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

A. A. Valcour* and R. C. Woodworth
Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405
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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.

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*Correspondence should be addressed to this author.

[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.

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EXPERIMENTAL PROCEDURES

Human serum transferrin was isolated from the Cohn IV fraction of human plasma as previously described (Penhallow et al., 1986). The N-terminal half-molecule of human transferrin was prepared by performing a 36-h digest of the iron-saturated protein with thermolysin, followed by purification by size exclusion on a Sephadex G-75 column as previously described (Lineback-Zins & Brew, 1980). The protein was purified to homogeneity by anion-exchange FPLC (Pharmacia) on a Polyanion SI column. Chemicals were of reagent grade unless otherwise specified. Gallium solutions in deuteriated water (D_2O) were prepared by dissolving $Ga(ClO_4)_3 \cdot 6H_2O$ in 10 mM $HClO_4$, followed by lyophilization and dissolution of the residue in D_2O. KCl and $K_2C_2O_4$ stock solutions were prepared by dissolving their anhydrous salts in D_2O.

Nuclear Magnetic Resonance Spectra. The ^{1}H NMR spectra were obtained at 250 MHz on the Bruker WM-250 NMR spectrometer, operating in the Fourier transform mode, in the Camille and Henry Dreyfus Nuclear Magnetic Resonance Laboratory, Department of Chemistry, University of Vermont. Most of the spectra recorded were derived from either 2048 or 4096 transients accumulated over a spectral width of 4 kHz. Spectra were obtained at 37 °C with a pulse width of 2.5 μs and a spectral aquisition time of 0.512 s. Convolution difference spectra with a line broadening of 4 Hz were constructed as described previously (Campbell et al., 1973). The weightings for convolution difference were varied to achieve maximum resolution.

Protein samples were prepared for ^{1}H NMR experiments by incubation in 99.8% D_2O at pH 5.0 and 22 °C for several hours, prior to lyophilization. This served to exchange labile peptide hydrogens with deuterium and thus flatten the base line in the spectral region where the resonances of imidazole C(2)H protons occur. The samples were then redissolved in 0.1 M KCl in D_2O to a final protein concentration of between 120 and 150 mg/mL. pH titrations were carried out by addition of 0.1 N DCl and 0.1 N NaOD [kept carbonate free by storage over $Ca(OD)_2$] through a rubber septum at the top of the NMR tube. Acetone was used as an internal standard, but the chemical shifts are reported relative to TSP. Addition of cacodylate, creatinine, 1-methylimidazole, and 2,4-dimethylimidazole to a final concentration of ca. 2 mM allowed pH determination for each sample directly from its ^{1}H NMR spectrum (Valcour & Woodworth, 1986). No attempt was made to correct for deuterium isotope effects, and pH values are reported as pH*. After initial sealing of the tube, the protein sample remained isolated from the environment throughout the course of the experiment.

Deuterium Exchange. Several studies have indicated that at alkaline pH the C(2) proton of histidine will undergo exchange with the protons, deuterons, and tritons of the solvent water (Markley & Cheung, 1973; Bradbury et al., 1980). Coordination of a metal to the histidine has been shown to substantially lower the rate of this exchange (Cass et al., 1979; Baldwin et al., 1979). We performed an experiment to determine the effect of gallium binding to HTF/2N on this exchange phenomenon. Two 80-mg HTF/2N samples were dissolved in 400 μL of 0.1 M ammonium bicarbonate in D_2O in separate NMR tubes. A saturating amount of $GaCl_3$ was added to one sample, and both samples were adjusted to ca. pH* 8.2. An initial spectrum was obtained, and the samples were incubated at 45 °C for 10 days. A spectrum of the sample that was subjected to D_2O exchange in the absence of metal was obtained. The metal was removed from the gallium-loaded sample, and spectra of this D_2O-exchanged apoprotein were obtained at a number of pH*s.

