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Transferrins

Hen ovo-transferrin, interaction with bicarbonate and iron uptake

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Fe(III) uptake by the iron-delivery and iron-scavenging protein, hen ovotransferrin has been investigated *in vitro* between pH 6.5 and 9. In the absence of any ferric chelate, apo-ovotransferrin loses two protons with $K_{1a} = 50 \pm 1$ nM and $K_{2a} = 4.0 \pm 0.1$ nM. These acid-base equilibria are independent of the interaction of the protein with bicarbonate. The interaction with bicarbonate occurs with two different affinity constants, $K_C = 9.95 \pm 0.15$ mM and $K_N = 110 \pm 10$ mM. $FeNAc_3$ exchanges its Fe(III) with the C-site of the protein in interaction with bicarbonate, direct rate constants $k_1 = 650 \pm 25\ M^{-1}\ s^{-1}$, reverse rate constant $k_{-1} = (6.0 \pm 0.1) \times 10^3\ M^{-1}\ s^{-1}$ and equilibrium constant $K_1 = 0.11 \pm 0.01$. This iron-protein intermediate loses then a single proton, $K_{3a} = 3.50 \pm 0.35$ nM, and undergoes a first change in conformation followed by a two or three proton loss, first order rate constant $k_2 = 0.30 \pm 0.01\ s^{-1}$. This induces a new modification in conformation followed by the loss of one or two protons, first order rate constant $k_3 = (1.50 \pm 0.05) \times 10^{-2}\ s^{-1}$. These modifications in the monoferric protein conformation are essential for iron uptake by the N-site of the protein. In the last step, the monoferric and diferric proteins attain their final state of equilibrium in about 15000 s.

The overall mechanism of iron uptake by ovotransferrin is similar but not identical to those of serum transferrin and lactoferrin. The rates involved are, however, closer to lactoferrin than serum transferrin, whereas the affinities for Fe(III) are lower than those of serum transferrin and lactoferrin. Does this imply that the metabolic function transferrins is more related to kinetics than to thermodynamics?

Keywords: transferrins; iron uptake.

Transferrins are involved in iron regulation and transport in vertebrates and some invertebrates such as worms or insects (Aisen, 1989). The transferrin family consists of two important branches: soluble glycoproteins and membrane melanotransferrin (Baker and Lindley, 1992). The soluble glycoproteins include serum transferrin, which transports iron from the blood stream and delivers it to the cytosol by receptor-mediated endocytosis (Dautry-Varsat et al., 1983), lactoferrin, which sequesters Fe(III) in biological media (Montreuil et al., 1960; Anderson et al., 1989) and ovotransferrin, which is assumed to be responsible for both transport and scavenging of Fe(III) in poultry (Kurokawa et al., 1995). The structures of the soluble transferrins were elucidated by X-ray diffraction. They all consist of a single chain of about 700 amino acids and 80 kDa arranged in two similar lobes linked by a small interlobe chain of about ten amino acids. In each lobe, iron is co-ordinated to the four lateral chains of two tyrosines, one histidine, one aspartate, and a carbonate or bicarbonate adjacent to an arginine (Bailey et al., 1988; Anderson et al., 1989; Zuccola, 1992; Kurokawa et al., 1995; Rawas et al., 1996; Moore et al., 1997). This carbonate constitutes the synergistic anion which gives these proteins affinities for iron in the $10^{20}\ M^{-1}$ range (Aisen, 1989; Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997). However, although soluble transferrins are very similar and present a high degree of similarity in their amino acid sequences (Baldwin, 1993), some differences exist in their structures (Anderson et al., 1989; Kurokawa et al., 1995; Rawas et al., 1996; Moore et al., 1997). These slight discrepancies are assumed to be responsible for the specificity of the metabolic function of each of these proteins (Baker and Lindley, 1992).

Lactoferrin is known to keep its iron when in acidic media, whereas serum transferrin loses its charge of iron in the acidic endosomes (Montreuil et al., 1960; Dautry-Varsat et al., 1983; El Hage Chahine and Pakdaman, 1995). In a recent series of articles, we proposed a general mechanism for iron uptake by

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Abbreviations. $FeNAc_3$, nitrilotriacetatoFe(III); c_1, analytical concentration of ovotransferrin; c_2, analytical concentration of $FeNAc_3$; c_3, analytical concentration of nitrilotriacetate; μ, ionic strength; OT, apo-ovotransferrin in an undefined state of protonation; OTH_m, protonated form of apo-ovotransferrin; SA, synergistic anion; $(SA)OT_CH_m$, the C-site of apo-ovotransferrin in interaction with the synergistic anion in the protonated form; $(SA)_2OTH_{m-2}$, apo-ovotransferrin in interaction with two synergistic anions in the deprotonated form; FeL, the iron chelate; L, the free chelating ligand; $(SA)OT_CH_mFe$ and $(SA)OT_CH_{m-l}Fe$, the transferrin C-site Fe(III) complex in the protonated and deprotonated form, respectively; $(SA)OTH_{m-l}Fe$ and $(SA)OTH_{m-q}Fe$, two intermediates monoferric ovotransferrin iron complexes in two different states of protonation; $(SA)OT'H_tFe$ and $(SA)OT'H_{t-v}Fe$, two other intermediates in different states of protonation; $(SA)OT'H_{t-v}Fe_2$, a diferric ovotransferrin intermediate; $(SA)OT''H_{t-v}Fe$ and $(SA)OT''H_{t-v}Fe_2$, the monoferric and the holoprotein in their final equilibrated states; T'_CH_3, the C-site of a protonated apotransferrin in interaction with the synergistic anion; T'_CH_3Fe and T'_CH_2Fe, monoferric intermediates in the protonated and deprotonated forms; $H_3T'_N$-T'_CH_2Fe, TH_5Fe, $TH_{5-m}Fe$, $T'H_{5-m}Fe$, $T'H_{3-m}Fe$ and $T'H_{4-m}Fe$, $T'H_{3-m}Fe_2$, other monoferric and diferric intermediates; $T''H_{3-m}Fe_2$, and $T''H_{3-m}Fe$, the holo and the monoferric transferrins in their final equilibrated states.

serum transferrin and lactoferrin from nitrilotriacetatoFe(III) ($FeNAc_3$). The first step of this mechanism is the interaction of the C-site of the protein with bicarbonate which is followed by a proton loss in the case of lactoferrin. In the second step $FeNAc_3$ exchanges Fe(III) very rapidly with the C-site of protein in interaction with the synergistic anion. This step is followed by a series of proton losses and changes in conformation lasting from a few seconds to hundreds of seconds, and allows the N-site to capture Fe(III). If Fe(III) is not available in the medium, the monoferric transferrin undergoes a last modification in conformation which occurs in about 4000 s for serum transferrin and 10 000 s for lactoferrin. This last step is also observed with the diferric transferrins. Iron uptake by serum transferrin is faster than that by lactoferrin. It, however, involves affinities for iron of the same order of magnitude (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998). Does this imply that the function of a transferrin, as iron-sequestering or iron transporting agent, is a matter of kinetics with lactotransferrin capable of holding iron longer than serum transferrin? What would then be the behaviour towards iron uptake of an iron-scavenger and an iron-deliverer such as ovotransferrin?

In this article, by using the methods and techniques of chemical relaxation (Eigen and DeMayer, 1973; Bernasconi, 1976), we report the mechanism of the interaction of ovotransferrin with bicarbonate and that of iron uptake by this protein in neutral aqueous media.

MATERIALS AND METHODS

KCl (Merck Suprapur), NaOH, and HCl (Merck Titrisol), EDTA (Merck Titriplex), $FeCl_3$, sodium carbonate (Normapur) and acetic acid (100%, Merck), nitrilotriacetic acid ($NAcH_3$) and Hepes (Aldrich), glycerol and boric acid (Sigma), acrylamide and APS (Ultra Pur, BRL), urea (GERBU), bromophenol blue (BioRad), Coomassie blue (Serva) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Promega) were used without further purification. Water and glassware were prepared as described previously (El Hage Chahine and Fain, 1993).

Protein purification. Ovotransferrin (Sigma) was purified and its purity and iron load were checked by spectrophotometric analysis and by urea-polyacrylamide gel electrophoresis, as previously described (Makey and Seal, 1976; Pakdaman and El Hage Chahine, 1997). All our experiments were performed with the purified apo-ovotransferrin.

Stock solutions. All stock solutions were used fresh. Apo-ovotransferrin concentrations (c_1) were checked spectrophotometrically by using a molecular extinction coefficient of 91 200 M^{-1} cm^{-1} at 280 nm (Hirose et al., 1995). Their concentrations ranged from 0.5 μM to 120 μM for the protein, from 5 mM to 400 mM for $NaHCO_3$ and from 20 mM to 50 mM for Hepes; ionic strengths (μ) were adjusted with KCl. The final pH values were adjusted to between 6.5 and 8.9 by adding microquantities of concentrated HCl and NaOH. Only the data acquired at $\mu = 0.2$ were taken into account for the kinetic and thermodynamic analysis.

pH measurements. pH values were measured at 25 ± 0.5°C with a Jenco pH-meter equipped with an Ingold calomel/glass combined microelectrode. Buffers used for pH standardisation were pH 7.00 and 10.01 (Sigma).

