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pH-Induced Conformational Transitions of Ferricytochrome *c*: A Carbon-13 and Deuterium Nuclear Magnetic Resonance Study†

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ABSTRACT: The acid and alkaline unfoldings of ferricytochrome *c* have been studied by ^{13}C and ^{2}H NMR spectroscopy employing native cytochrome *c* and cytochrome *c* in which the methyl groups of the methionyl residues have been enriched by 45% ^{13}C or ^{2}H. A p*K* of 3.4 ± 0.2 for the acid transition was determined from the natural abundance ^{13}C NMR spectra by following the collapse of a sharp singlet due to 10 threonine residues upon raising the pH from 2 to 5. This same transition was monitored by following the ^{2}H resonance due to Met-80 in deuterium-labeled cytochrome *c*. The progressive broadening of the ^{2}H resonance line width and decrease in the paramagnetic shift together with the ^{13}C NMR results indicate that the displacement of Met-80 as an axial heme iron ligand occurs concomitantly with the loss of the characteristic cytochrome fold. The presence of a paramagnetically shifted ^{2}H resonance due to Met-80 below pH 2, however, indicates that cytochrome *c* at low pH can form a loosely folded structure that allows Met-80 to come into close proximity with the heme iron. The alkaline transition was also monitored selectively by following the appearance in the region of diamagnetic ^{13}C shifts of the Met-80 resonance in ^{13}C-enriched cytochrome *c*. The ^{2}H resonance due to Met-80 in the deuterium-enriched protein disappears from its hyperfine-shifted upfield position without line broadening and reappears coincident with the Met-65 methyl resonance. The disappearance of both the Met-80 [^{13}C]- and [^{2}H]methyl resonances parallels the disappearance of the 695-nm absorption band with a p*K* of ~9. The ^{2}H resonance line widths proved to be sensitive indicators of the mobility of the methionyl residues.

Ferricytochrome *c* is known to form at least five stable conformational states which depends upon the ionization states of various functional groups within the protein (Dickerson & Timkovich, 1975). The pH-induced transitions between these conformations have been extensively studied by various spectroscopic methods including optical spectroscopy (Greenwood & Wilson, 1971; Drew & Dickerson, 1978; Davis et al., 1974; Babul & Stellwagen, 1972), EPR[1] (Lambeth, et al., 1973; Brautigan et al., 1977), and proton NMR (Gupta & Koenig, 1971; Morishima et al., 1977). Since the overall folding of the protein and the axial ligands of the heme iron mediate the oxidative-reductive properties of the protein, much attention has been focused on the unfolding of the native conformation which involves the displacement of the axial ligands Met-80 and His-18.

In this study, we have taken advantage of the sensitivity of the chemical shifts of ^{13}C NMR resonances in proteins to changes in tertiary structure to study the acid and alkaline unfolding of ferricytochrome *c*. In addition, we have prepared cytochrome *c* in which the methyl groups of Met-65 and Met-80 are isotopically enriched with either ^{13}C or ^{2}H. This has allowed the critical Met-80 group to be followed through the unfolding process by ^{13}C and ^{2}H NMR without interference from natural abundance background resonances.

Experimental Procedures

Materials. The cytochrome *c* employed for the natural abundance ^{13}C spectra was Sigma Type VI obtained from horse heart. The isotopically enriched ferricytochromes were prepared by established methods (Ando et al., 1966; Jones et al., 1975; Schejter & Aviram, 1972) with only minor modifications. Sigma Type VI protein was treated with either 90% ^{13}C- or ^{2}H-enriched methyl iodide at pH 3.0 and 25 °C for 24 h. At pH 3 the protein is partially unfolded thus allowing greater access to Met-80 which is normally buried within the protein interior. Only Met-65 and Met-80 are methylated since there are no free sulfhydryl groups, and free amines such as lysine are protonated at low pH. Demethylation of the methionyl residues was accomplished by incubation with dithiothreitol (DTT) at pH 10.5 for 48 h followed by elution on Sephadex G-25 with 0.02 M ammonium bicarbonate at pH 9-9.5. For final purification the samples were eluted on Amberlite CG-50 to remove forms of enriched cytochrome *c* that were not demethylated.

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[1] Abbreviations used: DTT, dithiotreitol; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Me_4Si, tetramethylsilane; CM, carboxymethyl.

The modified proteins were characterized by the following tests. The isotopically enriched cytochrome *c* exhibited the full extinction coefficients of the native protein at 530 and 695 nm in the oxidized form and at 520 and 550 nm in the reduced form. The best check for the native condition of the protein is its lack of reactivity with CO (Tsou, 1951). After CO was bubbled through a solution of the enriched protein in the reduced state for 1 h, the absorption at 550 nm decreased slightly, indicating the presence of ~5% denatured cytochrome *c*. The enzymic activity of the enriched protein was compared to that of the native protein by measuring the effect of addition of exogenous cytochrome *c* on the rate of oxygen consumption of cytochrome *c* depleted rat liver mitochondria (Jacobs & Sanadi, 1960). With succinate as the electron donor, the apparent K_m values observed were 1.8×10^{-9} and 1.6×10^{-9} M for the native and the isotopically enriched protein, respectively. Finally, the redox potentials for the enriched and native proteins were checked under identical conditions by the method of Margalit & Schejter (1973). These results gave $E_0 = 255$ mV for enriched cytochrome *c* and $E_0 = 263$ mV for native cytochrome *c*.

NMR Spectra. The ^{13}C spectra of the enriched and native cytochrome *c* were obtained on the NIH 270 spectrometer at 67.8 MHz. This instrument is a home-built system based on a Bruker 6.3 Tesla super-conducting magnet and a Nicolet 1180 data system. A spectral window of ±7575 Hz with 16K data points and a overall pulse repetion rate of 2.0 s were employed. The flip angle of the pulses was 90° with a pulse width of 25 μs. A 3-Hz digital line broadening function was applied to the signal before Fourier transformation. In the case of the natural abundance spectra, the sample solutions were 17 mM protein in 0.2 M NaCl and required 18 000 acquisitions which resulted in 10 h of spectrometer time for each spectrum. The ^{13}C-enriched samples were 5 mM protein in 0.2 M NaCl solutions and required 2 h of acquisition for a satisfactory signal. Proton noise decoupling was employed throughout, and the ambient probe temperature was 20 °C. The ^{2}H NMR spectra of deuterium-enriched cytochrome *c* were obtained in 10-mm tubes on the same instrument at 41.4 MHz. The sample solutions were 6 mM in protein and 0.1 M in NaCl. A spectral window of ±7575 Hz was used with 4K data points. A total of 27 000 scans with a pulse repetition rate of 0.135 s yielded a total acquisition time of 1 h. The sample temperature for the variable pH experiments was kept at 24 °C. For the variable temperature experiments, the temperature was monitored by inserting a glass-enclosed thermocouple of a digital thermometer directly into the sample after quickly ejecting the sample from the magnet. The natural abundance ^{2}H resonance from water was employed as a convenient secondary chemical shift reference (4.7 ppm). Due to the width of the observed resonances and the reference employed, the chemical shifts are reported only to a precision of ±0.1 ppm. Solution pH was adjusted by adding small amounts of dilute HCl or NaOH.

