^{1}H NMR Study of Effects of Synergistic Anion and Metal Ion Binding on pH Titration of the Histidinyl Side-Chain Residues of the Half-Molecules of Ovotransferrin†

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Received July 29, 1986; Revised Manuscript Received December 17, 1986

ABSTRACT: Separation of ovotransferrin into C-terminal (OTf/2C) and N-terminal (OTf/2N) half-molecules has made possible the resolution of all expected histidinyl C(2)H resonances by proton nuclear magnetic resonance at 250 MHz. The chemical shift of many of the resonances decreases with increasing pH, allowing construction of titration curves, whereas a few resonances fail to titrate. On formation of the $Ga^{III}OTf·2(C_2O_4)$ ternary complexes, two of the low-field C(2)H resonances in each half-molecule fail to titrate. This behavior implicates the imidazole groups giving rise to these resonances as ligands to the bound metal ion. A third C(2)H resonance in each half-molecule undergoes a marked reduction in pK'_a on formation of the ternary complex. The imidazole group displaying this resonance is implicated in a proton-relay scheme involved in binding the synergistic anion, oxalate, and a water of hydration on the bound metal ion. The titration curves for the various imidazole resonances have been fit to a four-parameter equation involving estimation of the pK'_a, the limiting chemical shift values, and a Hill constant n. Hill constants of <1 can be rationalized by correcting the titration curve for the charge Z on the protein as a function of pH and the work function w. The titration curve for the imidazole group in OTf/2C involved in the proton-relay scheme shows a value for $n>1$, which suggests positive cooperativity in the titration of this residue. The basis for this behavior cannot be rationalized at this time. ^{13}C NMR studies of [ζ-^{13}C]Arg-OTf suggest the Arg side chains may not be intimately invovled in formation of the ternary complex.

The transferrins (siderophillins) comprise a class of ca. 80 000-dalton glycoproteins each capable of binding at saturation two high-spin ferric ions and two synergistic anions, e.g., bicarbonate or carbonate. In previously reported proton nuclear magnetic resonance studies evidence was presented for the involvement of imidazole side chains of histidine as ligands to the bound metal ions and as possible binding residues for the synergistic anions (Alsaadi et al., 1981; Woodworth et al., 1977, 1983, 1984). Resolution of the C(2)H resonances of the imidazole side chains of ovotransferrin (OTf)[1] (conalbumin) from hens' egg white has been markedly improved by cleaving OTf into half-molecules and studying the N-terminal (OTf/2N) C-terminal (OTf/2C) half-molecules separately (Brown-Mason & Woodworth, 1984). This approach has allowed complete resolution of all the expected C(2)H resonances in each of the half-molecules and ready construction of pH titration curves for the individual C(2)H resonances. This had not been possible in holo-OTf owing to overlapping of resonances.

We report here improved fitting of the experimental titration data and a theoretical interpretation of the calculated Hill coefficients, and shifts in pK'_a for certain titration curves and propose the involvement of a histidinyl side chain in a proton relay between synergistic anion and a water of hydration.

Experimental Procedures

Ovotransferrin from hens' egg white and its half-molecules, OTf/2N and OTf/2C, were prepared as previously described (Brown-Mason & Woodworth, 1984). Chemicals were reagent grade unless otherwise specified. Stock 20 mM solutions of Ga(III) in D_2O were made by dissolving $Ga(ClO_4)_3·6H_2O$ in 10 mM $HClO_4$. A known volume of stock solution was lyophilized and the residue dissolved in D_2O and lyophilized. The final residue was dissolved in D_2O to the original solution volume. Stock 0.10 M solutions of KCl and $K_2C_2O_4$ were made from the anhydrous salts dissolved in D_2O. Titrations were carried out with ca. 0.1 N DCl (diluted from 12 N DCl) and ca. 0.1 N NaOD (diluted from 10% w/v NaOD). The 0.1 N NaOD was maintained carbonate free by storage over $Ca(OD)_2$, $Ca(OH)_2$ having been equilibrated with D_2O.