Spectrophotometric measurements. Spectrophotometric measurements were performed on a Perkin Elmer lambda 2 spectrophotometer equipped with a magnetic stirring device and a thermostated cell carrier with the temperature held in the sample cell at 25 ± 0.1 °C.

Fluorimetric measurements. Fluorimetric measurements were performed at 25 ± 0.5°C on an Aminco-Bowman series 2 Luminescence spectrometer equipped with thermostated cell carrier. Emission spectra were measured in the 300 to 420 nm range. Excitation wavelengths were set to 280 nm. Under the normal experimental conditions applied ($\mu = 0.2$, Hepes = 50 mM and 0.5 μM ≤ c_1 ≤ 2 μM) and at a given pH value, the intensity of the fluorescence emission at 333 nm was proportional to c_1.

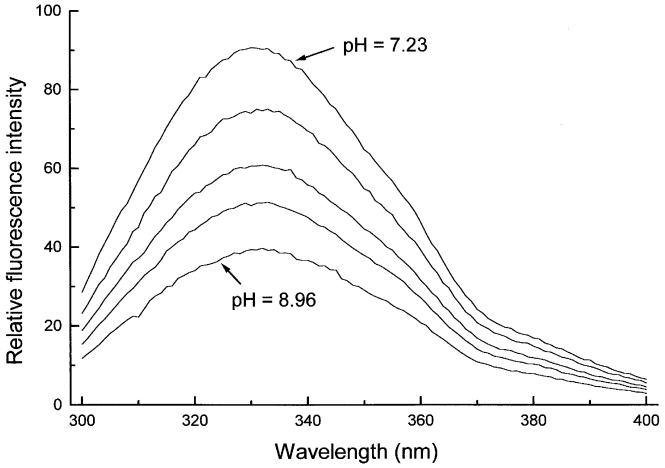

Fig. 1. Fluorescence emission spectra of ovotransferrin at different pH values. Measured with an excitation wavelength (λ_{ex} = 280 nm) at 25 ± 0.10 °C, 7.23 < pH < 8.96, μ = 0.2 for c_1 = 1.25 μM.

Stopped flow measurements. Fast kinetic measurements were performed with an SF 3L Hi-Tech stopped-flow spectrophotometer equipped with a temperature regulated bath 25 ± 0.5°C by mixing a $FeNAc_3$ solution with apo-ovotransferrin or with the monoferric C-site iron loaded ovotransferrin, as described previously (El Hage Chahine and Fain, 1994).

Mathematical formalism and signal analysis. The experimental conditions were set so as to permit the use of chemical relaxation formalism (Brouillard, 1980). All experimental signals were analysed as described elsewhere (El Hage Chahine and Fain, 1993). They all were pure mono- or multi-exponentials and were dealt with as relaxation modes (Bernasconi, 1976; Eigen and DeMayer, 1973).

RESULTS

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

Proton dissociation and interaction with bicarbonate. Transferrins present typical absorption and fluorescence emission spectra with fluorescence emission maxima (λ_{em}) in the 330−335 nm range when the proteins are excited with excitation maxima (λ_{ex}) in the 278−280 nm range (Lehrer, 1969; Pakdaman et al., 1998). In the 6.5 to 9.5 pH range, the intensity of the fluorescence emission of ovotransferrin depends on the acidity of the medium (Fig. 1). It decreases with increasing pH from a plateau in the 6 to 6.5 range to another plateau in the 9 to 9.5 range. Above pH 9.5, part of the protein seems to be irreversibly denatured. This is shown by the fact that the fluorescence intensity does not return to the expected value when the pH is decreased from above 9.5 to neutral. Therefore, as for serum transferrin and lactoferrin, all our experiments were performed at pH values of less than 9.3, and the results reported deal with reversible phenomena. Fluorescence intensity also decreases

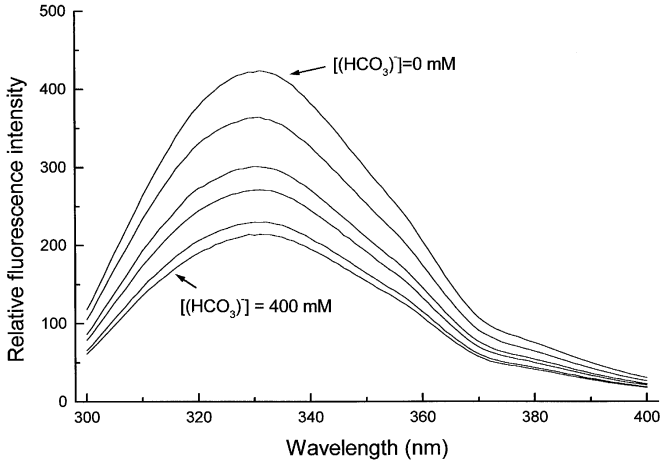

Fig. 2. Fluorescence emission spectra of ovotransferrin in the presence of 0 < [HCO_3^-] < 400 mM. Measured with an excitation wavelength (λ_{ex} = 280 nm) at 25 ± 0.10°C, pH 8.04 and μ = 0.5 for c_1 = 0.92 μM.

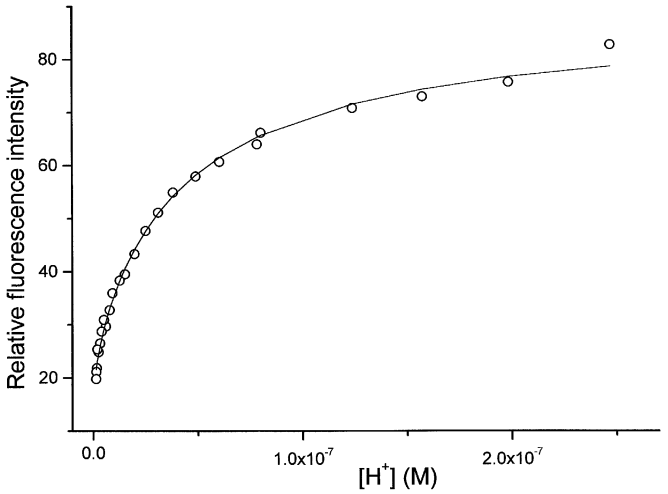

Fig. 3. Non-linear least-squares regression of F against $(f_1[H^+]^2 + f_3K_{1a}[H^+] + f_2K_{1a}K_{2a})c_1/([H^+]^2 + K_{1a}[H^+] + K_{1a}K_{2a})$ at 25±0.10°C and μ = 0.2. Reported between pH 6.65 and 8.94 at 25 ± 0.5°C and μ = 0.2 for c_0 = 1.25 μM and unknown f_2, K_{1a} and K_{2a}.

with increasing bicarbonate concentration at constant pH (Fig. 2). This intensity still varies with bicarbonate for HCO_3^- concentrations greater than 180 mM which with 20 mM of Hepes is the maximum concentration possible in order to keep an ionic strength of 0.2. However, at higher ionic strengths ($\mu = 0.5$), the fluorescence intensity ceases to vary for HCO_3^- concentrations greater than 400 mM. The experiments dealing with bicarbonate were performed at pH values greater than 7.3 in tightly stoppered cells to avoid any significant HCO_3^- concentration modification (Pakdaman et al., 1998).

Proton-dissociation of apo-ovotransferrin. In the absence or in the presence of bicarbonate, the decrease of the fluorescence intensity of ovotransferrin with pH can be approximated by two equations. If it is assumed that each of these equations describes a proton transfer (Eqns 1 and 2), the fluorescence intensity can be expressed as in Eqn (3) (El Hage Chahine et al., 1989)

$$OTH_{m-1} + H^+ \rightleftharpoons OTH_m \quad (1)$$

$$OTH_{m-2} + H^+ \rightleftharpoons OTH_{m-1} \quad (2)$$

$$F = (f_1[H^+]^2 + f_3K_{1a}[H^+] + f_2K_{1a}K_{2a})c_1/([H^+]^2 + K_{1a}[H^+] + K_{1a}K_{2a}) \quad (3)$$

where c_1 is the analytical concentration of ovotransferrin, OTH_m, OTH_{m-1} and OTH_{m-2} are the apo-ovotransferrin prototropic species present in the medium, m is an unknown number of protons likely to be lost, $K_{1a} = [H^+][OTH_{m-1}]/[OTH_m]$, $K_{2a} = [H^+][OTH_{m-2}]/[OTH_{m-1}]$, F is the fluorescence intensity, f_1, f_2 and f_3 are experimental factors which relate the fluorescence intensity to the concentrations of OTH_m, OTH_{m-1} and OTH_{m-2}, respectively. f_2 is unknown, f_1 and f_3 were measured in the absence of carbonate at pH 6.3 and pH 9.3, respectively.

A multiple non-linear regression of the data against Eqn (3) gave, in the absence and in the presence of $[HCO_3^-] = 50$ mM, the same $K_{1a} = 50 \pm 1$ nM and $K_{2a} = 4.0 \pm 0.1$ nM (Fig. 3). Therefore, in neutral and mildly basic media, ovotransferrin can exist as at least three prototropic species involving the two acid-base Eqns (1) and (2).

