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APOTRANSFERRIN PROTON DISSOCIATION AND INTERACTIONS WITH HYDROGENCARBONATE IN NEUTRAL MEDIA

LOUISA BELLOUNIS, ROWCHANAK PAKDAMAN AND JEAN MICHEL EL HAGE CHAHINE*
Institut de Topologie et de Dynamique des Systèmes, Université Paris 7, CNRS URA 34, 1 Rue Guy de La Brosse, 75005 Paris, France

A spectrofluorimetric titration analysis of the proton dissociation and the interactions of human serum transferrin with hydrogencarbonate was performed at pH 7·0–9·0. Apotransferrin loses a single proton probably per binding site with $K_a = (6{\cdot}80 \pm 0{\cdot}35) \times 10^{-9}$ M. This proton dissociation is independent of the hydrogencarbonate concentration. Apotransferrin does not interact with CO_3^{2-}. However, it interacts with two HCO_3^-, exhibiting two different affinity constants; the dissociation constant presumably for the C-site is $K_C = (4{\cdot}40 \pm 0{\cdot}15) \times 10^{-3}$ M and that for presumably the N-site is $K_N = (3{\cdot}60 \pm 0{\cdot}30) \times 10^{-2}$ M. These interactions are independent of pH and occur with the unprotonated and protonated apotransferrin species with the same low affinities. Such affinities are probably induced by ionic interactions involving the side chain of the arginine residues in each of the two binding sites. As for the proton dissociation, it can occur with one of the other side-chains of the amino acid residues of these binding sites.

1. INTRODUCTION

The proteins of the transferrin family constitute the major iron transport system in vertebrates.[1] Human serum transferrin is a single-chain bilobate glycoprotein of about 80 kD. Each lobe possesses one iron binding in which the metal is coordinated to four amino acid ligands. In the C-site of monoferric transferrin, these ligands are the side-chains of one aspartate residue (Asp 392), one histidine residue (His 385) and two tyrosines residues (Tyr 585 and Tyr 517). Iron is also close to a carbonate or a hydrogencarbonate adjacent to an arginine (Arg 456, Figure 1).[2–4] Moreover, in each iron binding cleft, a lysine lies in the neighbourhood of the iron atom.[2] Although the N- and C-binding sites are constituted of similar amino acid residues,[2–4] they have different thermodynamic and kinetic behaviours towards iron uptake or loss.[5–7] The C-binding site has a higher affinity for iron than N, as it reacts more rapidly with the metal during its uptake.[5–7]

Serum transferrin carries iron from the bloodstream across the plasma membrane barrier to cytosol.[8] It extracts the metal cation from low molecular mass chelates in neutral physiological media,[9,10] then interacts with a specific receptor at the plasma membrane. Protein and receptor are subsequently internalized by endocytosis in endosomes. These are then acidified to pH 5·6 and iron is released from the protein.[8] Iron extraction from the low molecular mass chelates needs the assistance of a third indispensable partner, carbonate or bicarbonate.[2–7,9–14] Indeed, synergistic carbonate or hydrogencarbonate is responsible for the extraordinarily high affinity of transferrin for Fe^{3+}.[11] Several reports deal with the interaction of the protein with carbonate in the absence of iron.[10,12–14] Harris *et al.*[12,13] estimated by the use of spectrophotometric techniques that the affinity of the sites for hydrogencarbonate are about 500 M^{-1} for a site and from 500–60 M^{-1} for the other site. More recently, using calorimetric techniques and a recombinant *N*-terminal half molecule of transferrin, Lin *et al.*[14] estimated that the affinity of the C-site for carbonate is about 250 M^{-1} while that of the N-site is too small to be detected. Neither study considered the state of protonation of transferrin and they were performed at a constant pH (7·4). This state of protonation can be of great importance for iron uptake.[5,6,15] Indeed, each binding site of transferrin loses three H^+ equivalents upon complex formation with iron[5–7] and the metal binding sites are affected by ionization of functional groups with an apparent pK_a value near physiological pH (*ca* 7·4).[15] Furthermore, proton transfers trigger iron uptake and loss by transferrin when carbonate is replaced by another synergistic anion, nitrilotriacetate.[5,6] Moreover, with hen ovotransferrin, protonation of the lysine residues seems to induce a

* Author for correspondence.

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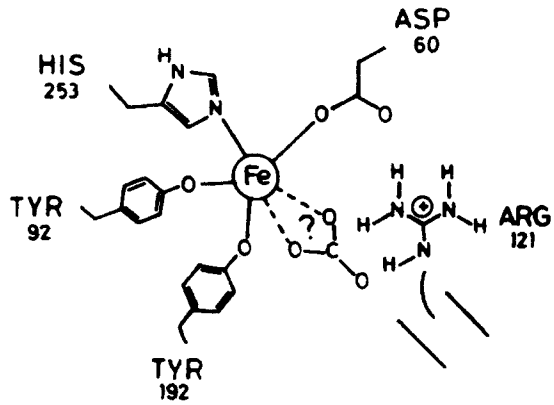

Figure 1. Schematic view of the C-site iron-binding site of monoferric transferrin

transition in the conformation of the binding site leading to iron loss.[16] In this work, we investigated by fluorimetric techniques[17] the state of protonation of apotransferrin and holotransferrin in neutral media and analysed its influence on the affinity of the protein for hydrogencarbonate in the absence of iron.