[^{13}C]Urea was purchased from MSD Isotopes, St. Louis, MO, and converted into the methylisourea by reaction with 5% excess dimethyl sulfate (Janus, 1955). The methylisourea was allowed to react with 10% excess copper–ornithine complex overnight (Greenstein & Winitz, 1961). Five grams of urea resulted in a final yield of 4.5 g of [ζ-^{13}C]Arg (31% of theory). The entire sample of [ζ-^{13}C]Arg was added to sufficient mixed chicken mash so as to dilute the enriched Arg 1:1 with the Arg contained in the diet. The mix was lightly labeled with [^{3}H]Arg so we could readily monitor the extent of incorporation of the ^{13}C-enriched Arg into the egg white protein. Eggs collected on days 2–5 showed usable levels of incorporation, so the OTf was isolated from the whites of these eggs for the ^{13}C studies. This labeling scheme is possible because birds lack a urea cycle and Arg is essential in the diet. In preliminary experiments we confirmed this lack of a urea cycle by feeding a mixture of [ζ-^{14}C]Arg and [2,3-^{3}H]Arg. The ratio of ^{14}C cpm to ^{3}H cpm was the same in the egg white as in the fed Arg, showing that the Arg is not cleaved to Orn for recycling through the urea cycle.

pH Titration. The ca. 1 mM OTf half-molecules were titrated in 5-mm NMR tubes by microliter additions of 0.1 N DCl or 0.1 N NaOD through a rubber septum. The pH

†Supported by USPHS Grant AM-21739.
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[1] Abbreviations: OTf, ovotransferrin; OTf/2C, C-terminal half-molecule of ovotransferrin; OTf/2N, N-terminal half-molecule of ovotransferrin; Orn, ornithine.

0006-2960/87/0426-3115$01.50/0

was measured by inserting a slender combination glass electrode (Microelectrodes, Inc., Londonderry, NH) into the NMR tube or by monitoring the chemical shift dependence on pH of internal markers (Valcour & Woodworth, 1986). Although these procedures were satisfactory for the oligosaccharide-containing OTf/2C, the carbohydrate-free OTf/2N tended to precipitate progressively during the titration. This problem was overcome by adjusting half of the OTf/2N sample to the low-pH limit and half to the high-pH limit in 1.5-mL Eppendorf tubes with rapid stirring. Admixture of varying amounts of the two samples served to adjust the pH without precipitation. When a glass electrode was used, the pH was measured before and after the accumulation of each NMR spectrum. Drift between measurements was usually less than 0.05 pH unit. The two values were averaged for data analysis. The glass electrode was restandardized during each spectral accumulation with a pair of standard buffers in H_2O of pH 4.03 and 6.97 or pH 6.97 and 9.09 depending on the pH range of the particular sample. All pH and NMR spectral measurements were made with the samples and buffers at 37 °C (310 K). pH values are reported as pH*, no correction having been made for deuterium isotope effects.

Protein samples in D_2O were adjusted to pH* 5 and allowed to equilibrate for several hours prior to lyophilization. The residue was redissolved in D_2O and lyophilized a second time. This scheme served not only to dilute out residual H_2O but also to exchange the peptide backbone hydrogens for deuterium, thus substantially decreasing the peptide proton background resonances in the imidazole C(2)H region. This procedure obviated the equilibration in 2 M [^{2}H]urea at high pH which had been used to exchange the peptide hydrogens in intact OTf (Alsaadi et al., 1981). As a consequence, all initial titrations were from low to high pH. At the end of an initial titration, e.g., OTf/2 plus 4-fold molar excess oxalate, however, a saturating amount of Ga(III) was added and the resulting $Ga^{III}OTf/2(C_2O_4)$ complex was titrated from high to low pH.

Nuclear Magnetic Resonance Spectra. Proton nuclear magnetic resonance spectra were recorded with a Bruker WM 250-MHz NMR spectrometer in the Camille and Henry Dreyfus NMR laboratory, Department of Chemistry, University of Vermont, operating in the Fourier transform mode with quadrature detection and a sweep width of 5000 Hz. The pulse width was 2.5–3 μs (22–30°), the acquisition time was 0.512 s, and there was no additional delay between pulses. Either 2048 or 4096 transients were accumulated and stored on magnetic disk. Convolution difference spectra (Campbell et al., 1973) with a line broadening of 4 or 8 Hz were constructed and plotted for interpretation of chemical shift values. The weighting for subtraction of the broadened from the original free induction decay (FID) was adjusted to give the least possible residual FID near zero acquisition time and the weighting (DC value) had to be changed as a function of the number of scans (NS). An internal standard of acetone was used, and all chemical shift values were then corrected to sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate (TSP), taken as zero. ^{13}C NMR spectra were run with a sweep width of 15 000 Hz and required 20K–80K transients. Probe temperature was 304 K. ^{13}C chemical shifts relate to tetramethylsilane (Me_4Si) as zero.