Results

^{13}C Spectra of Native Cytochrome c. The natural abundance ^{13}C NMR spectra of ferricytochrome *c* at 68 MHz are shown in Figure 1 at pH 2, 5, and 11. These spectra correspond to conformational states II, III, and IV in the terminology of Theorell & Åkesson (1941). Assignments of many of the resonances in the native conformation (state III) have been made previously (Oldfield & Allerhand, 1973; Oldfield et al., 1975) from spectra at 15 MHz. In general, the higher magnetic field employed in this study resulted in significantly improved resolution, and numerous resolved single-carbon

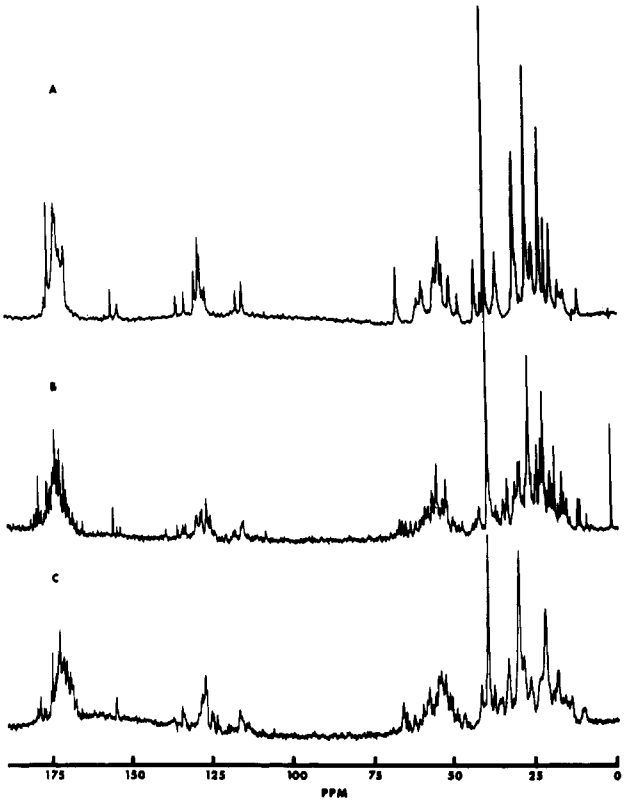

FIGURE 1: Natural abundance ^{13}C NMR spectra at 67.8 MHz of 17 mM solutions of horse heart cytochrome *c* in 0.1 M NaCl. (A) State II protein at pH 2.0. (B) State III protein at pH 5.0. (C) State IV protein at pH 11.0.

resonances appear in the aliphatic, aromatic, carboxyl, and carbonyl regions. In the aromatic region, however, the contribution of chemical shift anisotropy to the relaxation rate resulted in somewhat broader lines for the nonprotonated carbons. The carbon resonances of the heme and the axial iron ligands, Met-80 and His-18, are not observed in the paramagnetic ferric form of the protein due to extensive line broadening and large shifts which result from electron–nuclear dipolar and hyperfine contact interactions with the heme iron unpaired electrons.

Below pH ~3, much of the tertiary structure which forms the characteristic cytochrome fold in cytochromes of many species is lost. Comparison of the spectrum of the native conformation with many resolved resonances (Figure 1B) to the spectrum of the protein at pH 2 (Figure 1A) in which many of these resonances have become isochronous clearly shows that the protein has unfolded to a nearly random-coil structure. The partially folded pH 2 form (state II) reversibly refolds to the native conformation upon the addition of dilute base. At pH 3, however, the carbon spectrum is virtually indistinguishable from the spectrum of the protein at pH 2. Significant changes occur between pH 3 and 5, but the fine structure remains essentially unchanged from pH 5 to 8. A similar constancy was observed for the hyperfine shifted resonances in the proton spectra of native cytochrome *c* in the same pH range (Gupta & Koenig, 1971). At higher pH values, as shown for pH 11 in Figure 1C, many of the sharp resonances coalesce into somewhat broader resonances than observed in the loosely folded low-pH form. The lack of resolution may result from the presence of multiple forms of cytochrome *c* in slow equilibrium on the NMR time scale.

^{13}C-Enriched Cytochrome c. The ^{13}C NMR spectra of cytochrome *c* enriched by 45% ^{13}C in Met-65 and Met-80 methyl groups at neutral pH are shown in Figure 2. In the

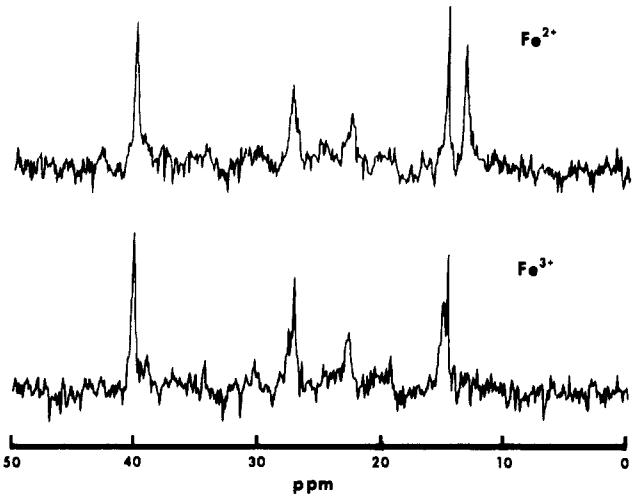

FIGURE 2: ^{13}C NMR spectra of reduced and oxidized cytochrome *c* enriched with 45% ^{13}C in the methyl groups of Met-65 and Met-80. Each spectrum resulted from a 1-h acquisition of a 7 mM solution of cytochrome *c* in 0.1 M NaCl. The spectrum of the reduced protein was obtained from the same sample as that of the oxidized protein after the addition of sodium dithionite. Chemical shift assignments are given in the text.

