Spectroscopic Studies on the Conformation of Cytochrome c and Apocytochrome c

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SUMMARY

The appearance of the downfield region of the PMR spectrum of apocytochrome c is consistent with that of an extensively disordered protein. The resonances of the three histidine C-2 protons are almost equivalent and have a pKₐ of 6.2. In contrast, this region of the ferricytochrome c PMR spectrum shows many sharp resonances, due to the tertiary structure of the protein and contact shifts from the heme group. The complete titration of one histidine residue, with a pKₐ of 6.41, can be determined in the holoprotein. At low pH, the resonance of a second histidine residue can be observed.

The acid-induced conformational transition of ferricytochrome c can be resolved into several component transitions by the use of different spectral parameters. Measurements of absorbance at 305 nm and fluorescence emission at 340 nm show a single cooperative transition with midpoint of about pH 2.5. These indices are closely related to the geometry of the heme group. The resonances of the two observable histidine residues change in a way suggesting denaturation only below pH 1.8, but indicate conformational changes at about pH 3. The resonances assigned to heme methyl groups, however, undergo significant changes between pH 3 and 4.

The reduced viscosity of acid-denatured ferricytochrome c and the fluorescence emission of its tryptophan residue increase markedly as salt concentration is lowered, indicating that the conformation of the acid denatured protein is sensitive to ionic strength.

Horse heart ferricytochrome c has been demonstrated by x-ray crystallography to have a compact globular structure with the heme group buried in a crevice (1). It is of importance to determine the extent to which this prosthetic group influences the folding of the protein. Recent studies using several hydrodynamic and spectroscopic techniques have presented evidence of the dependence of the compact native structure of cytochrome c on the presence of the porphyrin ring (2, 3). We have used high resolution proton magnetic resonance (PMR) spectroscopy as an additional means of probing the conformational properties of cytochrome c and of its apoprotein.

Cytochrome c is also known to undergo a reversible conformational transition at acid pH, and recently Babul and Stellwagen have documented that this major transition is highly cooperative, occurring over a narrow pH range with a transition midpoint of about 2.5 (4). We have studied this structural transformation with PMR spectroscopy as a probe of the chemical environment of the histidine residues and of the methyl groups of the heme prosthetic group, with fluorescence measurements of the emission of the solitary tryptophan residue, and with measurements of the Soret absorption of the heme group. Thus, it has been possible to monitor separately certain of the events which accompany the gross acid-induced conformational transition.

MATERIALS AND METHODS

Horse heart ferricytochrome c was purchased from Sigma and purified chromatographically as described by Fisher et al. (3). Apocytochrome c was prepared by a modification of the silver sulfate method (3). Samples for PMR studies were lyophilized from 99.7% D₂O and dissolved in 0.1 M NaCl-D₂O (100%, Bio-Rad). Solutions for PMR studies were made up to about 100 mg per ml. Concentrations were determined by amino acid analysis in studies to determine the number of histidine protons in titrating peaks.

PMR spectra were recorded at 220 MHz on a Varian Associates HR 220 Spectrometer with an ambient probe temperature of 22 ± 1°C. Time-averaging was done on a Varian C-1024 computer of average transients, as described previously (5, 6). All chemical shift values are from 6% tetramethylsilane in CDCl₃, with positive values downfield and negative values upfield. Difference spectra were obtained using a modification of a computer program which had been developed by Dr. John Markley (Purdue University) and modified by Mildred McNeel (National...
FIG. 1. The downfield region of 220 MHz PMR spectra of apocytochrome c in 0.1 M NaCl-D$_2$O at the pH values indicated. The assignment of resonances are: the resonance which shifts from 8.4 to 7.6 ppm, His C-2-H; the sharp resonance which shifts with pH from 7.12 ppm (pH 5.00) to 6.8 ppm (pH 6.85), His C-4-H; broad peaks 6.0 to 7.2 ppm, phenylalanine; other broad peaks 6.6 to 7.2 ppm, tyrosine and tryptophan. These resonances are probably broadened by viscosity and aggregation.

Institutes of Health) which normalized the area under the two curves and provided a horizontal base-line between the chosen extremities. Computer curve fitting of spectra to obtain area values and pH titration data to obtain pK$_a$ values was done as described (5). Values of pH are direct meter readings using a microcombination glass electrode (Ingold) inserted into the protein solution in the PMR tube.

