The influence of pH on the equilibrium distribution of iron between the metal-binding sites of human transferrin

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The dependence of the metal-binding properties of transferrin on pH in the pH 6–9 range was investigated by urea/polyacrylamide-gel electrophoresis. Equations are presented for calculating the relative values of the four conditional site constants for the stepwise binding of iron to the two sites of transferrin and for calculating the equilibrium distribution of the protein among the four principal forms, apotransferrin, the C-terminal and N-terminal monoferric transferrins and diferric transferrin. The relative affinity of iron for the two sites and the co-operativity of iron-binding follow characteristic 'pH titration' curves. A mathematical model that can account for the former behaviour is presented. In both cases the metal-binding sites are affected by the ionization of functional groups with apparent pK_a values near physiological pH approx. 7.4. There is strong positive co-operativity in the release of protons from these groups. The results indicate that pH must be accurately controlled in studies of the differential properties of the two sites of the transferrin molecule.

It is well known that the preferred site for the initial binding of iron by transferrin is partly determined by pH (Princiottto & Zapolski, 1975; Lestas, 1976; Chasteen *et al.*, 1977; Harris, 1977; Aisen *et al.*, 1978; Evans & Williams, 1978). Thus at pH 6.0 iron introduced as $(NH_4)_2SO_4 \cdot FeSO_4$ binds preferentially to the C-terminal site rather than to the N-terminal site, whereas at pH 8.5 it binds more rapidly to the N-terminal site. However, there is only limited information on the effect of pH on the distribution of iron between the two binding sites under equilibrium conditions (Aisen *et al.*, 1978), and the influence of pH in the physiological region has not been studied. The latter question may well be important in attempting to analyse the distribution of transferrin-bound iron that is observed in serum, where there is preferential occupation of the N-terminal site (Leibman & Aisen, 1979; Williams & Moreton, 1980). The present paper describes the influence of pH on the ratio of the iron-binding affinities of the two sites and on the co-operativity of iron binding.

Materials and methods

Preparation of transferrin

Human serum apotransferrin of stated 98% purity was purchased from Behringwerke A. G., Marburg-

am-Lahn, West Germany. Alternatively transferrin was isolated from outdated plasma by the method described for rabbit transferrin (Morgan, 1964; Baker *et al.*, 1968), except that a second chromatography step on DEAE-Sephadex A-50, with elution with a 0–0.4 M-NaCl gradient in 5 mM-Tris/HCl buffer, pH 8.5, was used in place of the preparative electrophoresis step. In some cases an additional step was used to remove traces of albumin. The impure protein (1.7 g) was dissolved in 25 ml of 0.1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.0, and passed down a column (2 cm $\times$ 3 cm) of Blue Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The adsorbed albumin was eluted with 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.0.

Transferrin was also prepared by affinity chromatography by using a modification of the method of van Eijk & van Noort (1976). A column (2.5 cm $\times$ 7.0 cm) of sheep anti-(human transferrin) immunoglobulin (100 mg) bound to 45 ml of CNBr-activated Sepharose 4B (March *et al.*, 1974) was equilibrated with 0.125 M-NaCl/0.1 M-Tris/HCl buffer, pH 7.4. Serum (20 ml) was passed through the column, and the adsorbed transferrin was eluted with 1 M- NH_3 . The pH of the eluted protein was immediately lowered to 9.6 by adding solid Tris hydrochloride, and the solution (50 ml) was then dialysed against two 40-fold volume changes of 0.125 M-NaCl/10 mM-Hepes [4-(2-hydroxyethyl)-1-

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piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.4, and concentrated to 7.5 ml on an Amicon model 52 ultrafiltration apparatus with a PM10 membrane. Approx. 35 mg of transferrin was obtained from 20 ml of serum by this method.

Apotransferrin was prepared as described previously (Warner & Weber, 1951; Evans & Williams, 1978). The molar concentration of transferrin was calculated from the absorbance at 278 nm, $A_{278}^{1\%} = 11.4$ (Aisen *et al.*, 1967), by assuming a molecular weight of 78 000.

Preparation of samples

For the determination of iron-binding constants, transferrin solutions (2 mg/ml) with iron saturations ranging from 15% to 75% were prepared by mixing stock solutions (20 mg/ml) of apotransferrin and diferric transferrin, together with appropriate volumes of 1 M-NaHCO₃, 1 M-Hepes/NaOH buffer, pH 7.4, 1 M-trisodium citrate and water to give a final volume of 1 ml with concentrations 1 mM-NaHCO₃, 1 mM-citrate and 50 mM-Hepes/NaOH buffer, pH 7.4. The citrate served as a mediator for the transfer of iron between the metal-binding sites of transferrin. The samples, in screw-capped vials, were incubated at 37°C for 3–6 days until equilibrium was reached, as shown by the lack of further change in the urea/polyacrylamide-gel electrophoresis patterns. Samples prepared by adding iron(II) or iron(III)-nitrilotriacetate to apotransferrin, procedures that generate monoferric species initially, required significantly longer to achieve equilibrium (typically 1–2 weeks). The pH of each sample was measured to 0.01 pH unit before and after incubation, by using a Pye-Unicam model 79 pH-meter equipped with an Ag/AgCl glass microelectrode. The pH change during incubation was usually less than 0.03 unit. The pH values quoted are for 37°C, the pH-meter being standardized at that temperature with pH 7.00 (37°C) buffer. The pH of most samples was measured at 20°C and corrected to 37°C by the empirical relationship $\text{pH}(37^\circ\text{C}) = \text{pH}(20^\circ\text{C}) - C$. For this buffer system at pH approx. 7.5, $C = 0.21$.

