Nuclear Magnetic Resonance Titration Curves of Histidine Ring Protons

V. COMPARATIVE STUDY OF CYTOCHROME c FROM THREE SPECIES AND THE ASSIGNMENT OF INDIVIDUAL PROTON RESONANCES

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SUMMARY

A single histidine C-2 proton resonance is seen to shift with change in pH in the NMR spectra of horse ferrocytochrome c, as previously found for horse ferricytochrome c (COHEN, J. S., FISHER, W. R., AND SCHECHTER, A. N. (1973) J. Biol. Chem. 249, 1113-1118). The pKα value (6.5) of this histidine residue in the reduced form is almost the same as that (6.4) in the oxidized form, and only a small upfield shift is observed in the reduced form, indicating a slightly more electronically shielded environment in this case. No titrating resonance is observed in the NMR spectra of tuna ferri- and ferrocytochrome c in the pH range 5 to 8. Since histidine residue 33 in horse cytochrome c is replaced by a tryptophan residue in the analogous position in tuna cytochrome c, it can be concluded that the titrating resonance observed for the horse protein can be assigned to histidine residue 33.

A sharp resonance appears in the spectra of tuna ferricytochrome c in the region of absorption of histidine ring C2 protons when the pH is adjusted below pH 3.5. This resonance can be assigned to histidine residue 26, the only unliganded histidine residue present (histidine 18 is liganded to the iron atom and does not absorb in this region). A similar resonance is observed under the same conditions in spectra of horse ferricytochrome c. Its attribution to histidine residue 26 enables a consistent assignment of the 2 observed histidine ring C2 proton resonances to the 2 unliganded histidine residues in the horse protein.

The imidazole side chain of histidine 26 is believed to be hydrogen-bonded to the peptide bond carbonyl oxygen atom of proline 44 (DICKERSON, R. E., et al. (1971) J. Biol. Chem. 246, 1511-1533). Presumably, the protonation of this imidazole side chain, leading to the breakage of this hydrogen bond, is one of the first steps in the acid-induced conformational transition of cytochrome c, which has a midpoint at pH 2.5.

A comparative study of the pH-dependent chemical shifts of resonances in the spectra of yeast ferricytochrome c enables the three C-2 proton resonances observed to be assigned to the 3 unliganded histidine residues present in this case (26, 33, 39). The similarity of the pH-induced chemical shifts of the analogous histidine C2 proton resonances in tuna, horse, and yeast cytochrome c indicates a similarity in the environments of the histidine residues in these cytochromes from widely divergent species.

In previous investigations of the conformation of horse ferricytochrome c by nuclear magnetic resonance spectroscopy, we observed a single titrating histidine ring C-2 proton resonance (1). McDonald and Phillips (2) have recently published an extensive analysis of NMR spectra of horse cytochrome c species. They also observed a titrating histidine C-2 proton resonance which they tentatively assigned to histidine residue 26 in the amino acid sequence.

The assignment of individual resonances in protein NMR spectra has aided considerably in the interpretation of spectral phenomena (3). We now describe the results of comparative studies of cytochrome c from three species, horse, tuna, and yeast, which enable us to assign this single titrating resonance to histidine residue 33. Comparison of other features of the NMR spectra, particularly in the pH range 3 to 3.5 of the acid transition (4, 5), enable other individual resonance assignments and conformational similarities to be delineated. In such comparisons, the imidazole side chain protons act as nondisturbing, sensitive probes for their immediate environments within the protein molecule.

EXPERIMENTAL PROCEDURE

Horse heart and tuna heart ferricytochrome c were purchased from Sigma Chemical Co. and yeast (Candida krusei) ferri cytochrome c from Calbiochem (preparation of Sankyo Co., Tokyo, Japan). No further purification was made; NMR spectra of horse ferricytochrome c were essentially identical with those from previous studies (1). Samples for NMR studies were lyophilized four or five times from D2O (99.7%, Merck) and dissolved in 0.1 M NaCl-D2O (100%, Bio-Rad) at concentrations of about 100 mg per ml (8 × 10⁻³ M).

NMR spectra were recorded at 220 MHz on a Varian Associates FT220 NMR system at an ambient probe temperature of 20 ± 1°C. A 90° pulse (40 μs) was used, usually with an acquisition time of 1.6 s, giving a resolution of 0.7 Hz per point for an 8 K point transform. A pulse delay of 2 to 4 s and a sensitivity enhancement factor of 0.6 s were usually used. With these conditions excellent...
spectra were usually obtained in 25 scans, taking a total time of 3 min. All chemical shifts are relative to 6% tetramethylsilane in carbon tetrachloride. Values of pH are direct meter readings using a microcombination glass electrode (Ingold) inserted into the protein solution in the NMR tube. No correction was necessary for the deuterium isotope effect on histidine ionizations (6). Adjustments of pH were made with solutions of 0.1 M NaOD and DCl (Merek) containing 0.1 M NaCl. Curve fitting of titration data using computer methods with a theoretical expression for a single proton association-dissociation equilibrium was employed as described previously (7).