Absorption measurements were made on a Cary 15 recording spectrophotometer using a 1-cm path length cuvette. Fluorescence measurements were made on an Hitachi-Perkin-Elmer SP-2 Fluorometer using 3-cc cuvettes with excitation at 280 nm (band pass = 7 nm); emission spectra from 290 to 400 nm (band pass = 9 nm) were recorded. pH adjustments and measurements were made in the cuvette; all samples had 0.1 M NaCl except when specified. Viscosity measurements were performed as previously described (3).

RESULTS

The high resolution PMR spectra of apocytochrome c in the aromatic region showed relatively little fine structure (Fig. 1). Resonances corresponding to chemically equivalent imidazole C-2, C-4, and aromatic protons were seen. These spectra are similar to those reported for various denatured proteins (6-10). The histidine C-2 and C-4 proton resonances were readily identified in each spectrum. At intermediate pH values two C-2 proton maxima were observed, suggesting that the histidine residues existed in slightly different environments. The pK$_a$ values obtained from the theoretical fits of the C-2 proton data (Fig. 2), including the two resolved maxima, were 6.21 ± 0.02 and 6.10 ± 0.02 (5). The area of the single peak at pH 5.00 was found to correspond to three protons (relative area = 3.05) by comparison with the spectrum of a standard histidine solution.

High resolution PMR spectra of the aromatic (low field) region of ferricytochrome c showed many sharp resonances (Fig. 3). These arise, in part, from selective shift effects on protein and porphyrin resonances, due to shielding by the porphyrin rings and the iron atom (11). The very low viscosity of cytochrome c (3) results in a low correlation time for the protein and also contributes to the formation of sharp, clearly resolved resonances. Sixteen time-averaged spectra at pH values between 4 and 9 were obtained, and the chemical shift values of the 19 peaks resolved in the aromatic region were plotted as a function of pH. Four peaks were observed to shift appreciably with pH. Only one

1 Of the remaining peaks, (δ = 6.8 ppm at pH 5.87) shifted 0.2 downfield below pH 5, another (δ = 7.7 at pH 5.87) shifted 0.2 ppm upfield above pH 5. The most downfield peak in this region (δ = 9.6 at pH 5.87) shifted downfield from 9.1 ppm at pH 4 to 9.7 ppm above pH 7. The origin of these three resonances is unknown.

FIG. 2. Titration curve of the C-2 proton resonance of apocytochrome c, corresponding to 3 protons at low pH, but showing a bifurcation at two intermediate pH values. The lines are theoretical fits assuming a simple proton association-dissociation equilibrium (5). The change of chemical shift on titration, 0.98 ppm, is very close to that in model compounds (21).

FIG. 3. The downfield region of 220 MHz PMR spectra of ferricytochrome c in 0.1 M NaCl-D$_2$O at the pH values indicated. The arrow indicates the position of the single titrating His C-2-H resonance at each pH value.
Fig. 5. Titration curve of the single C-2 proton resonance of ferrocytochrome c. The line is a theoretical fit as in Fig. 2. The change in chemical shift on titration was 1.25 ppm.

peak gave a clear titration curve characteristic of a histidine ring C-2 proton resonance, the identification of which was aided by taking differences between several pairs of spectra (Fig. 4) (12). The PMR titration curve of this peak is plotted in Fig. 5 and fitted to a theoretical curve for a simple proton association-dissociation equilibrium with a $pK_a$ value of 6.41 ± 0.02. The area of this peak in a protein solution of known concentration at pH 3.19, relative to that of the C-2 proton resonance of a standard solution of histidine, was found to be 1.05.

It was possible to obtain information about the acid-induced structural transition of cytochrome c by studying PMR spectra at low pH. Fig. 6 shows spectra of the protein below pH 3.5. At these pH values the C-2 proton discussed above was downfield (8.8 ppm) from the other aromatic resonances and corresponded to the protonated form of the imidazole ring. However, between pH 3.5 and 1 it shifted slightly upfield, then downfield, suggesting several proton-associated processes affecting its chemical environment (Fig. 7). An additional peak appeared at pH 3.19, and its position shifted further downfield upon decreasing the pH. This peak had an area of unity (0.95) relative to that of the titrating C-2 proton at pH 3.19. Presumably this resonance represents a second histidine C-2 proton in cytochrome c.

As the pH was lowered below 2, the apparent area of these two peaks decreased, and a single broad resonance was observed.
Fig. 6. The downfield region of 220 MHz PMR spectra of ferri-
cytochrome c at low pH values in 0.1 M NaCl-D2O. The most
downfield resonance (8.8 ppm at pH 3) is that which titrates at
higher pH (Fig. 5). The alteration of spectral characteristics
between pH 3.47 and 3.19 and pH 1.80 and 0.66 reflects separate
acid-dependent conformation transitions. The lowest spectrum
was obtained on returning the pH from 0.66 to 3.31.

