

Transferrin, is a Mixed Chelate-Protein Ternary Complex Involved in the Mechanism of Iron Uptake by Serum-transferrin *in Vitro*?

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Iron uptake by transferrin from triacetohydroxamatoFe(III) (Fe(AHA)₃) in the presence of bicarbonate has been investigated between pH 7 and 8.2. The protein transits from the opened apo- to the closed holoform by several steps with the accumulation of at least three kinetic intermediates. All these steps are accompanied by proton losses, probably occurring from the protein ligands and the side-chains involved in the interdomain H-bonding nets. The minor bihydroxamatoFe(III) species Fe(AHA)₂ exchanges its iron with the C-site of apotransferrin in interaction with bicarbonate without detectable formation of any intermediate protein-iron-ligand mixed complex; direct second-order rate constant $k_1 = 4.15(\pm 0.05) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The kinetic product loses a single proton and undergoes a modification in its conformation followed by the loss of two or three protons; first-order rate constant $k_2 = 3.25(\pm 0.15) \text{ s}^{-1}$. This induces a new modification in the conformation; first-order rate constant $k_3 = 5.90(\pm 0.30) \times 10^{-2} \text{ s}^{-1}$. This new modification in conformation rate controls iron uptake by the N-site of the protein and is followed by a single proton loss; $K_{3a} = 6.80 \text{ nM}$. Finally, the holoprotein or the monoferric transferrin in its thermodynamic equilibrated state is produced by a last modification in the conformation occurring in about 4000 seconds. But for the Fe(AHA)₃ dissociation and the involvement of Fe(AHA)₂ in the first step of iron uptake, this mechanism is identical to that reported for iron uptake from FeNac₃. This implies that the exchange of iron between a chelate and serum-transferrin occurs by a single general mechanism. The nature of the iron-providing chelate is only important for the first kinetic step of the exchange, which can be slowed to such an extent that it rate limits the exchange of iron.

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Introduction

The transferrin family constitutes the major iron transport and/or scavenging system in vertebrates

and some invertebrates (Aisen, 1998). Most of the transferrins are soluble glycoproteins which consist of a single chain of about 700 amino acid residues and 80 kDa (Aisen, 1998). They are bilobal and

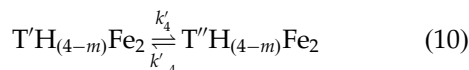
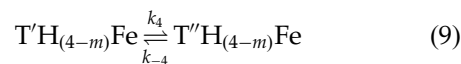
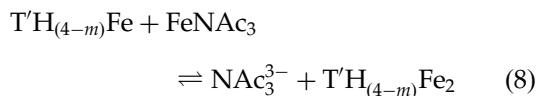
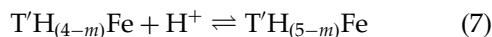
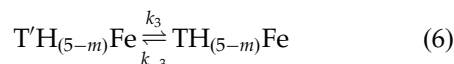
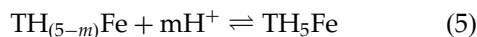
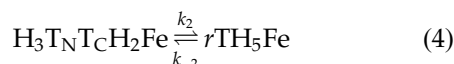
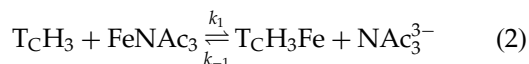
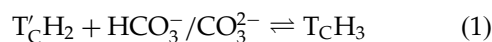
Abbreviations used: FeNac₃, nitrilotriacetatoiron(III); Fe(AHA)₃, triacetohydroxamatoiron(III); FeL, FeL₂ and FeL₃ the different chelates; L, chelating ligand; T_CH₂ the C-site of apotransferrin in neutral media and in the absence of carbonate with two protein ligands in the protonated form; T_CH₃, the C-site of apotransferrin interacting with bicarbonate; T_CH₃Fe and T_CH₂Fe, the first protein-iron intermediate in the protonated and deprotonated forms; H₃T_NT_CH₂Fe, TH₅Fe, TH_(5-m)Fe, T'H_(5-m)Fe and T'H_(4-m)Fe, intermediate monoferric complexes; T'H_(4-m)Fe₂, differic transferrin intermediates; T''H_(4-m)Fe and T''H_(4-m)Fe₂, monoferric and holotransferrin in the final equilibrated state; m, 2 or 3; T, transferrin in an undefined state; FeLT, the intermediate iron ternary mixed complex in an undefined state; FeT, the iron-transferrin complex in an undefined state; c₁, analytical apotransferrin concentration; c₂, analytical chelate concentration; c₃, analytical chelating ligand concentration; T_CH₃FeL₂, the iron-C-site intermediate ternary mixed complex.

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each lobe is organised to form a metal binding cleft or pocket in which iron is co-ordinated to four protein ligands and a synergistic carbonate anion (Jeffrey *et al.*, 1998; Moore *et al.*, 1997; Kurokawa *et al.*, 1995; Zuccola, 1992).

Serum-transferrins and ovotransferrins transport iron from neutral biological fluids to the cytoplasm by receptor-mediated endocytosis (Dautry Varsat *et al.*, 1983). In contrast, lactoferrins and again ovotransferrins behave as bacteriostatic iron scavengers (Moore *et al.*, 1997; Kurokawa *et al.*, 1995). In a recent series of articles, we investigated the kinetics and thermodynamics of iron uptake from nitrilotriacetatoFe(III) (FeNAC₃), by these three major iron transport and sequestration transferrins: serum-transferrin (Mechanism I), lactoferrin and ovotransferrin. We showed that iron uptake occurs by similar mechanisms with the three proteins with, however, important differences in the rates and in the number of protons lost during the uptake (El Hage Chahine & Fain, 1993; El Hage Chahine & Pakdaman, 1996; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998; Bou Abdallah & El Hage Chahine, 1998).

Mechanism I

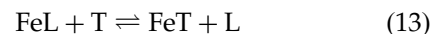
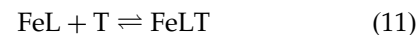


The first step in iron uptake by serum-transferrin is the exchange of Fe(III) between the low molecular mass chelate FeNAC₃, and the C-site of the protein

in interaction with bicarbonate (T_cH₃) to yield (T_cH₃Fe) a first C-site iron-loaded protein (equations (1) and (2)). It is followed by a proton loss yielding T_cH₂Fe or H₃T_NT_cH₂Fe (equation (3)). This intermediate undergoes a change in conformation (TH₅Fe, equation (4)) followed by two or three (*m*) proton losses (TH_(5-m)Fe, equation (5)) which affect both lobes of the protein. Another change in conformation (T'H_(5-m)Fe, equation (6)) followed by a new proton loss (T'H_(4-m)Fe, equation (7)) allow the N-site to capture a Fe(III) cation if it is available in the medium (equation (8)). It also allows the protein, whether monoferric or diferric, to attain its final state of equilibrium in a final conformation change which lasts about 4000 seconds at 25 °C (T''H_(4-m)Fe, T''H_(4-m)Fe₂, equations (9) and (10); Pakdaman & El Hage Chahine, 1996).