EXPERIMENTAL

Human serum apotransferrin (Sigma) was purified according to published procedures.[5,6] Holotransferrin was prepared by incubating a neutral solution of human apotransferrin (Sigma) (20 mg ml^{-1}) and Na_2CO_3 (20×10^{-3} M) with six equivalents of nitilotriacetatoiron [Fe(NTA)] for 1 h at 37 °C. This solution was then dialysed four times against neutral 0·2 M KCl solution and a fifth time against final buffer at 4 °C.[9,10] $NaHCO_3$, KCl (Merck, Suprapur), ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (H_4EDTA), NaOH and HCl (Merck, Titrisol), HEPES [*N*′-(2-hydroxyethyl)piperazine-*N*-ethanesulphonic acid] (Sigma or Aldrich) and nitrilotriacetic acid (H_3NTA) (Aldrich) were used without further purification. Water and glassware were prepared as described previously.[5,6]

Stock solutions. All stock solutions were freshly prepared. Transferrin concentrations (c_0) were checked spectrophotometrically.[5,6] Their concentrations ranged from 5×10^{-7} to 2×10^{-6} M for apotransferrin and holotransferrin and those of $NaHCO_3$ from 5×10^{-4} to 15×10^{-2} M. HEPES concentrations were 5×10^{-2} M and final ionic strengths were adjusted to 0·2 M with KCl. Since the proton-dissociation pK of carbonic acid is 6·35 ($CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H + HCO_3$), in order to avoid any decrease in hydrogencarbonate concentration by CO_2 loss, the buffered solutions were used fresh and were prepared in basic media (pH ≈ 9). The final pH values were then adjusted between 6·5 and 8·9 by adding micro quantities of concentrated HCl and NaOH. However, the pH of the solutions containing $NaHCO_3$ was never allowed to go below 7·2 and all experiments were performed in sealed cells.

pH measurements. pH values were measured, at 25 ± 0·5 °C, in the spectrophotometer cell with a Jenco pH-meter equipped with an Ingold combined microelectrode. Buffers used for pH standardization were pH 7·00 and 10·01 (Beckman).

Spectrophotometric measurements. Spectrophotometric measurements were performed at 25 ± 0·5 °C on a Cary C210 spectrophotometer equipped with a magnetic stirring device and a thermostated cell carrier.

Fluorimetric measurements. Fluorimetric measurements were performed at 25 ± 0·5 °C on a Perkin-Elmer LS 50 luminescence spectrometer equipped with a thermostated cell carrier and a magnetic stirring device. Emission spectra were measured in the range 300–420 nm. Excitation wavelengths were set at either 260 or 280 nm.

RESULTS

In neutral media when excited at 280 nm, the tryptophan excitation maximum, or at 260 nm in the tryptophan excitation area, apotransferrin fluoresces with a typical emission spectrum ($\lambda_{em\ max} = 338$ nm).[17] The fluorescence intensity of apotransferrin decreases with increasing pH from a plateau in the range 6·8–7·2 to another plateau in the range 9–9·3. Above pH 9·5, part of the protein seems to be irreversibly denatured, as 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, all our experiments were performed at pH < 9·0 and the reported results relate to reversible phenomena. The fluorescence intensity also decreases with increasing hydrogencarbonate concentration at constant pH (see Figure 3). This intensity still varies with hydrogencarbonate concentration at $[HCO_3^-] > 2 \times 10^{-2}$ M (approximately that of natural media), which indicates that the final hydrogencarbonate– apotransferrin equilibrium is not reached at these concentrations.

Since the state of charge of the protein is unknown, ion charges will not be shown.

Proton dissociation of apotransferrin in the vicinity of neutrality

Figure 2 implies that in addition to the interaction between apotransferrin and hydrogencarbonate (Figure 3), there is a proton transfer reaction occurring in the pH range of our experiments (7–9). In the absence of HCO_3^-, we shall assume that this proton dissociation

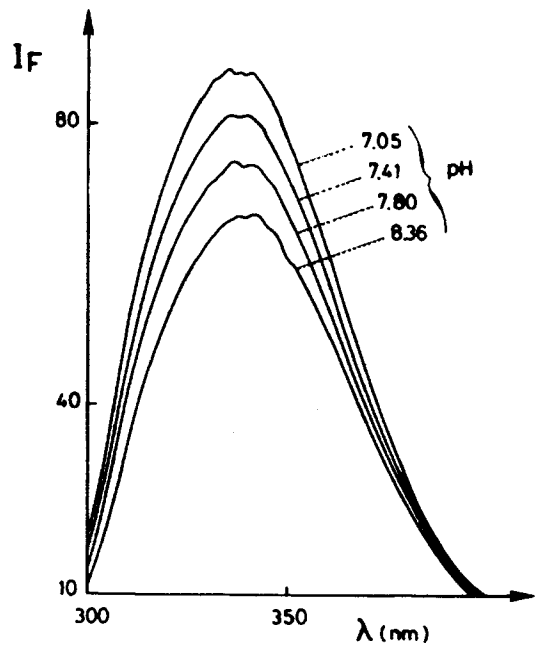

Figure 2. Fluorescence emission spectra of transferrin in pH range 7–9 with $\lambda_{ex} = 280$ nm, at $25 \pm 0{\cdot}5$ °C, $I = 0{\cdot}2$ and $c_0 = 1 \times 10^{-6}$ M