Buffers for pH studies in the range pH 6.0–9.0 were prepared by adding 4 M-NaOH dropwise to 50 ml of 50 mM-Mes (4-morpholine-ethanesulphonic acid)/50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-propanesulphonic acid] solution, with or without 0.5 M-NaCl, and removing 1.0 ml volumes at approx. 0.1 pH-unit intervals. Approx. 1.5 ml of NaOH solution was required to titrate the entire pH range. To each 1.0 ml volume was added 25 μ l each of diferric transferrin solution (40 mg/ml) and apotransferrin solution (40 mg/ml) and 20 μ l of 50 mM-trisodium citrate/50 mM-NaHCO₃. Measurements of pH and incubations were performed as described above. The temperature corrections

throughout the pH range were $C(\text{pH}) = 0.14$ (5.7), 0.16 (6.0), 0.17 (6.5), 0.20 (7.0) and 0.21 (7.5–8.5).

Diferric transferrin was prepared by adding a calculated volume of 0.1 M-(NH₄)₂SO₄·FeSO₄ in 10 mM-HCl [which stabilizes the stock Fe(II) solution against air oxidation] to a solution of apotransferrin in water or in buffer at a pH greater than 7 and leaving the mixture to stand until no further increase in A_{470} was observed (1–2 days). The iron saturation was checked by urea/polyacrylamide-gel electrophoresis, and more Fe(II) was added if necessary. The presence of excess iron was avoided and in most cases a saturation of 95% was used.

Urea/polyacrylamide-gel electrophoresis

This was performed as described by Williams & Moreton (1980), except that better resolution was obtained by using 0.334% (w/v) bisacrylamide and a voltage of 115 V for 22 h. Gels with 12.5 μ g of protein applied per 1 cm slot were stained with Coomassie Brilliant Blue R250 (Berg, 1969), and after destaining they were scanned at 560 nm with a Pye-Unicam 8-100 spectrophotometer.

Areas of the four transferrin peaks (Fig. 1) were determined either by triangulation (height of peak $\times$ width at the half-height) or by cutting out and weighing. The two methods agreed to within 3%. When the peaks had very irregular shapes (Fig. 2), the second method was preferred. The precision of determining mole fractions is $\pm 4.5\%$, based on replicate runs of the same sample containing all four species present in nearly equal amounts.

To check that this method gives a true picture of the distribution of transferrin among its four species, four samples were prepared in which the distributions were different. These samples consisted of apotransferrin, diferric transferrin and two samples of 45%-iron-saturated transferrin prepared by adding (NH₄)₂SO₄·FeSO₄ or Fe(III)-nitrilotriacetate to apotransferrin at pH 7.5. The latter two samples contained all four transferrin species but in different proportions. Each sample was run in quadruplicate and the total area was determined. When the same total amount of protein was applied to the gel, the total areas given by the four samples agreed to within 4%. Thus the four species of transferrin give the same colour yield.

The EDTA in the gel buffer does not cause redistribution of iron during electrophoresis, since a mixture of equal amounts of apotransferrin and diferric transferrin did not give rise to monoferric transferrins. Apotransferrin itself did not acquire iron during electrophoresis. The percentage iron-saturation of samples calculated from peak areas [eqn. (A-9) in Appendix A] was usually within 2.5% (absolute) of the value expected from the amount of iron added.

Results

Urea/polyacrylamide-gel electrophoresis

Fig. 1 shows the scan of an electrophoretic separation of the four species of transferrin, which in order of increasing mobility are the apoprotein, the C-terminal monoferric complex, the N-terminal monoferric complex and the diferric protein (Evans & Williams, 1978). In the present work these four species are designated Tf', Tf-Fe', Fe-Tf' and Fe-Tf-Fe' respectively.

Fig. 1. Spectrophotometric scan of a urea / polyacrylamide-gel electrophoretogram showing bands due to the four species of transferrin

Experimental details are given in the text. The choice of base-line and apportionment of area are indicated. Resolution was often better than shown here.

Fig. 2. Spectrophotometric scans of the Fe-Tf-Fe' band of the urea/polyacrylamide-gel electrophoretogram of a sample of transferrin at two percentages of iron-saturation

Experimental details are given in the text. The presence of more than one component is indicated. Protein was purified by anion-exchange chromatography.

The primes are added to designate that in solution each species exists as a number of subspecies that differ from each other in their states of protonation and their affinities for iron. Throughout the text, primes refer to apparent quantities.

Fig. 2 shows the diferric band of a sample transferrin, purified by anion-exchange chromatography, at two percentages of iron-saturation. The band contains at least four components, two of which have different affinities for iron. Heterogeneity was very pronounced with the protein purified by affinity chromatography and was also often observed with commercially supplied transferrin. This was most marked in the diferric transferrin band, but was sometimes seen in the other bands also. On the other hand, the transferrin bands in Rivanol-treated serum (Williams & Moreton, 1980) appeared to be homogeneous. This suggests that some denaturation of transferrin occurs readily during purification procedures. Maeda *et al.* (1977) have shown that cattle transferrin in serum exhibits complex heterogeneity, which depends in part on differences in sialic acid content and in part on internal cleavage of the polypeptide chain. Anderson & Anderson (1979) have obtained similar results for human serum transferrin by using two-dimensional electrophoresis.

