.07 ± 0.06.

Discussion

The Fe(nta) complex is present in aqueous media in the form of several species and can give dimers.[29] However, for the highest Fe(nta) concentration used in this work (≈1 × 10^{-3} mol dm^{-3}), Bates and Wernicke[10] showed that 85% of Fe(nta) is in the form of a monocomplex which reacts with the protein. Nevertheless, despite this we cannot directly neglect the possible influence of a very low concentration of dimer on complex formation with the protein. The break-up of the dimeric species of Fe(nta) is extremely fast.[29b] If the dimer were to break up during iron uptake, it would only affect the rate of reaction (4) (Fe uptake by the C site) by less than 10% at the highest iron concentrations used here. In the typical experimental c_2 concentration range of this work (0.2–5) × 10^{-4} mol dm^{-3}, this effect will be even more negligible. As for Fe uptake by N, this is controlled by a slow proton-transfer [reaction (19)] the rate of which depends only on [H] [equation (21)] and not on the concentration of Fe(nta). Therefore, the influence of dimer break-up seems to be negligible during iron uptake.

Near neutrality, iron uptake at the C site occurs by complex formation between T and Fe [reaction (4), K_1 = 1 × 10^{-5} mol dm^{-3}] and is accompanied by a deprotonation of the complex (pK_{1a} = 6.35). Kinetic data give access only to the second-order rate constant for complex formation, k_1 = 7.00 × 10^3 dm^3 mol^{-1} s^{-1}, which is rather high for complex formation with Fe^{III} even with more than one negative ligand.[30] If we assume that the binding sites of iron in T are similar to those of the other proteins of the transferrin family, with carbonate iron will be co-ordinated by four protein ligands (two phenolate oxygens of two tyrosines, the heterocyclic imidazole amine of the histidine lateral chain and the lateral carboxylate of an aspartate) and a single HCO_3^- or CO_3^{2-} anion bound to metal and adjacent to an arginine side chain.[2,3] The dissociation pK_a value of the phenol of tyrosine is 10.07, that of the aspartate lateral chain is 3.86 and that of histidine imidazolium is 6.0.[27] However, in the protein the pK_a values of these residues are not known. They can be quite different from those of the free amino acids.[31] In the present case, since nta is a trichelating ligand, the binding of Fe(nta) to T by complex formation with the protein necessarily induces a different distribution of the ligands in the co-ordination sphere of the metal.[32] This makes this complex different from that formed with free Fe^{III} and one carbonate. It can, moreover, explain the single proton loss involved in complex formation by mechanism B and implies that FeH_3T_C occurs with fewer ligands than the natural complex. Regardless of this, the more basic the ligand, the more stable is the complex.[32] Nevertheless, near neutrality, and before complex formation with the protein, the two phenols of the tyrosines and the imidazole of histidine are probably neutral while the carboxylate of aspartate is in the anionic form. This leads to a rather strong complex between the iron carboxylate and most probably phenols or phenolates and a weaker binding with imidazole.[32] This is confirmed by the fact that complex formation between Fe^{III} and tyrosine is accompanied by an absorption band at about 470 nm similar to that of Fig. 2.[33] That the pK_{1a} is 6.35 may suggest imidazolium deprotonation in FeH_3T_C. However, in this case complex formation would no longer occur according to mechanism B, because under our experimental conditions (8.4 ⩾ pH ⩾ 7) imidazole is already in the neutral form. It would, therefore, directly react with the protein by reaction (3). In transferrin the pK_a of tyrosine phenol deprotonation is unknown. It may, however, be as low as 6.35 in FeH_3T_C since a number of transition metals have the ability to enhance acidity which leads to a reduction of the pK_a values of the free ligands by several units in chelates.[34] If one of the tyrosine phenolates is engaged in the FeH_2T_C complex, in basic media (pH ≈ 10) binding of Fe to T would probably occur by reaction (3) in mechanism A, and the major species would always be FeH_2T_C whether produced according to mechanism A or B. Consequently, since in neutral media, FeH_2T_C is produced only according to mechanism B without any detectable involvement of H_2T_C, pK_{1a} cannot be the pK_{1a}' of tyrosine phenol deprotonation [reaction (2)]. In this case, $K_1' = [H_2T_C][Fe]/[FeH_2T_C] = K_1K_{1a}'/K_{1a} \approx 2.30 \times 10^{-9}$ mol dm^{-3}. The value of K_1' is much higher than that reported for binding to site C with synergistic carbonate (2 × 10^{-21} mol dm^{-3} at pH 7.4).[5] This confirms our proposals concerning the engagement of fewer protein ligands in FeH_2T_C.