Data Analysis and Curve Fitting. Data sets of pH vs. chemical shift were fitted by the BMD/PAR routine (Ralston, 1979), which carries out derivative-free nonlinear regression (maximum likelihood estimation), to the equation

$$pH = pK'_a + (1/n) \log [\alpha/(1 - \alpha)] \quad (1)$$

where $\alpha = (\delta_{max} - \delta_{obsd})/(\delta_{max} - \delta_{min})$ and n = the Hill coefficient. The analysis produces estimates of pK'_a, n, δ_{max}, δ_{min}, and the standard deviation (SD) of each parameter. Estimated parameters were then used to generate the lines drawn through the experimental points.

In an attempt to rationalize Hill coefficients of <1.0, the equation fitted (Edsall & Wyman, 1958) was

$$pH = pK'_a + \log [\alpha/(1 - \alpha)] - 0.868w\Delta Z \quad (2)$$

where w is the work function as usually defined and ΔZ is the charge difference between the pK'_a determined from fitting the data to eq 1 and the particular observed pH in an NMR titration. ΔZ was calculated from a titration of buffer-free OTf/2C in 0.1 N KCl at 25 °C carried out under computer control with the sample under N_2.

Results

The 250-MHz proton nuclear magnetic resonance convolution difference spectra for the OTf half-molecules at a pH showing maximum resolution of the imidazole C(2)H resonances are shown in Figure 1 with the imidazole resonances arbitrarily numbered from low to high field. These number designations are used to identify the titration curves and titration parameters in subsequent figures and in Table I. Note that the resonances of the five histidinyl residues in the C-terminal half and the seven histidinyl residues in the N-terminal half (Jeltsch & Chambon, 1982) have been resolved.

It is well established that a number of small organic molecules containing a free carboxyl group and an electron-donor function can serve as synergistic anions in lieu of carbonate (Schlabach & Bates, 1975; Woodwoorth et al., 1975). We chose to use oxalate in the present studies owing to its low pK'_a's, so that it would exist as a dianion over the pH range used; carbonate would be converted to CO_2 at pH 5 (Alsaadi et al., 1981). Because high-spin Fe(III) is paramagnetic, it broadens, often beyond recognition, NMR resonances of its ligands. As a consequence of this behavior with OTf (Woodworth et al., 1977) we substituted Ga(III) as the bound metal ion because of its diamagnetic character and coordination chemistry similar to Fe(III) (Alsaadi et al., 1981).

Figures 2 and 3 show the titration data and the computer-fitted curves using eq 1 for the titratable imidazole C(2)H resonances of OTf/2N and OTf/2C as the apoproteins, the apoproteins plus a 4-fold concentration of oxalate, and the $Ga^{III}OTf/2(C_2O_4)$ complexes. The parameters derived from the computer fits are listed in Table I.

Most resonances remained sharp throughout the titrations with a few notable exceptions. Resonances 1 and 2 of $Ga^{III}OTf/2C(C_2O_4)$ and resonances 1 and 3 of $Ga^{III}OTf/2N(C_2O_4)$ were broader than in the apoproteins and broadened more with increasing pH although they remained readily distinguishable. Resonance 3 of OTf/2C broadened appreciably at $pH > pK'_a$. The broadening was much less on titration of OTf/2C plus oxalate and hardly noticeable on titration of $Ga^{III}OTf/2C(C_2O_4)$.

Discussion

Resonances 1 and 2 of OTf/2C and resonances 1 and 3 of OTf/2N are little influenced by the presence of oxalate but fail to titrate in the Ga(III) complex. This behavior is taken as evidence that these resonances arise from imidazole side chains which serve as ligands to the specifically bound metal ions (Alsaadi et al., 1980; Campbell et al., 1975). Resonance 2 of OTf/2N is perturbed neither by oxalate nor by Ga(III) and therefore probably arises from an histidinyl residue remote from the metal ion/anion binding site. Resonance 9 of OTf/2C fails to titrate in the Ga(III) complex and so likely