In Mechanism I, iron exchange between FeNAC₃ and serum-transferrin occurs without the accumulation of any mixed protein-FeNAC₃ ternary intermediate complex. However, according to Bates and co-workers, transferrin interacts with the low molecular mass chelate (FeL) to yield an intermediate ternary complex (TFeL) which in turn loses L, the chelating agent (Mechanism II, equations (11) and (12); Bates *et al.*, 1967; Bates & Wernicke, 1971; Bates & Schlabach, 1975; Cowart *et al.*, 1982, 1986).

Mechanism II



Kinetic intermediates were detected during iron uptake from the acetohydroxamic acid and pyrophosphate complexes and were assumed to be the ternary intermediates TFeL (Cowart *et al.*, 1982, 1986). Iron release from holotransferrin was presumed promoted by anion binding to the protein and considered as possibly occurring by two different paths: (i) *via* the ternary intermediate (equations (11) and (12)); and/or (ii) without the involvement of this intermediate (equation (13); Egan *et al.*, 1992).

Before being targeted for degradation, a serum-transferrin molecule performs about 100 cycles of iron-delivery by endocytosis (Dautry-Varsat *et al.*, 1983; Crichton, 1991). Therefore, during its lifetime a serum-transferrin molecule interacts with iron about 100 times. Iron(III) is completely insoluble in neutral media where it can only exist in the complex form (Hancock & Martell, 1989). In order to be iron loaded, transferrins or any other iron transport systems such as siderophores are constrained to extract iron from a soluble complex (Crichton, 1991). We, therefore, believe that the understanding of the general mechanism of formation of a complex between iron(III) *in vitro* and transferrins is of importance for the comprehension of the

metabolic role of these proteins in regulating iron *in vivo*.

Here we use the methods and techniques of chemical relaxation to re-examine iron uptake from triacetohydroxamatoiron(III), Fe(AHA)_3 , by serum-transferrin.

Results

For reasons of simplicity and lack of knowledge of the state of the charge in the binding sites of serum-transferrin near neutrality, the charges of the protein species involved are not given.

When two neutral solutions ($7.00 \leq \text{pH} \leq 8.2$) of apotransferrin and Fe(AHA)_3 containing bicarbonate are mixed rapidly, four kinetic phenomena are observed by fluorescence emission detection and two by absorption detection (Figures 1 to 3). The first kinetic phenomenon occurs in the 100 ms to one second range, is detected by an exponential decrease in the fluorescence intensity and leads to the accumulation of a first kinetic product (Figure 1(a)). Its rate depends on the analytical protein concentration (c_1), complex concentration (c_2), analytical AHA ligand concentration (c_3), bicarbonate concentration and pH. Under our experimental conditions, this process is not detected by absorption spectroscopy. In the second kinetic phenomenon, the first kinetic intermediate yields a second kinetic product (Figures 1(b)-(d), 2 and 3). This is detected as an exponential increase in absorption with time in the 330 nm or 460 nm range (Figure 1(b)) or a slight decrease of the fluorescence emission intensity. The rate of this process depends only on pH and seems to be independent of c_1 , c_2 , c_3 and $[\text{HCO}_3^-]$. The second kinetic intermediate yields, in turn, a third kinetic product (Figure 1(c) to (e)). This process occurs in the 100 second range. It is revealed by an exponential increase in absorption with time for $320 \text{ nm} \leq \lambda \leq 380 \text{ nm}$ and for $\lambda \geq 480 \text{ nm}$ or its decrease for $380 \text{ nm} \leq \lambda \leq 470 \text{ nm}$ (Figures 1(c), (d) and 3). It is also detected as an exponential decrease in the emission fluorescence intensity (Figure 1(e)). Finally, the third kinetic intermediate gives the thermodynamic product (Figure 1(e)). This last kinetic phenomenon is detected as an exponential decrease in the fluorescence emission intensity with time (Figure 1(e)). It is complete in about 4000 seconds and is independent of the concentrations of the species present in the medium. The fluorescence emission spectra measured after mixing apotransferrin with one equivalent or five equivalents of Fe(AHA)_3 at the end of each kinetic process, and before the beginning of the following phenomenon, and those measured at the thermodynamic equilibrium (Figure 2) are identical to those measured after the mixing of apotransferrin with FeNAC_3 (Pakdaman & El Hage Chahine, 1996). However, the absorption spectra measured under the same conditions are very different from those reported with FeNAC_3 . Nevertheless, when

serum-transferrin interacts with only one equivalent of Fe(AHA)_3 , the absorption spectrum of the first kinetic intermediate at the end of second process (Figure 1(b) and (c)) is identical to that reported under the same conditions with FeNAC_3 (Figure 3; Pakdaman & El Hage Chahine, 1996).

All results are reported at an ionic strength $\mu = 0.2$. However, in order to test the salt or ionic strength effect on iron uptake (Williams *et al.*, 1982; Aisen, 1998) some experiments were performed at ionic strengths varying from 0.1 to 0.5 with KCl concentrations varying from 50 to 400 mM. The results were identical to those obtained at $\mu = 0.2$. Therefore, under our experimental conditions, the salt effect was not observed.

First kinetic process

As with iron uptake from FeNAC_3 , we will assume that the kinetic product accumulated at the end of the fast process shown in Figure 1(a) only involves iron uptake by the C-site of the protein (Pakdaman & El Hage Chahine, 1996). At fixed pH, c_1 and c_3 , with c_2 varying from $10c_1$ to $100c_1$, there is a linear relationship between the experimental reciprocal relaxation times ($(\tau_1)^{-1}$) related to this first fast kinetic process shown in Figure 1(a) and $(c_2 - c_1)$. This relationship was also observed during iron uptake from FeNAC_3 by serum-transferrin, lactoferrin and ovotransferrin (Bou Abdallah & El Hage Chahine, 1998; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998). Furthermore, at fixed pH, c_1 and $c_2 = 10c_1$, with c_3 varying from $5c_2$ to $50c_2$, there is another linear relationship between experimental $(\tau_1)^{-1}$ and $1/c_3$. This relationship was not observed during iron uptake from FeNAC_3 . Although under our experimental conditions ($7.00 < \text{pH} < 8.2$) FeL_3 Fe(AHA)_3 is the major acetohydroxamatoFe(III) species, it is in a fast equilibrium with the minor FeL_2 species which is in equilibrium with the very minor FeL species (equations (14) and (15); Martell & Smith, 1989; Schwarzenbach & Schwarzenbach, 1963; Monzyk & Crumbliss, 1979; Birus *et al.*, 1985). Furthermore, if FeL_3 was directly involved in the uptake of iron by serum-transferrin, there would not be a linear relationship between the experimental $(\tau_1)^{-1}$ and $1/[\text{L}]$. This linear relationship implies that FeL_2 is the species involved in this uptake. Indeed, if FeL were the species involved in the uptake of iron, a linear relationship between $(\tau_1)^{-1}$ and $1/[\text{L}]^2$ would be observed (Bernasconi, 1976). These facts led us to envisage two possible mechanisms for the first step of iron uptake by the C-site of serum-transferrin in interaction with bicarbonate (T_CH_3) from Fe(AHA)_3 . In Mechanism III, an intermediate ternary complex is formed, whereas in Mechanism IV, this complex is not formed. As with FeNAC_3 , iron uptake from acetohydroxamatoFe(III) will be followed by proton loss (Bou Abdallah & El Hage Chahine, 1998; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998):