The heterogeneity observed in the present work is not due to differences in sialic acid contents, since neuraminidase treatment only decreased the mobility of diferric transferrin but did not remove the heterogeneity. Further evidence that the presence or absence of sialic acid does not affect the metal-binding sites comes from e.p.r. spectroscopy. Identical vanadium X-band e.p.r. signals are given by the VO^{2+} complexes of transferrin and asialotransferrin. Similarly, hen serum transferrin and hen ovotransferrin, which are thought to differ only in their carbohydrate contents (Williams, 1962; Lee *et al.*, 1978), also give identical vanadium e.p.r. spectra (N. D. Chasteen & S. Heaphy, unpublished work). The unexplained splittings in the Q-band e.p.r. spectrum reported for the VO^{2+} -transferrin complex (White & Chasteen, 1979) could be due to the sample heterogeneity observed in the present study.

Determination of site constants for iron-binding

The equilibrium distribution of the four species of transferrin as a function of iron-saturation is shown in Fig. 3. The ratios of the four site constants that describe the stepwise binding of iron to transferrin can be calculated from the mole fractions of the four species present at any percentage of saturation (Appendix A). These conditional constants are designated k'_{1N} and k'_{1C} for the binding of iron to species Tf' to form species Fe-Tf' and Tf-Fe' respectively, and k'_{2N} and k'_{2C} for the binding of iron

Fig. 3. Distribution of transferrin among the four species as a function of percentage iron saturation. Experimental details are given in the text. Continuous lines are calculated from the equilibrium equations [eqns. (A-11)–(A-14) in Appendix A] by using the relative values of the conditional site constants given in the Results section. Conditions: 1 mM- NaHCO_3 /1 mM-sodium citrate/10 mM-Hepes buffer, pH 7.40 (37°C). Protein was purified by anion-exchange chromatography.

Fig. 4. Dependence of the site preference factor, k'_{IN}/k'_{IC} , on pH. Experimental details are given in the text. Continuous lines are calculated from eqn. (B-12) in Appendix B by using the parameters given in Table 1. Conditions: 1 mM- NaHCO_3 /1 mM-sodium citrate/50 mM-Mes/50 mM-Hepes/50 mM-Hepes buffer, pH (37°C) at the values indicated, with and without 0.5 M-NaCl. Protein was obtained from Behringwerke A.G.

to species Tf-Fe' and Fe-Tf' respectively to form species Fe-Tf-Fe'. From eqn. (A-8) in Appendix A we obtain $k'_{1N}:k'_{1C}:k'_{2N}:k'_{2C} = 1.00:2.50 \pm 0.30:0.66 \pm 0.07:1.60 \pm 0.30$ for the averages and standard deviations of the eight samples with iron saturations over 20% under the conditions given in Fig. 3. These ratios are a direct measure of the relative affinity of iron for the two sites, k'_{1N}/k'_{1C} ($= k'_{2N}/k'_{2C}$), and of the co-operativity of the sequential binding of iron, k'_{2N}/k'_{1N} ($= k'_{2C}/k'_{1C}$).

The curves in Fig. 3 were calculated from the equilibrium equations [eqns. (A-11)–(A-14) in Appendix A] by using the average values for the binding constants given above. The satisfactory fit of the curves to the observed points is a further indication that the system has reached equilibrium (see the Materials and methods section).

pH-dependence of the affinity of iron for the two sites

Fig. 4 shows the pH-dependence of the site-preference factor, k'_{1N}/k'_{1C} (Appendix A), in the presence and absence of 0.5 M-NaCl. The data follow a characteristic pH-titration curve. Below the pH transition zone, binding to the C-terminal site is preferred in either solution. At pH values above the transition zone the sites have approximately equal affinity for iron in the absence of NaCl, whereas with NaCl the N-terminal site is favoured by nearly 2:1. In the pH range 6.0–6.5 the pH profiles are the same in the presence and in the absence of NaCl. At pH values below 6.1 there is no observable iron binding at the N-terminal site.

The data of Fig. 4 cast in the form of the modified Henderson–Hasselbalch equation [eqn. (1)]:

$$\log \left[\frac{\frac{k'_{1N}}{k'_{1C}}}{\frac{k'_{1N}}{k'_{1C}}} \right] = -n' \cdot \text{pH} + n' \cdot \text{p}K'_a \quad (1)$$

are presented in Fig. 5. In eqn. (1) k'_{1N}/k'_{1C} and k^h_{1N}/k^h_{1C} are the limiting values of k'_{1N}/k'_{1C} at pH values below and above the pH transition zone respectively. A linear regression yields the apparent values and 95% confidence intervals $n' = 1.78 \pm 0.24$, $\text{p}K'_a = 7.72 \pm 0.04$ (correlation coefficient -0.971) and $n' = 1.96 \pm 0.08$, $\text{p}K'_a = 7.44 \pm 0.01$ (correlation coefficient -0.999) for the NaCl-free and 0.5 M-NaCl samples respectively.