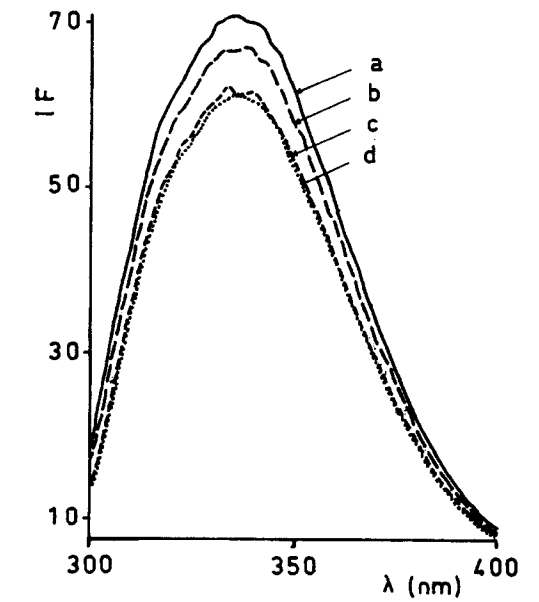

Figure 3. Fluorescence emission spectra of transferrin at pH 7·35 (a) and in the presence of three different hydrogencarbonate concentrations, (b) 1×10^{-2}, (c) 10×10^{-2} and (d) 15×10^{-2} M, with $\lambda_{ex} = 280$ nm, at $25 \pm 0{\cdot}5$ °C, $I = 0{\cdot}2$, $c_0 = 1 \times 10^{-6}$ M

involves one or several side-chains of the amino acid residues.

$$T + nH \rightleftharpoons TH_n \quad (1)$$

with $K_a = [T][H]^n/[TH_n]$, n being the number of non-identical amino acid side-chains involved in reaction (1).

At a low protein concentration, the fluorescence intensity can be expressed as[18]:

$$F = f_1[T] + f_2[TH_n] \quad (2)$$

where F is the fluorescence intensity and f_1 and f_2 are the experimental factors which relate the fluorescence at 338 nm to [T] and [TH_n], respectively.

The analytical concentration of apotransferrin is expressed as

$$c_0 = [TH_n] + [T] \quad (3)$$

At pH < 7, where $[TH_n] \gg [T]$, the initial fluorescence intensity (F_0) is expressed as

$$F_0 = f_2[TH_n] = f_2 c_0 \quad (4)$$

From K_a and equations (2)–(5), we can easily derive a Benesi and Hildebrandt type relationship which can be expressed as[5,6]

$$\Delta f/\Delta F = 1/c_0 + [H]^n/K_a c_0 \quad (5)$$

with $\Delta F = F_0 - F$ and $\Delta f = f_2 - f_1$.

A linear least-squares regression of $[H]^n$ (pH 7·0–9·0) against $\Delta f/\Delta F$ is only achieved for $n = 1$ (Figure 4). From the slope of the regression line, a single proton dissociation constant for apotransferrin is determined: $K_a = (6{\cdot}70 \pm 0{\cdot}1) \times 10^{-9}$ M.

The fact that the intensities of the fluorescence spectra of T (pH ≈ 9) and TH (pH ≈ 7) decrease with increasing [HCO_3^-] led us to assume that the T and TH species can both interact with hydrogencarbonate:

$$TH + 2HCO_3^- \rightleftharpoons T''H \quad (6)$$

$$T''H \rightleftharpoons T'' + H \quad (7)$$

$$T + 2HCO_3^- \rightleftharpoons T'' \quad (8)$$

In this mechanism, we assume that both the N- and the C-sites of apotransferrin interact with a hydrogencarbonate molecule.