Horse and tuna ferrocytochrome c were prepared in the following manner. Sodium ascorbate (0.1 M) in D$_2$O containing 0.1 M NaCl was added to the lyophilized D$_2$O-exchanged ferri forms, as prepared above. The final ascorbate concentration was 0.02 M. The criterion used to verify complete reaction was based on the ratio of the visible absorption maxima 550-520 nm. Visible absorption spectra of the samples of each protein in the presence of 0.02 M ascorbate and 0.1 M Tris, pH 7.8, and also in the presence of a large excess of ascorbate in the same buffer were taken with a Cary 14 scanning spectrophotometer. The ratios for both samples of horse cytochrome c were identical (1.73) and compared well with the value (1.74) found by Schejter et al. (8) for reduced horse cytochrome c in phosphate buffer, pH 7.4. The ratios for tuna ferrocytochrome c were 1.84 and 1.86 in the presence of 0.02 M ascorbate and a large excess, respectively. All spectra were taken under a nitrogen atmosphere at 25°C. A value of 27.6 was used for the millimolar extinction coefficient at 550 nm for the reduced form. This can be partly attributed to the absence of the effect of the unpaired electron spin in the reduced form. This represents a somewhat more electronically shielded environment, with no major changes in adjacent charges indicated for this histidine C-2 proton in horse ferrocytochrome c.

NMR spectra of tuna ferro- and ferricytochrome c are shown in Fig. 3. In neither form is any resonance observed to shift in this same region of the spectrum and in the pH range in which a pK value for a histidine residue is generally found (6). The absence of a titrating resonance in the reduced and oxidized form of tuna cytochrome c and the presence of such a resonance in both forms of horse cytochrome c (Table I) would tend to indicate the assignment of this titrating resonance to the extra histidine residue present in the primary structure of the horse protein. This histidine is located at position 33 and replaces a tryptophan residue at that analogous position in the amino acid sequence of the tuna protein (10).

RESULTS

The downfield region of NMR spectra of horse ferrocytochrome c at different pH values are shown in Fig. 1. One titrating resonance corresponding to a histidine ring C-2 proton and one corresponding to a C-4 proton (6) are clearly observed. Much of the fine structure which was present in this region of the spectrum of horse ferrocyanochrome c (1) is absent in the spectrum of the reduced form. This can be partly attributed to the absence of the effect of the unpaired electron spin in the reduced form. Thus, the single titrating C-2 proton resonance is more readily seen in this case, and its NMR titration curve is readily plotted (Fig. 2). (The C-4 proton becomes obscured in the region of aromatic resonances.) These data indicate the presence of a single titratable histidine residue with a pK$_a$ value of 6.54 ± 0.02. This pK$_a$ value is very close to that found for a very similar titrating resonance in the oxidized form (Table I). There is a small upfield shift of the whole titration curve in the reduced form. This represents a somewhat more electronically shielded environment, with no major changes in adjacent charges indicated for this histidine C-2 proton in horse ferrocytochrome c.

Fig. 2. Titration curve of the histidine ring C-2 proton resonance of horse ferrocytochrome c. ——, theoretical fit assuming a simple proton association-dissociation equilibrium (7). ---, theoretical fit for the oxidized form (1).

<table>
<thead>
<tr>
<th>Cytochrome c</th>
<th>His present in sequence</th>
<th>His C-2-H titrating*</th>
<th>pK$_a$</th>
<th>$\Delta^{ppm} \pm 0.01$</th>
<th>$\delta_{min}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuna (Fe$^{II}$ and Fe$^{III}$)</td>
<td>18, 26</td>
<td>None</td>
<td>6.54 ± 0.02</td>
<td>1.25</td>
<td>7.59</td>
</tr>
<tr>
<td>Horse Fe$^{II}$</td>
<td>18, 26, 33</td>
<td>33</td>
<td>6.41 ± 0.02</td>
<td>1.23</td>
<td>7.42</td>
</tr>
<tr>
<td>Yeast (Fe$^{III}$)</td>
<td>18, 20, 33, 39</td>
<td>39</td>
<td>6.74 ± 0.01</td>
<td>1.11</td>
<td>7.41</td>
</tr>
</tbody>
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* His-18 is liganded to the iron atom and does not titrate.

$\Delta$ is the change of chemical shift on titration and $\delta_{min}$ the chemical shift value at high pH (see ref. 6).