A model for the observed pH-dependence of k'_{1N}/k'_{1C} is presented in Appendix B. According to this model, two functional groups per molecule of transferrin undergo ionization to bring about a change in the relative iron-binding affinities of the two sites. The curves in Fig. 4, which are calculated from the resultant equation [eqn. (B-12) in Appendix

Fig. 5. Henderson–Hasselbalch plots for the two curves in Fig. 4

$\log [\text{Acid}]/[\text{base}]$ is given by the left-hand side of eqn. (1). Continuous lines are linear regressions of eqn. (1) with the resultant parameters given in the Results section. Broken lines are calculated from eqns. (2) and (3) for different choices of the K_2/K_1 ratio and $K'_a = \sqrt{K_1 K_2}$. Conditions were as given in Fig. 4.

Table 1. Parameters for the pH-dependence of the site preference factor

See Appendix B for definition of parameters.

Parameter	NaCl-free	0.5 M-NaCl
$\text{p}\bar{K}_{a,C}^*$	7.72	7.44
$\text{p}\bar{K}_{a,N}^\dagger$	7.11	6.70
$\text{p}\bar{K}_a$	<7.11	<6.70
n'	1.78	1.96
k'_{1N}/k'_{1C}	0.063	0.061
k^h_{1N}/k^h_{1C}	1.03	1.85

* $\text{p}\bar{K}_{a,C} = \text{p}K'_a$ [eqns. (1) and (B-12) (in Appendix B)].

† Calculated from eqn. (B-10) in Appendix B.

B] for a two-proton ionization, fit the observed data very well. The experimental values of the parameters of the model are summarized in Table 1.

It is useful to consider transferrin in terms of the

general equation for the ionization of a diprotic acid (Edsall & Wyman, 1958):

$$\bar{h} = \frac{\frac{K_1}{[H^+]} + \frac{2K_1K_2}{[H^+]^2}}{1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2}} \quad (2)$$

where $\bar{h}$ is the average number of protons ionized per molecule during titration with base and K_1 and K_2 are the successive macroscopic ionization constants. Eqn. (B-12) (in Appendix B) of the model is essentially equivalent to eqn. (2). The corresponding Henderson–Hasselbalch equation is:

$$\log \left[\frac{2 - \bar{h}}{\bar{h}} \right] = -n' \cdot \text{pH} + n' \cdot \text{p}K'_a \quad (3)$$

For the case where the two ionizing groups are equivalent, the macroscopic constants are related by the statistical factor, $K_1 = 4K_2$. In this case a Henderson–Hasselbalch plot will give the observed values $n' = 1$ and $\text{p}K'_a = (\text{p}K_1 + \text{p}K_2)/2$, which is indistinguishable from the case where there is only one ionizing group: $n' = 1$ and $\text{p}K'_a = \text{p}K_a$. Fig. 5 shows Henderson–Hasselbalch lines for various choices of K_2/K_1 . The $K_2 = 0.25K_1$ line of Fig. 5 is expected for either two equivalent ionizing groups or for a single ionizing group. However, for monoferric transferrin we observe that $n' \sim 2$, which can occur only when $K_2 \gg K_1$ in eqn. (2). Thus proton ionization in transferrin shows high positive co-operativity. Again the observed $\text{p}K'_a = (\text{p}K_1 + \text{p}K_2)/2$. Conservatively, we estimate that for the protein $K_2/K_1 > 10$.

pH-dependence of metal-binding co-operativity

The co-operativity factor k'_{2N}/k'_{1N} (Appendix A) as a function of pH is shown in Fig. 6. For non-interacting sites $k'_{2N}/k'_{1N} = 1.0$, but this is clearly not the case with transferrin, where in the absence of NaCl there is negative co-operativity in the pH range 6.5–8.0, changing to positive co-operativity at higher pH values. In the presence of 0.5 M-NaCl negative co-operativity changes to non-co-operativity in the pH range 6.5–6.9 and then changes to positive co-operativity at pH values over 7.5. Again, the data follow an acid–base pH profile. The corresponding Henderson–Hasselbalch equation is given by:

$$\log \left[\frac{\frac{k^h_{2N}}{k^h_{1N}} - \frac{k'_{2N}}{k'_{1N}}}{\frac{k'_{2N}}{k'_{1N}} - \frac{k^h_{2N}}{k^h_{1N}}} \right] = -n' \cdot \text{pH} + n' \cdot \text{p}K'_a \quad (4)$$

where k^h_{2N}/k^h_{1N} and k'_{2N}/k'_{1N} are the observed co-operativity factors above and below the tran-

Fig. 6. Dependence of the co-operativity factor, k'_{2N}/k'_{1N} , on pH

Continuous lines are calculated from eqn. (5) with $n' = 4$, $\text{p}K'_a = 8.09$ and 7.78 , $k^h_{2N}/k^h_{1N} = 0.64$ and 0.97 , and $k^h_{2N}/k^h_{1N} = 1.46$ and 1.94 for the NaCl-free and 0.5 M-NaCl solutions respectively. The broken portion of the 0.5 M-NaCl curve suggests an additional pH transition not accounted for by eqn. (5). Conditions were as given in Fig. 4.

sition region respectively. Linear regressions of the data in the pH range 7.5–8.5 plotted according to eqn. (4) give $n' = 3.07 \pm 0.25$, $\text{p}K'_a = 8.09 \pm 0.13$ (correlation coefficient -0.971) and $n' = 4.33 \pm 0.03$, $\text{p}K'_a = 7.78 \pm 0.02$ (correlation coefficient -0.998) for the NaCl-free and 0.5 M-NaCl solutions respectively, the latter data being the more reliable.