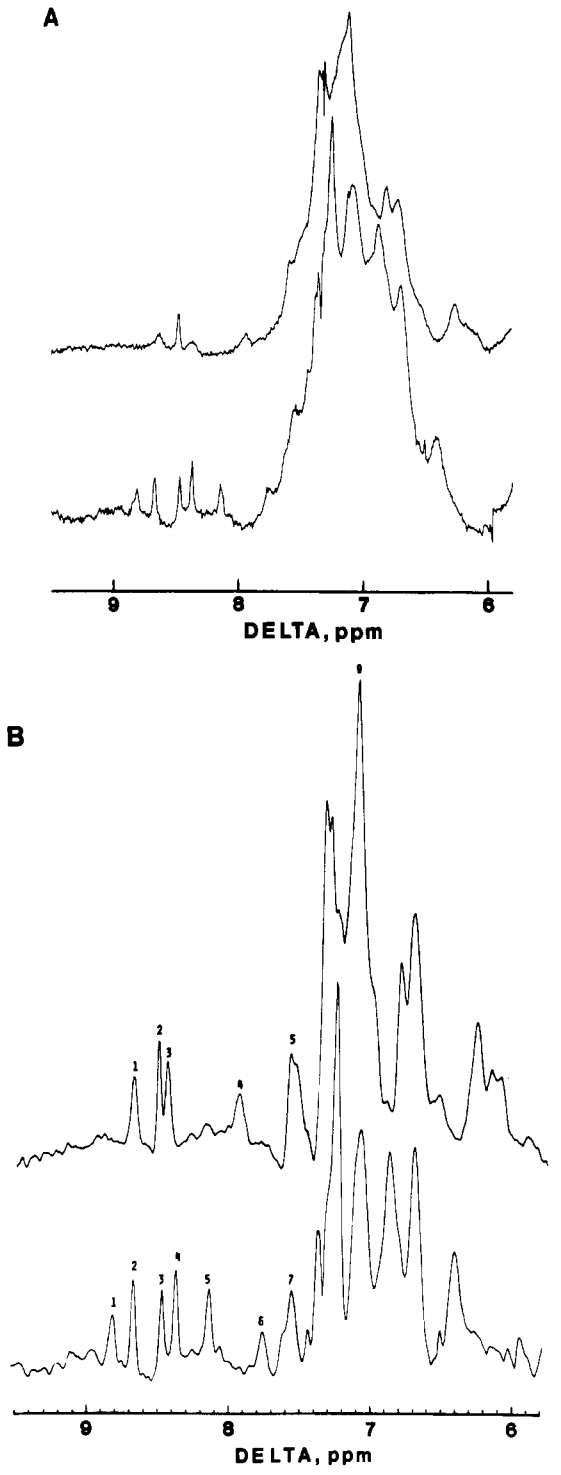


FIGURE 1: (A) Proton nuclear magnetic resonance spectra at 250 MHz of (top trace) OTf/2C, pH* 5.7, and (bottom trace) OTf/2N, pH* 6.3. (B) Convolution difference spectra of (top trace) OTf/2C, pH* 5.7, and (bottom trace) OTf/2N, pH* 6.3. Numbers arbitrarily assigned to the resonances from low to high field are used in the following figures, in Table I, and in the text to index the resonances.