Fig. 7. Chemical shift changes of the resolved C-2 proton
resonances shown in Fig. 6. The solid line joins the data points
for the broad resonance of presumed completely denatured histi-
dine. ×, points regenerated on increasing pH.

(Fig. 6). This transition occurred between pH 1.80 and 0.66.
Upon back titration by addition of alkali, these two resonances
were again resolved, indicating reversibility of this transition.
The absence of the broad resonances present before denaturation
indicated that these were due to N—H protons, which had ex-
changed for deuterium during denaturation.

Two well resolved resonances were observed in the upfield
region of the spectra of cytochrome c at pH 7.5 (Fig. 8), and these
had relative areas of 1:1. They were seen to coalesce in a broad
peak between pH 4.0 and 3.7. It was impossible to measure the
areas of these resonances accurately in the transition region due
to their overlap and broadness. This transition was also com-
pletely reversible.

The acid transition was studied by measurements of the Soret
spectrum as a function of pH from 7.0 to 1.0. The results were

Fig. 8. Portion of the upfield region of 220 MHz spectra of ferri-
cytochrome c in 0.1 M NaCl-D2O at the pH values indicated.
These resonances have been attributed to heme-methyl groups

identical to those of Babul and Stellwagen (4) with an isosbestic
point at 405 nm. A plot of per cent change at 395 nm (Fig. 9)
as a function of pH showed a cooperative transition with a mid-
point at pH 2.5, as they had found.

Fluorescence emission spectra showed almost complete quench-
ing above pH 4. This is presumably due to nonradiative energy
transfer from the single tryptophan residue and from the four
tyrosine residues to the heme group. As the pH was lowered,
emission characteristic of these residues was observed. Fig. 9
also shows the fluorescence emission at 340 nm, normalized to
100% change, as a function of pH. The transition is almost
identical to that measured by absorption spectroscopy.

It has been shown by viscosity measurements that 6 M guani-
dine hydrochloride causes a structural change in cytochrome c
so that it approaches random coil behavior (3). Similarly, the
heme quenching of tryptophan fluorescence is abolished by this
solvent (3). To test if the acid state was so randomized, guani-
dine hydrochloride was added to the acidified protein to make it
6 M. It was found that the intrinsic fluorescence (after correction
for dilution) doubled, indicated residual quenching interactions
in the acidified cytochrome c.

These quenching interactions, at pH 1.9, proved very sensitive
to the ionic strength of the solution (Fig. 10A) for, as the con-
studies of native cytochrome c have concentrated mainly on the
groups during a denaturation process (6, 10). Previous PMR
means of monitoring detailed changes in individual atoms or
centration at pH 1.9, 25", and protein concentration 6.8 X 10^-4
value increased to 6.9 ml per g (Fig. 10B). It is evident that the molecular domain of the protein increases strikingly at low ionic strength, presumably due to intramolecular charge repulsion. Both the fluorescence and viscosity data indicate that, although ferricytochrome c undergoes an acid structural transition in 0.1 M NaCl, the protein still maintains some compact structure. Only when the ionic strength of the acidified solution is reduced does the protein unfold in the manner demonstrated by Babul and Stellwagen (4).

Fig. 10. A, changes in fluorescence emission of ferricytochrome c at 340 nm, with 290 nm excitation, at pH 1.9 as a function of NaCl concentration. Relative fluorescence emission is expressed as a percentage of the maximum emission occurring in the absence of NaCl. Protein concentration, 5 X 10^-8 M. B, changes in the reduced viscosity of ferricytochrome c as a function of NaCl concentration at pH 1.9, 25", and protein concentration 6.8 X 10^-4 M.

centration of NaCl was varied from about 0.13 M toward 0, the fluorescence emission increased, approaching that value found in 6 M guanidine hydrochloride at pH 1.9. The shape of the acid-induced transition and its midpoint, however, were relatively insensitive to the NaCl concentration.

To clarify further the effect of salt on the conformation of acid-denatured ferricytochrome c, the reduced viscosity of the protein was measured in water and after addition of increments of NaCl (Fig. 10B). It is evident that the molecular domain of the protein increases strikingly at low ionic strength, presumably due to intramolecular charge repulsion. Both the fluorescence and viscosity data indicate that, although ferricytochrome c undergoes an acid structural transition in 0.1 M NaCl, the protein still maintains some compact structure. Only when the ionic strength of the acidified solution is reduced does the protein unfold in the manner demonstrated by Babul and Stellwagen (4).