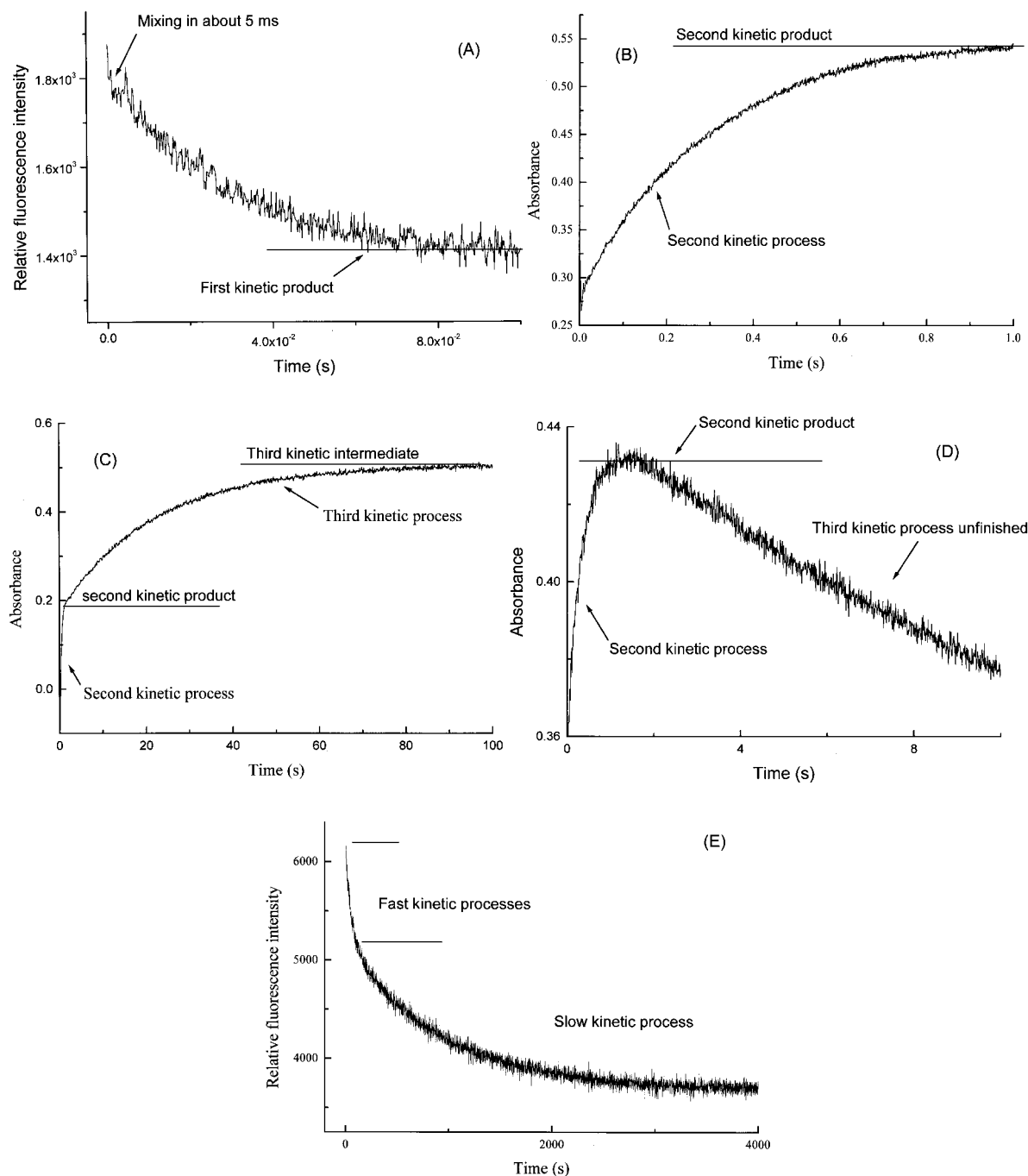


Figure 1. Fluorescence and absorption variation with time when a neutral solution of serum-transferrin is mixed with a neutral solution of acetohydroxamatoFe(III) at $25(\pm 0.5)^\circ\text{C}$ and $\mu = 0.2$. (a) Mono-exponential decrease of the fluorescence intensity ($320\text{ nm} < \lambda < 350\text{ nm}$ with $\lambda_{\text{ex}} = 280\text{ nm}$) over 0.1 second during a stopped-flow experiment for $c_1 = 4\ \mu\text{M}$, $c_2 = 0.1\ \text{mM}$, $c_3 = 0$, pH 7.92 and $[\text{HCO}_3^-] = 20\ \text{mM}$. (b) Mono-exponential increase of absorption at $\lambda = 330\text{ nm}$ over one second during a stopped-flow experiment for $c_1 = 38.6\ \mu\text{M}$, $c_2 = 0.45\ \text{mM}$, $c_3 = 0$, $[\text{HCO}_3^-] = 20\ \text{mM}$ and pH 7.60. (c) Bi-exponential increase of absorption at $\lambda = 330\text{ nm}$ during a stopped-flow experiment for $c_1 = 38.6\ \mu\text{M}$, $c_2 = 0.45\ \text{mM}$, $c_3 = 0$, $[\text{HCO}_3^-] = 20\ \text{mM}$ and pH 7.80. (d) Multi-exponential variation of the absorption at $\lambda = 400\text{ nm}$ over ten seconds under the same conditions as (b). (e) Multi-exponential decrease of the fluorescence intensity at $\lambda = 330\text{ nm}$ with $\lambda_{\text{ex}} = 280\text{ nm}$ over 4000 seconds after a fast $[\text{Fe}(\text{AHA})_3]$ -jump for $c_1 = 1\ \mu\text{M}$, $c_2 = 12\ \mu\text{M}$, $c_3 = 0$, $[\text{HCO}_3^-] = 15\ \text{mM}$ and pH 7.60.

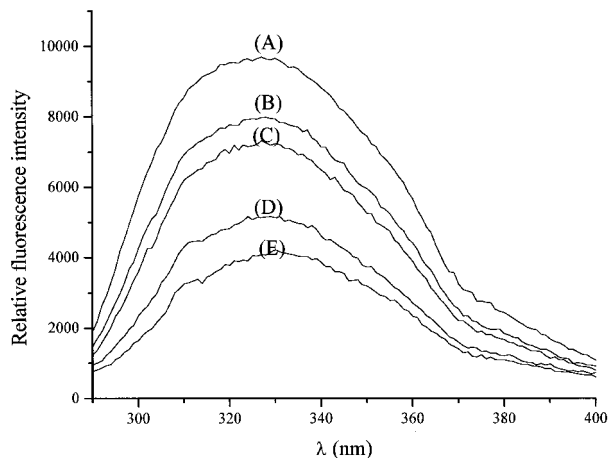
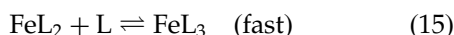
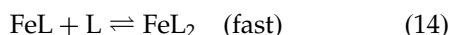
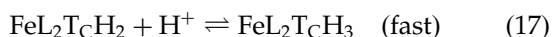
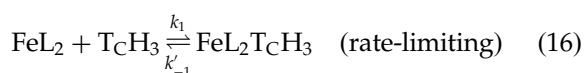


Figure 2. Fluorescence emission spectra of the serum-transferrin species involved in the mechanism of iron uptake from $\text{Fe}(\text{AHA})_3$ with $\lambda_{\text{ex}} = 280 \text{ nm}$ at $25(\pm 0.1)^\circ\text{C}$, $\mu = 0.2$ and $\text{pH } 7.50$ for $c_1 = 1 \mu\text{M}$ and $[\text{HCO}_3^-] = 20 \text{ mM}$, (a) at $c_2 = 0$, (b) ten seconds and (c) two hours after the addition of $c_2 = 1.1c_1$. (d) and (e) are recorded (with an acquisition time of four seconds) ten seconds and two hours after the addition of $c_2 = 5c_1$. The emission maxima are of 325 nm for (a) and 329 nm for (b), respectively.