Spectrophotometric pH Titration. HTF and HTF/2N samples were dissolved in a 5 mM Mes/5 mM Tris/0.1 M KCl solution to a final concentration of approximately 1 mg/mL. All buffers were maintained iron free by incubation with Chelex X-100 resin (Bio-Rad), and all glassware was cleaned with 50% nitric acid. Samples were titrated from high to low pH with 0.1 M HCl (diluted from constant-boiling HCl) delivered by a Hamilton syringe and microtitrator. Sample pH was monitored continuously by a Ross 81-03 combination pH electrode attached to a Corning 155 pH/ion meter. The samples were stirred continuously and were maintained at 25 °C in water-jacketed cuvettes. Absorbances were measured by using a Cary 219 spectrophotometer equipped with timer and wavelength programer accessories (Varian). A 10-min delay was maintained between addition of titrant and measurement of sample absorbance. The data are presented in terms of millimolar extinction coefficient by using $E^{1\%}_{280}$ values of 10.00 (Michaud, 1967) for HTF and 10.2 for HTF/2N (Zak et al., 1983) and molecular weights of 35 600 for HTF/2N (Lineback-Zins & Brew, 1980) and 79 550 for HTF (MacGillivray et al., 1982).

Data Analysis and Curve Fitting. Deuterium ion concentration, $[H^+]$, vs. chemical shift, δ_{obsd}, was fitted directly to the equation:

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

where $\alpha = (\delta_{max} - \delta_{obsd})/(\delta_{max} - \delta_{min})$ and n = the Hill coefficient. The nonlinear least-squares modeling was performed with MLAB on-line modeling laboratory (Knott, 1979) on a DEC-20 computer.

RESULTS

^{1}H NMR Spectra of Histidines of Apo-HTF/2N. In apo-HTF/2N the resonances of C(4) protons occurred in the region of major aromatic intensity and could not be well resolved. The C(2) protons were well resolved from all other protein resonances and at certain pH*s could be resolved from each other (Figure 1). Figure 2 shows the titration data for these residues and the computer-generated least-squares fit curves for each residue. The parameters for these fits are listed in Table I.

Histidines 1 and 2 were at lower field and had higher pK'_a's than the other residues of the apoprotein. As the pH* was increased, both resonances broadened considerably to the point where neither peak could be discerned at pH*s >7. Lack of data over most of the titration curve of these resonances led to dependency values near 1.0 for the parameters of the fits and resulted in large errors. Resonances 3–5 tended to overlap over much of the titration range, making them difficult to resolve at some pH*s. Resonance 6 had peculiar titration behavior in that it had a low pK'_a and very low slope. Resonances 7 and 8 did not titrate at all and were broader than other resonances, making them difficult to discern even in convolution difference spectra. These resonances were probably derived from histidinyl residues buried within the hydrophobic interior of the protein. Such residues would not titrate because they would have no interaction with the solvent and would be broadened due to an decreased relaxation time caused by their inability to rotate freely.

The rate of exchange of C(2) protons of certain histidinyl residues was substantially decreased by metal binding. After incubation of the apoprotein for 10 days at 45 °C the ^{1}H NMR spectra showed complete loss of intensity of all resonances corresponding to histidinyl C(2) protons. When the protein that was exchanged in the gallium-loaded form was