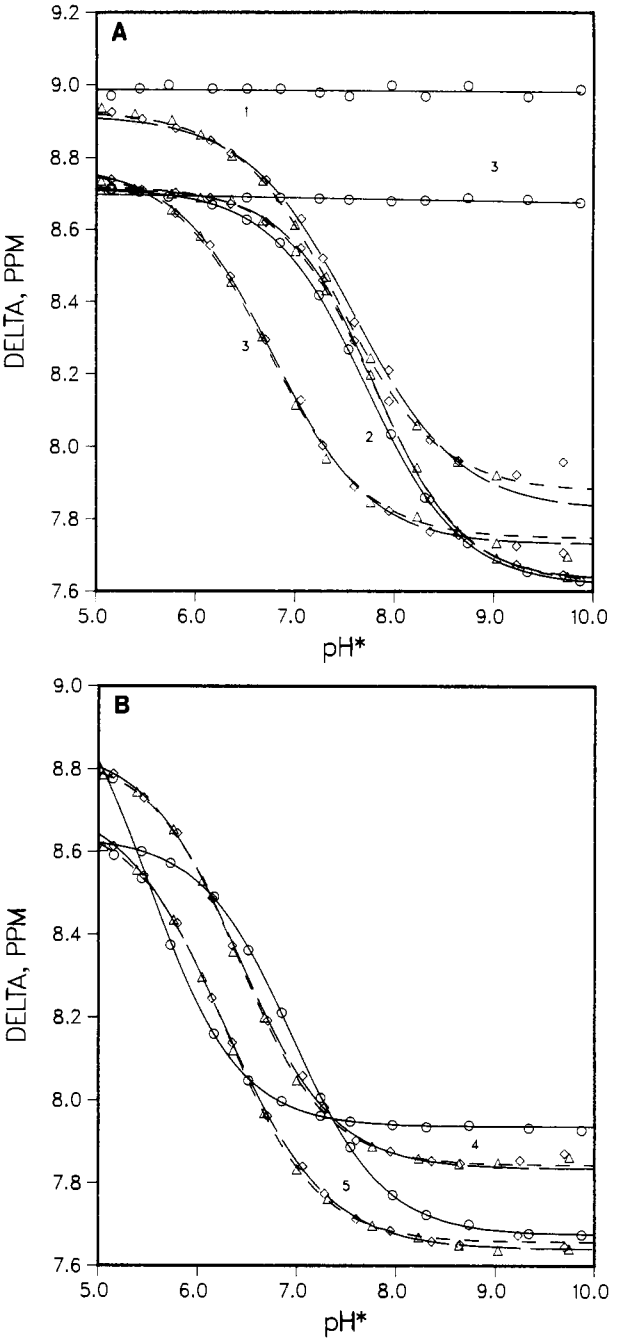


FIGURE 2: Titration curves for the C(2)H imidazole resonances in (◇, --) OTf/2N, (△, ---) OTf/2N + $4C_2O_4^{2-}$, and (○, —) Ga^{III}OTf/2N(C_2O_4). Titration curves are segregated into panels A and B for clarity. Lines were fit to the data as described in the text. The protein concentration was ca. 1 mM in 0.1 M KCl and D_2O. The temperature was 310 K.

arises from the C(4)H of the imidazole side chain for resonance 1 or 2. The pK'_a and slope estimates are not good enough to make the assignment at this time. The determination ought to be possible on the basis of double-resonance experiments. Resonances 4 and 5 of OTf/2C and resonances 6 and 7 of OTf/2N failed to titrate and may arise from internal histidinyl residues not accessible to solvent in the pH range studied.

Resonance 1 of OTf/2C serves as a "normally titrating" imidazolium group. Although its pK'_{eff} is nearly 1 pH unit higher than the pK'_{int} for an imidazolium side chain, owing probably to negatively charged neighboring side chains, it has a Hill coefficient of <1.0, which can be accounted for reasonably well by invoking the work function, w, required to add or remove a unit charge from the protein of charge Z. The mean square error obtained on fitting the data to eq 1 with n set to 1.0 is 0.0533, whereas the mean square error on fitting the data to eq 1 with n as an adjustable parameter or to eq 2 was 0.0136.

Resonance 3 of OTf/2C exhibits remarkable behavior inasmuch as its Hill coefficient is >1.0 in all three chemical states and its pK'_a decreases significantly on formation of the Ga(III) complex (Figure 2A, Table I). It is probably resonance E previously reported for $(Ga^{III})_2$OTf(C_2O_4) (Alsaadi

Table I: Estimated Parameters for the NMR Titration of C(2)H Histidinyl Resonances in Half-Molecules of Ovotransferrin

sample	resonance	pK'_a (±SD)	n (±SD)	δ_{max} (±SD)	δ_{min} (±SD)
OTf/2C	1	7.02 (±0.09)	0.85 (±0.17)	8.769 (±0.030)	7.860 (±0.053)
	2	7.02 (±0.05)	0.98 (±0.09)	8.524 (±0.010)	7.666 (±0.020)
	3	6.26 (±0.02)	1.58 (±0.07)	8.463 (±0.001)	7.736 (±0.001)
	9	6.57 (±0.01)	1.16 (±0.01)	7.109 (±0.0001)	6.960 (±0.0003)
OTf/2C + $4C_2O_4^{2-}$	1	7.12 (±0.06)	1.15 (±0.13)	8.782 (±0.006)	7.901 (±0.016)
	2	7.05 (±0.01)	1.02 (±0.02)	8.539 (±0.002)	7.660 (±0.004)
	3	6.29 (±0.10)	1.53 (±0.23)	8.463 (±0.005)	7.713 (±0.002)
	9	6.64 (±0.16)	1.37 (±0.13)	7.105 (±0.003)	6.943 (±0.003)
$Ga^{III}OTf/2C(C_2O_4)$	1				
	2				
	3	4.66 (±0.10)	1.04 (±0.13)	8.450 (±0.000)[a]	7.763 (±0.001)
	9				
OTf/2N	1	7.57 (±0.04)	0.83 (±0.07)	8.914 (±0.016)	7.833 (±0.033)
	2	7.80 (±0.02)	0.99 (±0.03)	8.711 (±0.004)	7.633 (±0.002)
	3	6.79 (±0.03)	0.91 (±0.04)	8.769 (±0.012)	7.733 (±0.004)
	4	6.42 (±0.02)	0.92 (±0.05)	8.852 (±0.013)	7.835 (±0.008)
	5	6.27 (±0.02)	0.85 (±0.02)	8.724 (±0.013)	7.641 (±0.002)
OTf/2N + $4(C_2O_4)$	1	7.43 (±0.02)	0.86 (±0.03)	8.926 (±0.009)	7.877 (±0.007)
	2	7.79 (±0.03)	0.95 (±0.05)	8.716 (±0.008)	7.626 (±0.004)
	3	6.75 (±0.04)	0.89 (±0.05)	8.772 (±0.018)	7.748 (±0.006)
	4	6.42 (±0.01)	0.98 (±0.02)	8.830 (±0.004)	7.843 (±0.002)
	5	6.29 (±0.01)	0.95 (±0.02)	8.677 (±0.006)	7.658 (±0.003)
$Ga^{III}OTf/2N(C_2O_4)$	1				
	2	7.73 (±0.01)	0.91 (±0.01)	8.709 (±0.002)	7.617 (±0.001)
	3				
	4	5.24 (±0.13)	0.84 (±0.05)	9.475 (±0.203)	7.930 (±0.003)
	5	6.95 (±0.01)	0.95 (±0.01)	8.634 (±0.002)	7.676 (±0.003)