Interaction of apo-ovotransferrin with bicarbonate. At constant pH, the fluorescence intensity of ovotransferrin decreases with HCO_3^- concentration to attain a plateau for HCO_3^- concentration of ≈400 mM (Fig. 2). To a first approximation, this fluorescence decrease can be fitted by two lines, each of which has a slope independent of pH. This implies that the interaction with bicarbonate is independent of the state of protonation of the ovotransferrin as reported for serum transferrin (Bellounis et al., 1996). We may also assume to a first approximation, as in the case of serum transferrin and lactoferrin, that the interaction with bicarbonate occurs with both sites, with however, one of the sites (presumably the C-site) presenting a much greater affinity for the synergistic anion (Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997). We can therefore write for the C-site:

$$OTH_m + HCO_3^- \rightleftharpoons (SA)OTH_m \quad (4)$$

$$OTH_{m-1} + HCO_3^- \rightleftharpoons (SA)OTH_{m-1} \quad (5)$$

$$OTH_{m-2} + HCO_3^- \rightleftharpoons (SA)OTH_{m-2}. \quad (6)$$

Several series of experiments were performed. Two were with $[HCO_3^-]$ varying from 0 to 40 mM at fixed pH 7.3 and pH 9.0 where the major species present in the medium are OTH_m in acidic and OTH_{m-2} in basic media. Experimental data obeyed for each pH value Eqn (7) (Pakdaman and El Hage Chahine, 1997).

$$\Delta f/\Delta F = 1/c_1 + K_C/(c_1[HCO_3^-]) \quad (7)$$

in which, $\Delta F = F - F_0$, with F_0 being the fluorescence intensity measured in the absence of bicarbonate and F the fluorescence intensity; $\Delta f = f_4 - f_1$ in the vicinity of pH 7, or $f_6 - f_3$ in the vicinity of pH 9, with f_4 and f_6 the experimental factors which relate the fluorescence intensity to the concentrations of species $(SA)OTH_m$ and $(SA)OTH_{m-2}$, respectively. f_4 was measured at pH 6.5 with an initial analytical concentration of HCO_3^- of 50 mM under a CO_2 saturated atmosphere (Pakdaman and El Hage Chahine, 1997). f_6 was approximated from the fluorescence emission spectrum of apo-ovotransferrin at pH 9.3 and HCO_3^- concentration of 50 mM.

From both linear regressions, identical $K_C = 9.95 \pm 0.15$ mM were determined, implying that, contrary to lactoferrin and similar to serum transferrin (Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997), the interaction of the ovotransferrin site with the highest affinity for HCO_3^- does not involve any acid-base reaction (Fig. 4). If we assume that the second site of ovotransferrin also interacts with bicarbonate at high HCO_3^- concentration and that this interaction is much weaker than that with the other site and independent of H^+ concentration, we can write

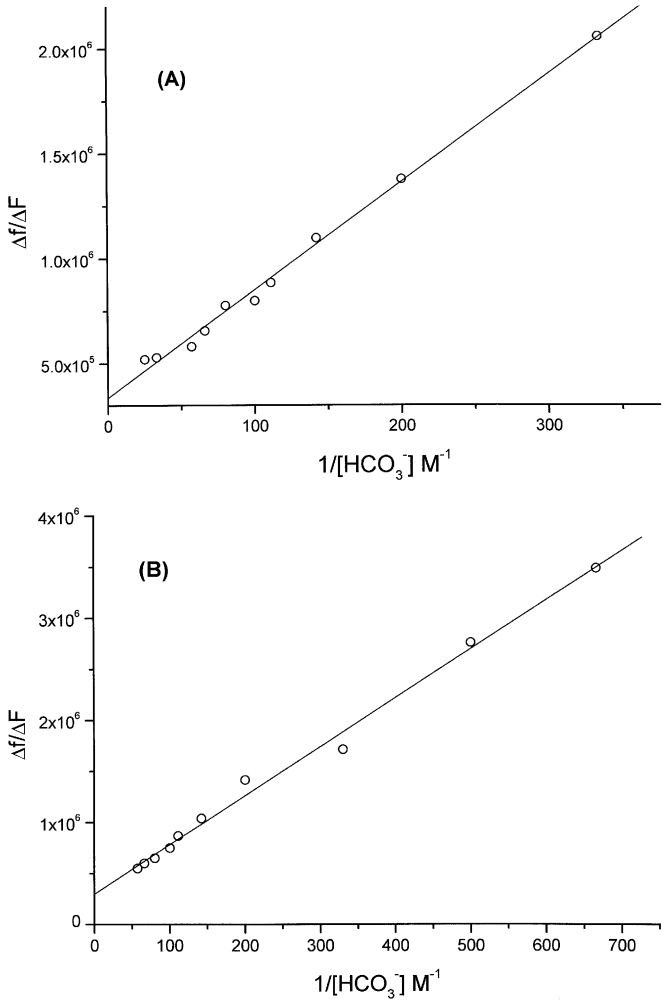

Fig. 4. Plot of $\Delta f/\Delta F$ against $1/(c_1[HCO_3^-])$ at 25±0.1 °C, μ = 0.2. Reported for (A) at pH 8.98 for c_1 = 1.9 μM, intercept, $(3.50 \pm 0.50)\times10^5$ M^{-1}; slope, $(5.2 \pm 0.2)\times10^3$; r = 0.99115 and (B) at pH = 7.35 for c_1 = 1.9 μM, intercept, $(3.00 \pm 0.40)\times10^5$ M^{-1}; slope, $(4.85 \pm 0.15)\times10^3$; r = 0.99646.

at pH ≈9 where apo-ovotransferrin has the $(SA)OTH_{m-2}$ structure:

$$(SA)OTH_{m-2} + HCO_3^- \rightleftharpoons (SA)_2OTH_{m-2}\,. \qquad (8)$$

In this case the fluorescence intensity will be expressed as (El Hage Chahine et al., 1989):

$$F = (f_1K_CK_N + f_4K_N[HCO_3^-] + f_5[HCO_3^-]^2)c_1/$$
$$(K_CK_N + K_N[HCO_3^-] + [HCO_3^-]^2) \qquad (9)$$

with $K_N = [(SA)OTH_{m-2}][HCO_3^-]/[(SA)_2OTH_{m-2}]$ and f_5, the experimental factor which relates fluorescence intensity to $[(SA)_2OTH_{m-2}]$; f_5 was measured at $\mu = 0.5$ for HCO_3^- concentration = 400 mM.

A multiple non-linear regression with least-squares adjustment of the data against Eqn (9) in which K_C and f_1 are known gave $k_N = 110 \pm 10$ mM (Fig. 5). Besides f_5, all the data used for this fit were acquired at $\mu = 0.2$ for HCO_3^- concentration of less than 150 mM at pH 9.05.

Iron uptake by ovotransferrin. When a neutral solution of apo-ovotransferrin is rapidly mixed with a neutral solution of $FeNAc_3$ in the presence of bicarbonate, four kinetic processes are detected (Fig. 6). Although 10 to 30 fold slower, these processes resemble those already reported for serum transferrin and

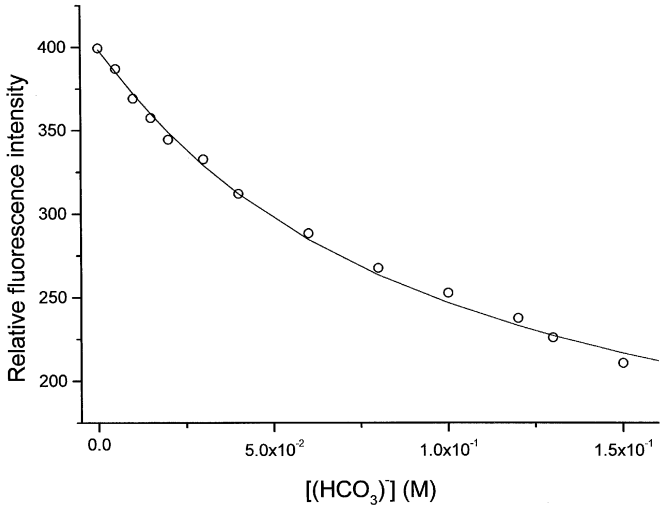

Fig. 5. Non-linear least-squares regression of F against $(f_1K_CK_N + f_4K_N[HCO_3^-] + f_5[HCO_3^-]^2)c_1/(K_CK_N + K_N[HCO_3^-] + [HCO_3^-]^2)$. Reported at 25 ± 0.1 °C, μ = 0.2 and pH = 9.05 with K_2 and f_2 unknown.

lactoferrin (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998). The first process detected by the stopped flow techniques occurs in the 2 to 5 s range as an exponential increase of absorbance. Its amplitude never reaches that expected for the C-site iron loaded ovotransferrin (Fig. 6A). Both the rate and amplitude of this fast process depend on c_1, c_2 (nitrilotriacetato-Fe(III) concentration), c_3 (nitrilotriacetate concentration), HCO_3^- concentration and pH. The second phenomenon is also an exponential increase of the absorbance or decrease of the fluorescence emission intensity. It occurs in the 50-s range and its rate depends only on pH (Fig. 6B and C). These two processes are detected in the 400 to 500 nm absorption region and in the 300 to 400 nm emission range for an excitation λ_{ex} = 280 nm. This implies that the iron load by the C-site does not reach its final state of equilibrium at the end of the two fast processes of Fig. 6. When $c_2 > c_1$, the fluorescence emission spectra measured under the same conditions at the end of the second fast process of Fig. 6A and B are not those of the holoprotein or those of the C-site iron loaded transferrin (Fig. 7). The third kinetic process occurs in the 300−500-s range (Fig. 6C). It is detected by fluorescence emission in the 300 to 400 nm range with an excitation wavelength λ_{ex} = 280 nm and by absorption spectroscopy in the 400 to 500 nm range. The fourth process occurs in the 15000 s range and its rate seems to be independent of pH, c_1, c_2 and HCO_3^- concentration within the limits of uncertainty.