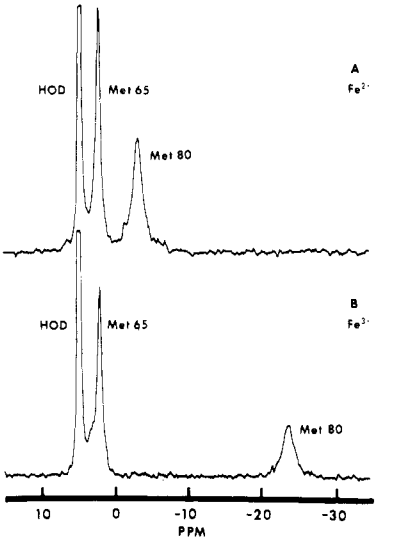

FIGURE 3: 41.4-MHz ^2H NMR spectra of reduced (A) and oxidized (B) cytochrome *c* enriched with 45% ^2H in the methyl groups of Met-65 and Met-80. Each spectrum resulted from a 1-h acquisition on 5 mM solutions of cytochrome *c* in 0.1 M NaCl at pH 7. The spectrum of the reduced protein was obtained on the same sample as the oxidized protein after addition of sodium dithionite and adjustment of the pH. Chemical shift assignments are given in the text.

diamagnetic reduced form, the Met-80 methyl resonance at 13.0 ppm appears upfield from the Met-65 peak at 14.5 ppm as a result of a substantial ring current shift due to its close proximity to the heme. The Met-65 methyl is more than 16 Å removed from the heme and is thus unperturbed by interactions with the heme. In the ferric form of the protein, electron–nuclear dipolar and hyperfine contact interactions cause the Met-80 methyl resonance to be shifted away from the normal range of diamagnetic shifts and broadened beyond detection. No paramagnetically shifted ^{13}C resonances have been observed for cytochrome *c* (Wüthrich, 1976). The Met-65 resonance in the spectrum of the ferric protein remains close to its position in the spectrum of the reduced form (14.0 ppm). A second smaller resonance at 14.5 ppm in the ferricytochrome *c* spectrum varied in intensity depending on the prior history of the sample. Extended treatment at high pH or extended chromatographic purification of the sample usually resulted in an increase in the intensity of this resonance at neutral pH. Titration of the sample through the alkaline transition revealed that this resonance results from Met-80 in a conformation of the oxidized form of the protein with the Fe–S linkage disrupted. The sharp resonance at 40.0 ppm in both spectra in Figure 2 is due to natural abundance methylene carbons of the 19 lysyl residues.

^2H-Enriched Cytochrome c. The ^2H spectra of [[ϵ-$^2H_3$]-Met-65,[ϵ-$^2H_3$]Met-80]cytochrome *c* in the oxidized and reduced forms are shown in Figure 3. The sharp low-field resonance results from natural abundance deuterium in water (~17 mM HOD) and was assigned a chemical shift of 4.7 ppm. The methyl-d_3 groups in the protein give rise to resonances at 1.8 and −3.3 ppm in the reduced protein and 1.8 and −24.4 ppm in the oxidized protein. (The negative sign indicates shifts to higher field.) The resonance at 1.8 ppm in spectra of the oxidized and reduced forms corresponds to the normal methyl chemical shift region and thus results from either Met-65 or Met-80 in which the Fe–S bond is broken and the methyl is removed from the heme environment. The resonance at −3.3 ppm results from a substantial ring current shift on the Met-80 methyl in close proximity to the heme in the diamagnetic reduced state. In the oxidized paramagnetic form, the methyl-d_3 resonance is shifted by electron–nuclear dipolar and hyperfine contact shifts, in addition to the diamagnetic heme ring currents, to −24.2 ppm. The assignments of these resonances are in agreement with proton shifts previously reported (Gupta & Koenig, 1971; Gupta & Redfield, 1972; McDonald & Phillips, 1973; Morishima et al., 1977). The Met-65 resonance is not distinguishable in proton spectra since it is obscured in the aliphatic envelope. Thus, the methyl proton shift is also extablished here, since the proton and deuteron chemical shifts are the same in ppm, except for small secondary isotope effects.

The integrated intensities of each resonance line relative to the Met-80 resonance at −3.3 ppm in the reduced form were measured for both the oxidized and reduced forms in two different preparations of cytochrome *c* at pH 7. The −3.3 ppm resonance was used as the basis of comparison of peak areas since Met-80 is not easily displaced by exogenous ligands in the reduced form, and the ratio of peak areas should thus give the enrichment levels at Met-65 and Met-80. From these results it appears that Met-80 is enriched by 65–85% of the level of enrichment of Met-65 depending on the exact conditions of the preparation. These proportions are based on the assumption that all Met-80 residues are bound in the reduced form. This difference in the relative enrichments must reflect the relative reaction rates toward methyl iodide in the alkylation reaction. Since the alkylation was performed at pH 3, close to the acid transition for the unfolding of cytochrome *c*, less than complete alkylation of Met-80 may indicate a partial shielding of this group even at pH 3.

As in the case of deuterated acetyl methyl groups in sperm whale apomyoglobin reconstituted with 2,4-diacetyldeuterohemin (Oster et al., 1975), the ^2H resonance line widths of the deuterated methionyl methyls in cytochrome *c* exhibit considerable motional narrowing due to internal rotation about the CH_2–S and S–CH_3 bonds. For a C–D bond which reorients with a protein the size of cytochrome *c* as a whole without internal motion, the ^2H line widths can be as large as 1000 Hz (Wooten & Cohen, 1979). The line widths observed for the methionyl methyl-d_3 groups, however, are in the range of 25–65 Hz. The resonance line widths, T_2 values, and effective correlation times for the methionyl methyl groups in the oxidized and reduced forms of the protein at neutral

Table I: Apparent pKs for the Alkaline Transition and Spectral Parameters at Neutral pH Determined from ^{2}H Spectra of ^{2}H-Enriched Cytochrome c

sample	pK[a]	chemical shifts (ppm)	$\Delta\nu_{1/2}$ (Hz)[b]	τ_{eff} (10^{-10} × s)[c]
1	8.9 ± 0.2	1.8	25	1.8
		−3.3	53	3.9
		−24.0	65	4.8
2	8.8 ± 0.03	1.8	24	1.8
		−3.3	51	3.7
		−24.8	55	4.1

[a] Determined from both the increase in area of the resonance at 1.8 ppm and the decrease in area of the resonance at −24 ppm. [b] Resonance line width at half-maximum height. [c] As computed from eq 1.

pH and 24 °C are given in Table I. The effective correlation time, τ_{eff}, has been estimated from

$$1/\tau_{eff} = \frac{2\Delta\nu_{1/2}}{3\pi(e^2Qq/h)^2} \quad (1)$$

where $\Delta\nu_{1/2}$ is the line width at half-height and e^2Qq/h is the deuteron quadrupole coupling constant which was taken to be 170 kHz (Wooten et al., 1979). Quadrupole coupling constants are not available for substituted amino acids, but most deuterium-substituted methyl groups give constants in the range 165–170 ± 10 kHz (Mantsch et al., 1978). Equation 1 assumes that the reorientation rate is sufficient to place τ_{eff} in the region of extreme motional narrowing.