We have not formulated a model to account for the behaviour illustrated in Fig. 6, but the data fit an equation of the general form:

$$\frac{k'_{2N}}{k'_{1N}} = \frac{\frac{k^h_{2N}}{k^h_{1N}} - \frac{k'_{2N}}{k'_{1N}}}{1 + \frac{K'^{n'}}{[H^+]^{n'}}} + \frac{\frac{k^h_{2N}}{k^h_{1N}}}{1 + \frac{K'^{n'}}{[H^+]^{n'}}} \quad (5)$$

with $n' \sim 4$. Equations of this type are obtained when the sequential ionization of functional groups shows highly positive co-operativity [see, for example, the limiting case of eqn. 38 in Edsall & Wyman (1958)]. Therefore the transition from negative to positive co-operativity in iron-binding by transferrin is associated with positive co-operativity in proton

ionization. The continuous lines in Fig. 6 were calculated from eqn. (5) with $n' = 4$ and other parameters given in the legend.

Discussion

The iron-binding properties of transferrin follow well-defined pH titration curves (Figs. 4 and 6), which shows that highly specific structural changes occur that influence the metal-binding sites. Consistent with this is the fact that the effect of NaCl is seen only at pH values above the transition region. It seems unlikely that a change in the identity of the ligands bound to the metal is involved or that the ionizing groups themselves are ligands, because the pH-dependence of the difference in free energy of binding to the two sites is small. This is given by:

$$\Delta G_{\text{pH}}^0 = \Delta(\Delta G^0)^h - \Delta(\Delta G^0)^l = RT \cdot \ln \left[\frac{k_{\text{IN}}^h/k_{\text{IC}}^h}{k_{\text{IN}}^l/k_{\text{IC}}^l} \right] \quad (6)$$

where the $\Delta(\Delta G^0)^l$ terms are the difference in standard free energies of iron binding to the two sites at pH values above and below the transition region. The $\Delta G_{\text{pH}}^0 = 7-9$ kJ/mol obtained from eqn. (6) is less than the 25 kJ/mol energy of a hydrogen bond (Pimentel & McClellan, 1960). Furthermore, the e.p.r. spectrum of the iron centres is relatively invariant over this pH range. The pH effects may be due to transitions between conformational substates of the protein in which the configuration of the ligands about the metal are slightly different. Although this is difficult to prove, there is ample indirect evidence that under different conditions transferrin exists in different conformational states; however, knowledge about their structures is lacking (Chasteen *et al.*, 1977; Evans & Williams, 1980; Baldwin, 1980; Casey & Chasteen, 1980, and references cited therein).

The ionized groups observed here are probably histidine and/or tyrosine residues. Titration data for both apotransferrin and diferric transferrin suggest that two or three histidine residues and one tyrosine residue are ionized in the pH range 7-8 (Hazen, 1962). For the related protein apo-ovotransferrin approx. seven groups are titrated in the pH range 7-8 (Wishnia *et al.*, 1961). These are probably the imidazole groups of histidine residues. A recent proton n.m.r. study of ovotransferrin (Alsaadi *et al.*, 1980) shows the presence of histidine residues with unusually high pK_a values, comparable with those observed in the present work. Several other studies suggest the presence of histidine residues at or near the metal-binding sites of transferrin (Bezkorovainy & Grohlich, 1971; Krysteva *et al.*, 1975; Mazurier *et al.*, 1977; Zweier & Aisen, 1977; Zweier *et al.*, 1979).

It is not possible from our results alone to determine whether one or both sites are influenced by pH, since only the ratio of binding constants is measured. However, Aisen *et al.* (1978) have measured the absolute values of the conditional site constants k'_{IN} and k'_{IC} at pH 6.7 and pH 7.4 and found that they both increase with pH, the increase being greater for k'_{IN} than for k'_{IC} (the 'thermodynamic' site constants decrease with increasing pH, k_{IC} decreasing more than k_{IN}). This is consistent with our observation that $k'_{\text{IN}}/k'_{\text{IC}}$ is greater at high pH values.

According to the model in Appendix B, the proton ionization of transferrin can be described by the three constants $\bar{K}_a$, $\bar{K}_{a,N}$ and $\bar{K}_{a,C}$ (Table 1). These are the ionization constants for apotransferrin and the N-terminal and C-terminal monoferric transferrins respectively. The last two constants can be measured from Fig. 4 (see Appendix B), but $\bar{K}_a$ must be inferred from other data. It is necessary to know the values of $k_{\text{IN}}^h/k_{\text{IN}}^l$ and $k_{\text{IC}}^h/k_{\text{IC}}^l$ in eqns. (B-8) and (B-9) (in Appendix B) from which $\bar{K}_a$ can be calculated. Since the 'thermodynamic' site constants obtained by Aisen *et al.* (1978) are smaller at higher pH, we conclude that both ratios are less than 1. It follows from eqns. (B-8), (B-9) and (B-10) in Appendix B that $p\bar{K}_a < p\bar{K}_{a,N} < p\bar{K}_{a,C}$, and therefore that $p\bar{K}_a < 7.11$ (Table 1), a result that is consistent with the ionization of histidine. With small metal complexes one invariably observes that the pK_a of the free ligand is greater than that of the complex (Smith & Martell, 1975). Since this is the opposite of what is observed with transferrin, it is further evidence that the ionizing functional groups are not metal ligands in the protein.