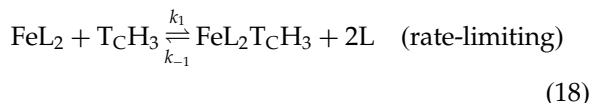
with $K_{\text{FeL}} = [\text{FeL}_2][\text{L}]/[\text{FeL}_3] = 63 \text{ nM}$ (Martell & Smith, 1989; Schwarzenbach & Schwarzenbach, 1963).

Mechanism III



with $K'_{1a} = [\text{FeL}_2\text{TcH}_2][\text{H}^+]/[\text{FeL}_2\text{TcH}_3]$

Mechanism IV



with $K_{1a} = [\text{FeTcH}_2][\text{H}^+]/[\text{FeTcH}_3] = 16 \text{ nM}$ (Pakdaman & El Hage Chahine, 1996).

The reciprocal relaxation time equations associated with equations (16) and (18) when each is rate-limiting can be expressed by equations (19) and (21), respectively (see the Appendix):

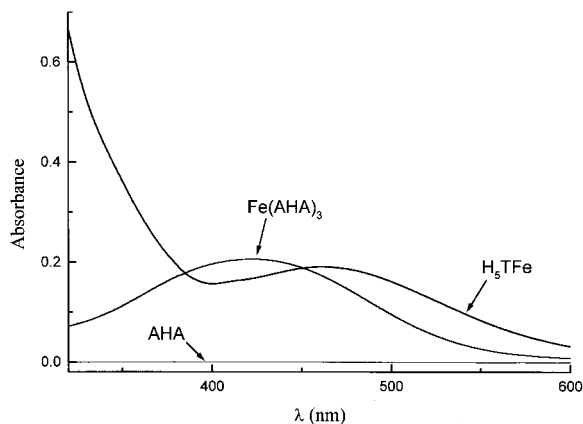


Figure 3. Absorption spectra of AHA, H_5TFe and $\text{Fe}(\text{AHA})_3$. The H_5TFeT spectrum was recorded in ten seconds after mixing serum-transferrin with either one equivalent of FeNAC_3 (seven) or $\text{Fe}(\text{AHA})_3$ at $25(\pm 0.1)^\circ\text{C}$ for $c_1 = 87 \mu\text{M}$, $c_2 = 100 \mu\text{M}$, $c_3 = 1.2 \text{ mM}$, $\text{pH } 7.10$ and $[\text{HCO}_3^-] = 20 \text{ mM}$ at $\mu = 0.2$.

$$(\tau_1)^{-1} = k'_{-1}[\text{H}^+]/K'_{1a} + k'_1(\beta'[\text{FeL}_2]/\alpha + [\text{TcH}_3]K_{\text{FeL}}\beta'/[\text{L}]) \quad (19)$$

which under our experimental conditions ($c_2 > 10c_1$ and $0 \leq c_3 \leq 50c_2$) can be expressed as:

$$(\tau_1)^{-1} = k'_{-1}[\text{H}^+]/K'_{1a} + k'_1\beta'K_{\text{FeL}}c_2/[\alpha(c_3 + 3c_1)] \quad (20)$$

$$(\tau_1)^{-1} = k_{-1}([\text{L}]^2[\text{H}^+]/K_{1a} + 6[\text{L}][\text{FeTcH}_3]\beta) + k_1(\beta[\text{FeL}_2]/\alpha + [\text{TcH}_3]K_{\text{FeL}}\beta'/[\text{L}]) \quad (21)$$

which can be expressed as:

$$(\tau_1)^{-1}/\{[\text{H}^+](c_3 + 9c_1)(c_3 + 3c_1)\} = k_{-1}/K_{1a} + k_1\beta K_{\text{FeL}}c_2/\{\alpha[\text{H}^+](c_3 + 3c_1)^2(c_3 + 9c_1)\} \quad (22)$$

with $\alpha = 1 + [\text{HCO}_3^-]/K_{\text{HCO}_3^-}$, $\beta' = 1 + [\text{H}^+]/K'_{1a}$ and $\beta = 1 + [\text{H}^+]/K_{1a}$. K_{1a} and K'_{1a} are considered to be very similar ($K_{1a} \approx K'_{1a}$) because they deal with proton transfers occurring on the same sites and $K_{\text{HCO}_3^-} = [\text{TcH}_2][\text{HCO}_3^-]/[\text{TcH}_3] = 4.4 \text{ mM}$ (Pakdaman & El Hage Chahine, 1996; Bellounis *et al.*, 1996).

Equation (20) is not obeyed by the experimental data. Mechanism III is, therefore, discarded.

A very good linear least-squares regression of the experimental data against equation (22) is obtained (Figure 4). From the slope of the best regression line $k_1 = 4.15(\pm 0.05) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ is determined with k_{-1} much too small to be measured.

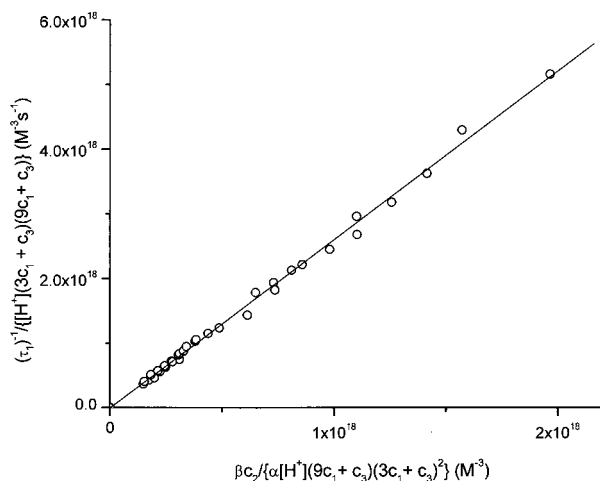


Figure 4. Plot of $(\tau_1)^{-1}/\{[H^+](3c_1 + c_2)(9c_1 + c_2)\}$ against $\beta c_2/\{\alpha[H^+](c_3 + 9c_1)(c_3 + 3c_1)^2\}$ at $25(\pm 0.5)^\circ\text{C}$ for $\mu = 0.2$, for 53 experimental points with $3 \mu\text{M} \leq c_1 \leq 6 \mu\text{M}$, $50 \mu\text{M} \leq c_2 \leq 0.2 \text{ mM}$, $0 \leq c_3 \leq 2 \text{ mM}$, $15 \text{ mM} \leq [\text{HCO}_3^-] \leq 35 \text{ mM}$ and $7.10 \leq \text{pH} \leq 8.00$. Intercept, $-0.04(\pm 2) \times 10^{16} \text{ M}^{-3} \text{ s}^{-1}$; slope, $2.60(\pm 0.03) \text{ s}^{-1}$; $r = 0.99882$.