The first kinetic step. As for serum transferrin or lactoferrin, when an $FeNAc_3$ solution is mixed with apo-ovotransferrin, the first kinetic process of Fig. 6A can describe a rate-limiting iron exchange between the protein and the chelate (Eqn 10) followed by Eqn (11) (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998). The latter is probably a diffusion-controlled proton-loss which should occur in the microsecond range (Eigen and DeMayer, 1973: Bernasconi, 1976).

$$(SA)OT_CH_m + FeL \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (SA)OT_CH_mFe + L \quad \text{(rate-limiting)} \qquad (10)$$

$$(SA)OT_CH_{m-l}Fe + l\,H^+ \rightleftharpoons (SA)OT_CH_mFe \quad \text{(very fast)} \qquad (11)$$

with m, an unknown number of protons susceptible to be lost and l, the number of protons lost during the first step of Fig. 6A and B.

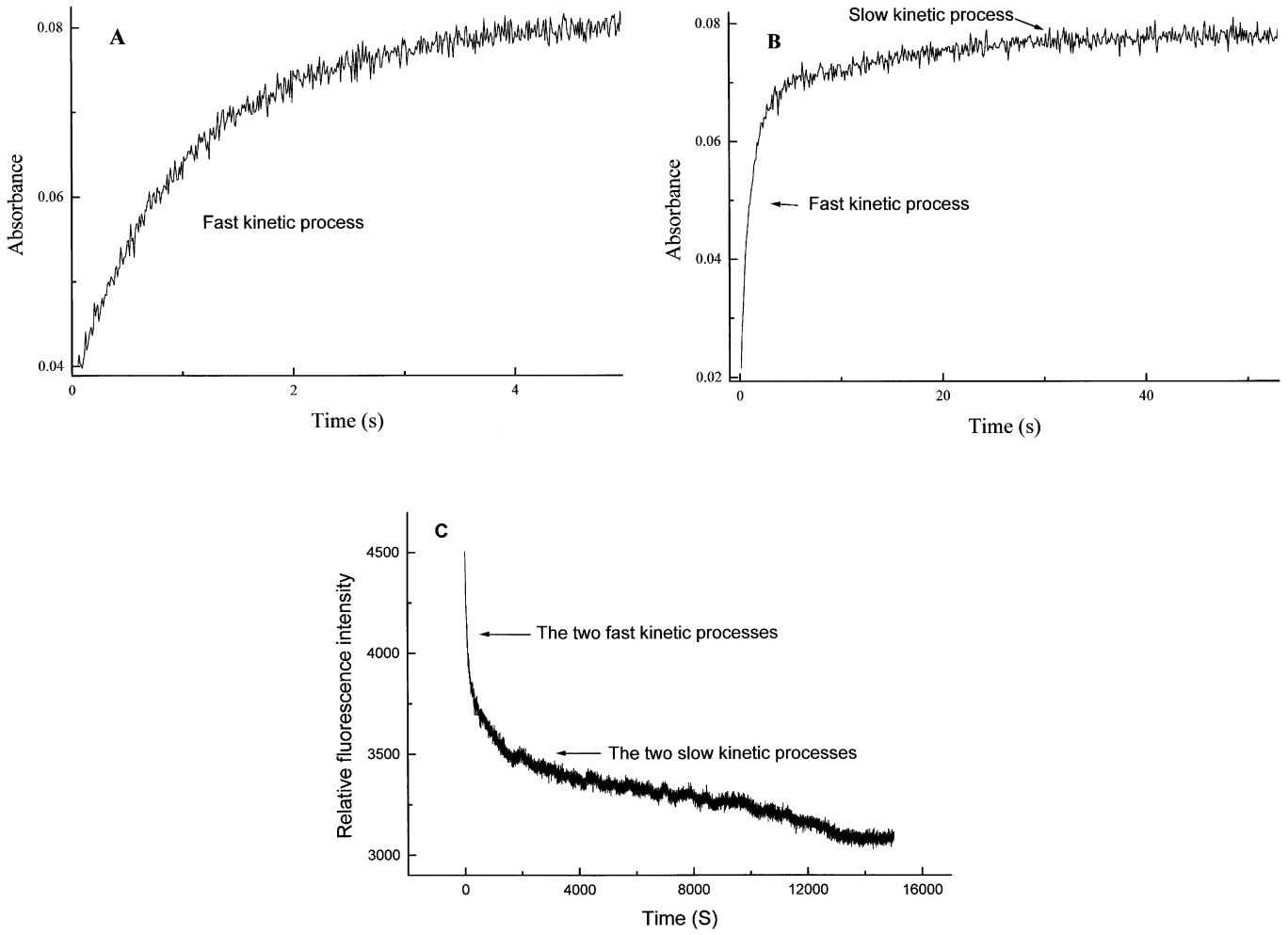

Fig. 6. Increase of absorbance and decrease of fluorescence subsequent to a rapid [$FeNAc_3$]-jump on an ovotransferrin solution at 25±0.5°C and μ = 0.2. (A) Exponential increase of absorbance at λ = 465 nm recorded in 0.5 s when an apo-ovotransferrin solution (c_1 = 43.5 μM) is mixed with $FeNAc_3$ (c_2 = 0.35 mM) in the presence of NAc_3 (c_3 = 0 μM) and bicarbonate ([HCO_3^-] = 20 mM) at pH = 8.17 ± 0.02. (B) A multiexponential increase of absorbance recorded in 50 seconds under the same experimental conditions as A. (C) A multiexponential decrease of fluorescence at an emission wavelength (λ_{em} = 330 nm) for an excitation wavelength (λ_{ex} = 280 nm) recorded in 15000 s when an apo-ovotransferrin solution (c_1 = 0.7 μM) is mixed with $FeNAc_3$ (c_2 = 5.6 μM) at pH = 8.07 ± 0.02.

Table 1. The $(c_2 - c_1)/\alpha(2c_1 + c_3)$, the corresponding $(\tau_1)^{-1}/(2c_1 + c_3)$ and the deduced k_{obs} values. Reported at 25 ± 0.5°C, and μ = 0.2 at three fixed pH and [HCO_3^-] values. The linear regressions of $(c_2 - c_1)/\alpha(2c_1 + c_3)$ against $(\tau_1)^{-1}/(2c_1 + c_3)$ gave; (A) intercept, (5.90 ± 0.50)×10^3 M^{-1} s^{-1}; slope, 520 ± 20 M^{-1} s^{-1}; r = 0.99975; (B) intercept, (6.8 ± 0.5)×10^3 M^{-1} s^{-1}; slope, 560 ± 30 M^{-1} s^{-1}; r = 0.99932; (C) intercept, (6.0 ± 0.5)×10^3 M^{-1} s^{-1}; slope, 630 ± 15 M^{-1} s^{-1}; r = 0.99991; (D) intercept, (6.6 ± 0.7)×10^3 M^{-1} s^{-1}; slope, 715 ± 35 M^{-1} s^{-1}; r = 0.99910; (E) intercept, (6.5 ± 0.5)×10^3 M^{-1} s^{-1}; slope, 790 ± 30 M^{-1} s^{-1}; r = 0.99954.

(A) pH = 8.03 [HCO_3^-] = 20 mM $c_1 = 9.76\times10^{-5}$ M		(B) pH = 8.17 [HCO_3^-] = 20 mM $c_1 = 8.71\times10^{-5}$ M		(C) pH = 8,30 [HCO_3^-] = 20 mM $c_1 = 1.16\times10^{-5}$ M		(D) pH = 8,42 [HCO_3^-] = 20 mM $c_1 = 8.58\times10^{-5}$ M		(E) pH = 8,50 [HCO_3^-] = 20 mM $c_1 = 6.73\times10^{-5}$ M	
$\frac{(\tau_1)^{-1}}{2c_1}$	$c_2 - c_1/2c_1$	$\frac{(\tau_1)^{-1}}{2c_1}$	$c_2 - c_1/2c_1$	$\frac{(\tau_1)^{-1}}{2c_1}$	$c_2 - c_1/2c_1$	$\frac{(\tau_1)^{-1}}{2c_1}$	$c_2 - c_1/2c_1$	$\frac{(\tau_1)^{-1}}{2c_1}$	$c_2 - c_1/2c_1$
6147	0.52	8725	3.49	6465.5	0.79	9126	3.497	7281	0.98
6660	1.55	9070	5	6983	1.65	9522	3.998	8024	1.73
7787	3.6	9759	5.24	7845	2.94	9907	4.5	8470	2.47
8310	4.62	10103	5.98	8362	3.81	10198	5	9064	3.21
9580	7.18	10448	6.5	9741	5.96	10606	5.49	10847	5.44
		11022	7.48			10886	5.991	12035	6.93
		11481	8.45			11305	6.493		
K_{obs} = 518.996		K_{obs} = 563.882		K_{obs} = 634.988		K_{obs} = 713.555		K_{obs} = 789.714	