The interesting feature of mechanism B is that Fe(nta) binding to site C occurs in mildly acidic media with average affinity for iron, but its occurrence allows the probable deprotonation of a tyrosine phenol ligand leading to an increase in the affinity of the binding site for the metal. What happens when synergistic carbonate is involved in the binding is difficult to predict. However, since in the latter case binding occurs with the loss of three protons, it most probably involves the deprotonation of the second tyrosine phenol of the binding site, and perhaps that of HCO_3^- to CO_3^{2-}, as already suggested elsewhere.[5]

Although iron is bound to the N site of transferrin by the same protein ligands as the C site, iron binding to the N-terminal site occurs in more basic media,[4,7] and is controlled by a slow proton transfer. Slow proton transfers can usually be associated with transition-metal complex dissociation,[34,35] with proton-induced change of protein conformations,[36] and with the dissociations of sterically hindered protonation sites.[37] All these cases can totally or separately apply to binding of Fe(nta) to the N site of T. It should be pointed out that Fe(nta) binding to site C is much faster than that to N and is more favoured thermodynamically. Therefore, binding to N cannot be detected unless the C site is already iron-saturated. Consequently, we are able to analyse Fe(nta) binding not to apotransferrin but

Table 2 Rate and equilibrium constants for the uptake of Fe(nta) by transferrin

Reaction	Direct rate constant/ $dm^3 mol^{-1} s^{-1}$	Reverse rate constant/s^{-1}	Equilibrium constant/ $mol dm^{-3}$
$H_3T_C + Fe \rightleftharpoons FeH_3T_C$	7.00×10^3		1.00×10^{-5}
$FeH_2T_C + H \rightleftharpoons FeH_3T_C$			4.5×10^{-7}
$Fe + H_2T_C \rightleftharpoons FeH_2T_C$			2.3×10^{-9}
$Fe + T_NH_3 \rightleftharpoons T_NH_3Fe$			
$T_NH_2Fe + H \rightleftharpoons T_NH_3Fe$	4.5×10^6	0.40	8.5×10^{-8}

only to a protein whose *N* site conformation may have been modified by iron binding to site *C*.[14]

Table 2 summarizes our results and shows that the *C* binding site in transferrin, because of its four protein ligands, possesses several degrees of affinity for Fe(nta). These affinities are dependent on the state of protonation of the protein ligands. This can be of importance in understanding the mechanism of iron uptake by T in neutral media and its release in mildly acidic media. Indeed, in order to deplete iron by a competing chelating agent from the extraordinarily stable natural proteic complex, the concentration of this competing agent should be several orders of magnitude larger than that of citrate, pyrophosphate or any other species in natural media.[27] Therefore, the protonation of the protein ligands probably diminishes the affinity of the protein for iron rendering its depletion by the chelating agents present in the medium thermodynamically possible.

Conclusion

In this work, we propose a general mechanism for uptake of Fe(nta) by transferrin. Besides the fact that this mechanism partly includes that of Bates and co-workers[4,9] with Fe(nta) 'ternary' complex formation, it highlights the important role played by the sequential deprotonation of the amino acid ligands engaged in the process of complex formation between the protein and Fe^{III}. Our analysis cannot be directly transposed *in vivo*, not merely because of the absence of carbonate but also because of the absence of the protein receptor from the medium.[38] However, we believe that our proposals reflect some of the basic principles which govern iron transfer from chelates to transferrin in the presence of carbonate.