The analytical concentration of apotransferrin in the presence of hydrogencarbonate can be expressed as

$$c_0 = [T] + [TH] + [T''H] + [T''] \quad (9)$$

Apotransferrin binds carbonate with two affinity constants in the range 500–50 M^{-1}.[12,13] We can therefore consider that the apparent affinity constant of the protein for hydrogencarbonate $\{K \approx ([T''] + [T''H])/[HCO_3^-]^2([T] + [TH])\}$ would be in the range 10^4–10^3 M^{-2}. For a hydrogencarbonate concentration $c_1 \approx 0{\cdot}15$ M and a protein concentration $1 \times 10^{-6} \leq c_0 \leq 1 \times 10^{-5}$ M the protein will be mostly

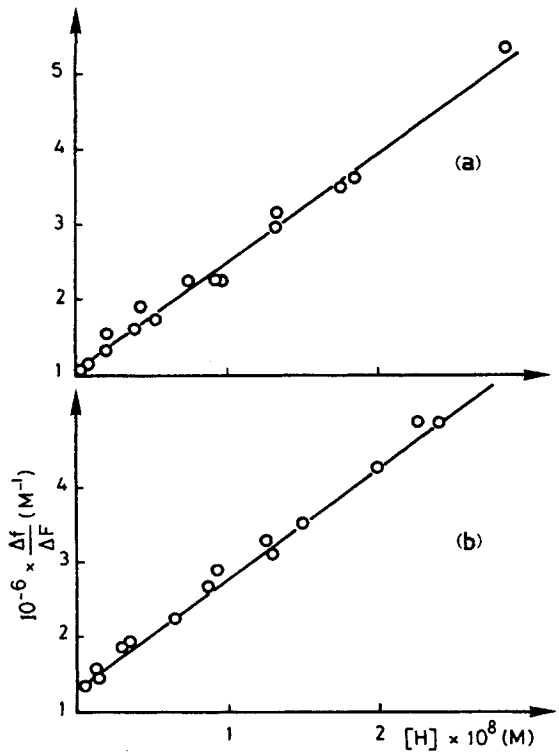

Figure 4. Plot of $\Delta f/\Delta F$ vs $[H^+]$ at 25 ±0·5 °C, $I = 0{\cdot}2$ M. (a) $c_0 = 1 \times 10^{-6}$ M: intercept, $(1{\cdot}25 \pm 0{\cdot}15) \times 10^6$ M^{-1}; slope, $(1{\cdot}50 \pm 0{\cdot}05) \times 10^{14}$ M^{-2}; $r = 0{\cdot}9948$; (b) $c_1 = 0{\cdot}1$ M and $c_0 = 1 \times 10^{-6}$ M: intercept, $(1{\cdot}05 \pm 0{\cdot}10) \times 10^6$ M^{-1}; slope, $(1{\cdot}45 \pm 0{\cdot}05) \times 10^{14}$ M^{-1}; $r = 0{\cdot}9938$

under the T″ and/or the T″H forms because in this case $([T''] + [T''H]) \approx c_0 c_1^2 K/(1 + c_1^2 K) \approx c_0$.[12-14] This allows us to simplify equation (9) to

$$c_0 \approx [T''H] + [T''] \quad (10)$$

Therefore, the fluorescence intensity would obey the equation

$$F = f_3[T''H] + f_4[T''] \quad (14)$$

in which f_3 is related to [T″H] and f_4 is related to [T″].

From $K_{2a} = [H][T'']/[T''H]$ and equations (10) and (11), we derive the equation

$$\Delta f'/\Delta F = 1/c_0 + [H]/K_{2a}\, c_0 \quad (12)$$

with $\Delta f' = f_3 - f_4$.

Experiments were performed at constant hydrogencarbonate concentration ($c_1 = 0{\cdot}1$ M and $c_0 = 1 \times 10^{-6}$ M) and variable pH. A linear least-squares regression plot of $\Delta f/\Delta F$ vs [H] [Equation (12)] was very satisfactory [Figure 4(b)]. From the slope of the regression line, $K_{2a} = (6{\cdot}90 \pm 0{\cdot}15) \times 10^{-9}$ M is determined. This K_{2a} value equals, within the limits of uncertainty, the K_a determined in the absence of hydrogencarbonate.

The interactions of hydrogencarbonate with apotransferrin in the vicinity of neutrality

The fact that $K_a \approx K_{2a} \approx 6{\cdot}80 \times 10^{-9}$ M leads us to assume that in the presence of a high hydrogencarbonate concentration at pH ≥ 7·0, the apotransferrin is mostly in the TH and T″H forms; near pH 9·0 it would be in the T and T″ forms. According to Lin *et al.*,[14] the affinity of the C-site for hydrogencarbonate is presumably much greater than that of the N-site. To a first approximation, we assume that binding of hydrogencarbonate to apotransferrin is sequential: it would first occur with the site presenting the highest affinity for the anion and would be followed by the binding with the other site. We can, therefore, write for the protonated species (pH ≈ 7)

$$TH + HCO_3^- \rightleftharpoons T'H \quad K_{1C} = [TH][HCO_3^-]/[T'H] \quad (13)$$

$$TH + HCO_3^- \rightleftharpoons T''H \quad K_{1N} = [T'H][HCO_3^-]/[T''H] \quad (14)$$

T′H is a transferrin the C-site of which interacts with hydrogencarbonate whereas in T″H both sites interact with hydrogencarbonate.