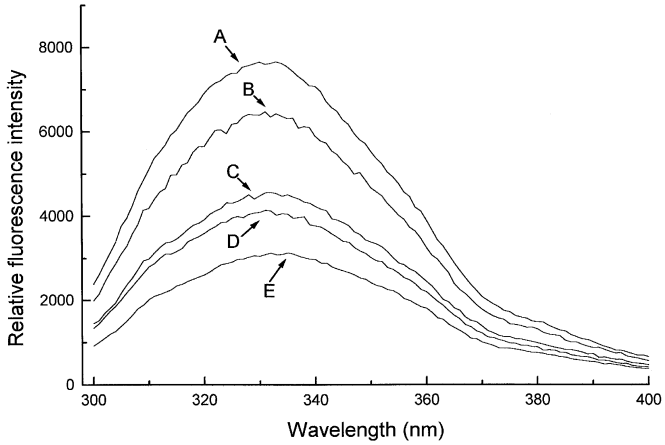

Fig. 7. Emission fluorescence spectra of apo- and iron loaded ovotransferrin species at 25±0.5 °C, pH 8.07 and μ = 0.2 for c_1 = 0.7 μM. Measured for (A) apo-ovotransferrin, (B) 50 s after fast mixing, with $c_2 = c_1$, (C) 15000 s later, (D) 50 s after mixing, with $c_2 = 10c_1$ and (E) 15000 s later.

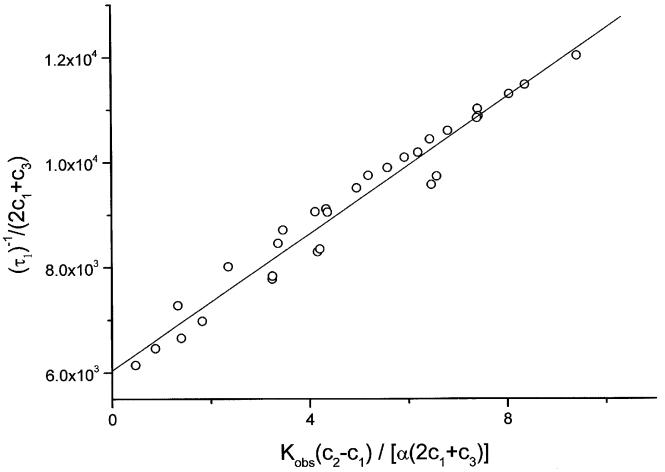

Fig. 8. Plot of $(\tau_1)^{-1}/(2c_1 + c_3)$ against $\beta(c_2 - c_1)/[\alpha(2c_1 + c_3)]$. Reported at 25 ± 0.5°C, μ = 0.2, 30 μM ≤ c_1 ≤ 60 μM, 50 μM ≤ c_2 ≤ 0.65 mM and 7.40 ≤ pH ≤ 8.90. Intercept, $(6.0 \pm 0.1)\times10^{-3}$ M^{-1} s^{-1}; slope, 650 ± 25 M^{-1} s^{-1}; r = 0.9805.

The reciprocal relaxation time equation associated with rate-limiting Eqn (10) can be expressed as (Pakdaman and El Hage Chahine, 1996):

$$(\tau_1)^{-1}/(2c_1 + c_3) = k_{-1} + k_{obs}(c_2 - c_1)/[\alpha(2c_1 + c_3)] \quad (12)$$

with

$$k_{obs} = k_1\beta \quad (13)$$

in which, $\alpha = (1 + K_C/[HCO_3^-])$ and $\beta = (1 + K_{3a}/[H^+]^l)$.

Eqn (13) can also be written as Eqn (14):

$$k_{obs} = k_1 + k_1K_{3a}/[H^+]^l \quad (14)$$

where $K_{3a} = [(SA)OT_CH_{m-l}Fe][H^+]^l/[(SA)OT_CH_mFe]$

Five series of experiments were performed at five fixed pH values. Five very good linear least-squares regressions of the data related to each fixed pH against Eqn (11) were obtained (Table 1). These led to five different K_{obs} values and gave the same $k_{-1} = (6.0 \pm 0.1)\times10^3$ M^{-1} s^{-1} (Table 1). The best linear least-squares regression of the K_{obs} against $1/[H^+]^l$ was only obtained for $l = 1$ from the slope and the intercept k_1 = 650 ± 25 M^{-1} s^{-1} and K_{3a} = 3.50 ± 0.35 nM. This gave $K_1 = k_1/k_{-1} = [(SA)OT_CH_mFe][L]/[(SA)OT_CH_m][FeL] = 0.11 \pm 0.01$. This first step in the uptake of iron was further checked by another series of experiments performed at different pH, c_1, c_2 and c_3 values. A good linear least-squares regression of the data against Eqn (12) was obtained and gave the same k_1 and k_{-1} values (Fig. 8).

Therefore, the first step in iron-uptake by the C-site of ovotransferrin occurs by similar paths than those of serum transferrin and lactoferrin, with however, lower rate constants and lower affinities for Fe(III).

The second kinetic step. The second kinetic step in iron uptake by ovotransferrin (Fig. 6B) is much slower than that reported for serum transferrin and lactoferrin. It occurs in the 50 s range and, as for serum transferrin and lactoferrin, it was ascribed to a change of conformation of the protein followed by m proton losses (Eqns 15 and 16; Pakdaman and El Hage Chahine, 1996).

$$(SA)OT_CH_{m-l}Fe \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (SA)OTH_{m-l}Fe \quad \text{(rate-limiting)} \quad (15)$$

$$(SA)OTH_{m-q}Fe + (q + 1)H^+ \rightleftharpoons (SA)OTH_{m-l}Fe \quad \text{(fast)} \quad (16)$$

where $(SA)OTH_{m-l}Fe$ is the product of a rate-limiting modification in the conformation affecting both sites of $(SA)OT_CH_{m-l}Fe$ (Eqn 15) which is followed by $q + 1$ proton-losses (Eqn 16).

The reciprocal relaxation time equation associated with Eqn (15) can be expressed as (Pakdaman and El Hage Chahine, 1996):

$$(\tau_2)^{-1} = k_2\{K_{3a}/(K_{3a} + [H^+])\} + k_{-2}[H^+]^{q+1}/K_{4a}. \quad (17)$$

Eqn (17) can also be written as Eqn (18).

$$(\tau_2)^{-1}/[H^+]^{q+1} = k_2\{K_{3a}/(K_{3a} + [H^+])\,[H^+]^{q+1}\} + k_{-2}/K_{4a} \quad (18)$$

with $K_{4a} = [(SA)OTH_{m-q}Fe][H^+]^{q+1}/[(SA)OTH_{m-l}Fe]$.

Good linear least-squares regressions of the data against Eqn (18) were obtained for $q + 1 = 2$ or 3 and gave k_2 = 0.30 ± 0.01 s^{-1} (Fig. 9). Therefore, as for serum transferrin and lactoferrin, the modification in the protein conformation described by the second kinetic step (Fig. 6A) is accompanied by the loss of two or three protons.

The third and fourth kinetic processes. The third kinetic process of Fig. 6B and C is also similar with that detected for serum transferrin (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998). It is, however, slower and occurs in the 500 s range. As for serum transferrin and lactoferrin, it can be ascribed to a modification in the conformation of the iron loaded C-site of the protein accompanied by an acid-base reaction, also affecting the N-lobe and allowing it to capture Fe(III) if available in the medium (Eqns 19−21). There is, however, a major difference between the prototropic behaviour of serum transferrin or lactoferrin and ovotransferrin (Eqn 20). The latter loses two protons in the vicinity of neutrality instead of the single proton lost by the two other transferrins (Pakdaman and El Hage Chahine; 1996; Pakdaman et al., 1998).

$$(SA)OTH_tFe \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (SA)OT'H_tFe \quad \text{(rate-limiting)} \quad (19)$$

$$(SA)OT'H_{t-v}Fe + vH^+ \rightleftharpoons (SA)OT'H_tFe \quad \text{(very fast)} \quad (20)$$

$$(SA)OT'H_{t-v}Fe + FeL \rightleftharpoons (SA)OT'H_{t-v}Fe_2 + L \quad \text{(fast)} \quad (21)$$