Second kinetic process

The rate of the second kinetic process shown in Figures 1(b) and (c) depends on nothing other than pH. This process is identical to that observed during serum-transferrin iron uptake from FeNAC_3 . We, therefore, ascribe it to equation (4) followed by acid-base equation (5). The reciprocal relaxation time associated with equation (4) is expressed as equation (23) (Pakdaman & El Hage Chahine, 1996):

$$(\tau_2)^{-1} = k_2 K_{1a}/(K_{1a} + [\text{H}^+]) + k_{-2}[\text{H}^+]^m/K_{2a} \quad (23)$$

which can be expressed as equation (24):

$$(\tau_2)^{-1}/[\text{H}^+]^m = k_2 K_{1a}/(K_{1a} + [\text{H}^+])[\text{H}^+]^m + k_{-2}/K_{2a} \quad (24)$$

with $K_{2a} = [\text{TH}_{(5-m)}\text{Fe}][\text{H}^+]^m/[\text{TH}_5\text{Fe}]$.

A multi-linear least-squares regression of the experimental data against equation (23) shows that $m > 1$. Linear least-squares regressions of the data against equation (24) are satisfactory for $m = 2$ and $m = 3$; both gave $k_2 = 3.25(\pm 0.15) \text{ s}^{-1}$ (Figure 5). This value is, within the limits of uncertainty, equal to that reported for serum-transferrin iron uptake from FeNAC_3 (Pakdaman & El Hage Chahine, 1996).

Third kinetic process

The absorption amplitude of the second kinetic process shown in Figure 1(d) varies with λ (Figure 3). $\text{Fe}(\text{AHA})_3$ and AHA have typical absorption spectra in the wavelength range at

which our experiments were performed (Figure 3). The amplitude of a kinetic process is expressed as the sum of the contributions of the absorption variation of all the species involved (Dubois *et al.*, 1992). This explains the amplitude decrease at $330 \text{ nm} \leq \lambda \leq 480 \text{ nm}$ and its increase at $\lambda \geq 480 \text{ nm}$. When $c_1 \approx c_2$, $\text{Fe}(\text{AHA})_3$ reacts completely with serum transferrin. In this case, the spectrum measured at the end of the second kinetic process and before the beginning of the third process (Figure 1(c)) is that of FeT_cH_5 (Pakdaman & El Hage Chahine, 1996). This absorption spectrum matches that reported by Cowart *et al.* (1982) under very low bicarbonate concentrations and similar conditions of concentrations (Figure 3). The rates of these kinetic processes are identical to those reported for serum-transferrin iron uptake from FeNAC_3 . The experimental reciprocal relaxation times, $(\tau_3)^{-1}$, obey equation (25) which is associated with equation (6) when followed by acid-base equation (7) (Pakdaman & El Hage Chahine, 1996):

$$(\tau_3)^{-1}/[\text{H}^+] = k_3 K_{3a}/([\text{H}^+] + K_{3a})[\text{H}^+] + k_{-3}/K_{3a} \quad (25)$$

with $K_{3a} = [\text{T}'\text{H}_{(4-m)}\text{Fe}][\text{H}^+]/[\text{T}'\text{H}_{(5-m)}\text{Fe}] = 6.8 \text{ nM}$ (Pakdaman & El Hage Chahine, 1996).

A linear least-squares regression of the experimental fluorescence data against equation (25) gave $k_3 = 5.90(\pm 0.30) \times 10^{-2} \text{ s}^{-1}$ (Figure 6). This value is, within the limits of uncertainty, equal to that reported for serum-transferrin iron uptake from FeNAC_3 (Pakdaman & El Hage Chahine, 1996).

The fourth kinetic process

The fourth kinetic process is only observed by fluorescence and occurs in about 4000 seconds (Figure 1(d)). Its reciprocal relaxation time is independent of the concentration of the species present in the medium. It therefore describes a first-order kinetic process (Bernasconi, 1976). This process is identical to that observed in the case of iron uptake by serum-transferrin in the presence of FeNAC_3 . Subsequently, it can be associated with equations (9) and (10).

Discussion

In Table 1 we summarise the mechanisms of serum-transferrin iron uptake from triacetoxyamatoFe(III) and nitrilotriacetatoFe(III) (Pakdaman & El Hage Chahine, 1996). Under our experimental conditions, $7.00 \leq \text{pH} \leq 8.2$, acetoxyamatoFe(III) is mostly in the $\text{Fe}(\text{AHA})_3$ form (Schwarzenbach & Schwarzenbach, 1963). However, at $\text{pH} \geq 8$ part of the chelate can be hydroxylated (Brown & Chidambaram, 1978). This does not seem to affect our kinetic analysis, mostly because iron uptake by serum-transferrin occurs from the

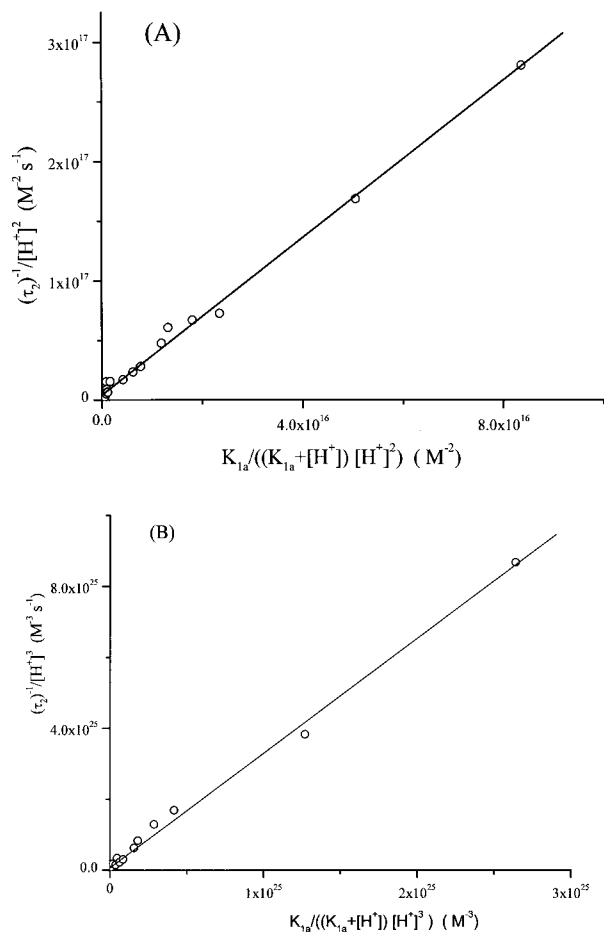


Figure 5. Plot of $(\tau_2)^{-1}/[\text{H}^+]^m$ against $K_{1a}/(K_{1a} + [\text{H}^+])[\text{H}^+]^m$, for 22 experimental points at $25(\pm 0.5)^\circ\text{C}$, $\mu = 0.2$, with $17.6 \mu\text{M} \leq c_1 \leq 48.3 \mu\text{M}$, $79.5 \mu\text{M} \leq c_2 \leq 0.43 \text{ mM}$ and $25 \text{ mM} \leq [\text{HCO}_3^-] \leq 15 \text{ mM}$. (a) For $m = 2$: intercept, $4.0(\pm 4.0) \times 10^{-15} \text{ M}^{-2} \text{ s}^{-1}$; slope, $3.30(\pm 0.15) \text{ s}^{-1}$; $r = 0.9984$. (b) For $m = 3$: intercept, $7(\pm 7) \times 10^{23} \text{ M}^{-3} \text{ s}^{-1}$; slope, $3.25(\pm 0.15) \text{ s}^{-1}$; $r = 0.9976$.

minor $\text{Fe}(\text{AHA})_2$ species (Table 1 and equations (18)).