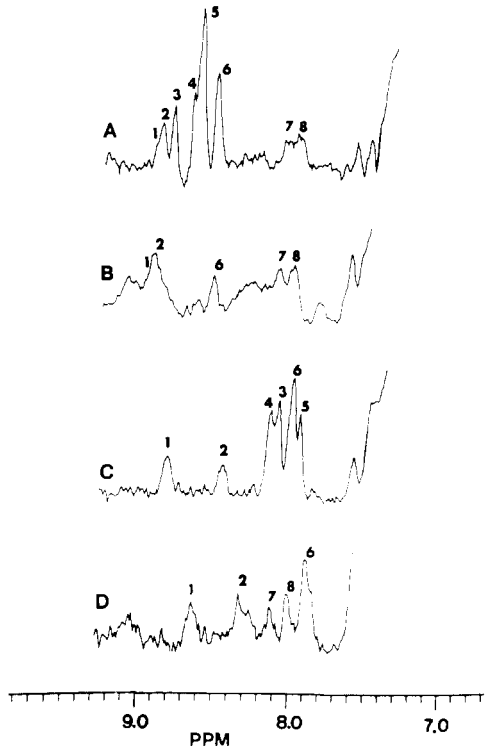

FIGURE 1: Convolution difference spectra of the histidinyl C(2)H region of apo-HTF/2N. Spectra A and C are spectra of apo-HTF/2N at pH* 4.55 and 6.56, respectively. Spectra B and D are spectra of HTF/2N samples that were subjected to a 10-day deuterium exchange in the gallium-loaded form, after which the metal was removed. Spectrum B is at pH* 4.53, and spectrum D is at pH* 6.84.

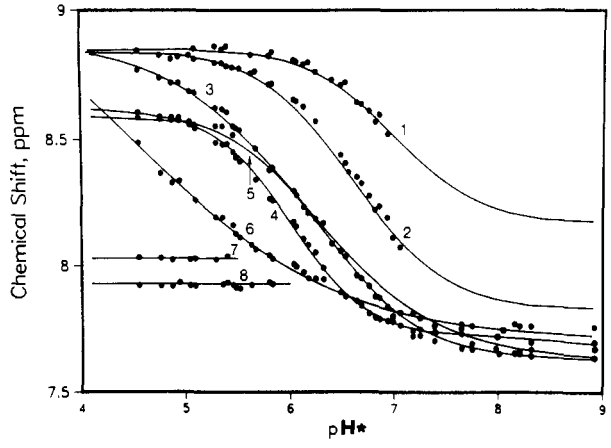

FIGURE 2: Proton nuclear magnetic resonance/pH* titration curves of histidinyl C(2)H resonances of apo-HTF/2N.

made metal and free, complete loss of resonances 3–5 was observed while resonances 1, 2, and 6–8 were protected (Figure 1). Resonances 7 and 8 are thought to arise from buried residues and as such would not be expected to exchange. Residues 1, 2, and 6 were not completely protected, and at least 16 000 transients were collected to obtain the spectra shown in Figure 1B,D. Resonance 7 and 8 are enhanced relative to the unexchanged protein for this reason.

Spectra of Histidines of Gallium-Loaded HTF/2N. The addition of Ga(III) to HTF/2N in the presence of excess oxalate caused distinct changes in the ^{1}H NMR titration behavior of the imidazolium C(2) protons though addition of anion alone caused no effect. In all cases the samples were gallium-loaded at high pH* and titrated to low pH*. The titration behavior was biphasic in that spectral resolution was very good at pH* >6.0, but the quality of the spectra (and the sample) deteriorated at pH* <6.0. The samples began

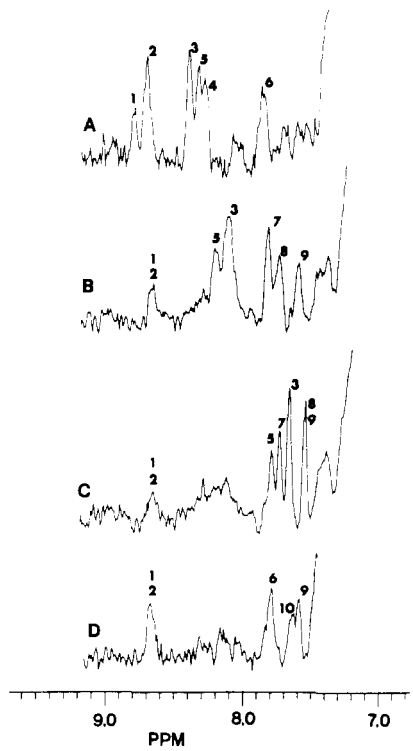

FIGURE 3: Convolution difference spectra of the histidinyl C(2)H region of gallium-loaded HTF/2N. Spectra A, B, and C are spectra of gallium-loaded HTF/2N at pH* 5.75, 6.22, and 7.48, respectively. Spectrum D is the spectrum of an HTF/2N sample that was subjected to a 10-day deuterium exchange in the gallium-loaded form. The pH* of sample D is 7.26.