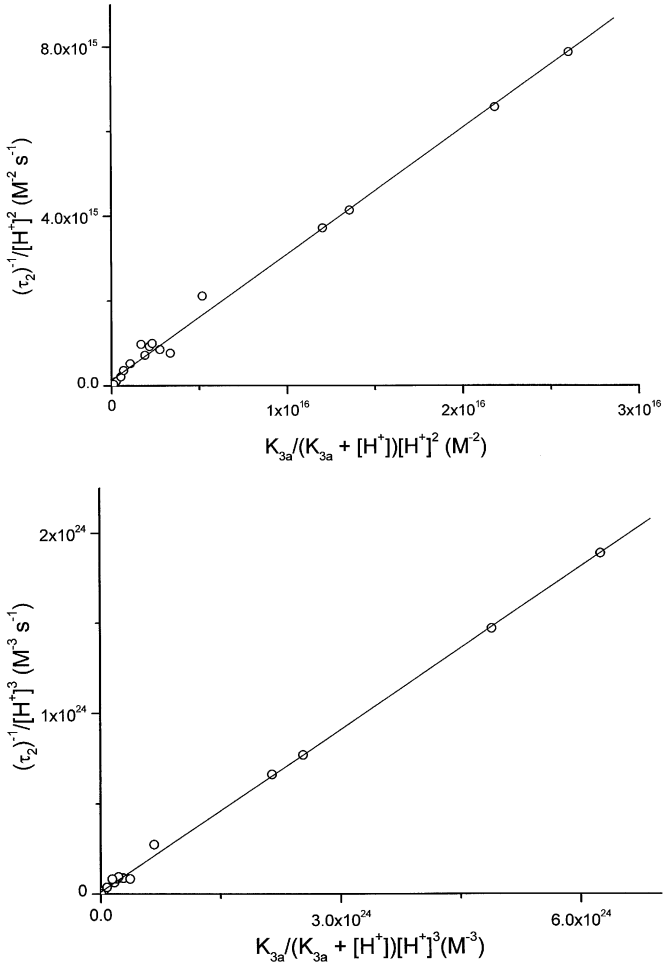

Fig. 9. Plot of $(\tau_2)^{-1}/[H^+]^{q+1}$ against $\{K_{3a}/(K_{3a} + [H^+])\ [H^+]^{q+1}\}$. Reported at 25 ± 0.5 °C, $\mu = 0.2$, 25 μM $\leqslant c_1 \leqslant$ 60 μM, 0.13 mM $\leqslant c_2 \leqslant$ 0.80 mM and 7.35 $\leqslant$ pH $\leqslant$ 8.38. (A) $q + 1 = 2$, intercept, $(7 \pm 3) \times 10^{13}$ M^{-2} s^{-1}; slope, 0.30 ± 0.01 s^{-1}; $r = 0.99679$. (B) $q + 1 = 3$, intercept, $(6 \pm 3) \times 10^{21}$ M^{-3} s^{-1}, slope, 0.30 ± 0.01 s^{-1}, $r = 0.99927$.

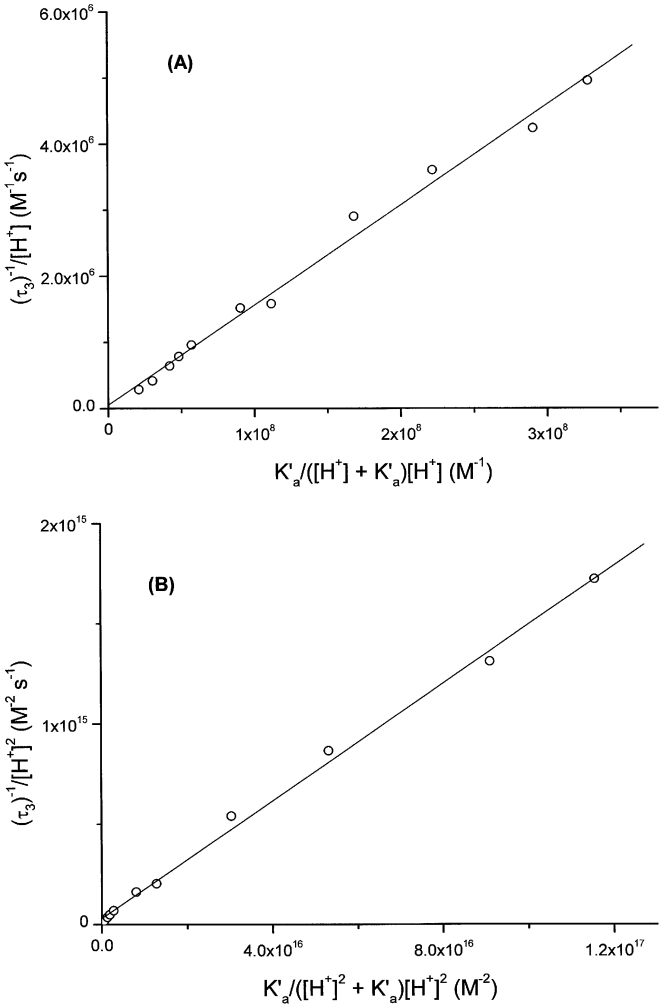

Fig. 10. Plot of $(\tau_3)^{-1}/[H^+]^{\upsilon}$ against $K'_a([H^+]^{\upsilon} + K'_a)[H^+]^{\upsilon}$. Reported at 25 ± 0.5 °C, $\mu = 0.2$ and $7.52 \leq$ pH ≤ 8.54 for $0.7\ \mu M \leq c_1 \leq 1.5\ \mu M$ and 3.5 μM $\leq c_2 \leq$ 30 μM. (A) $\upsilon = 1$ with $K'_a = K_{1a}$, intercept, $(6 \pm 8) \times 10^4$ M^{-1} s^{-1}; slope, $(1.50 \pm 0.05) \times 10^{-2}$ s^{-1}; $r = 0.99564$; (B) $\upsilon = 2$ with $K'_a = K_{1a}K_{2a}$; intercept, $(3.20 \pm 1.40) \times 10^{13}$ M^{-2} s^{-1}; slope, $(1.45 \pm 0.05) \times 10^{-2}$ s^{-1}, $r = 0.99827$.

where (SA)OT′H_tFe an intermediate produced by rate-limiting change of conformation allowing the monoferric ovotransferrin to capture a second Fe(III) if available, with $t = m - q$ and υ, the number of protons lost by the intermediate.

The reciprocal relaxation time equation associated with Eqn (19) if rate-limiting can be expressed as Eqn (22) (Pakdaman and El Hage Chahine, 1996):

$$(\tau_3)^{-1}/[H^+]^{\upsilon} = k_3K'_a/([H^+]^{\upsilon} + K'_a)[H^+]^{\upsilon} + k_{-3}/K'_a \qquad (22)$$

with $K'_a = K_{1a}K_{2a}$ when $\upsilon = 2$ and $K'_a = K_{1a}$ or K_{2a} for $\upsilon = 1$. A linear least-squares regression of the experimental data is only obtained for $K'_a = K_{1a}$ when $\upsilon = 1$ as it was also obtained for $\upsilon = 2$. From the slope of the regression lines, the same $k_3 = (1.50 \pm 0.05) \times 10^{-2}\ s^{-1}$ is determined (Fig. 10).

As for serum transferrin and lactoferrin, the rate of the fourth kinetic process is always constant and does not vary with the concentration of any of the species present in the medium. Eqns (23) and (24), therefore, describe a true first-order kinetic processes (Eigen and DeMayer, 1973; Bernasconi, 1976).

$$(SA)OT'H_{t-\upsilon}Fe_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} (SA)OT''H_{t-\upsilon}Fe_2 \qquad (23)$$

$$(SA)OT'H_{t-\upsilon}Fe \underset{k'_{-4}}{\overset{k'_4}{\rightleftharpoons}} (SA)OT''H_{t-\upsilon}Fe\ . \qquad (24)$$

The reciprocal relaxation time equation associated Eqns (23) and (24) is expressed as Eqn (25) (Pakdaman and El Hage Chahine, 1996):

$$(\tau_4)^{-1} = k_4 + k'_4 + k_{-4} + k'_{-4}\ . \qquad (25)$$

This equation clearly indicates that the experimental $(\tau_4)^{-1}$ should be constant as observed. This probably implies another modification in the conformation of the protein when its C-site is charged with iron. Whether this modification is followed by proton loss cannot be established at this stage. All that can be said is that (SA)OT′$H_{t-\upsilon}$Fe or (SA)OT′$H_{t-\upsilon}Fe_2$ does not reach the final state of equilibrium as an, only C-site iron loaded transferrin, or as a holoprotein before the end of this slow process, as shown by the fluorescence emission spectra (Fig. 7).

Role of the ionic strength. All the reported experimental results were obtained at a constant temperature 25 ± 0.5 °C and a constant ionic strength ($\mu = 0.2$). However, the interaction of transferrin with iron seems to depend on the ionic strength and on Cl^- concentration (Williams et al., 1982). One series of experiments was, therefore, performed with μ varying from 0.1 to 0.5 and KCl concentration varying from 50 to 450 mM (data not shown). These results were identical with those reported in this

article. Therefore, under our experimental conditions (μ = 0.2, 20 mM $\leqslant$ [Hepes] $\leqslant$ 100 mM and 100 mM $\leqslant$ [KCl] $\leqslant$ 180 mM), the kinetic runs are not affected by Cl^- concentration.

DISCUSSION

As shown for serum transferrin and lactoferrin, at pH ≈8, the presence of two nitrilitriacetatoFe(III) species in the medium [more than 70% $FeNAc_3(OH)$ and less than 30% of the $FeNAc_3(OH)_2$] does not affect our kinetic analysis (El Hage Chahine and Fain, 1993; Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998). Contrary to what was observed with lactoferrin, and in agreement with what was observed for serum transferrin, the interaction of apo-ovotransferrin with bicarbonate is independent of the state of protonation of the protein and does not involve any proton transfer (Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997). The affinity of both sites of ovotransferrin for bicarbonate are about threefold lower than those of serum transferrin (Bellounis et al., 1996). Our results indicate that in natural physiological conditions (pH 7.4, HCO_3^- concentration ≈20 mM), only 10% of presumably the C-site of apo-ovotransferrin interacts with bicarbonate, whereas 30% of the same site of serum transferrin and about the same percentage for lactoferrin interact with the synergistic anion (Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997). Since the interaction with bicarbonate is a prerequisite for iron uptake (Aisen, 1989; Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998), the affinity of the C-site of ovotransferrin for iron may be lower than that of serum transferrin and lactoferrin. This is actually confirmed by the fact that the affinity of ovotransferrin for iron after the metal exchange between $FeNAc_3$ and the C-site (Fig. 6A) is lower than those we reported for serum transferrin and lactoferrin (Table 2).