Acid Transition. The transition from the unfolded low-pH form to the native conformation restores both Met-80 and His-18 as axial heme iron ligands. As mentioned earlier, the spectrum at pH 3 is not significantly different from the spectrum at pH 2. This is true in spite of the fact that His-18 and Met-80 are known to be displaced from the heme iron below pH 2.5. The lack of observable differences is to some degree misleading since a small number of residues in the vicinity of the heme which may be involved in the acid transition may by unobservable due to the paramagnetic interactions with the heme iron. Above pH 3, however, significant changes are observed in the fine structure of the aliphatic portion of the spectrum. The effect of the transition from a loosely folded state with one or two protein residues bound to the heme to the native conformational state with both His-18 and Met-80 bound in most clearly seen in the region of the ^{13}C spectra shown in Figure 4. The 10 threonine β carbons (there are no serine residues in horse heart cytochrome c) give rise to a single sharp resonance at 67.3 ppm at pH 3 which progressively diminishes in intensity as the pH is raised. Multiple resonances concomitantly appear at higher field which correspond to the individual threonine residues as they move into more hydrophobic and thus magnetically shielded environments. The apparent pK for the transition estimated from the spectra in Figure 4 is ~3.4 ± 0.2. Less specific changes can be seen in the α-carbon envelope. Stellwagen & Babul (1975) monitored the same transition by following changes in the optical Soret band at 395 nm which yielded pKs ranging from 3.2 to 3.7 as the chloride concentration was increased from 0.2 to 3.0 M. The 695-nm band also reappears with a characteristic pK of 3.2, indicating that this transition involves restoration of Met-80 as an axial heme ligand (Drew & Dickerson, 1978).

The ^{2}H NMR spectra of the deuterium-enriched cytochrome c show directly the participation of Met-80 in this transition (Figure 5). The Met-80 methyl resonance shifts from −14.1 upfield from Me_4Si at pH 3 to −23.9 ppm at pH 4 with a concomitant decrease in the line width as detailed

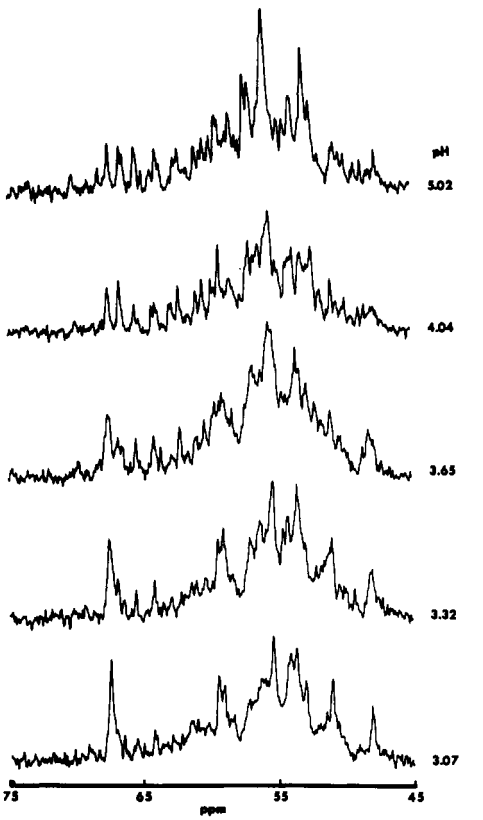

FIGURE 4: Spectral changes in natural abundance ^{13}C NMR spectra of ferricytochrome c due to the acid transition. The envelope from 45 to 50 ppm results principally from protein α carbons. The sharp resonance at 67.3 ppm in the spectrum at pH 3 results from the 10 threonine β carbons.

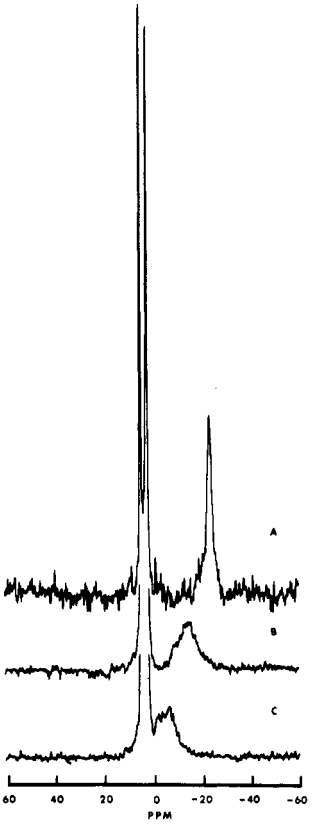

FIGURE 5: Spectral changes in ^{2}H NMR spectra of ^{2}H-enriched cytochrome c due to the acid transition. (A) pH 4.1. (B) pH 2.9. (C) pH 1.85.

Table II: ^2H Spectral Parameters for the Met-80 CH_3 Resonance as a Function of pH in the Acid Transition of Ferricytochrome *c*

pH	$\Delta\nu$ (Hz)	chemical shift (ppm)
7.10	65	−23.9
4.10	82	−23.9
2.95	288	−14.1
1.85	243	−6.4

[a] Preparation 1 from Table I.

in Table II. The asymmetric non-Lorentzian shape of the resonance at pH 3 suggests that there may be more than one conformation of bound Met-80 between pH 3 and pH 4. This is consistent with the appearance of a second paramagnetically shifted proton methyl resonance due to Met-80 below pH 4 in the ^1H NMR spectrum of native cytochrome *c* (Morishima et al., 1977).

Alkaline Transition. Above pH 8, cytochrome *c* undergoes a two-phase transition in which a proton is rapidly lost, followed by a comparably slower conformational rearrangement (Davis et al., 1974). The overall p*K* for the transition is 9.0, but the intrinsic p*K* for the ionizing group is 11.0. Thus, either a tyrosyl, lysyl, or arginyl group must be involved. Met-80 is displaced from the heme as demonstrated by the disappearnce of the 695-nm band (Greenwood & Wilson, 1971) to give a low-spin species. The identity of the sixth heme ligand in this high-pH form has not been identified with certainty, but Lys-79 appears to be a reasonable choice. (Brautigan et al., 1977; Gupta & Koenig, 1971; Morishima et al., 1977; Smith & Millet, 1980). A low-spin form without the 695-nm band can be formed, however, even when Lys-79 has been chemically modified, rendering it unavailable for binding to the heme iron (Pettigrew et al., 1976). In such a case, a free hydroxyl may be the sixth ligand.