[a] Because only the lower half of the sigmoid titration curve is experimentally accessible, the computer fitting routine assumes a preset value for δ_{max}, which was based on the δ_{max} for OTf/2C.

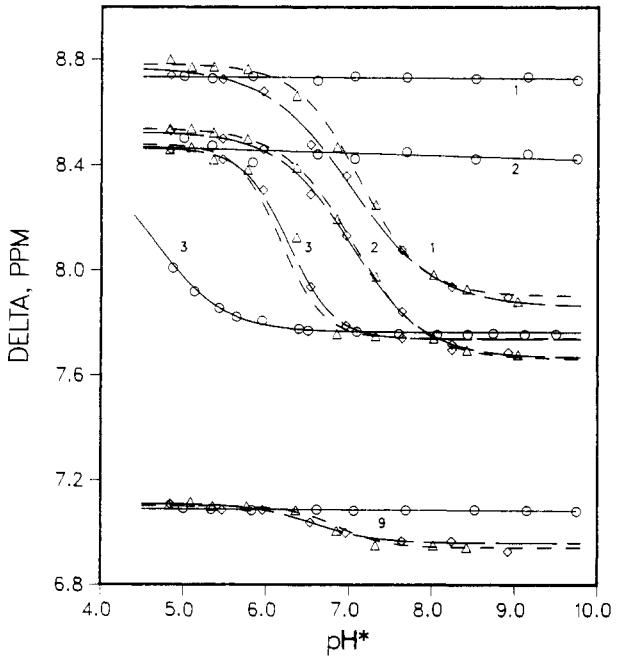


FIGURE 3: Titration curves for the C(2)H imidazole resonances and one C(4)H imidazole resonance in (◊, --) OTf/2C, (△, ---) OTf/2C + $4C_2O_4^{2-}$, and (○, —) $Ga^{III}OTf/2C(C_2O_4)$. The protein concentration was ca. 1 mM in 0.1 M KCl and D_2O. T was 310 K.

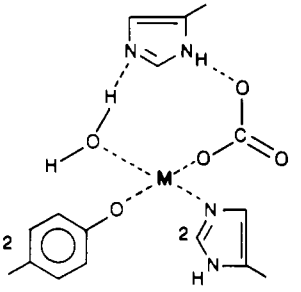
et al., 1981). Our initial report of a decrease in the pK'_a of this resonance on addition of oxalate (Woodworth et al., 1984) has not been corroborated by further study at either a 4-fold or a 25-fold ratio of oxalate to OTf/2C. It turns out in retrospect that the sample which gave rise to the apparent decrease in pK'_a on addition of oxalate had been preequilibrated in 2 M [^{2}H]urea in D_2O at pH 8 in order to exchange peptide protons (Alsaadi et al., 1981). Possibly this treatment altered the protein so as to change its titration behavior.

Although the Hill coefficients for most of the other resonances approach 1.0 and are greater than that for resonance 1 of OTf/2C with its "accountable" slope of 0.85, the slope of >1.0 for resonance 3 of OTf/2C suggests a cooperative process in the ionization of this imidazolium group similar to the so-called "triggered ionization" (Laskowski & Scheraga, 1954). Theoretical considerations of the way in which neighboring groups may participate in the titration of an imidazolium group suggest that such a cooperative titration can be accounted for by the simultaneous titration of two imidazolium groups hydrogen bonded to the same carboxylate side chain (Laskowski & Scheraga, 1954) or by a conformational change in the protein such that the pK'_a of a critical side chain is lower in the base-stabilized conformation than in the acid-stabilized conformation (Tanford, 1961). The first possibility is unlikely in the present case as resonances 1–3 integrate for 1 proton each on direct Fourier transformation of the FID. A conformational change has been found in ovotransferrin in the pH range 4–5 (Wishnia et al., 1961) but not in the range 5–7. It is likely that some not yet identified group cotitrates with the histidinyl side chain giving rise to resonance 3.