Serum apotransferrin and apolactoferrin are both engaged in one acid-base reaction independent of HCO_3^- concentration. In the presence of bicarbonate, another proton loss from the apolactoferrin interacting with the synergistic anion is detected (Bellounis et al., 1996; Pakdaman and El Hage Chahine, 1997). With ovotransferrin, we detect two bicarbonate-independent acid-base reactions leading to the loss of two protons in mildly basic media (pH ≈8). Whether both these two proton losses occur from one lobe of ovotransferrin or whether they concern both lobes of the protein cannot be determined at this stage.

Ovotransferrin is believed to be an iron-scavenging and iron-transport system in poultry (Kurokawa et al., 1995; Mason et al., 1996). It is, therefore, assumed to play the role of both serum transferrin and lactoferrin in mammals. From the mechanistic standpoint, there are great similarities between ovotransferrin, lactoferrin and serum transferrin in their kinetic behaviour towards iron uptake. However, the rates of the reactions involved in iron uptake by ovotransferrin are from 3 to 50 times lower than for serum transferrin and from 2 to 10 times lower than for lactoferrin (Table 2). In the three proteins, the first step in iron uptake from $FeNAc_3$ is the exchange of iron between the chelate and the C-site of the protein in interaction with bicarbonate (Table 2). This process is followed by two other modifications in the protein conformation, accompanied by three to five proton losses and allowing the N-site to capture another Fe(III) from $FeNAc_3$ if it is available. Finally, a last step lasting about four hours for ovotransferrin allows the monoferric species or the diferric species to attain their final state of equilibrium.

The rates involved in iron uptake by ovotransferrin are closer to those of lactoferrin than those of serum transferrin (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998), whereas the affinities involved in this iron uptake are the lowest when

Table 2. The mechanism of iron exchange between $FeNAc_3$, human serumtransferrin, bovine lactoferrin (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998) and hen ovotransferrin.

Reaction	Direct rate constant			Reverse rate constant			Equilibrium constant		
	serum transferrin	lactoferrin	ovotransferrin	serum transferrin	lactoferrin	ovotransferrin	serum transferrin	lactoferrin	ovotrans-ferrin
				$M^{-1}\ s^{-1}$					
$T'_CH_3 + FeL \rightleftharpoons T'_CH_3Fe + L$	$8.0\times10^4\ M^{-1}\ s^{-1}$	$4.9\times10^4\ M^{-1}\ s^{-1}$	$6.50\times10^2\ M^{-1}\ s^{-1}$	7.50×10^4	1.80×10^5	6.05×10^3	1	0.25	0.11
$T'_CH_2Fe + H^+ \rightleftharpoons T'_CH_3Fe$							16 nM	17 nM	3.5 nM
$H_3T_N\text{-}T'_CH_2Fe \rightleftharpoons TH_5Fe$	2.80 s^{-1}	1.00 s^{-1}	0.3 s^{-1}						
$mH^+ + TH_{5-m}Fe \rightleftharpoons TH_5Fe$									
$TH_{5-m}Fe \rightleftharpoons T'H_{5-m}Fe$	$6.30\times10^{-2}\ s^{-1}$	$8.75\times10^{-3}\ s^{-1}$	$1.50\times10^{-2}\ s^{-1}$						
$T'H_{3-m}Fe + FeL \rightleftharpoons L + T'H_{3-m}Fe_2$									
$T'H_{4-m}Fe + H^+ \rightleftharpoons T'H_{5-m}Fe$							6.8 nM	7.94 nM	50 nM
$T'H_{3-m}Fe + H^+ \rightleftharpoons T'H_{4-m}Fe$									4 nM
$T'H_{3-m}Fe_2 \rightleftharpoons T''H_{3-m}Fe_2$									
$T'H_{3-m}Fe \rightleftharpoons T''H_{3-m}Fe$									

compared to serum transferrin or lactoferrin. In this case, the affinity of the protein for iron would not be the factor that determines the iron sequestration and or transport role of the protein. The capacity of the protein to keep its iron longer in neutral media can partly control this metabolic function. Iron loss from transferrins in favour of a competing ligand will be controlled by the slowest step of the mechanism of Table 2. This step occurs in about 5000 s for serum transferrin, 10 000 s for lactoferrin and more than 15 000 s for ovotransferrin. Therefore, in neutral media serum transferrin would lose its iron more rapidly to a siderophore, for example, than lactoferrin or ovotransferrin. In this case its efficiency as an iron-scavenging agent will be lower than that of the other two transferrins. In contrast, in acidic media, serum holotransferrin and holo-ovotransferrin easily lose their iron load (El Hage Chahine and Pakdaman, 1995 and unpublished data), whereas lactoferrin is known to keep its iron load even at pH ≈2 (Montreuil et al., 1960). Therefore, the behaviour of ovotransferrin towards iron release in acidic media is comparable to that of the iron-deliverer serum transferrin and not to that of the iron-scavenger lactoferrin.

The structures of transferrins are different depending on whether the proteins are in the apo- or holo-form (Baker and Lindley, 1992; Mecklenburg et al., 1997). In the apo-form of transferrins, the two lobes are mostly in an open conformation (Baker and Lindley, 1992). In the holo-form, the lobes are in a closed conformation in which the two domains of each site act as pairs of jaws enveloping one Fe(III) and one bicarbonate or carbonate (Anderson et al., 1989; Zuccola, 1992; Kurokawa et al., 1995). In most soluble transferrins, such as bovine and human lactoferrins, rabbit and human serum transferrins and hen and duck ovotransferrins, the metal environment and the binding ligands are identical (Bailey et al., 1988; Anderson et al., 1989; Zuccola, 1992; Kurokawa et al., 1995; Rawas et al., 1996; Moore et al., 1997). In the open apo-form, the lobes of transferrins are accessible to the outside aqueous medium (Baker and Lindley, 1992). This suggests that the pK_a values of the lateral chains of the aminoacid residues in each binding site of the apoprotein open conformation should be close to those in water (Russel et al., 1987). Therefore, in the apo-form, the side-chains of aspartate and glutamate are probably deprotonated because their proton dissociation pK_a values in water are close to 4. As for the lysines, they would be protonated and thereby positively charged because their proton dissociation pK_a values are close to 10. In the C-site of ovotransferrin, there are six interdomain H-bonds (two Asp-Thr, Gln-Lys, Asn-Asn, Asp-Ser and Glu-Ser), whereas four are found for the C-site of bovine lactoferrin (Ser-Asn, two Asp-Thr and Thr-Thr) and an Asp-Arg-Lys triplet in that of serum transferrin (Zuccola, 1992; Dewan et al., 1993; Kurokawa et al., 1995; Moore et al., 1997). If we assume that protonations of the side-chains involved in these interdomain interactions weaken these H-bonds (Zundel, 1976), the stability of the closed conformation of the C-lobe of hololactoferrin would be less sensitive to pH than that of ovotransferrin or serum transferrin. Indeed, in the open form of lactoferrin, where water is accessible, the protonation of Thr, Ser and Asn of the Ser-Asn and Thr-Thr H-bonds should occur in very acidic media and in the vicinity of pH 4 for the Asp engaged in the Asp-Thr couple. In contrast, in ovotransferrin, the protonation of the Asp and Glu engaged in the two Asp-Thr, the Asp-Ser and the Glu-Ser H-bonds would occur in the pH 4 range and in serum transferrin the protonation of the Asp of the Asp-Arg-Lys triplet will also occur, as proposed by Dewan et al. in 1993, at rather high pH values. This is supported by the fact that lactoferrins are known to hold their charge of iron at very low pH values (Montreuil et al., 1960), whereas holo-ovotransferrin and serum holotransferrin completely lose their iron charge in seconds at pH 4 (unpublished experiments and El Hage Chahine and Pakdaman, 1995). This alone cannot, however, explain the discrepancies in the affinities of the transferrins for Fe(III), because in all these transferrins complex formation occurs with the same protein ligands, the same synergistic anion, with the same overall geometry and in quite similar environments.