The conservation of mass implies

$$c_0 = [TH] + [T'H] + [T''H] \quad (15)$$

From equation (15), K_{1C} and K_{1N}, we can easily derive the equations

$$1/[T'H] = 1/c_0 + K_{1C}/c_0[HCO_3^-] + [HCO_3^-]/c_0 K_{1N} \quad (16)$$

and

$$1/[T''H] = 1/c_0 + K_{1N}/c_0[HCO_3^-] + K_{1N}K_{1C}/c_0[HCO_3^-]^2 \quad (17)$$

Since presumably $K_{1C} \ll K_{1N}$,[14] at low bicarbonate concentration ($c_1 \approx K_{1C}$)[14] equation (16) simplifies to

$$1/[T'H] = 1/c_0 + K_{1C}/c_0[HCO_3^-] \quad (18)$$

The fluorescence intensity can be expressed as

$$F = f_2[TH] + f_5[T'H] + f_4[T''H] \quad (19)$$

where f_5 is an experimental factor related to [T′H].

From equations (15), (19) and (18), we can derive

$$1/\Delta F = 1/\Delta F_{0a} + K_{1C}/\Delta F_{0a}[HCO_3^-] \quad (20)$$

with $\Delta F_{0a} = c_0(f_2 - f_5)$, where f_5 is an experimental factor related to [T′H]; f_2 is directly determined from the initial fluorescence intensity in the absence of hydrogencarbonate whereas ΔF_{0a} is the intercept of the line relating to equation (20).

At high $[HCO_3^-]$, the N-site starts to interact with hydrogencarbonate. From equations (15), (17) and (19) at $[HCO_3^-] > K_{1C}$, equation (21) is derived:

$$\Delta F/c_0 = \Delta f_1 + [HCO_3^-]\Delta f_2/K_{1N} - [HCO_3^-]\Delta F/K_{1N}c_0 \quad (21)$$

with $\Delta f_1 = f_2 - f_5$ and $\Delta f_2 = f_2 - f_4$.

In more basic media (pH ≈ 9), the protein is mostly in the T form, both sites of which can interact with hydrogencarbonate. Moreover, since the affinity of the C-site for the latter is much higher than that of the N-site,[14] we can write

$$T + HCO_3^- \rightleftharpoons T' \quad K_{2C} = [T][HCO_3^-]/[T'] \quad (22)$$

$$T' + HCO_3^- \rightleftharpoons T'' \quad K_{2N} = [T][HCO_3^-]/[T''] \quad (23)$$

The same mathematical treatment as that applied to equations (18)–(21) yields the equations

$$1/\Delta F = 1/\Delta F_{0b} + K_{2C}/\Delta F_{0b}[HCO_3^-] \quad (24)$$

and

$$\Delta F/c_0 = \Delta f_3 + [HCO_3^-]\Delta f_4/K_{2N} - [HCO_3^-]\Delta F/K_{2N}c_0 \quad (25)$$

in which $\Delta f_3 = (f_1 - f_6)$, $\Delta f_4 = (f_1 - f_4)$ and $\Delta F_{0b} = \Delta f_4 c_0$, which is the intercept of the line relating to equation (24); f_6 is an experimental value relating to [T'].

Linear least-squares regression plots of $1/\Delta F$ vs $1/[HCO_3^-]$ [equations (18) and (24)], related to the interaction of hydrogencarbonate with the C-sites of species T and TH, are satisfactory (Figures 5 and 6). From the slope and intercept of the regression lines, $K_{1C} \approx K_{2C} \approx (4{\cdot}40 \pm 0{\cdot}15) \times 10^{-3}$ M are determined. As for site N, a multilinear regression of the data versus

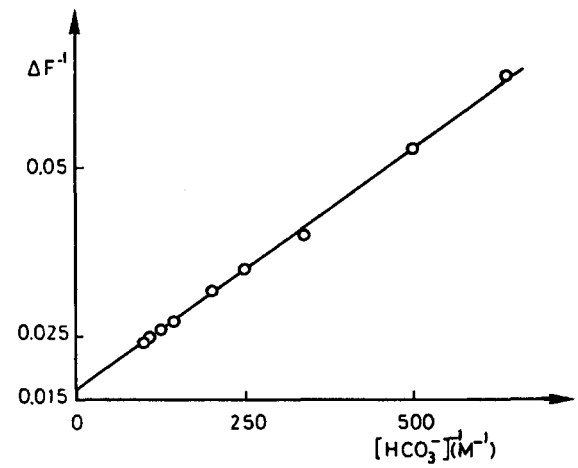

Figure 6. Plot $1/\Delta F$ vs $1/[HCO_3^-]$ at 25 ± 0·5 °C, I = 0·2 M, $c_0 = 1 \times 10^{-6}$ M, $c_1 < 1 \times 10^{-2}$ M and pH = 8·79. Intercept, $(1{\cdot}65 \pm 0{\cdot}15) \times 10^{-2}$ M; slope, $(7{\cdot}20 \pm 0{\cdot}05) \times 10^{-5}$ M; r = 0·9985

equations (21) and (25) shows that the factors $\Delta F/K_N$ do not affect linearity and can be neglected. Therefore, two satisfactory linear least-squares regression plots of $\Delta F/c_0$ dealing with the interaction of apotransferrin with hydrogencarbonate ($c_1 > 1 \times 10^{-2}$ M) vs $[HCO_3^-]$ (Figures 7 and 8) are obtained and give $K_{1N} \approx K_{2N} \approx (3{\cdot}6 \pm 0{\cdot}3) \times 10^{-2}$ M.