TABLE I

Properties of titrating histidine residues in cytochromes

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</tr>
</tbody>
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* From Ref. 1.
Spectra of horse and tuna ferricytochrome c taken at the pH range of the acid conformational transition\(^1\) (4, 5) are compared in Fig. 4. Previous work has described the quite sudden appearance of a second single proton resonance in this region of the spectrum of horse ferricytochrome c between pH 3.5 to 3.2 (1). The sample used in the present investigation was well exchanged with D\(_2\)O, and lacked some of the broader slowly exchanging \(\text{N}-\text{H}\) resonances present in the previously reported spectra, which appeared to somewhat reduce the apparent sharp cooperative nature of the appearance of this resonance. Its position, somewhat upfield from the single titrating histidine C-2 proton resonance observed, and its coalescence with the totally denatured C-2 proton resonance at pH \(\leq 1\), indicated that this resonance is also derived from a histidine C-2 proton (1). In the case of tuna ferricytochrome c spectra only the upfield resonance is observed and exhibits essentially the same properties as that in the horse protein. In view of the fact that histidine 26 is the only unliganded histidine residue present in tuna cytochrome c, this resonance may be assigned to the C-2 proton of histidine 26 in both proteins. Reduction of the pH of horse ferrocyanochrome c to a value of 3 results in autoxidation. The area of the single titrating resonance was seen to decrease while in close proximity to it a similar resonance was observed to increase in area. This peak was 0.07 ppm upfield and presumably represented the single titrating resonance observed in horse ferricytochrome c. These observations tend to confirm the identity of these resonances with a single histidine residue in the amino acid sequence. The chemical shift separation is less than that (0.17 ppm) indicated by a direct comparison of the two sets of data (Fig. 3). This difference may be ascribed to variations in the bulk diamagnetic susceptibility commonly observed from one protein sample to another (normally about 0.1 ppm).

\(^1\) Measurement of the acid induced conformational change by absorbance at 395 nm by the method of Babul and Stellwagen (5) indicated a small difference in the midpoint of the curves when the protein was dissolved in D\(_2\)O and H\(_2\)O. Measurements of pH as uncorrected meter readings (6) gave values for the midpoints of 2.37 (D\(_2\)O) and 2.45 (H\(_2\)O).
Fig. 5. The downfield region of 220-MHz NMR spectra of yeast ferricytochrome c in 0.1 M NaCl-D$_2$O at several pH values. Three individual histidine C-2 proton resonances are observed (pH 3.51), but only one titrates throughout the pH range (pH 7.14).

Fig. 6. Titration curves of the histidine ring C-2 proton resonance of yeast ferricytochrome c. The most downfield resonance broadened sharply at pH 6 and its whole curve could not be delineated. The most upfield resonance behaved very similarly to those of the horse and tuna proteins at pH 3 to 3.5 (Fig. 4).

DISCUSSION

High resolution NMR studies of proteins have benefited greatly from the assignment of resolved resonances to individual atoms in a protein molecule (3). The first such unequivocal assignment was that of the single titrating ring C-2 proton resonance of histidine 15 in hen egg white lysozyme (11). The first comparison between homologous proteins differing in a single histidine residue to obtain such an assignment utilized nucleases from two distinct strains of Staphylococcus aureus (12).

There is a great deal of homology between the amino acid sequences of cytochrome c from many widely divergent species (13). All cytochrome c's have a histidine residue at position 18 in the polypeptide chain which is liganded to the iron atom in the heme moiety. This results in a contact shift of the ring protons to the far downfield portion of the spectrum (14) and they were not observed in the present work. Most other cytochrome c's have histidine residues at positions 26 and 33. However, variants are observed in some species, and in tuna cytochrome c, for example, histidine is replaced by a tryptophan residue at position 33 (10).

We have previously described a single titrating histidine C-2 proton resonance in horse ferricytochrome c (1). This is consistent with the much earlier observation of Theorell of a single normally titrating histidine residue using potentiometric methods (15). The present work reports a titrating resonance (Figs. 1 and 2) with very similar characteristics in the reduced form of the horse protein (Table I). Unless an unusual conformational change accompanies the oxidation-reduction process which completely masks a normally titrating histidine residue and unmasks a nontitrating residue, it is reasonable to conclude that these represent the same histidine residue. The only differences found are a slight increase in pK value and a decrease in chemical shift representing a slightly more electronically shielded environment in the reduced form. Also, the observation of a slow conversion between these resonances as ferricytochrome c autoxidizes at pH 3 tends to confirm their common origin.

Careful comparison of spectra of tuna cytochrome c in both the oxidized and reduced forms reveals no titrating resonance between pH 5 and 8 as that observed in the horse protein (Fig. 3). Tuna cytochrome c contains only one unliganded histidine residue at position 26 in the amino acid sequence. Hence, the titrating resonance in the spectra of horse cytochrome c may be assigned to the histidine residue at position 33 in the amino acid sequence.