The only difference between the two mechanisms of Table 1 lies in the first step and in the fact that with $\text{Fe}(\text{AHA})_3$, the first kinetic process is detected by fluorescence emission and not by absorption. This explains why this step was not detected by Cowart *et al.*, (1982) who used only absorption spectroscopy. Indeed, an analysis of the kinetics of serum-transferrin iron uptake from acetohydroxamato $\text{Fe}(\text{III})$ by stopped-flow with absorption detection only shows the existence of two kinetic processes (Figures 1(c) and (d)) instead of the four detected by fluorescence emission. The first fluorescence detected process (Figure 1(a)) is fast. The direct second-order rate constant, k_1 , measured in the presence of $\text{Fe}(\text{AHA})_3$ (Table 1 and equation (18)) is higher than that reported with FeNAC_3 (Table 1 and equation (2)). This, added to the fact that the reverse rate constant, k_{-1} ,

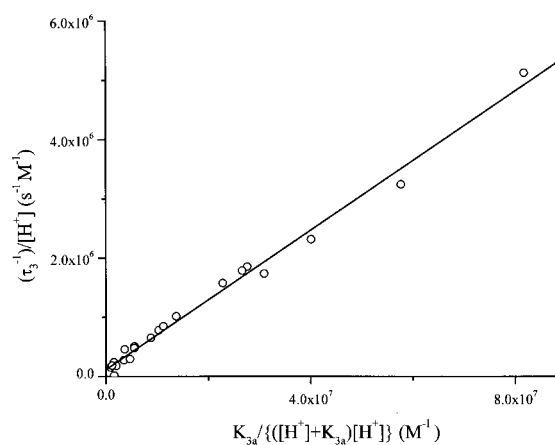


Figure 6. Plot of $(\tau_3)^{-1}/[\text{H}^+]$ against $K_{3a}/([\text{H}^+] + K_{3a})[\text{H}^+]$, for 21 experimental points at $25(\pm 0.5)^\circ\text{C}$ and $\mu = 0.2$ with $0.8 \mu\text{M} \leq c_1 \leq 4 \mu\text{M}$, $4 \mu\text{M} \leq c_2 \leq 50 \mu\text{M}$ and $10 \text{ mM} \leq [\text{HCO}_3^-] \leq 40 \text{ mM}$. Intercept, $1.2(\pm 0.80) \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$; slope, $5.90(\pm 0.30) \times 10^{-2} \text{ s}^{-1}$; $r = 0.99508$.

is too small to be measured, seems to imply that the affinity of $(\text{T}_\text{C}\text{H}_3)$, the C-site of serum-transferrin in interaction with bicarbonate (k_1/k_{-1}), for $\text{Fe}(\text{AHA})_2$ is higher than for FeNAC_3 . With both chelators this step is fast and occurs without the accumulation of any ternary chelate-protein intermediate. It, nevertheless, leads to the accumulation of a first kinetic intermediate (Figure 1(a)) and is followed by a single proton loss (Table 1 and equations (2), (18) and (3)). This kinetic product is responsible for all the subsequent processes, which lead to the accumulation of two other kinetic products and, finally, to the C-site iron-loaded and/or the holo-protein (Table 1 and equations (4) to (10)). This mechanism was reported for the three major proteins of the transferrin family serum-transferrin, lactoferrin and ovotransferrin (Bou Abdallah & El Hage Chahine, 1998; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998).

The first kinetic step detected by absorption spectroscopy by us and by Cowart *et al.*, (1982) corresponds to the second kinetic process detected by fluorescence spectroscopy. This step leads to the accumulation of a kinetic product (Figure 1(c) and (d)), which was assumed by Cowart *et al.* (1982) to be the ternary transferrin-acetohydroxamato- $\text{Fe}(\text{III})$ intermediate complex. We show that this second kinetic intermediate (Figure 1(b)) cannot be ascribed to a ternary intermediate complex between the protein and the chelate. The fluorescence emission spectrum of this intermediate is that of kinetic product TH_5Fe (Figure 2(b)). It is formed with the same elementary rate constant whether $\text{Fe}(\text{III})$ uptake occurs from FeNAC_3 or $\text{Fe}(\text{AHA})_3$ (Table 1, equations (4), (23) and (24) and Figures 5). This intermediate is produced by a modification in the conformation of both the C and N-sites of monoferric serum-transferrin, which is

Table 1. The mechanisms of iron uptake by serum-transferrin from Fe(AHA)₃ and FeNAC₃

Reaction	Direct rate constant ^a L = FeNAC ₃	Forward rate constant L = Fe(AHA) ₃	Reverse rate constant L = FeNAC ₃	Equilibrium constant
FeL + L ⇌ FeL ₂	(14)			
FeL ₂ + L ⇌ FeL ₃	(15)			
T _C H ₂ + HCO ₃ ⁻ /CO ₃ ²⁻ ⇌ T _C H ₃	(1)			4.4 mM ^a
FeL ₂ + T _C H ₃ ⇌ FeT _C H ₃ + 2L	(18)	8.0 × 10 ⁴ M ⁻¹ s ⁻¹	4.15(±0.05) × 10 ⁷ M ⁻¹ s ⁻¹	
T _C H ₃ + FeL ⇌ T _C H ₃ Fe + L	(2)			7.5 × 10 ⁴ M ⁻¹ s ⁻¹
T _C H ₃ Fe ⇌ T _C H ₂ Fe + H ⁺	(3)			16 nM ^a
H ₃ T _N T _C H ₂ Fe ⇌ TH ₅ Fe	(4)	2.80 s ⁻¹	3.25(±0.15) s ⁻¹	
TH _(5-m) Fe + m H ⁺ ⇌ TH ₅ Fe	(5)			
T'H _(5-m) Fe ⇌ TH _(5-m) Fe	(6)	6.30 × 10 ⁻² s ⁻¹	5.90(±0.30) × 10 ⁻² s ⁻¹	
T'H _(4-m) Fe + H ⁺ ⇌ T'H _(5-m) Fe	(7)			6.8 nM ^a
T'H _(4-m) Fe + FeL ⇌ L + T'H _(4-m) Fe ₂	(8)			
T'H _(4-m) Fe ⇌ T''H _(4-m) Fe	(9)			
T'H _(4-m) Fe ₂ ⇌ T''H _(4-m) Fe ₂	(10)			

^a Pakdaman & El Hage Chahine (1996).

followed by the loss of two or three protons (Table 1 and equations (4) and (5)).