The strong positive co-operativity ($K_2 > K_1$) in proton ionization of the monoferric transferrins is unexpected, since normally the removal of the second proton from a diprotic acid is more difficult than the first ($K_2 \ll K_1$). This implies that in the protein the ionization of the first proton causes structural changes that facilitate the ionization of the second proton. For example, a hydrogen bond could be broken or a buried group exposed to the solvent. Since $p\bar{K}_a < 7.11$ for apotransferrin, this suggests that the intrinsic pK_a values of the ionizing groups are well below the pH transition region. This would favour 'spontaneous' dissociation.

The co-operativity of iron-binding depends on the state of ionization of the protein and ranges from negative to positive (Fig. 6). Therefore interactions between the metal-binding domains must occur, as other studies also suggest (Woodworth *et al.*, 1975; Aisen *et al.*, 1978; Casey & Chasteen, 1980; Evans & Williams, 1980; Zweier, 1978). It could be an oversimplification to attribute the value of $n' \sim 4$ obtained in the Henderson-Hasselbalch plot for iron-binding co-operativity to the ionization of four

functional groups, because the co-operativity factor k'_{2N}/k'_{1N} involves products of concentrations so that the actual number of ionizing groups might be less than four. The observed pK'_a for co-operativity is probably a composite of constants describing proton ionizations of the apo-, monoferric and diferric forms of transferrin. Given the limited data available, it is not possible to say whether these groups are distinct from those that influence the site preference factor k'_{1N}/k'_{1C} . NaCl at 0.5 M lowers the apparent pK'_a by 0.3 in each case. A decrease in pK'_a with increasing ionic strength is to be expected for proteins above their isoelectric points (Edsall & Wyman, 1958).

The relative values for the apparent site constants at pH 7.4 at 37°C obtained in the present work are in reasonable agreement with those calculated from the absolute values obtained by Aisen *et al.* (1978), which give $k_{1N}:k_{1C}:k_{2N}:k_{2C} = 1.00:5.90 \pm 2.10:0.41 \pm 0.14:2.40 \pm 0.80$ for 25°C and 30 mM-citrate/0.1 M-NaNO₃/10 mM-Hepes/NaOH buffer, pH 7.4. Lower temperature is known to favour the binding of iron to the C-terminal site (Williams & Moreton, 1980; J. Williams, N. D. Chasteen & K. Moreton, unpublished work). Under the experimental conditions used in both laboratories iron prefers to bind to the C-terminal site, and with small negative co-operativity.

The acid-base chemistry of transferrin is not completely understood, and therefore the model for the pH-dependent behaviour of the metal-binding sites described in the present paper may require modification as more data become available. It is, however, clear that, in studies of transferrin performed near the physiological pH of 7.4, where the metal-binding properties of the protein change rapidly with changes of pH, attention should be paid to the accurate control of pH. Whether the functional groups responsible for these changes have some function *in vivo* remains to be seen.

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APPENDIX A

Site constants for iron-binding to transferrin

Metal-binding to the two sites of transferrin is described by four site constants (Aisen *et al.*, 1978). We write the appropriate equilibria in simplified form as:

where the k'_i constants are conditional (apparent) site constants dependent on temperature, pH, ionic strength, bicarbonate and other factors that influence iron-binding. Species Tf' , $Fe-Tf'$, $Tf-Fe'$ and $Fe-Tf-Fe'$ represent apotransferrin, the two monoferric transferrins with iron bound in the *N*-terminal and *C*-terminal sites and diferric transferrin respectively, each of which is composed of more than one form in solution designated collectively by the prime. The conditional site constants are given by:

$$k'_{1N} = \frac{[Fe-Tf']}{[Fe][Tf']} \quad (A-1)$$

$$k'_{1C} = \frac{[Tf-Fe']}{[Fe][Tf']} \quad (A-2)$$

$$k'_{2N} = \frac{[Fe-Tf-Fe']}{[Fe][Tf-Fe']} \quad (A-3)$$

$$k'_{2C} = \frac{[Fe-Tf-Fe']}{[Fe][Fe-Tf']} \quad (A-4)$$

where $[Fe-Tf']$ is the analytical concentration of all transferrin species with iron bound in the *N*-terminal site. The other concentrations are defined similarly.

Because the sum of the standard free energies of a cyclic process is zero, only three of the four constants are independent. By combining eqns. (A-1)–(A-4), we obtain eqns. (A-5) and (A-6):

$$\frac{k'_{1N}}{k'_{1C}} = \frac{k'_{2N}}{k'_{2C}} \quad (A-5)$$

$$\frac{k'_{2N}}{k'_{1N}} = \frac{k'_{2C}}{k'_{1C}} \quad (A-6)$$

The site preference factor k'_{1N}/k'_{1C} of eqn. (A-5) expresses the relative affinity of iron for the *N*-terminal and *C*-terminal binding sites. The cooperativity factor k'_{2N}/k'_{1N} of eqn. (A-6) expresses

the relative ease with which iron binds in a given site when the other site is occupied versus when it is empty. Determination of the absolute values for the k'_i constants requires a knowledge of the unbound iron concentration. However, their relative values can be readily calculated from the mole fractions of the four forms of transferrin. Since we have a constant-volume system, eqn. (A-1) can be re-arranged to give eqn. (A-7):

$$\frac{[Fe-Tf']}{[Tf']} = \frac{X_N}{X_A} = k'_{1N}[Fe] \quad (A-7)$$

where X_N and X_A are the mole fractions of *N*-terminal monoferric transferrin and apotransferrin respectively.