Although arginyl and lysyl side chains have been invoked as likely candidates for ligands to the synergistic anions on the basis of difference chemical modification studies of arginine in apotransferrin and diferric transferrin (Rogers et al., 1977), on the basis of ^{13}C NMR studies in $Fe_2Tf(^{13}CO_3)_2$ (Zweier et al., 1981), and by analogy with anion binding in various proteins (Riordan, 1977), there is little direct evidence for the involvement of arginine or lysine in binding of synergistic anions to the transferins. To this end, we synthesized [ζ-^{13}C]Arg and fed it to laying hens, which in turn incorporated it at ca. 50% efficiency into egg white protein. The OTf isolated from these eggs gave a single sharp resonance at 159.5 ppm relative to Me_4Si that was at least 10 times more intense than the natural abundance ^{13}C resonances for the protein. Formation of the $Fe_2OTf(CO_3)_2$ complex of this protein led to no changes in chemical shift or line broadening for the [ζ-^{13}C]Arg resonance. Similar results were obtained for the Pr(III) and Eu(III) complexes. Some paramagnetic broadening by the bound iron might have been expected for arginyl

side chains in the binding sites. Although ^{13}C chemical shifts are rather insensitive to environmental changes (Oldfield et al., 1975), we had expected to shift out resonances of any binding site [ζ-^{13}C]Arg from the single resonance in apo-OTf (28 Arg residues) in the Pr(III) or Eu(III) complexes.

The substantial decrease in pK'_a of resonance 3 of OTf/2C on the addition of Ga(III) can be rationalized if one presumes that the imidazole side chain lies in the binding site region. Then the binding of the tripositive Ga(III) ion ought to repel electrostatically a proton associated with the imidazolium group (Woodworth et al., 1984). Such a rationalization fails to explain why this residue would be in the binding site region at all, but the presence of this third histidinyl residue is consistent with the observation of three such highly conserved residues in the putative binding site regions (Chasteen, 1983). We propose a role for this imidazole group as the putative base in the proton-relay model for the iron-transferrin–carbonate ternary complex recently proposed (Carrano et al., 1985). If the base, B, in their model is taken to be imidazole, a general structure for the ternary complex would be



I

This structure takes into account coordination of a bound metal ion by two phenolate side chains (Tan & Woodworth, 1969; Pecoraro et al., 1981), two unprotonated imidazole side chains [this study and the following paper (Valcour & Woodworth, 1987)], the synergistic anion (Woodworth et al., 1975; Schlabach & Bates, 1975; Zweier et al., 1982), and a water of hydration (Koenig & Schillinger, 1969; Villafranca et al., 1976; Carrano et al., 1985; Bertini et al., 1985). Furthermore, structure I is consistent with the titration behavior of resonance 3 in $Ga^{III}OTf/2C(C_2O_4)$. In the proton-relay model this imidazole group is formally bonded to a single proton in such a way that the nonprotein ligands to the metal are either water and carbonate (as in structure I) or hydroxide and bicarbonate. Thus the chemical shift of resonance 3 ought to be that of the imidazole base as observed above pH 5. The presence of the positive metal center and stability of the proton-relay structure serve to make this imidazole a weaker base such that it accepts a proton only at much lower pH than it does in the absence of bound metal and anion. This appreciably lowered pK'_a for resonance 3 appears to be independent of the synergistic anion for the series carbonate, oxalate, and malonate (Woodworth, 1986). As these anions have rather disparate second pK'_a's, this result suggests that initial attack by a proton may be on some ligand other than the synergistic anion, e.g., imidazole, phenolate, or water. Nevertheless, exchange of bound with free synergistic anion is acid catalyzed (Woodworth et al., 1975). The decrease of ca. 1.8 pH units in pK'_a on going from OTf/2C to $Ga^{III}OTf/2C(C_2O_4)$ may account for the greater resistance to acid removal of the metal ion in the C-terminal site relative to that in the N-terminal site of holo-OTf. Although resonance 4 of OTf/2N exhibits a similar, if smaller, decrease in pK'_a on addition of Ga(III), resonance 5 exhibits the opposite effect, its pK'_a rising by ca. 0.7 pH unit, possibly balancing the pK'_a change of resonance 4. Resonance 4 may arise from an imidazole group in OTf/2N involved in the proton-relay scheme depicted in structure I.