T and TH are in rapid equilibrium. One of these species may, therefore, have a much higher affinity for bicarbonate than the other, which would always lead via

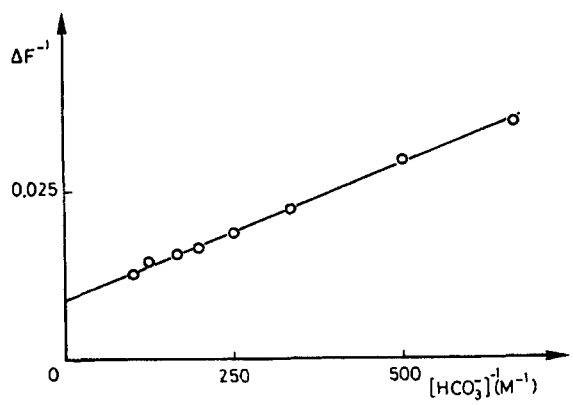

Figure 5. Plot $1/\Delta F$ vs $1/[HCO_3^-]$ at 25 ± 0·5 °C, I = 0·2 M, $c_0 = 1 \times 10^{-6}$ M, $c_1 < 1 \times 10^{-2}$ M and pH = 7·05. Intercept, $(9{\cdot}00 \pm 0{\cdot}45) \times 10^{-3}$; slope, $(4{\cdot}00 \pm 0{\cdot}05) \times 10^{-5}$ M; r = 0·9980

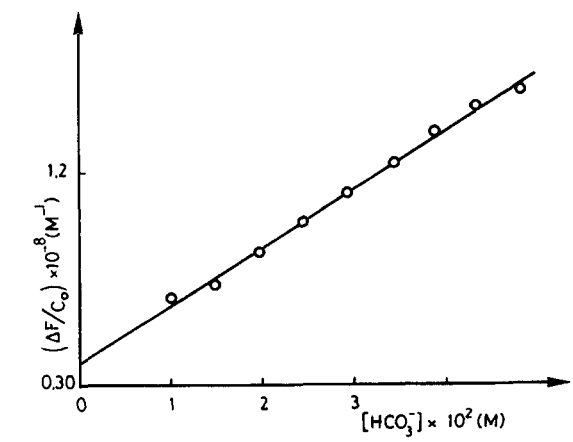

Figure 7. Plot of $\Delta F/c_0$ vs $[HCO_3^-]$ at 25 ± 0·5 °C, I = 0·2 M, $c_0 = 1 \times 10^{-6}$ M, $c_1 > 1 \times 10^{-2}$ M and pH = 7·14. Intercept, $(3{\cdot}55 \pm 0{\cdot}15) \times 10^7$ M^{-1}; slope, $(2{\cdot}55 \pm 0{\cdot}10) \times 10^9$ M^{-2}; r = 0·9982

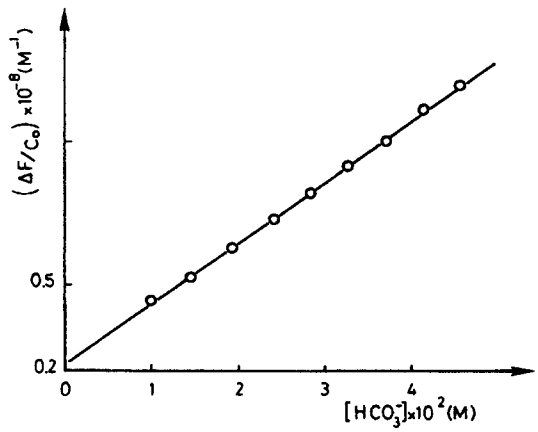

Figure 8. Plot of $\Delta F/c_0$ vs $[HCO_3^-]$ at 25 ± 0·5 °C, $I = 0{\cdot}2$ M, $c_0 = 1 \times 10^{-6}$ M, $c_1 > 1 \times 10^{-2}$ M and pH = 8·79. Intercept, $(2{\cdot}15 \pm 0{\cdot}15) \times 10^7$ M^{-1}; slope, $(2{\cdot}15 \pm 0{\cdot}05) \times 10^9$ M^{-2}; $r = 0{\cdot}9988$

fast proton transfer to the same final products:

$$T' + H \rightleftharpoons T'H \quad (26)$$

If the affinity of TH for bicarbonate is higher than that of T, the relationship between the fluorescence variation and the concentrations of the species present in the medium would be expressed as[5,6]

$$1/\Delta F = 1/\Delta F_{0a} + K_{1C}[H]/\Delta F_{0a}(K'_a + [H])[HCO_3^-] \quad (27)$$

with $K'_a = [H][T']/[T'H]$.