The third kinetic process detected by fluorescence is identical to the second process detected by absorption for serum-transferrin iron uptake from Fe(AHA)₃ (Figure 1(c) and (d)). This process was ascribed by Cowart *et al.* (1982) to ligand loss from the ternary chelate-protein complex. However, under our experimental conditions, this process cannot be ascribed to any ligand loss from any intermediate ternary complex. It leads after proton loss to kinetic intermediates T'H_(4-m)Fe and/or T'H_(4-m)Fe₂ as clearly shown by the fluorescence spectra (Figure 2(b) and (d)). This process is identical to that observed with the uptake of iron by serum-transferrin, as indicated by the identical formation rate constant, *k*₃ (Table 1 and equations (6) to (8)). It is followed by a proton loss and rate-limits Fe(III) uptake by the N-site of the protein (Table 1 and equation (7)). As for the fourth kinetic processes, they were only detected by fluorescence spectroscopy; they last about 4000 seconds and are strictly identical to those observed during iron uptake from FeNAC₃ (Pakdaman & El Hage Chahine, 1996). They were not reported by Cowart *et al.* (1982). We ascribe them to a last modification in the conformation of the C-site monoferric or diferric iron-loaded serum-transferrin during which the iron-loaded species attain their final state of equilibrium as holo- or monoferric proteins.

We were able to sequentially detect the four kinetic processes involved in the uptake of iron by transferrin because, in the cases of Fe(AHA)₃ and FeNAC₃ the first kinetic steps always described the uptake of iron by the C-site of the protein in interaction with bicarbonate. However, if these Fe(III) uptakes were slower than the last modifications in the conformation of the metal-loaded protein (Table 1 and equations (9) and (10)), they can become rate limiting and only one step would be observed for the uptake of iron. This is probably

the case with some chelates such as the ferric citrate where the uptake of iron by serum-transferrin lasts several hours and where the first step in the uptake is that of the depolymerisation of the iron citrate chelate (Bates *et al.*, 1967). We, therefore, believe that *in vitro* iron uptake by serum-transferrin occurs by the same mechanism regardless of the nature of the iron-providing chelate. Only the differences in the rates of the uptake would be due to the nature of the chelate. Does all this imply the formation of the ternary intermediate protein-metal-chelator complex, as shown during iron loss in mildly acidic media from a decarbonated protein in a different state of conformation and protonation than the holoprotein in neutral media (El Hage Chahine & Pakdaman, 1995) and suggested in neutral media (Bates *et al.*, 1967; Bates & Schlabach, 1975; Cowart *et al.*, 1982, 1986; Egan *et al.*, 1992)? This question cannot be answered directly at this stage. Indeed, although when absorption detection is used, our experimental signals are similar to those reported by Cowart *et al.* (1982), our conclusions differ markedly from theirs. We cannot, however, neglect the involvement of a ternary complex in iron uptake by transferrins. Our results only imply that this intermediate, if it exists, does not accumulate as a kinetic product (Bou Abdallah & El Hage Chahine, 1998; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998).

In neutral media, transferrins transit from a both lobes opened apo conformation, to a C-lobe closed and an N-lobe opened in the monoferric C-site iron-loaded protein and to finally the holoprotein in which both lobes are in a closed conformation (Moore *et al.*, 1997; Jeffrey *et al.*, 1998; Zuccola, 1992). In both lobes, iron is co-ordinated to two phenolates of two tyrosine residues, to one imidazol of a histidine residue, to a carboxylate of an aspartate residue and to a synergistic carbonate (Moore *et al.*, 1997; Jeffrey *et al.*, 1998; Zuccola, 1992). In the opened conformation of the apo form,

these ligands are in contact with the bulk aqueous medium. The C-site of the apo form is, moreover, in interaction with hydrogen-carbonate and not carbonate (Bellounis *et al.*, 1996; Pakdaman & El Hage Chahine, 1997; Bou Abdallah & El Hage Chahine, 1998). Furthermore in the opened apo form, the acid-base pK_a value of the protein ligands, specially the two phenols, should be close to those in aqueous media (about 10), and would be, subsequently, protonated (El Hage Chahine & Pakdaman, 1995; El Hage Chahine & Fain, 1993, 1994; Russel *et al.*, 1987). When in the vicinity of pH 7 the protein interacts with iron, its structure shifts from the opened apo configuration to the closed holo configuration in which the protein ligands and the synergistic anion are in the deprotonated form (Moore *et al.*, 1997; Jeffrey *et al.*, 1998; Zuccola, 1992). This occurs in the four kinetic steps shown in Figure 1 and as described in Table 1. The environment of the protein ligands and the bicarbonate are, consequently, considerably altered by chelation and by water expulsion from the binding cleft (El Hage Chahine & Fain, 1993, 1994; Bou Abdallah & El Hage Chahine, 1998; Russel *et al.*, 1987; Hancock & Martell, 1989). This process is well illustrated in the absence of bicarbonate. Indeed, when NAC_3 is the only synergistic anion present in the medium, serum-transferrin interacts with FeNAC_3 to form a mixed FeNAC_3 -transferrin complex in which NAC_3 can be readily replaced by carbonate upon addition of bicarbonate (El Hage Chahine & Fain, 1993; El Hage Chahine & Pakdaman, 1996). A deprotonation pK_a value of 6.34 was reported during the first step of FeNAC_3 uptake by serum-transferrin in the absence of bicarbonate and was ascribed to that of the phenols of the tyrosine engaged in the mixed complex formation (El Hage Chahine & Fain, 1993). Addition of bicarbonate leads to two other deprotonations detected (average $pK_a = 7.2$) upon the substitution of NAC_3 by carbonate in the mixed FeNAC_3 -transferrin complex to yield the holoprotein (El Hage Chahine & Pakdaman, 1996). We can, therefore, ascribe the proton transfers which follow the modifications in conformation during the uptake of iron by serum-transferrin (equations (3), (5) and (7)) to the deprotonation of bicarbonate and the phenols of the tyrosine residues engaged in complex formation. Because a protonated ligand, such as phenol or hydrogen carbonate, lacks affinity for Fe(III) (Eigen & Wilkins, 1965; Wilkins, 1976; Hancock & Martell, 1989), the first fast step would involve the interaction of the metal with the available non-protonated carboxylate of aspartate and or imidazol of histidine. This is followed by the loss of a single proton probably provided by bicarbonate (equation (3)). The two domains of the C-lobe would then start enveloping iron, this being accompanied by the loss of two protons from the remaining phenols (equation (5)). The third proton-loss (equation (7)) may be provided by one of the side-chains of the ligands involved in the interdomain H-bonding network (Moore *et al.*,

1997; Kurokawa *et al.*, 1995). These first three steps allow the protein to attain a conformation in which the N-site extracts iron from the chelate by a mechanism similar to that reported for the C-site (Pakdaman & El Hage Chahine, 1996; Bou Abdallah & El Hage Chahine, 1998). The iron-loaded serum-transferrin reaches afterward its final equilibrated state in a last step during which all the interlobe interactions occur stabilising further the closed holo structure (Table 1 and equations (9) and (10)). This last step is two- to threefold slower with the scavenging agents lactoferrin and ovotransferrin (Bou Abdallah & El Hage Chahine, 1998). It, therefore, rate-limits iron loss from the protein to a competing ligand or a siderophore. This is confirmed by most of the results from the literature, where iron loss to a competing chelating agent in neutral media is slower with the iron scavenger lactoferrin than with the iron deliverer serum-transferrin (Egan *et al.*, 1992; Chung & Raymond, 1993). This may imply that the metabolic function of transferrins in iron scavenging and/or delivery is related to the kinetics of this last process.