The natural abundance ^{13}C NMR spectrum of the state IV protein at pH 11 is shown in Figure 1C. The loss of spectral structure indicates at least partial unfolding of the structure. The aliphatic region of the spectrum most vividly shows the effects of this transition (Figure 6). Many aliphatic residues with unique microenvironments become magnetically equivalent in the high-pH form.

As in the case of the acid transition, the ^{13}C and ^2H isotopically enriched cytochromes allow the Met-80 and Met-65 methyl groups to be monitored through the alkaline transition without interference from natural abundance background resonances. In the ^{13}C NMR spectra, the [^{13}C]methyl resonance of Met-80 at 14.5 ppm appears only when it is displaced from the Fe(III) atom. The increase in peak intensity of the Met-80 resonance with increasing pH parallels the disappearance of the 695-nm optical band as shown in Figure 7. The apparent p*K* for the transition determined from the ^{13}C NMR spectra is 9.1. The Met-65 resonance at 14.2 ppm is unaffected throughout the titration. The p*K* for the alkaline transition is some preparations was significantly <9.0. Further, some residual intensity for the Met-80 ^{13}C resonance in all preparations remained after the pH had been raised to pH 11.0 and then lowered to neutral pH. Because the alkaline transition was not completely reversible as detected by this method, only data obtained while raising the pH of a freshly prepared sample were employed in Figure 7 and for the estimation of the transition p*K*. A similar effect was observed in the ^2H spectra of the ^2H-enriched cytochrome *c* as discussed below. The depression of the p*K* is indicative of a weakened crevice structure. A more dramatic change in the p*K* was observed for fully maleylated cytochrome *c* (Schejter et al., 1979) in which many of the normal electrostatic interactions which

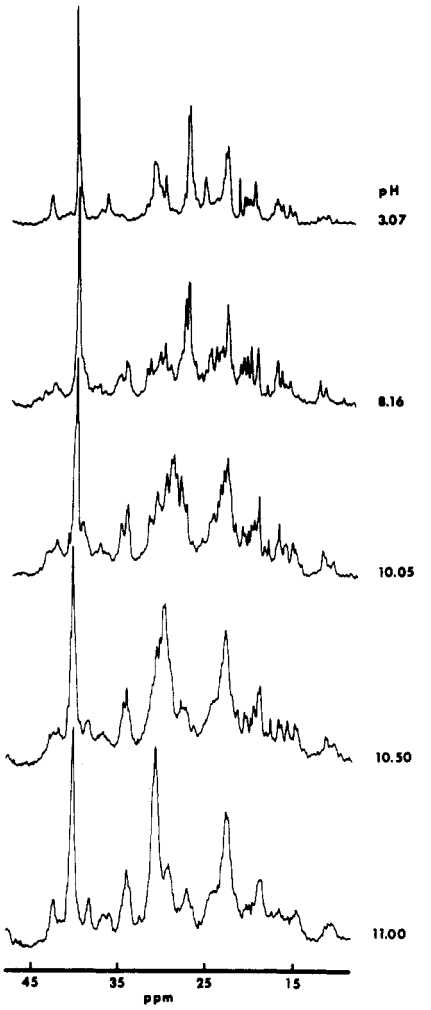

FIGURE 6: Aliphatic region of natural abundance ^{13}C NMR spectra of ferricytochrome *c* in the alkaline transition. The spectrum of the state II protein at pH 3 is shown for comparison with state IV protein at pH 11.0.

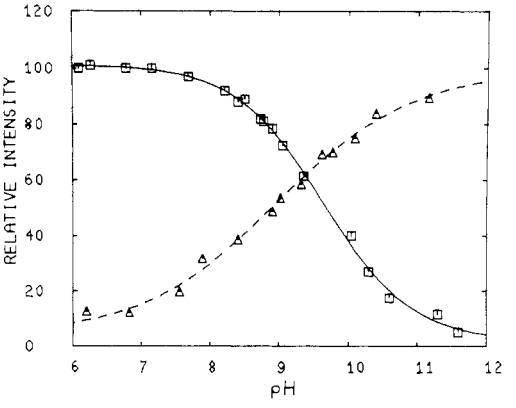

FIGURE 7: Alkaline transition as monitored by disappearance of the 695-nm optical absorption band (□) and appearance of the [^{13}C]methyl resonance of Met-80 in the ^{13}C spectrum of ^{13}C-enriched cytochrome *c* (△).

stabilize the tertiary structure are altered. Is is possible that prolonged treatment at high pH can lead to deamidation or other modifications which result in an alteration in the net charge of the protein. Samples purified by elution on CM-cellulose invariably exhibited unusual values for the p*K* which may reflect similar alterations in ionizable groups in the native protein structure. The isotopically enriched cytochrome *c* preparations which exhibited depressed p*K* values were excluded from this study.

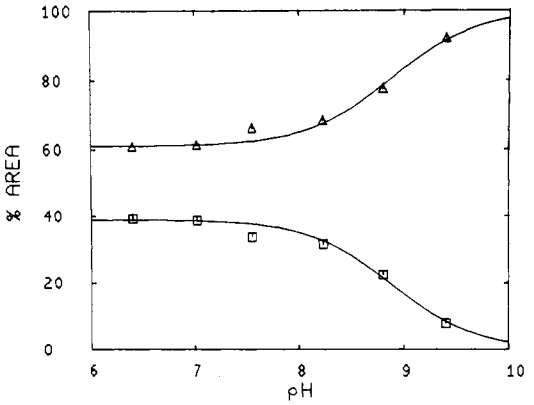

FIGURE 8: Alkaline transition as monitored by ^2H NMR spectra of ^2H-enriched cytochrome *c*. The top trace (△) is due to the 1.8-ppm resonance (Met-65 plus displaced Met-80), and the bottom trace (□) is due to the resonance at −24.2 ppm (heme-bound Met-80).