On the other hand, if the affinity of T towards HCO_3^- [reaction (22)] is higher than that of TH, the relationship between the fluorescence variation and the concentrations of the other species is expressed as[5,6]

$$1/\Delta F_{0b} = 1/\Delta F_{0b} + (K_{2C}K_a + [H]K_{2C})/\Delta F_{0b}[HCO_3^-] \quad (28)$$

Both equations (27) and (28) are pH dependent and are, therefore, not in agreement with the experimental data.

Proton dissociation of holotransferrin

Iron uptake by transferrin in the presence of hydrogencarbonate is accompanied by important quenching of fluorescence attributed to holotransferrin formation.[17] The fluorescence spectrum of holotransferrin does not vary with pH or with HCO_3^- in the pH range 6–9.

DISCUSSION

El Hage Chahine and Fain[5,6] showed that when carbonate is replaced by NTA, iron uptake by apotransferrin is followed by a single proton loss per binding site ($pK_a = 6{\cdot}35$ for the C-site) probably occurring from the phenol of one of the two tyrosine residues. They assumed that the complex formation between the protein and NTA leads to a shift in the pK_a from basic to acidic, probably at the tyrosine phenol, as observed during complex formation with some transition metals and during some enzymic and proteic reactions.[5,6,19–21] Moreover, they estimated that the three H^+ lost during iron uptake in the presence of hydrogencarbonate are probably provided by the two phenols of the tyrosine residues and the hydrogencarbonate. The single proton dissociation observed with apotransferrin ($pK_a = 8{\cdot}17$) is not detected with holotransferrin. This can indicate that the proton loss has already occurred during iron uptake by the protein. We therefore assume that this proton is provided by the binding sites. As with NTA, this can lead to a protein ligand pK_a shift from basic to acidic.[5,6] Apotransferrin exists in the vicinity of neutrality in a deprotonated and a protonated form in equilibrium [reaction (1), Table 1], both interacting with hydrogencarbonate [reactions (6) and (8)]. Acid–base reaction (1) ($pK_a = 8{\cdot}17$) involves a single proton transfer and is independent of the interaction with hydrogencarbonate. This pK_a value is higher than that reported by Chasteen and Williams[15] for the ionization of the protein sites upon iron uptake ($pK = 7{\cdot}4$). The N- and C-binding sites of apotransferrin contain the same amino acids.[2–4] Subsequently, the proton dissociation with $n = 1$ [reaction (1)] can occur in each binding site with an identical amino acid side-chain.[22] However, although similar, the C- and N-binding sites of apotransferrin seem to have dissimilar conformations, the 'closed' structure of the C-site and the 'open' one of the N-site of apolactotransferrin.[3] In this case, the environment of the amino acid residues would not be identical for the two binding sites and since the pK_a value reported for reaction (1) involves a single proton transfer, it would then apply to only one of the two iron-binding areas of the protein.

In neutral media and in each of the two C- and N-binding areas of apotransferrin, several amino acid side-chain functional groups are present: two phenols of two tyrosines, one imidazole of a histidine, one guanidinium moiety of an arginine and a protonated

Table 1. Mechanism of apotransferrin proton dissociation and interaction with hydrogencarbonate

Reaction	Dissociation constant (M)
$T + HCO_3^- \rightleftharpoons TH$	$6{\cdot}80 \times 10^{-9}$
$T + HCO_3^- \rightleftharpoons T'$	$4{\cdot}40 \times 10^{-3}$
$TH + HCO_3^- \rightleftharpoons T'H$	$4{\cdot}40 \times 10^{-3}$
$T' + HCO_3^- \rightleftharpoons T''$	$3{\cdot}60 \times 10^{-2}$
$T'H + HCO_3^- \rightleftharpoons T''H$	$3{\cdot}60 \times 10^{-2}$
$T'' + H \rightleftharpoons T''H$	$6{\cdot}80 \times 10^{-9}$

amino group of a lysine.[2-4] In the holoprotein the carbonate or hydrogencarbonate probably interacts with an arginine.[2,4] Furthermore, iron is close to a lysine which, however, does not seem to be involved in binding with the metal.[2] On the other hand, we have recently estimated that in the apoprotein, the side-chain of aspartate is probably in the carboxylate form and that of histidine in the imidazole form.[5] Furthermore, a recent approach to the uptake or release of iron by hen ovotransferrin involved the proton dissociation of the side-chain of a lysine with a pK_a of about 5·6. This was assumed to induce a change in the conformation of the binding site, allowing the interaction with the metal.[16] Therefore, the assignment of the pK_a to the proton dissociation of one of the side-chains in one or both binding sites is virtually impossible at this stage.