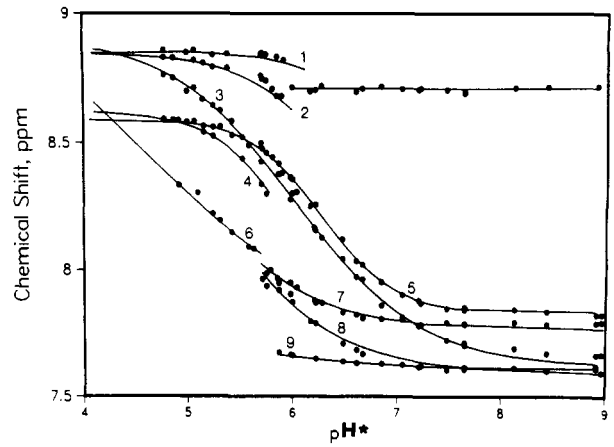

FIGURE 4: Proton nuclear magnetic resonance/pH* titration curves of histidinyl C(2)H resonances of gallium-loaded HTF/2N. Least-squares fit curves at pH*, greater than 6.0 correspond to fits of the gallium-loaded protein titration data. At pH* lower than 6.0 the fits of the apoprotein titration data are shown.

to precipitate at lower pH*, and many transients were needed to obtain well-resolved spectra. The spectra of the gallium-loaded protein are displayed in Figure 3. The titration data and least-squares fits are displayed in Figure 4, and the parameters are listed in Table I.

In spectra of the gallium complex, resonances 1 and 2 merged into one broad peak that failed to titrate until pH* 6.0. Below pH* 6.0 this nontitrating resonance split into a pair of resonances that titrated similarly to apoprotein resonances 1 and 2. There are five other residues that did titrate over the entire pH range. Resonance 3 of the gallium complex titrated similarly to resonance 3 of the apoprotein, probably indicating that they correspond to the same histidine and that this histidine was unaffected by metal binding. The other four

Table I

sample	resonance	pK'_a (±SD)	n (±SD)	δ_{max} (±SD)	δ_{min} (±SD)
apo-HTF/2N	1	7.00 (±0.38)	1.00 (±70.7)	8.846 (±0.176)	8.162 (±0.421)
	2	6.59 (±0.04)	0.94 (±0.04)	8.849 (±0.003)	7.820 (±0.009)
	3	6.08 (±0.09)	0.66 (±0.07)	8.889 (±0.062)	7.624 (±0.015)
	4	6.33 (±0.01)	0.92 (±0.03)	8.619 (±0.005)	7.629 (±0.002)
	5	6.00 (±0.02)	1.17 (±0.05)	8.614 (±0.005)	7.716 (±0.011)
	6	4.49 (±0.88)	0.44 (±0.11)	9.289 (±0.802)	7.708 (±0.031)
	7			8.029 (±0.004)[a]	
	8			7.929 (±0.012)[a]	
apo-HTF/2N (D_2O exchanged)	1	7.00 (±0.91)	1.00 (±83.1)	8.820 (±0.351)	8.162 (±0.294)
	2	6.60 (±0.07)	0.97 (±0.15)	8.848 (±0.007)	7.913 (±0.078)
	6	4.61 (±0.71)	0.50 (±0.09)	9.169 (±0.052)	7.713 (±0.091)
	7			8.047 (±0.007)[a]	
	8			7.946 (±0.010)[a]	
Ga^{III}HTF/2N(C_2O_4)	1			8.712 (±0.013)[a]	
	2			8.712 (±0.013)[a]	
	3	6.07 (±0.03)	0.72 (±0.04)	8.918 (±0.033)	7.619 (±0.016)
	5	6.27 (±0.36)	1.22 (±0.33)	8.592 (±0.411)	7.827 (±0.011)
	7	5.07 (±0.29)	0.78 (±0.12)	8.818 (±0.247)	7.768 (±0.007)
	8	4.83 (±0.28)	0.69 (±0.15)	9.590 (±2.553)	7.593 (±0.003)
	9	5.69 (±0.58)	0.73 (±0.36)	7.734 (±0.055)	7.609 (±0.006)
Ga^{III}HTF/2N(C_2O_4) (D_2O exchanged)	1			8.709 (±0.021)[a]	
	9	5.77 (±0.21)	0.75 (±0.09)	7.727 (±0.018)	7.611 (±0.004)
	6			7.841 (±0.006)[a]	
	10			7.679 (±0.005)[a]	