While the displacement of Met-80 from the heme by lowering solution pH causes the shifted methyl resonance in the ^2H NMR spectra of ^2H-enriched cytochrome *c* to be substantially broadened, by comparison, the Met-80 ^2H resonance line width does not significantly change during the alkaline transition. As the solution pH is increased, the area of the shifted resonance decreases while the area of the Met-65 methyl-d_3 resonance at 1.8 ppm increases. This enables the transition to be readily monitored by measurement of resonance areas as shown in Figure 8. The apparent p*K* value determined from the ^2H NMR titrations was 8.9, in good agreement with the p*K* determined by monitoring the 695-nm absorption band. It was usually found that the relative areas of the Met-65 and Met-80 resonances were somewhat different after the pH had been raised to above pH 10 and subsequently lowered to neutral pH. For example, a sample of deuterium-substituted cytochrome in which the solution pH was raised from pH 7 to 10.5 for several hours, and then reduced to pH 7, showed a Met-80 resonance with only about half its original intensity. Apparently, the protein gradually denatures irreversibly upon extended treatment at high pH.

Temperature Effects on ^2H Resonances. The ^2H NMR spectrum of deuterium-enriched cytochrome *c* was observed at temperatures ranging from 0 to 50 °C at pH 7. The dipolar and hyperfine contact contributions to the chemical shift are inversely proportional to temperature, and the high-field Met-80 resonance exhibits the expected linear dependence with reciprocal temperature with a slope of −9200 ± 300 and an intercept of 6.7 ± 1. The chemical shift of the Met-65 resonance at 1.8 ppm is essentially independent of temperature. A monotonic increase in the Met-80 line width occurs with heating above 30 °C until the resonance becomes too broad to observe at ~60 °C. A progressive decrease in the 695-nm absorption band with increasing temperature (Schejter & George, 1964; Kaminsky et al., 1973) indicates that the Met-80 heme iron bond is weakened or disrupted upon heating. This result suggests that the extreme ^2H line width observed at elevated temperature results from the rapid interconversion between multiple conformational isomers.

Discussion

Application of ^{13}C and ^2H NMR with Isotopic Enrichment to Proteins. The ^{13}C NMR spectra of proteins are especially sensitive to changes in tertiary structure. This is true because the various intramolecular interactions in proteins, such as hydrogen bonding, hydrophobic and electrostatic attractions and repulsions, and aromatic ring currents, contribute to the unperturbed covalent chemical shifts of individual carbon resonances. Consequently, there is a greater dispersion of chemical shifts for carbon atoms in a protein in its native conformation than is observed for the corresponding random-coil polypeptide. The ^{13}C NMR spectra of ferricytochrome *c* in this study reveal the effects of the pH-induced unfolding on many side chains and detect the approximate midpoints for the transitions which were previously monitored principally by alterations of the electron spin delocalization of the heme as detected by changes in the absorbance or EPR spectra or by changes in the hyperfine shifted resonances in the proton NMR spectra.

It is always desirable to monitor the influence of individual side chains, but this is frequently precluded in natural abundance ^{13}C NMR spectra because of the surfeit of protein resonances that obscure individual carbon resonances. In the case of cytochrome *c*, the resonances of Met-80, which is intimately involved in both the acid and alkaline transition, are not observable even when it is displaced from the heme, and paramagnetic interactions do not preclude their appearance. Recently developed synthetic methods have allowed specific sites to be enriched with ^{13}C, thus allowing various groups to be distinguished above the background of natural abundance resonances. Usually enrichment also has meant chemical modification, as with carboxymethylation (Eakin et al., 1975) or guanidination (Stellwagen et al., 1977), but under certain conditions the ε methyl of methionyl residues in proteins may be isotopically enriched and yield the native protein (Jones et al., 1976). The preparation of cytochrome *c* with isotopically enriched methionyl residues (Schejter et al., 1978) is especially fortuitous because of the critical role that Met-80 plays as an axial ligand of the heme iron. It was hoped that enhanced levels of ^{13}C would allow the detection of the paramagnetically shifted Met-80 methyl resonance, but no resonance due to the ^{13}C-enriched methyl group was detected in the native conformation. Although this result was somewhat disappointing, the presence of the Met-80 resonance in the region of diamagnetic shifts was an unambiguous indication that the Met-80–heme iron bond had been severed.

An alternative approach for investigating the Met-80 ligand is to enrich the methionyl methyls with ^2H rather than ^{13}C and observe the deuterium spectrum. Previously, there have been few reports of deuterium incorporation into protein amino acid residues for ^2H NMR, and these have principally involved solid-phase studies (Rothgels & Oldfield, 1981; Jelinski et al., 1980). Isotropic solution deuterium NMR offers several advantages over carbon or proton NMR. Due to the extremely low natural abundance of ^2H (0.0156%), the ^2H NMR spectra of enriched molecules can be observed without interference from background resonances. Unlike ^1H NMR, the chemical shift assignments are unambigous because a limited number of specific substitutions are usually made. Furthermore, the dominance of quadrupolar relaxation over other relaxation mechanisms results in line widths which are highly sensitive to local mobility and insensitive to paramagnetic centers or dissolved paramagnetic ions (Wooten & Cohen, 1979). Unfortunately, this advantage is partially offset because the small nuclear moment of ^2H results in deuterium chemical shifts (in hertz) which are smaller than their corresponding proton shifts by a factor of 6.5. This problem has been ameliorated somewhat by the high magnetic fields available in the current generation of NMR spectrometers.

Mobility of Methionyl Residues. The deuterium resonance line widths of the Met-65 and Met-80 resonances in the spectra of the protein at neutral pH (Figure 3) differ substantially (24–25 Hz vs. 55–65 Hz, respectively). It is reasonable to

expect that the Met-65 resonance should be more narrow because the Met-65 residue is on the periphery of the molecule and is thus less motionally restricted, whereas the Met-80 residue is buried within the heme pocket. Further, the Met-80 sulfur is bound to the heme iron, thus eliminating one degree of motional freedom. Before any conclusion can be drawn about the relative mobility of the two methionyl methyl groups, however, the possibility of paramagnetic broadening of the Met-80 resonance must be considered. In the reduced protein the heme iron is diamagnetic and thus makes no contribution to the Met-80 deuteron relaxation. In several samples, the Met-80 resonance line width in the reduced form measured slightly less than that in the oxidized form. The uncertainty (10–20%) in the measured line widths makes it difficult to conclude from this result that paramagnetic relaxation can contribute significantly to the Met-80 relaxation. There is also the intriguing possibility that the mobility of the Met-80 methyl group is actually greater in the reduced protein. Another method, consequently, is desirable for estimating the contributions to the relaxation rates.