The role of hydrogencarbonate is imperative for iron uptake by the protein in physiological media.[5-14] With other synergistic anions, such as nitrilotriacetate, the affinity of the protein for the metal drops dramatically from 10^{21} to 10^{10} M^{-1}.[5] Furthermore, in the absence of synergistic anions, this affinity is completely collapsed.[10] According to Bates and co-workers,[9,10] iron uptake occurs by the extraction of the metal from a highly stable low molecular mass chelate by the following mechanism:

$$\mathrm{HCO_3^-/CO_3^{2-}}$$

$$\mathrm{T + FeL \rightleftharpoons TFeL \rightleftharpoons TFe + L} \qquad (29)$$

In order to shift the equilibrium towards the iron-loaded protein, the affinity of the protein–synergistic anion adduct should necessarily be higher than the affinity of the chelating agent for iron. This can only be achieved with hydrogencarbonate.[9-11]

The proton dissociation pK of hydrogencarbonate into carbonate is 10·25 [reaction (30)]:

$$\mathrm{CO_3^{2-} + H \rightleftharpoons HCO_3^-} \qquad (30)$$

It is not known whether hydrogencarbonate or carbonate molecules are involved in the binding with iron [reaction (30)].[1,4-7] However, if CO_3^{2-} is involved in reactions (13) and (22) instead of HCO_3^-, the fluorescence variation in equations (20), (21), (24) and (25) would depend on $[CO_3^{2-}]$. $[CO_3^{2-}]$ can easily be determined from $K_{3a} = 5{\cdot}6 \times 10^{-11}$ M $= [CO_3^{2-}][H]/[HCO_3^-]$ as $[CO_3^{2-}] = [HCO_3]K_{3a}/[H]$. In this case, for example, equation (20) would become

$$1/\Delta F = 1/\Delta F_{0a} + K_{1C}[HCO_3^-]K_{3a}/\Delta F_{0a}[H] \qquad (31)$$

Subsequently, under our experimental conditions (pH 7·0–9·0), an interaction between apotransferrin and carbonate should be pH dependent. The fact that $K_{1C} \approx K_{2C}$ and $K_{1N} \approx K_{2N}$ (Table 1) and that these values were measured in the vicinities of pH 7·0 and 9·0 preclude any detectable interaction between the apotransferrin and carbonate in the pH range of this work. This, of course, does not exclude the possible involvement of CO_3^{2-} in the binding cleft of the holoprotein, especially that iron uptake by each site of apotransferrin is accompanied by a loss of three proton equivalents, one of which may be provided by a hydrogencarbonate proton dissociation.[1,5] Nevertheless, only hydrogencarbonate interacts with the protein. This interaction seems weak for a site, presumably C-, and very weak for another site, presumably N- ($K_{1C} \approx K_{2C} \approx 4{\cdot}40 \times 10^{-3}$ M, $K_{1N} \approx K_{2N} \approx 3{\cdot}60 \times 10^{-2}$ M; Table 1). This possibly describes an ionic interaction.[23] This interaction most likely occurs between the arginines of the binding sites and the hydrogencarbonates. This can be confirmed by the proximity of the arginines and HCO_3^- or CO_3^{2-} in the holoprotein binding clefts, as shown by the x-ray diagrams of several holoproteins of the transferrin family.[2-4]

The assumption of sequential binding of hydrogencarbonate to apotransferrin and the attribution of K_{1C} to the C-site and K_{1N} to the N-site are based on the observations of Lin *et al.*,[14] who estimated that carbonate binding to the N-site is too weak to be detected. On the other hand, Harris *et al.*[13] measured close hydrogencarbonate binding constants for both the N- and C-terminal monoferric transferrin. They assumed that the difference in the binding affinities for hydrogencarbonate can come from a statistical effect.[13] Our results show that the interaction of hydrogencarbonate with the protein occurs with two affinity constants. We cannot at this stage of our research ascribe each of these constants to a particular binding site because it has not yet been established whether the thermodynamic inequivalence of the sites towards binding hydrogencarbonate is the result of an interaction occurring with two non-identical binding sites or a strong negative cooperative effect occurring with identical sites. K_{1C} is close to that reported by Lin *et al.*,[14] whereas both K_{1C} and K_{1N} differ from those of Harris *et al.*[12,13] This is probably due to the experimental conditions under which our constants were measured ($\mu = 0{\cdot}2$ M, [HEPES] = 0·05 M). These conditions are different from those of Harris *et al.* (0·1 M HEPES buffer).[12,13] Indeed, they reported a slight salt effect with HEPES, which may explain these differences.

The results in Table 1 cannot be transposed to the natural medium where apotransferrin interacts with the low molecular mass chelates of the bloodstream. However, the fact that the affinities of the protein for hydrogencarbonate are very low indicates that in the bloodstream, the hydrogencarbonate concentration (*ca* 20 mM) is not sufficient to saturate the two binding sites of apotransferrin. Can this, at least partly, explain the higher affinity of the C-site for iron in natural media and does it indicate an anti-cooperativity of the sites upon interaction with carbonate and perhaps with iron? These questions are difficult to answer to and remain to be investigated. Moreover, little is known about the

structure of the transferrin–hydrogencarbonate adduct. However, the changes in the fluorescence spectrum of apotransferrin on interaction with HCO_3^- may indicate a change in the molecular environment of protein side-chains of the binding sites.[24] This possible hydrogencarbonate-induced change of conformation of the binding site combined with the proton dissociation of apotransferrin can represent a necessary requirement for iron uptake by the protein.

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