[a] Chemical shifts of nontitrating peaks.

titrating residues were numbered 5, 7, 8, and 9, but there was no basis on which to assign these to specific resonances of the apoprotein. The small chemical shift change exhibited by resonance 9 and its position at high field relative to the other resonances indicated that it may arise from a C(4) proton. A conformational change occurring subsequent to metal binding may have shifted a C(4) resonance out of the aromatic region.

The protein that had been subjected to deuterium exchange in the metal-loaded form was titrated from high to low pH* in that form. A total of 16 000 transients was accumulated to obtain the high-resolution spectrum displayed in Figure 3. The spectral quality deteriorated at low pH*, and it was not possible to obtain spectra below pH* 6.0. Deuterium exchange simplified the spectra by eliminating resonances 3, 5, 7, and 8. Resonances 1 and 2, which formed one broad, nontitrating peak in the gallium complex, were protected. Resonance 9 remained and maintained the same titration behavior as was observed in the unexchanged sample. This was consistent with resonance 9 coming from an imidazole C(4) proton. The deuterium exchange spectra were especially useful because they allowed two nontitrating resonances to be observed. Resonance 6 was difficult to see in the unexchanged sample spectra because it was broad and nontitrating and lay beneath the sharper resonances 3, 5, 7, and 8. The situation was complicated further because resonance 6 was not present in the spectra of the unexchanged protein at pH* below 6.0. The chemical shift of resonance 6 is constant and approximately equal to the lower asymptote of resonance 6 of the apoprotein. Since the C(2) proton of histidine 6 is also protected from deuterium exchange, we believe that resonance 6 comes from the same residue. Resonance 10 was located in a region where it should have been observable at some pH values in the spectra of the unexchanged metal-loaded protein. It was observed in the exchanged sample spectra for the same reason that resonances 7 and 8 were observed in the HTF/2N–D_2O exchange spectra; the longer accumulation time enhances small resonances. Resonance 10 is probably from a buried histidinyl residue.

The biphasic nature of the gallium complex titration could be explained if the complex had become unstable at pH* <6.0 and started to dissociate. Spectra of the apoprotein and gallium-loaded protein were very similar at pH*s <6.0; e.g., resonances 1 and 2 started to titrate and resonances 3–6 could be accounted for (Figure 4). To determine if the gallium complex was actually dissociating at pH* <6.0, we decided to monitor the gallium binding state as a function of pH. Attempts to measure gallium binding to HTF and HTF/2N through the use of urea gels (Makey & Seal, 1976) failed because the gallium-loaded proteins are not resolved from the apoprotein. The high concentration of urea in the gels probably destablizes the gallium complex and causes the metal to dissociate. The iron-loaded complexes of these two proteins did electrophorese differently than their metal-free counterparts in the same urea gel system. An increase in UV absorbance at 242 nm has been used as an indicator of gallium binding to HTF (Harris & Pecoraro, 1983). We measured the millimolar absorptivity of the gallium-loaded and apo forms of HTF and HTF/2N as a function of pH at 242 and 250 nm (Figure 5). The absorbance of the gallium complex was higher than that of the apoprotein and remained so until pH 6.0 was reached, at which point the absorbance started to decrease to that of the apoprotein. This was true for both HTF and HTF/2N. These data indicate that the gallium did