Similar expressions can be written from eqns. (A-2)–(A-4). The relative values for the four conditional binding constants are then given by:

$$k'_{1N} : k'_{1C} : k'_{2N} : k'_{2C} = \frac{X_N}{X_A} : \frac{X_C}{X_A} : \frac{X_D}{X_C} : \frac{X_D}{X_N} \quad (A-8)$$

where X_C and X_D are the mole fractions of the *C*-terminal monoferric and diferric transferrins respectively. Here $X_A + X_N + X_C + X_D = 1$. The X_i values can be determined from the relative areas of the four bands observed on the urea/polyacrylamide-gel electrophoretogram (Fig. 1 of the main paper).

The percentage iron saturation of the sample is given by:

$$\text{Saturation (\%)} = 50(X_N + X_C + 2X_D) \quad (A-9)$$

Note that at 50% saturation the amounts of apotransferrin and diferric transferrin must be equal regardless of the values of the binding constants.

The distribution of the four species of transferrin depends on the concentration of free iron. For X_A we write:

$$X_A = \frac{[Tf']}{[Tf'] + [Fe-Tf'] + [Tf-Fe'] + [Fe-Tf-Fe']} \quad (A-10)$$

Substitution of eqns. (A-1), (A-2) and (A-4) into eqn. (A-10) gives:

$$X_A = \frac{1}{1 + (k'_{1N} + k'_{1C})[Fe] + k'_{1N} k'_{2C}[Fe]^2} \quad (A-11)$$

Similarly, expressions for X_N , X_C and X_D are given by eqns. (A-12)–(A-14):

$$X_N = \frac{1}{1 + \frac{k'_{1C}}{k'_{1N}} + \frac{1}{k'_{1N}[\text{Fe}]} + k'_{2C}[\text{Fe}]} \quad (\text{A-12})$$

$$X_C = \frac{1}{1 + \frac{k'_{1N}}{k'_{1C}} + \frac{1}{k'_{1C}[\text{Fe}]} + k'_{2N}[\text{Fe}]} \quad (\text{A-13})$$

$$X_D = \frac{1}{1 + \frac{k'_{2C} + k'_{2N}}{k'_{2C} k'_{2N}[\text{Fe}]} + \frac{1}{k'_{1C} k'_{1N}[\text{Fe}]^2}} \quad (\text{A-14})$$

When the k'_i constants are expressed in relative values, $[\text{Fe}]$ becomes a 'dummy' variable proportional to the concentration of free iron. For a given set of k' values, substitution of a value for $[\text{Fe}]$ (0 to 10 times the largest k') into eqns. (A-11)–(A-14) gives the distribution of transferrin among its four species. The corresponding percentage saturation is calculated from eqn. (A-9). Typical results of these calculations are shown in Fig. 3 of the main paper.

Reference

Aisen, P., Leibman, A. & Zweier, J. L. (1978) *J. Biol. Chem.* **253**, 1930–1937

APPENDIX B

pH-dependence of the site preference factor

Consider the case where the three species of transferrin Tf' , $\text{Fe-Tf}'$ and $\text{Tf-Fe}'$ each exist in protonated and unprotonated forms that differ in the ionization of two functional groups, i.e. $(\text{H}_2\text{Tf})^z$, Tf^{z-2} , $\text{Fe}(\text{HCO}_3)(\text{H}_2\text{Tf})^{z-1}$, $\text{Fe}(\text{HCO}_3)\text{Tf}^{z-3}$, $(\text{H}_2\text{Tf})(\text{HCO}_3)\text{Fe}^{z-1}$ and $\text{Tf}(\text{HCO}_3)\text{Fe}^{z-3}$ respectively, where z denotes electronic charge. Here the

When the Fe^{3+} binds to apotransferrin one HCO_3^- ion is consumed, three protons are liberated, and the protein gains one unit of negative charge (Aisen *et al.*, 1978, and references cited therein). Accordingly, we can write the following reactions for iron-binding to the low-pH and high-pH species of transferrin:

bound anion, HCO_3^- , is shown explicitly and the parenthetic notation $(\text{H}_2\text{Tf})^z$ designates that the location of the ionizing functional groups within the transferrin molecule is undetermined. Furthermore, let us assume that the ionization changes the affinities of the metal sites for iron.

where the superscripts l and h on the 'thermodynamic' site constants denote binding at low pH and high pH respectively, i.e. below and above the pH of transition between the protonated and unprotonated forms. The equilibria that describe the proton ionization are given by:

(Over the pH 6–9 range of the experiments reported here, more than two groups on the protein must be ionizing. However, in our simplified treatment, only the groups that influence the differential strength of iron binding to the two sites are considered.) Here $\bar{K}_a$, $\bar{K}_{a,N}$ and $\bar{K}_{a,C}$ are the root-mean-square values of the successive ionization constants for each reaction, and reflect the strengths of acid dissociation in apotransferrin and *N*-terminal and *C*-terminal monoferric transferrins respectively. By combining eqns. (B-1), (B-2), (B-5) and (B-6) and eqns. (B-3), (B-4), (B-5) and (B-7) respectively, eqns. (B-8) and (B-9) are obtained:

$$\frac{k_{1N}^h}{k_{1N}^l} = \frac{\bar{K}_{a,N}^2}{\bar{K}_a^2} \quad (\text{B-8})$$

$$\frac{k_{1C}^h}{k_{1C}^l} = \frac{\bar{K}_{a,C}^2}{\bar{K}_a^2} \quad (\text{B-9})$$

These two equations show the explicit dependence of the metal-binding site constants on the proton ionization constants and express the fact that strength of metal-binding and proton ionization are coupled. Only when the proton ionization constant is changed by binding of the metal in the *N*-terminal site, i.e. $\bar{K}_a \neq \bar{K}_{a,N}$ in eqn. (B-8), will there be a pH effect on the site constant, i.e. $k_{1N}^l \neq k_{1N}^h$. The same is true for the *C*-terminal site [eqn. (B-9)]. In order for there to be a differential effect on the two sites $\bar{K}_{a,N}$ must not equal $\bar{K}_{a,C}$.