Wüthrich (1976) has estimated the total contribution of paramagnetic relaxation to the proton line width of the Met-80 methyl in native cytochrome *c* to be ~40 Hz. As discussed by Johnson & Everett (1972), in the absence of excessive broadening due to quadrupolar relaxation, the resonance line width of a deuteron in a paramagnetic complex is expected to be less than its proton counterpart by a factor of 42.4. The estimated paramagnetic contribution to the deuteron line width of deuterium-substituted Met-80 in ferricytochrome *c* is thus <1 Hz. Even if the full line width of the proton resonance of 100–110 Hz (Burns & La Mar, 1979) resulted from paramagnetic interactions, as opposed to proton–proton dipolar interactions, for example, the comparable deuterium line width would be only ~2.4 Hz. Finally, since dipolar relaxation between deuterons is more than 2 orders of magnitude less than dipolar relaxation between protons, the deuteron resonance line width must be due largely to quadrupolar relaxation alone. Thus, the observed difference between the deuteron line widths of the Met-65 and Met-80 resonances can be attributed largely to the difference in mobility at the two sites.

Because the Met-80 sulfur atom is coordinated to the heme iron, the CH_2–S internal rotation is effectively frozen out, and the Met-80 methyl relaxation thus results from the overall tumbling of the protein as well as internal rotation about the S–CH_3 bond. The correlation time for rotation about this bond, τ_{S-CH_3}, can be estimated from the relation (Lyerla et al., 1974; Deslauriers & Smith, 1977)

$$1/\tau_{eff}(\text{Met-80}) = 1/\tau_0 + 1/\tau_{S-CH_3} \quad (2)$$

where τ_0, the overall correlation time, has been estimated to be 10^{-8} s (Eakin et al., 1975) and τ_{eff}, calculated from eq 1 and the observed line widths of 55–65 Hz, falls in the range of $4.2–5.0 \times 10^{-10}$ s. A similar relation can be written for the correlation time for the rotation of the Met-65 methyl group

$$1/\tau_{eff}(\text{Met-65}) = 1/\tau_0 + 1/\tau_{S-CH_3} + 1/\tau_{CH_2-S} \quad (3)$$

which includes the motion about the CH_2–S bond.

From eq 2 and 3, it is readily apparent that

$$1/\tau_{CH_2-S}(\text{Met-65}) = 1/\tau_{eff}(\text{Met-65}) - 1/\tau_{eff}(\text{Met-80}) \quad (4)$$

if the rates of methyl rotation at the two sites are the same. On the basis of the observed line width of 25 Hz, τ_{eff}(Met-65) is 1.8×10^{-10} s, and eq 4 thus predicts that τ_{CH_2-S} falls within the range of $2.9–3.3 \times 10^{-10}$ s. Intuitively, it would not be expected that reorientation about the CH_2–S bond would require less time than methyl rotations. For any line width of the Met-80 resonance greater than twice the Met-65 resonance line width, the estimated correlation time for motion of the Met-65 CH_2–S bond is shorter than the assumed methyl correlation time. Consequently, the methyl rotation rate at Met-65 must, in fact, be slightly greater than the methyl rotation of Met-80. Nevertheless, the near equality of the correlation times for motion about the CH_2–S and S–CH_3 bonds suggests that the CH_2–S bond of Met-65 undergoes essentially free internal rotation, a result which is consistent with certain methionyl residues in dihydrofolate reductase (Blakley et al., 1978). A more accurate estimation of internal rotation times must be obtained by fitting the observed relaxation rates to various models for reorientation which include internal rotation.

Deuterium Resonance Intensities and Line Broadening in ^2H-Enriched Cytochrome c. The ^2H resonance line width of the Met-80 methyl group in the deuterium-substituted cytochrome *c* proved to be a sensitive indicator of the types of processes giving rise to the Met-80 displacement at low and high pH. In the acid transition, the Met-80 resonance line width broadens dramatically whereas in the alkaline displacement the line width remains unchanged as the integrated intensity decreases. Clearly, in the alkaline form Met-80 is effectively blocked from interaction with the heme since no exchange broadening is observed, and the complete loss of the paramagnetic and ring current shifts causes the Met-80 resonance to reappear in the region of diamagnetic shifts.

The progressive increase of the Met-80 ^2H line width with decreasing pH suggests that Met-80 is in competition with other ligands. On the basis of solvent T_1 relaxation measurements, Gupta & Koenig (1971) have demonstrated that water molecules are readily accessible to the heme iron at low pH. Due to the loss of the 695-nm absorption and the known access of solvent molecules, it is reasonable to expect that the Met-80 residue is rapidly jumping in and out of the heme environment, resulting in lifetime broadening of the resonance line due to chemical exchange. Indeed, a measurable increase in the line width occurs at the very beginning of the transition (pH ~5).

There are, however, several other possible mechanisms which can lead to line broadening. The paramagnetic contribution to the line width is proportional to $S(S + 1)$, where S is the total electron spin, and to T_{1e} the electron spin–lattice relaxation time (Wüthrich, 1976). At low pH the heme iron converts from an $S = {}^1/_2$ to either an $S = {}^3/_2$ or ${}^5/_2$ state (Boeri et al., 1953; Theorell & Åkesson, 1941) or to a mixture of $S = {}^1/_2$ and $S = {}^5/_2$ states (Lanir & Aviram, 1975), and T_{1e} may change by as much as 1 order of magnitude (La Mar, 1979). Although the paramagnetic broadening in the low-spin form is no more than 1–2 Hz, the increase in line width could be as much as 500 Hz depending on the change in T_{1e}. This ignores the fact that the electron–nuclear dipolar contribution to the nuclear relaxation rate (and thus the line width) is also inversely proportional to r^6, where r is the distance from the interacting nucleus and the heme iron. Since r undoubtedly increases in the acid transition, the actual broadening due to the change in spin state is probably much less. Finally, the electron–nuclear dipolar ("psuedocontact") and the heme ring current contributions to the chemical shift are inversely proportional to r^3. Any slightly different configuration of the S–CH_3 group will result in a slightly different shift and thus contribute to the apparent line width. The movement of the Met-80 resonance toward higher field at low pH, however, also indicates a significant increase in the average Fe–S distance

and increases the likelihood that chemical exchange is occurring. It is worth noting here that the hyperfine shifted resonances in proton spectra of native cytochrome *c* are broadened beyond detection below pH 2 due to the strong paramagnetic interactions; thus the application of ^2H NMR and ^2H labeling provides information which cannot easily be obtained by proton NMR.