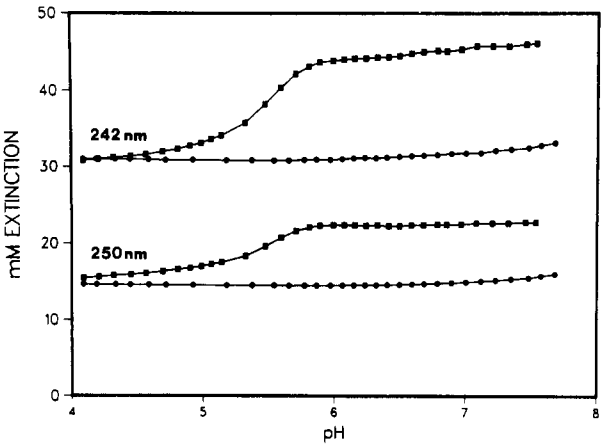

FIGURE 5: Millimolar extinction coefficient of HTF/2N plotted as a function of pH. Titrations were from high to low pH. (■) Gallium-loaded HTF/2N; (●) apo-HTF/2N.

dissociate from HTF/2N at pH <6.0 and that this behavior is consistent with that of the holoprotein.

Discussion

In earlier studies of intact ovotransferrin and HTF, ^1H NMR was used to implicate histidines as metal ligands, but limitations of resolution and sensitivity prevented the analysis of individual C(2)H histidinyl resonances, allowing only groups of histidines to be followed (Alsaadi et al., 1981; Woodworth et al., 1977). The present study takes advantage of the fact that the HTF/2N fragment is less than half of the size of the HTF molecule. Sensitivity was enhanced due to the fact that a higher molar concentration of the half-molecule could be obtained without prohibitive increases in viscosity, and an improvement in resolution was seen because HTF/2N has nine histidines (Lineback-Zins & Brew, 1980) and HTF has nineteen (MacGillivray et al., 1982). Convolution difference was applied to improve resolution further and allowed us to chart the titration of eight of the nine expected resonances of HTF/2N. We analyzed ^1H NMR spectra directly in an attempt to discover broad resonances that would not be seen after convolution difference, but the resolution in these spectra was so poor that no furthur information was obtained.

A series of small organic molecules that contain a free carboxyl group proximal to a functional group capable of binding iron have been shown to substitute for carbonate as the synergistic anion in metal binding to transferrin (Schlabach & Bates, 1975; Woodworth et al., 1975). Because carbonate is converted to CO_2 at pHs below 5.0, we chose to use oxalate in 4-fold molar excess as the obligate anion (Alsaadi et al., 1981). Oxalate has low pK'_a's and exists as a dianion throughout the pH range studied. High-spin ferric iron is a paramagnetic metal and as such tends to broaden beyond recognition the NMR resonances of its ligands. To avoid this complication, we chose to substitute Ga(III) as the bound metal since it is diamagnetic and shows binding kinetics similar to those of iron binding to HTF (Harris & Pecoraro, 1983).

The deprotonation of the imidazolium nitrogen of histidine with increasing pH results in a continuous decrease in the C(2)H and C(4)H chemical shifts (Markley, 1975). A marked change in the pH titration behavior of a histidinyl residue is expected on ligation of its imidazole nitrogen to a metal ion (Cass et al., 1977). When the metal binds stably through the pK'_a region of the imidazole, the environment of the C(2) proton remains constant as compared to the environment of the C(2) proton of an imidazole with a dissociable proton. Metal binding should inhibit motion of the histidine involved in binding and broaden its NMR resonance. These circumstances would account for the titration behavior exhibited by resonances 1 and 2 of HTF/2N at pH* >6.0. The fact that these two resonances were protected from deuterium exchange in the gallium-loaded protein further justifies the statement that they are ligands to the metal (Cass et al., 1979). In the apoprotein, these resonances broaden considerably at high pH*, which might indicate that the histidines of the binding site are in slow exchange between two different conformations. EPR studies show evidence for two distinct binding site conformations for the metal-loaded transferrin (Campbell & Chasteen, 1977), and the ^1H NMR observations might indicate a similar situation in the apo molecule.