We recently attributed iron release in serum transferrin to the proton-assisted loss of the synergistic anion, which is followed by the sequential protonations of the side-chains of the aminoacid ligands of the metal (El Hage Chahine and Fain, 1994; El Hage Chahine and Pakdaman, 1995). When one of the ligands protonates, it loses its affinity for iron, permitting a possible partial opening of the binding cleft. We reported for serum transferrin a final slow proton transfer, controlling iron release from the C-site and occurring with pK_a≈4. We ascribed this pK_a to the aspartate ligand acid-base equilibrium and we assumed that the observed slow proton transfer controls the access of water to the ligand in acidic media and, therefore, the transition of the C-site binding cleft from the final closed iron-containing structure to the opened apo-form (El Hage Chahine and Fain, 1994; El Hage Chahine and Pakdaman, 1995). In contrast, in order to form the metal coordination sphere, the two domains of each lobe of the proteins should adopt a position approaching the ligands from the metal and thereby help the two domains of the binding cleft to envelop iron, as also observed for some siderophores (Caudle et al., 1995). The bonds involved in Fe(III) complex formation with phenolate are much stronger than any H-bond (Hancock and Martell, 1989). They would, therefore, be responsible for the closure of the binding cleft upon complex formation and this even if the interdomain H-bonds play a conformation stabilising role. We thus assume that, in the presence of bicarbonate, which neutralises the positive charges in the neighbourhood of the co-ordination sphere (Baker and Lindley, 1992), complex formation lowers the pK_a of one of the phenols of the two tyrosine ligands (El Hage Chahine and Fain, 1993). This can initiate the closure of the binding cleft to surround iron, and can account for the first proton loss detected in the fast step (Fig. 6A). The beginning of the cleft closure and the chelating effect can trigger the other tyrosine side chain deprotonation by lowering the other phenol pK_a (Wilkins, 1976; Caudle et al., 1995, El Hage Chahine and Fain, 1993). This would increase the affinity of the ligand for iron, thereby enhancing complex formation which cannot occur unless the conformation of the binding cleft changes from open to closed conformation. This can also trigger complex formation in the other lobe of the protein as shown by rate-limiting step 2 (Fig. 6B). This step is followed by other conformational changes involving interdomain interactions which increase the affinity of the protein for the metal (Fig. 6C and D). This can also account for the discrepancies in proton losses which occur during the uptake of iron by the different transferrins (Pakdaman and El Hage Chahine, 1996; Pakdaman et al., 1998).

Conclusion. Iron uptake by the C-site of soluble transferrins triggers a series of changes in the conformation which allow the protein to achieve two interdependent goals. In the first, the N-site captures a second iron, if available in the medium, and then very slowly attains its final state of equilibrium. Otherwise, in the absence of any available iron, the monoferric protein also attains its thermodynamic equilibrated state by a pathway similar to that of the diferric species.

Although the overall mechanisms of iron uptake by soluble transferrins are very similar, the dynamic and thermodynamic behaviour of these proteins seem to be related to their metabolic function. In the iron-scavengers lactoferrin and ovotransferrin, the uptake of Fe(III) is two to three times slower than that in

the iron-deliverer, serum transferrin. In contrast, the affinity for Fe(III) of the iron-deliverer, serum transferrin, is higher than that of the iron-scavenger and deliverer, ovotransferrin. Does this imply that iron-transport or sequestration are more related to kinetics than thermodynamics?

REFERENCES

Aisen, P. (1989) Physical biochemistry of the transferrins − Update, 1984−1988, *Phys. Bioinorg. Chem. SER 5*, Iron Carriers and Iron Proteins (Loehr, T., ed.) pp. 353−371, VCH, New York.

Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W. & Baker, E. N. (1989) Structure of Human lactoferrin − Crystallographic structure analysis and refinement at 2.8 Å resolution, *J. Mol. Biol. 209*, 711−734.

Bailey, S., Evans, W. E., Garrat, R. C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R. & Watson, J. L. (1988) Molecular structure of serum transferrin at 3.3 Å resolution, *Biochemistry 27*, 5804−5812.

Baker, E. N. & Lindley, P. F. (1992) New perspectives on the structure and function of transferrins, *Inorg. Biochem. 47*, 147−160.

Baldwin, G. S. (1993) Comparison of transferrin sequences from different species, *Comp. Biochem. Physiol. 106B*, 203−218.

Bellounis, L., Pakdaman, R. & El Hage Chahine, J. M. (1996) Apotransferrin proton dissociation and interactions with bicarbonate in neutral media, *J. Phys. Org. Chem. 9*, 111−118.

Bernasconi, C. F. (1976) *Relaxation kinetics*, Academic Press, New York.

Brouillard, R. (1980) How much an equilibrium can be shifted in a chemical relaxation experiment, *J. Chem. Soc. Faraday Trans. 176*, 583−587.

Caudle, M. T., Caldwell, C. D. & Crumbliss, A. L. (1995) Dihydroxamic acid complexes of iron(III) − ligand pK_a and coordinated hydrolysis constants, *Inorg. Chimica Acta 240*, 519−525.

Dautry-Varsat, A., Cienchanover, A. & Lodish, H. F. (1983) pH and recycling of transferrin during receptor-mediated endocytosis, *Proc. Natl Acad. Sci. USA 80*, 2258−2262.

Dewan, J. C., Mikami, B., Hirose, M. & Sacchettini, J. C. (1993) Structural evidence for pH-sensitive trigger in the hen ovotransferrin N-lobe. Implication for transferrin iron-release, *Biochemistry 32*, 11963−11968.

Eigen, M. & DeMayer, L. (1973) in *Techniques of Chemistry, Part 2, vol. 6* (Weissenberger, A. & Hammes, G., eds) Wiley, New York.

El Hage Chahine, J. M. & Fain, D. (1993) The mechanism of iron-transferrin interactions. Uptake of the iron nitrilotriacetic acid complex, *J. Chem. Soc. Dalton Trans.*, 3137−3143.

El Hage Chahine, J. M. & Fain, D. (1994) The mechanism of iron release from transferrin − Slow proton induced loss of nitrilotriacetatoiron(III) complex in acidic media, *Eur. J. Biochem. 223*, 581−587.

El Hage Chahine, J. M. & Pakdaman, R. (1995) Transferrin, a mechanism for iron release, *Eur. J. Biochem. 230*, 1101−1110.

El Hage Chahine, J. M. (1990) Kinetics and thermodynamics of the structural transformations of thiamine in aqueous media. Part 5 − Interaction of thiamine with SDS micelles, *J. Chem. Soc. Perkin Trans. 2*, 1045−1050.

Hancock, R. D. & Martell, A. E. (1989) Ligand design for selective complexation of metal ions in aqueous solutions, *Chem. Rev. 89*, 1875−1914.

Hirose, J., Fujiwara, H., Magarifuchi, T., Iguti, Y., Iwamoto, H., Kominami, S. & Hiromi, K. (1996) Copper binding selectivity of N- and C-sites in serum (human) and ovotransferrin, *Biochim. Biophys. Acta 1296*, 103−111.

Kurokawa, H., Mikami, B. & Hirose, M. (1995) Crystal structure of diferric hen ovo-transferrin at 2.4 Å resolution, *J. Mol. Biol. 254*, 196−207.

Lehrer, S. S. (1969) Fluorescence and absorption studies of the binding of copper and iron to transferrin, *J. Biol. Chem. 244*, 3613−3617.

Makey, D. G. & Seal, U. S. (1976) The detection of four molecular forms of Human transferrin during the iron-binding process, *Biochim. Biophys. Acta 453*, 250−256.

Mecklenburg, S. L., Donohoe, R. J. & Olah, G. A. (1997) Tertiary structural changes and iron release from human serum transferrin, *J. Mol. Biol. 270*, 739−750.

Montreuil, J., Tonnelat, J. & Mullet, S. (1960) *Préparation et propriétés de la lactosidérophiline du lait de femme*, *Biochim. Biophys. 45*, 413−421.

Moore, A. S., Anderson, B. F., Groom, C. R., Haridas, M. & Baker, E. N. (1997) Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution, *J. Mol. Biol. 274*, 222−2236.

Pakdaman, R. & El Hage Chahine, J. M. (1996) A mechanism for iron uptake by transferrin, *Eur. J. Biochem. 236*, 922−931.

Pakdaman, R. & El Hage Chahine, J. M. (1997) Transferrin − the interaction of lactoferrin with hydrogen carbonate, *Eur. J. Biochem. 249*, 149−155.

Pakdaman, R., Petitjean, M. & El Hage Chahine, J. M. (1998) Transferrins − a mechanism for iron uptake by lactoferrin, *Eur. J. Biochem.*, in the press.

Rawas, A., Muirhead, H. & Williams, J. (1996) Structure of diferric duck ovo-transferrin at 2.35 Å resolution, *Acta Crystallogr. D52*, 631−640.

Russel, A. J., Thomas, P. G. & Fersht, A. R. (1987) Electrostatic effects on modification of charged groups in the active site cleft of subtilisin by protein engineering, *J. Mol. Biol. 193*, 803−813.

Wilkins, R. G. (1976) *The study of kinetics and mechanism of reactions of transition metal complexes*, Allyn and Bacon, Boston.

Williams, J., Chasteen, N. B. & Moreton, K. (1982) The effect of salt concentration on the iron-binding properties of human transferrin, *Biochem. J. 201*, 527−532.

Zuccola, H. J. (1992) *The crystal structure of monoferric human serum transferrin*, Ph.D. Thesis (Georgia Institute of Technology) UMI, Ann Arbor.

Zundel, G. (1976) Easily polarizable hydrogen bonds-their interaction with the environment-IR continuum and anomalous large proton conductivity, in *The hydrogen bond* (Schuster, P., Zundel, G. & Sandorfy, C., eds) pp. 683−766, Elsevier, New York.