DISCUSSION

The PMR spectra of apocytochrome c and the measurements of an area corresponding to three protons for the single C-2 resonance at low pH showed that the environments of the three histidine residues were closely similar (Fig. 1). In addition, the lack of fine structure in the rest of the aromatic region is characteristic of denatured proteins (6-10). These findings suggest that the protein exists in a disordered state. They complement previous studies of the viscosity and of the absorption, fluorescence, and circular dichroism spectra of horse heart apocytochrome c, indicating that the porphyrin is necessary to stabilize the native conformation (2, 3).

The splitting of the C-2 peak at intermediate pH values indicates some detectable difference in the environment of the histidines. Possibly this may arise from different neighboring amino acids in the primary sequence or from residual tertiary structure. The apoprotein in its extensively disordered state may contain areas of local structure and is presumably not an idealized random coil.

Apart from enabling a qualitative distinction between grossly different conformational forms of a protein, PMR provides a means of monitoring detailed changes in individual atoms or groups during a denaturation process (6, 10). Previous PMR studies of native cytochrome c have concentrated mainly on the resonances which are shifted to high or low field by paramagnetic effects of the unpaired electron on the heme group in the oxidized state (13-19). One study of the effects of pH and temperature on these resonances has recently been reported (20).

The interpretation of the PMR spectra of ferricytochrome c in the aromatic region was made difficult because of the plethora of resonances. That these arise from the compact folded protein structure as well as the presence of the prosthetic group was shown by the specific differences in the downfield region compared to spectra of the apoprotein. One peak could be clearly resolved and identified as a single histidine resonance by measurements of its area and titration properties. It had a pK of 6.4, which is close to that of free histidine in solution (21). At pH 3.19 another peak of similar area appeared, which probably arises from a second histidine residue. These peaks probably result from histidine residues 26 and 33, because histidine residue 18, which is liganded to the iron atom, is presumably shifted further downfield (14). However, the specific identity of each resonance is not certain. These two resonances approach each other as the pH is lowered. A single broad peak, seen in the pH 0.66 spectrum, increases in area at the expense of these two peaks. This broad resonance presumably corresponds to the equivalent protons in the acid-denatured form of cytochrome c. This peak is broadened probably as a result of the increased viscosity in the denatured state. The over-all transition was shown to be reversible by regeneration of the two discrete resonances at higher pH (Fig. 6).

Obervation of the resolved resonances in the upfield region of the spectra indicated a significant transition occurring between pH 4.0 and 3.7 (Fig. 8). The resonances in this region are believed to arise, in the oxidized form of the protein, from methyl groups on the heme (7). The PMR results thus would indicate some modification of the structure of the heme crevice occurring at pH values more alkaline than those affecting the properties of the two observable histidine C-2 proton resonances.

These acid-induced changes in the electromagnetic properties of several residues in the protein should be compared to the changes in the absorption and fluorescence properties of the molecule. We have found, in confirmation of the absorption results of Babul and Stellwagen (4), that by these latter criteria the transition is highly cooperative with a midpoint at pH 2.5. These parameters reflect primarily the environment of the heme pocket (the tryptophan is ordinarily in proximity to one of the isopropyl substituents of the porphyrin ring (1)). The marked changes in the histidine C-2 proton resonances occur at lower pH values than those parameters which reflect the heme environment. Thus, as with previous studies of staphylococcal nuclease (6) and ribonuclease (22), studies of several spectral indices, especially PMR, can show evidence of detectable intermediates in equilibrium studies of an acid-induced structural transition.

Sensitivity of the acid denatured state of cytochrome c to ionic strength has been observed by several techniques (4, 23, 24) as well as for several other proteins, such as staphylococcal nuclease (25). It is likely that the sensitivity of the fluorescence emission at 340 nm of acidified cytochrome c to NaCl concentration, as well as the large change in reduced viscosity, indicate dependence of the conformation of the acid denatured state on ionic strength. Electrostatic repulsion among the protonated groups of the protein at low pH would be expected to increase as ionic strength is

3 Recently, in studies with tuna cytochrome c which lacks histidine residue at position 33, it has been possible to assign the titrating resonance to that residue and the other to histidine residue 26 (J. S. Cohen and M. B. Hayes, unpublished results).
lowered, and this could increase the average separation of the heme group from the tryptophan residue and thus increase the quantum yield of that residue. Charge-induced unfolding would account for the high viscosity reported for cytochrome c in water at pH 2.0 (4) and confirmed in the present study.

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