The presence of two histidinyl residues per binding site, which act as ligands to the metal, is consistent with previous findings. It has been suggested that a third histidinyl residue might play a role in anion binding. This hypothesis is based upon the results from previous ^1H NMR experiments on ovotransferrin (Alsaadi et al., 1981; Woodworth, 1986; Woodworth et al., 1987) and the observation of three highly conserved histidinyl residues in the putative metal binding domains of HTF, ovotransferrin, and lactoferrin (Chasteen, 1983). A third histidinyl residue of HTF/2N, the one responsible for resonance 6, does not titrate and is protected from deuterium exchange in the metal-loaded form. It is different from the two residues that were implicated as direct metal ligands in that it has a low pK'_a and much lower slope. The low pK'_a could be caused by the presence of a positive center in the vicinity of the histidinyl imidazole, which would be consistent with the imidazole being involved in anion binding. Studies performed on the half-molecules of ovotransferrin gave results similar to those found for HTF/2N (Woodworth et al., 1987). In that case, the residue determined to be involved in anion binding was found to have a lowered pK'_a in the metal-loaded state. This might also be the case for the residue of HTF/2N, but technical problems involving metal complex dissociation prevented the determination of spectra at low pH*. It has been proposed that the low-pK'_a histidine might serve as the base in the proton-relay scheme postulated for the iron–transferrin–carbonate complex (Carrano et al., 1985; Woodworth et al., 1987). The histidine would act as a general base pulling the anion proton toward itself and delivering another proton to hydroxide, forming water or vice versa. This is not a complete transfer, and both entities are probably in intermediate ionic states. The stability of the charge-relay system and the presence of positively charged metal would lower the affinity of the imidazole for a proton and consequently its pK'_a. The position of resonance 6 in the gallium-loaded spectra indicates that histidine 6 remains unprotonated through the entire pH range in which spectra were obtainable. This finding is consistent with histidine 6 being the base in the putative charge-relay system that binds the anion to the protein.

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Inhibition of the Gelation of Extracellular and Intracellular Hemoglobin S by Selective Acetylation with Methyl Acetyl Phosphate†

Hiroshi Ueno, Lennette J. Benjamin, Maria A. Pospischil, and James M. Manning*

The Rockefeller University, New York, New York 10021

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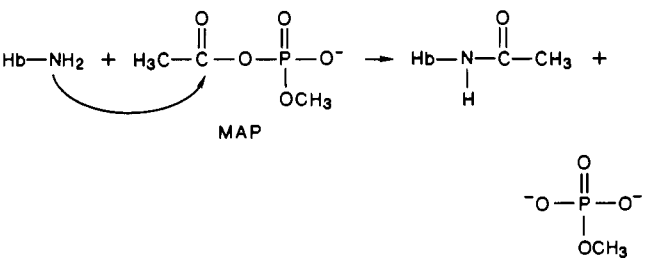
ABSTRACT: Methyl acetyl phosphate binds to the 2,3-diphosphoglycerate (2,3-DPG) binding site of hemoglobin and selectively acetylates three amino groups at or near that site. The subsequent binding of 2,3-DPG is thus impeded. When intact sickle cells are exposed to methyl acetyl phosphate, their abnormally high density under anaerobic conditions is reduced to the density range of oxygenated, nonsickling erythrocytes. This change is probably due to a combination of direct and indirect effects induced by the specific acetylation. The direct effect is on the solubility of deoxyhemoglobin S, which is increased from 17 g/dL for unmodified hemoglobin S to 22 g/dL for acetylated hemoglobin S at pH 6.8. Acetylated hemoglobin S does not gel at pH 7.4, up to a concentration of 32 g/dL. The indirect effect could be due to the decreased binding of 2,3-DPG to deoxyhemoglobin S within the sickle erythrocyte, thus hindering the conversion of oxyhemoglobin S to the gelling form, deoxyhemoglobin S.

Methyl acetyl phosphate (MAP),[1] a monoanionic acetylating agent, was designed (Kluger & Tsui, 1980) for the active site of those enzymes that bind small anions and have a susceptible nucleophile nearby. We have previously shown that MAP reacts with hemoglobin A (HbA) in a very specific fashion (Ueno et al., 1986). Thus, for oxy-HbA there were only three amino groups per $\alpha\beta$ dimer that were acetylated, and these were Val-1, Lys-82, and Lys-144 of the β-chain.



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