Appendix

In all the biexponential processes, the first time constant was always at least six times smaller than the second. This allowed the use of substitution methods to derive the reciprocal relaxation times involved in the uptake and release of Fe(nta) by T.[23-25]

Derivation of Equations (6) *and* (8).—If complex formation between Fe(nta) and T obeys Mechanism A, the Beer–Lambert law can be expressed by equation (A1) where ε_3 is the molar

$$A = \varepsilon_1[FeH_{3-n}T_C] + \varepsilon_2[H_3T_C] + \varepsilon_2'[H_{3-n}T_C] + \varepsilon_3[Fe] \quad (A1)$$

absorption coefficient of free Fe(nta).

At $\lambda = 465$ nm we do not detect any modifications in the absorption of the transferrin between pH 6.3 and 7.4. We therefore assume that $\varepsilon_2 \approx \varepsilon_2'$. Moreover, $\varepsilon_3 \ll \varepsilon_2'$, ε_1 and for $c_2 < c_1$, $[Fe] < [FeH_{3-n}T_C]$. From equation (A1), K_{1a}', K_1' and conservation of mass [equations (A2) and (A3)] we derive

$$c_1 = [FeH_{3-n}T_C] + [H_3T_C] + [H_{3-n}T_C] \quad (A2)$$

$$c_2 = [Fe] + [FeH_{3-n}T_C] \quad (A3)$$

equation (6). If complex formation obeys Mechanism B, the Beer–Lambert law can be expressed by equation (A4) in which

$$A = \varepsilon_1[FeH_{n-3}T_C] + \varepsilon_1'[FeH_3T_C] + \varepsilon_2[H_3T_C] + \varepsilon_3[Fe] \quad (A4)$$

ε_1' is the molar absorption coefficient of FeH_3T_C.

Since at $\lambda = 465$ nm we do not detect any variations in the absorption of iron–protein complex, we assume that $\varepsilon_1 \approx \varepsilon_1'$. Mass conservation [equations (A5) and (A6)] and the

$$c_1 = [FeH_3T_C] + [FeH_{3-n}T_C] + [H_3T_C] \quad (A5)$$

$$c_2 = [FeH_3T_C] + [FeH_{3-n}T_C] + [Fe] \quad (A6)$$

expressions for K_1 and K_{1a} allow us to derive equation (8).

Derivation of Rate Equation (11).—The rate equation associated with reaction (4) can be expressed by equation (A7). When

$$-d[FeH_2T_C]dt = -k_1[Fe][H_3T_C] + k_{-1}[FeH_3T_C] \quad (A7)$$

applied on Mechanism B, the formalism of chemical relaxation is only valid for $c_2 < c_1$ and for $c_2 > 3c_1$.[29] From conservation of mass, relations (A8) and (A9) hold. The acid–base reaction (5)

$$\Delta[FeH_3T_C] + \Delta[FeH_2T_C] + \Delta[H_3T_C] = 0 \quad (A8)$$

$$\Delta[Fe] + \Delta[FeH_3T_C] + \Delta[FeH_2T_C] = 0 \quad (A9)$$

can be considered to be in a constant state of equilibrium during reaction (4) [equation (A10)].

$$\Delta[FeH_3T_C] = [FeH_2T_C]\Delta[H]/K_{1a} + [H]\Delta[FeH_2T_C]/K_{1a} \quad (A10)$$

In buffered media, reciprocal relaxation-time equation (11) associated with reaction (4) is determined from equations (A7)–(A10).

Equations (13) and (14) are derived from analytical expressions of equilibrium constants K_1 and K_2 and from mass conservation equations (A11) and (A12).