By combining eqns. (B-1)–(B-4) and (B-6) and (B-7), the overall process of metal binding and proton dissociation can be written in a cyclic fashion:

where the equilibrium constants are written for a clockwise cyclic process. It follows from eqns. (B-8) and (B-9) that:

$$\frac{\bar{K}_{a,C}^2}{\bar{K}_{a,N}^2} \cdot \frac{k_{1N}^h}{k_{1C}^h} \cdot \frac{k_{1C}^l}{k_{1N}^l} = 1 \quad (\text{B-10})$$

Therefore only five of the six equilibrium constants are independent; this is a consequence of the cyclic equilibrium.

The observed site preference factor is given by:

$$\begin{aligned} \frac{k_{1N}'}{k_{1C}'} &= \frac{X_N}{X_C} = \frac{[\text{Fe-Tf}']}{[\text{Tf-Fe}']} \\ &= \frac{[\text{Fe}(\text{HCO}_3)(\text{H}_2\text{Tf})^{z-1}] + [\text{Fe}(\text{HCO}_3)\text{Tf}^{z-3}]}{[(\text{H}_2\text{Tf})(\text{HCO}_3)\text{Fe}^{z-1}] + [\text{Tf}(\text{HCO}_3)\text{Fe}^{z-3}]} \end{aligned} \quad (\text{B-11})$$

Rearrangement of eqn. (B-11) and substitution of eqns. (B-1) through eqns. (B-4) and (B-7) for the appropriate concentrations gives the $[\text{H}^+]$ -dependence of the site preference factor expressed in eqn. (B-12):

$$\frac{k_{1N}'}{k_{1C}'} = \frac{k_{1N}^l/k_{1C}^l}{1 + \frac{\bar{K}_{a,C}^2}{[\text{H}^+]^2}} + \frac{k_{1N}^h/k_{1C}^h}{1 + \frac{[\text{H}^+]^2}{\bar{K}_{a,C}^2}} \quad (\text{B-12})$$

The limiting values of k_{1N}^l/k_{1C}^l are k_{1N}^l/k_{1C}^l and k_{1N}^h/k_{1C}^h below and above the pH transition defined by $\bar{K}_{a,C}$.

Eqn. (B-12) can also be written in terms of $\bar{K}_{a,N}$ by substituting

$$\bar{K}_{a,C}^2 = \bar{K}_{a,N}^2 \cdot \frac{k_{1N}^l}{k_{1C}^l} \cdot \frac{k_{1C}^h}{k_{1N}^h}$$

from eqn. (B-10). Eqn. (B-12) fits the experimental data of Fig. 4 of the main paper.

One can also derive expressions for the dependence of the 'thermodynamic' binding constants on $[\text{H}^+]$. For *N*-terminal site binding, k_{1N} is given by:

$$\begin{aligned} k_{1N} &= \frac{[\text{FeTf}'][\text{H}^+]^3}{[\text{Tf}'][\text{HCO}_3^-][\text{Fe}^{3+}]} \\ &= \frac{[\text{Fe}(\text{HCO}_3)\text{Tf}^{z-3}] + [\text{Fe}(\text{HCO}_3)(\text{H}_2\text{Tf})^{z-1}]}{[\text{Tf}^{z-2}] + [\text{H}_2\text{Tf}^z]} \\ &\quad \times \frac{[\text{H}^+]^3}{[\text{HCO}_3^-][\text{Fe}^{3+}]} \end{aligned} \quad (\text{B-13})$$

Rearrangement of eqn. (B-13) and substitution of eqns. (B-1), (B-2) and (B-5) for the concentrations gives:

$$k_{1N} = \frac{k_{1N}^l}{1 + \frac{\bar{K}_a^2}{[\text{H}^+]^2}} + \frac{k_{1N}^h}{1 + \frac{[\text{H}^+]^2}{\bar{K}_a^2}} \quad (\text{B-14})$$

Similarly for k_{1C} we obtain eqn. (B-15):

$$k_{1C} = \frac{k_{1C}^l}{1 + \frac{\bar{K}_a^2}{[\text{H}^+]^2}} + \frac{k_{1C}^h}{1 + \frac{[\text{H}^+]^2}{\bar{K}_a^2}} \quad (\text{B-15})$$

Note that the conditional and 'thermodynamic' constants are equivalent when expressed as relative values because the $[\text{H}^+]^3/[\text{HCO}_3^-]$ factor cancels. Eqn. (B-12) can be obtained by taking the ratio of eqns. (B-14) and (B-15). The absolute values for k_{1N} and k_{1C} depend on pH in accordance with eqns. (B-14) and (B-15) only if the ionization of the two functional groups is the only effect operating. This has not been established in the present study, since only relative values have been measured.

Reference

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