An unusual result involving the integrated resonance intensities appeared in the ^2H NMR spectra of deuterium-enriched cytochrome *c* which has been difficult to explain on the basis of available data. Whenever a sample of the ferric protein was reduced, the integrated intensity of the Met-80 resonance approximately doubled (Figure 3). This was true even in samples that gave normal p*K*s for the alkaline transition and in spite of the excellent characterization of the protein that indicated a completely native preparation (see Experimental Procedures). Since the Met-80 methyl resonance always appears as a three-proton resonance in the ^1H NMR spectrum of native cytochrome *c* (McDonald & Phillips, 1973; Burns & La Mar, 1979), it would seem likely that a portion of these preparations is slightly denatured. It has long been known that Met-80 is more tightly bound to the reduced than to the oxidized iron (Margoliash & Schejter, 1966). Thus, a minor modification in the protein that might prevent Met-80 from binding in the ferric form would nonetheless allow an intact iron–sulfur bond in the more compact reduced form. Another possibility is that once Met-80 has been displaced from the heme, as during alkylation at pH 3, it does not necessarily return to the same configuration whenever the iron–sulfur linkage is restored. As discussed earlier, if a distribution of configurations were present in a nonnative conformation, then the variation in the *S*-methyl orientation would result in different paramagnetic shifts and thus cause the resonance intensity to be smeared out. The reason for this speculation is that the full extinction coefficient for the 695-nm band, indicating an intact iron–sulfur bond, was observed for the deuterium-substituted cytochrome *c*. Whatever the case, the portion of bound Met-80's observed in the ^2H NMR spectra must be due to native cytochrome *c* both because it exhibits a normal p*K* for the alkaline transition and because its paramagnetic shift corresponds to the proton shift of Met-80 in normal cytochrome *c*.

Tertiary Structure in the Low-pH Form of Cytochrome c. The ^{13}C NMR spectrum of native cytochrome *c* below pH 2 gives little evidence that significant tertiary structure exists at this low pH. In fact, the pH 2 spectrum in Figure 1A closely resembles the putative spectrum of a random-coil protein with the same amino acid sequence as cytochrome *c* (Wüthrich, 1976). Other physical evidence also indicates the unfolding of the polypeptide chain. The visible Soret band maximum is shifted from 410 in the low-spin native form at pH 4 to 396 nm in a mixed-spin form at pH 2 (Drew & Dickerson, 1978), and the 695-nm absorption band which signals an intact bond between the heme iron and the Met-80 sulfur in the native state disappears. Further, the reduced viscosity of native cytochrome *c* at pH 2 is typical of an extended-chain structure (Babul & Stellwagen, 1972). These results are consistent with the loosely folded structure indicated by the carbon spectrum.

There are several indications, however, that cytochrome *c* retains a significant degree of tertiary structure below pH 2. The absence of resolved single-carbon resonances in the ^{13}C NMR spectrum at pH 3 indicates that there are relatively few interactions between amino acid side chains. Nonetheless, no ^{13}C resonance for the Met-80 methyl group in ^{13}C-enriched cytochrome *c* was observed at pH 2 (Schejter et al., 1978) while an upfield-shifted ^2H resonance was observed for Met-80 in ^2H-enriched cytochrome *c* even at pH 1.8. These results indicate that the Met-80 group remains proximal to the heme although it is likely that there is a great deal of motional flexibility present (vide supra). In the alkylation of the deuterium preparation at pH 3, the Met-65 site was enriched with deuterium to a slightly higher level than the Met-80 site ($\sim$ 20%). It appears that Met-80 is partially shielded from the alkylating reagent perhaps by its closeness to the heme face. Finally, the low-field C-2 proton resonances of His-26 and His-33 do not become fully equivalent, as would be the case in a completely random-coil protein, until pH <1 (Cohen & Hayes, 1974).

The addition of chloride ions at pH 2 reduces the paramagnetism of ferricytochrome *c* to a value typical of the $S = {}^3/_2$ state (Boeri et al., 1953). This behavior, together with concomitant spectral changes, was originally attributed to the proton-induced breakage of the bonds between the iron and axial protein ligands, followed by the formation of bonds between the metal and one or two chlorides (Boeri et al., 1953; Stellwagen & Babul, 1975). Lanir and Aviram, however, found that the spectroscopic changes induced by chloride were also obtained by other ions, including perchlorate, which is well-known for its inability to bind metals, in substantially lower concentrations (Aviram, 1973; Lanir & Aviram, 1975). The effect of increasing concentrations of anions at this low pH is not, apparently, due to their direct binding to the iron, but to conformational changes that may partly restore the protein–iron coordination. This is based on the observation that the 695-nm band characteristic of the heme iron–methionine sulfur bond in ferricytochrome *c* disappears upon acidification but is partially restored by the addition of chloride or perchlorate (Aviram, 1973; Greenwood & Wilson, 1971). Furthermore, the anion methanesulfonate causes the same spectroscopic changes but does not bind the iron directly (Lanir & Aviram, 1975). Thus, the interaction between the heme iron and the Met-80 sulfur atom must not be completely disrupted even at this low pH. This is particularly true under the conditions of the NMR experiments that require substantial concentrations of protein and, consequently, of the anion that is introduced as acid. Previously, it has been shown that Met-80 is an axial ligand in the reduced form of cobalt-substituted cytochrome *c* at pH 1 (Dickinson & Chien, 1975).

There are no readily apparent changes in the ^{13}C or ^2H NMR spectra that can be attributed to the protonation and displacement of His-18, which can occur with a p*K* lower than 1.2, depending on the chloride concentration. The re-formation of the Met-80 heme iron linkage as determined from the appearance of the 695-nm band (Drew & Dickerson, 1978) and the full upfield shift of the Met-80 deuterium resonance, however, coincides with the appearance of multiple ^{13}C resonances due to individual threonine residues in distinct protein environments. The binding of Met-80 to the heme iron apparently occurs simultaneously with the complete refolding to the native globular structure. The p*K* for the transition as determined by each of these methods is $\sim 3.4 \pm 0.2$. The absence of a paramagnetically shifted ^2H resonance due to Met-80 implies that the exchange rate between the high-pH form and the native conformation is slow and strongly favors the form with unbound Met-80. This result is consistent with the kinetic determination of the rate constants for exchange in the alkaline isomerization (Davis et al., 1974) and NMR transfer of saturation studies (Gupta et al., 1972).

Conclusions

Below pH 2, ferricytochrome *c* has a very loosely folded structure which allows competition between Met-80 and solvent water molecules for the axial heme iron ligand site. The process of refolding to the native conformation in the acid transition coincides with the re-formation of the Met-80 sulfur–heme iron bond. In the alkaline transition there are no indications of chemical exchange between heme-bound and unbound states of Met-80. The methyl groups of Met-65 and Met-80 in ferricytochrome *c* when enriched with deuterium give ^{2}H NMR resonances which relax predominately by the quadrupolar mechanism at neutral pH even in the presence of the paramagnetic heme iron, and the ^{2}H line widths reflect the relative mobilities of the methionyl methyl groups.

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