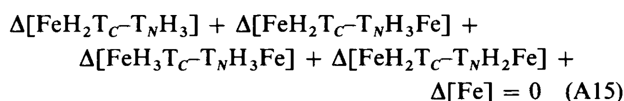
$$c_1 = [H_3T_C] + [FeH_3T_C] + [FeH_2T_C] \quad (A11)$$

$$c_2 = [Fe] + [FeH_3T_C] + [FeH_2T_C] \quad (A12)$$

Derivation of Rate Equations (20) *and* (21).—The rate equation associated with reaction (18), if this is rate-limiting, can be expressed by equation (A13). Conservation of mass gives equations (A14) and (A15). If acid–base reactions (17) and (19)

$$-d[FeH_2T_C\text{–}T_NH_2Fe]/dt = -k_2[Fe][FeH_2T_C\text{–}T_NH_3] + k_{-2}[FeH_2T_C\text{–}T_NH_3Fe] \quad (A13)$$

$$\Delta[FeH_3T_C\text{–}T_NH_3] + \Delta[FeH_2T_C\text{–}T_NH_3Fe] + \Delta[FeH_3T_C\text{–}T_NH_3Fe] + \Delta[FeH_2T_C\text{–}T_NH_2Fe] = 0 \quad (A14)$$



$$\Delta[\mathrm{FeH_2T_C{-}T_NH_3}] + \Delta[\mathrm{FeH_2T_C{-}T_NH_3Fe}] + \Delta[\mathrm{FeH_3T_C{-}T_NH_3Fe}] + \Delta[\mathrm{FeH_2T_C{-}T_NH_2Fe}] + \Delta[\mathrm{Fe}] = 0 \quad (A15)$$

are in a constant state of equilibrium during reaction (18) one obtain equations (A16) and (A17). The value of $\tau_2'^{-1}$ [equation

$$\Delta[\mathrm{FeH_2T_C{-}T_NH_3Fe}] = [\mathrm{H}]\Delta[\mathrm{FeH_2T_C{-}T_NH_2Fe}]/K_{2a} + [\mathrm{FeH_2T_C{-}T_NH_2Fe}]\Delta[\mathrm{H}]/K_{2a} \quad (A16)$$

$$\Delta[\mathrm{FeH_3T_C{-}T_NH_3Fe}] = [\mathrm{FeH_2T_C{-}T_NH_3Fe}]\Delta[\mathrm{H}]/K_{1a}'' + [\mathrm{H}]\Delta[\mathrm{FeH_2T_C{-}T_NH_3Fe}]/K_{1a}'' \quad (A17)$$

(20)] is derived from equations (A13)–(A17). The rate equation associated with reaction (19), if this is rate-limiting, is given by equation (A18).

$$-\mathrm{d}[\mathrm{FeH_2T_C{-}T_NH_2Fe}]/\mathrm{d}t = k_{2a}[\mathrm{FeH_2T_C{-}T_NH_2Fe}][\mathrm{H}] - k_{-2a}[\mathrm{FeH_2T_C{-}T_NH_3Fe}] \quad (A18)$$

Since the second complex is in a semi-equilibrated state we can derive equations (A19) and (A20).

$$\Delta[\mathrm{FeH_2T_C{-}T_NH_3Fe}] = [\mathrm{FeH_2T_C{-}T_NH_3}]\Delta[\mathrm{Fe}]/K_2 + [\mathrm{Fe}]\Delta[\mathrm{FeH_2T_C{-}T_NH_3}]/K_2 \quad (A19)$$

$$\Delta[\mathrm{FeH_3T_C{-}T_NH_3Fe}] = [\mathrm{FeH_3T_C{-}T_NH_3}]\Delta[\mathrm{Fe}]/K_2 + [\mathrm{Fe}]\Delta[\mathrm{FeH_3T_C{-}T_NH_3}]/K_2 \quad (A20)$$

From these equations we derived the reciprocal relaxation-time equation associated with reaction (19) [equation (21)].

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