Conclusion

In vitro, iron uptake by serum-transferrin from a chelate occurs without the accumulation of a kinetic ternary protein-chelate product. The nature of the iron-providing chelate does not seem to affect this thermodynamic equilibrium. It may, however, modify the kinetics by slowing the first step to such an extent that it becomes rate limiting.

Materials and Methods

Better than 98% pure human serum apotransferrin (Sigma, lot 47H9318) was further purified and purity checked by published procedures (El Hage Chahine & Fain, 1993). KCl (Merck Suprapur), NaOH, and HCl (Merck Titrisol), EDTA (Merck Titriplex), FeCl_3 , acetic acid and sodium carbonate (Merck), nitrilotriacetic acid ($\text{N}(\text{AcH})_3$), and Hepes (Aldrich), glycerol, acetohydroxamic acid ($\text{H}(\text{AHA})$), urea and boric acid (Sigma), acrylamide, bromophenol blue, Coomassie blue and TEMED (Boehringer Mannheim) were used without further purification. Water and glassware were prepared as described (El Hage Chahine & Fain, 1993).

Stock solutions

All stock solutions were used fresh. They were prepared in previously boiled bidistilled water. The Hepes concentration in neutral buffers was 50 mM and final pH values were adjusted to between pH 7 and 8 with microquantities of concentrated HCl or NaOH. Transferrin concentrations (c_1) were checked spectrophotometrically (El Hage Chahine & Fain, 1993). They ranged from 0.5 μM to 0.13 mM. $\text{Fe}(\text{AHA})_3$ solutions (5 mM) were prepared by mixing three parts of a neutral solution of acetohydroxamic acid with one part of an equimolar solution of FeCl_3 at pH 2. The pH was slowly raised to pH 6 with microquantities of 10 M NaOH. These solutions were further diluted to the required final $\text{Fe}(\text{AHA})_3$ concentrations in the buffers. The NaHCO_3 concentrations

ranged from 5 mM to 30 mM in the apotransferrin and Fe(AHA)₃ solutions. All final ionic strengths were adjusted to 0.2 M with KCl.

Iron-loaded transferrins

The holoprotein was obtained in the presence of 20 mM bicarbonate when apotransferrin was allowed to interact for several hours with more than two equivalents of Fe(AHA)₃ or FeNAC₃. The only C-site iron-loaded serum transferrin was obtained under similar conditions when the protein interacted with only one equivalent of Fe(AHA)₃ or FeNAC₃. This iron load was always checked by polyacrylamide/urea gel electrophoresis (El Hage Chahine & Pakdaman, 1995; Makey & Seal, 1976).

Spectrophotometric measurements

Absorption measurements were performed at 25(±0.1)°C on a Perkin Elmer Lambda 2 spectrophotometer equipped with a magnetic stirring device and thermostatted cell carrier. Fluorimetric measurements were performed at 25(±0.1)°C on a Aminco-Bowman series 2 luminescence spectrometer equipped with a thermostatted cell carrier. Emission spectra were measured in the 300 nm to 420 nm range. The excitation wavelength was set to 280 nm (Lehrer, 1969; Pakdaman & El Hage Chahine, 1996).

Stopped-flow measurements

Fast kinetic measurements were performed on an SF 3L Hi-Tech stopped-flow spectrophotometer equipped with two different light sources and a thermostatted bath at 25(±0.5)°C, as described (Bou Abdallah & El Hage Chahine, 1999).

Mathematical formalism and signal analysis

The experimental conditions were set so as to permit the use of chemical relaxation formalism; all experimental signals were analysed as described elsewhere (El Hage Chahine & Fain, 1993; Eigen & DeMaeyer, 1963).

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Appendix

The substitution methods were used to derive the reciprocal relaxation time equations (Bernasconi, 1976; Eigen & DeMaeyer, 1963).

Derivation of equation (19)

The rate equation of equation (16), the conservation of mass and the constant state of equilibrium of equations (1), (14), (15) and (17) when equation (16) is assumed to be rate-limiting and the fact that $(\text{FeL}) \ll (\text{FeL}_2)$ and (FeL_3) allow us to write:

$$-d[\text{FeL}_2\text{TCH}_2]/dt = k'_{-1}[\text{FeL}_2\text{TCH}_3] - k'_1[\text{FeL}_2][\text{TCH}_3] \quad (26)$$

$$\Delta[\text{FeL}_2\text{TCH}_2] + \Delta[\text{FeL}_2\text{TCH}_3] + \Delta[\text{TCH}_3] + \Delta[\text{T}'\text{CH}_2] = 0 \quad (27)$$

$$\Delta[\text{FeL}_2\text{TCH}_2] + \Delta[\text{FeL}_2\text{TCH}_3] + \Delta[\text{FeL}_3] + \Delta[\text{FeL}_2] = 0 \quad (28)$$

$$\Delta[\text{L}] + 3\Delta[\text{FeL}_3] + 2\Delta[\text{FeL}_2] + 2(\Delta[\text{FeL}_2\text{TCH}_2] + \Delta[\text{FeL}_2\text{TCH}_3]) = 0 \quad (29)$$

$$\Delta[\text{TCH}_3] = [\text{HCO}_3^-]\Delta[\text{T}'\text{CH}_2]/K_{\text{HCO}_3}^- + [\text{T}'\text{CH}_2]\Delta[\text{HCO}_3^-]/K_{\text{HCO}_3}^- \quad (30)$$

$$\Delta[\text{FeL}_2\text{TCH}_3] = [\text{H}^+]\Delta[\text{FeL}_2\text{TCH}_2]/K'_{1a} + [\text{FeL}_2\text{TCH}_2]\Delta[\text{H}^+]/K'_{1a} \quad (31)$$

$$\Delta[\text{FeL}_3] = [\text{L}]\Delta[\text{FeL}_2]/K_{\text{FeL}} + [\text{FeL}_2]\Delta[\text{L}]/K_{\text{FeL}} \quad (32)$$

From equations (26) to (32), equation (19) is derived.

Derivation of equation (21)

The conservation of mass and the constant state of equilibrium (K_{1a}) of equation (3) when equation (18) is assumed rate-limiting allow us to write:

$$-d[\text{FeTCH}_2]/dt = k_{-1}[\text{FeTCH}_3][\text{L}]^2 - k_1[\text{FeL}_2][\text{TCH}_3] \quad (33)$$

$$\Delta[\text{FeTCH}_2] + \Delta[\text{FeTCH}_3] + \Delta[\text{TCH}_3] + \Delta[\text{T}'\text{CH}_2] = 0 \quad (34)$$

$$\Delta[\text{FeTCH}_2] + \Delta[\text{FeTCH}_3] + \Delta[\text{FeL}_2] + \Delta[\text{FeL}_3] = 0 \quad (35)$$

$$2\Delta[\text{FeL}_2] + 3\Delta[\text{FeL}_3] + \Delta[\text{L}] = 0 \quad (36)$$

$$\Delta[\text{FeT}_c\text{H}_3] = [\text{H}^+]\Delta[\text{FeT}_c\text{H}_2]/K_{1a} + [\text{FeT}_c\text{H}_2]\Delta[\text{H}^+]/K_{1a} \quad (37)$$

From equations (30), (32) and (33) to (37), equation (21) is derived.

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