Between pH 3.5 and 3 a single sharp resonance appears in the downfield region (8.54 ppm) in the spectra of tuna ferricytochrome c (Fig. 4). A very similar phenomenon is seen to occur in the horse protein. This must be considered one of the first steps in the acid-induced conformational transition (4). The over-all transition is seen as highly cooperative by techniques which monitor essentially the structure of the heme pocket (1, 5) and has a midpoint at pH 2.5. Histidine residue 18 would not, therefore, be expected to be cleaved from the iron atom until this pH value, and previous NMR studies indicate that the histidine resonances do not become equivalent, indicating complete denaturation, until pH < 1.8 (1). In view of the fact that the resonance observed at pH < 3.2 can be ascribed to a second discrete histidine C-2 proton (1), and tuna cytochrome c has only 1 unliganded histidine, this acid-induced peak must arise from histidine 26. This enables a consistent assignment of these two individual resonances in the NMR spectra to 2 histidine residues in horse cytochrome c.

The regions surrounding the 2 histidine residues in question in the structure of horse ferricytochrome c as determined by x-ray crystallography (16) are shown in Figs. 7 and 8. Although both histidine residues are near the surface of the molecule, the environment of histidine 26 appears to be more crowded, particu-
FIG. 7. An 8 Å section centered on the imidazole ring of histidine residue 26 in the x-ray crystallographic structure of horse ferricytochrome c (16). This was generated using the NIH-XRAY molecular modeling system (20). The exterior of the protein is at the top.

FIG. 8. An 8 Å section centered on the imidazole ring of histidine residue 33 of horse ferricytochrome c (see Fig. 7).

larly with hydrophobic residues such as tyrosine 46 and prolines 30 and 44, compared to the environment of histidine 33. Also, histidine 26 appears to be hydrogen-bonded to the carbonyl group of proline 44 (15, 16). On this basis, one would expect histidine 33 to be the more freely titrating histidine residue. This is consistent with our assignment for the one titrating histidine resonance but contrasts with the recent tentative conclusion of McDonald and Phillips (2), on the basis of the protein structures determined by x-ray crystallography, that the titrating resonance arises from histidine 26. In arriving at their conclusion, it appears that they neglected the fact that position 33 in tuna ferrocytochrome c is a tryptophan residue. This is buried in a hydrophobic pocket (17) in contrast to the accessibility of histidine 33 found in the horse ferricytochrome c structure (Fig. 8). Thus, the salient difference manifested here for residue 33 is not that between oxidation states but rather that between cytochromes from different species with different primary sequences. It is to be anticipated that in the structure of horse ferrocytochrome c histidine 33 will be solvent accessible and in tuna ferricytochrome c tryptophan 33 will be buried, much as these residues are in their respective alternate oxidation-reduction forms. Apart from providing a cautionary note to the use of x-ray structures of crystals to rationalize NMR observations in solution, our results emphasize the need for careful experimental comparisons among homologous proteins from different species, such as in the case of the myoglobin (18), before individual resonance assignments can be considered acceptable.

The relatively hydrophobic nature of the pocket in which histidine 26 is found would probably result in an upfield shift of the resonances of the imidazole side chain. The fact that the resonance assigned to this residue appears to be broadened above pH 3.0 (Fig. 4) would indicate that the imidazole side chain is held in a rigid configuration by the hydrogen bond in the native conformation, resulting in efficient inter-residue dipolar proton relaxation. A protonation process at about pH 3.2, perhaps involving this side chain, would appear to result in the disruption of this hydrogen bond as one of the first steps in the acid-induced conformational transition. The C-2 proton resonance then shifts in a cooperative manner over a narrow pH range to its normal downfield position.

The acid-induced conformational change involving histidine 26 occurs almost identically for the three cytochromes studied here (Figs. 4 to 6). This can be considered to indicate a highly specific environment for the side chain of histidine 26 which is probably conserved in the structure of these and other cytochrome c's. In this respect, it is interesting to note that position 26 is almost invariant in the amino acid sequence of many cytochrome c's (one case of substitution with glutamine) while position 33 is quite variable (five substitutions) (13).

In comparing the results for the cytochrome c from yeast with results for the cytochromes from the organisms higher on the phylogenetic scale, several similarities are evident. While other NMR results indicate some differences in the environments of homologous amino acid side chains in cytochromes from different species (2), our results indicate a high degree of similarity for the environments of 2 homologous histidine residues in three cytochromes from divergent species. In this sense, NMR may be used as a tool to monitor the degree of evolutionary change in the electronic environment of a residue in a series of homolo-
gous proteins, which may not be the same as the more commonly employed parameter of sequence evolution (19).

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REFERENCES
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