Our suggestion that binding of synergistic anions and metal ions to ovotransferrin is an ordered process (Woodworth et al., 1975)

$$\text{OTf} + \text{anion} \rightleftarrows \text{OTf(anion)}$$

$$\text{OTf(anion)} + M^{n+} \rightleftarrows \text{(M)OTf(anion)}$$

has recently been confirmed, extended, and quantified for the binding of carbonate, phosphate, sulfate, and vanadate to human transferrin (Harris, 1985) and for binding of gallium to human lactoferrin (Harris, 1986). In the former study Harris reported a negative difference spectrum in the 242- and 294-nm regions on addition of specifically bound anions to apotransferrin and concluded that the anion may interact initially with binding site phenol side chains via hydrogen bonding. Likewise, the proton-relay model implies hydrogen bonding of anion to a specific imidazole group. The fact that only one or two synergistic or synergistic-like anions bind per transferrin molecule (Woodworth et al., 1975; Harris, 1985) means that specific recognition for these anions occurs and implies multidentate association to the anion. Indeed the binding of this anion may bring the metal binding ligands into a conformation necessary for binding of the metal ion.

Registry No. Ga, 7440-55-3; $(CO_2^-)_2$, 338-70-5; L-histidine, 71-00-1.

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Proton Nuclear Magnetic Resonance Spectroscopy of Human Transferrin N-Terminal Half-Molecule: Titration and Hydrogen–Deuterium Exchange†

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Received July 29, 1986; Revised Manuscript Received December 17, 1986

ABSTRACT: The binding of Ga(III) to the proteolytically derived N-terminal half-molecule of human transferrin (HTF/2N) was studied by proton nuclear magnetic resonance spectroscopy. The pH-dependent titration curves of the histidinyl C(2) proton chemical shifts were altered upon formation of the Ga^{III}HTF/2N(C_2O_4) ternary complex. Two high-pK'_a histidines failed to titrate when the metal and synergistic anion formed a complex with the protein. These results implicated two histidinyl residues as direct ligands to the metal. The rates of hydrogen–deuterium exchange for the C(2) protons of certain histidinyl residues were substantially decreased by metal ion binding. The two ligand histidines were protected from exchange, and a third, low-pK'_a, histidinyl residue was protected. We propose that this third histidinyl residue is involved in anion binding and may serve as the base in the putative proton-relay scheme proposed for complex formation.

Human serum transferrin (HTF)[1] is a member of an important class of iron binding proteins found in the physiological fluids of vertebrates (Aisen & Listowski, 1980). The transferrin molecule consists of a single polypeptide chain comprising two similar globular domains each of which binds one ferric ion (Gorinsky et al., 1982; Abola et al., 1982). Metal binding requires the concomitant binding of an anion which in the physiological case is carbonate or bicarbonate (Koenig & Schillinger, 1969; Pecoraro et al., 1981). Investigations directed at determining the ligands that bind each ferric ion have implicated two histidines (Rogers et al., 1977; Alsaadi et al., 1981), two tyrosines (Tan & Woodworth, 1969; Pecoraro et al., 1981), one water or hydroxyl (Bertini et al., 1975; Carrano et al., 1985), and the obligate anion (Woodworth et al., 1975; Schlabach & Bates, 1975; Zweier et al., 1981). Proton NMR studies performed on ovotransferrin indicated that a third histidine might be involved in anion–protein interactions (Alsaadi et al., 1981; Woodworth et al., 1987). We have improved the resolution of the proton nuclear magnetic resonance (^{1}H NMR) spectra of HTF by studying its proteolytically derived N-terminal half-molecule (HTF/2N) (Lineback-Zins et al., 1980). pH* titration curves were constructed for the C(2) protons of the histidinyl residues of the N-terminal domain of HTF. We performed deuterium exchange upon the apo-HTF/2N and gallium-loaded HTF/2N. These techniques allowed us to characterize further the involvement of histidinyl residues in metal binding by HTF.

†Supported by USPHS Grants AM-21739 and 5429-25-4.
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[1] Abbreviations: HTF, human serum transferrin; HTF/2N, N-terminal half-molecule of human transferrin; NMR, nuclear magnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TSP, sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate; FPLC, fast protein liquid chromatography; EPR, electron paramagnetic resonance.

0006-2960/87/0426-3120$01.50/0