The Nature of the Thermal Equilibrium Affecting the Iron Coordination of Ferric Cytochrome *c*†

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ABSTRACT: In cytochrome *c*, ligation of the heme iron by the methionine-80 sulfur plays a major role in determining the structure and the thermodynamic stability of the protein. In the ferric state, this bond is reversibly broken by moderately acid or alkaline pH's (p*K*'s 2.5 and 9.4, respectively) and by exogenous ligands. NMR studies of horse ferricytochrome *c* in which the Met-65 and Met-80 methyl groups were chemically enriched with ^{13}C demonstrate that, at 59 °C, a temperature at which the protein is still folded, the sulfur−iron bond is already partially broken. This structural change corresponds to the reversible disappearance upon moderate heating of the 695 nm band, characteristic of the sulfur−iron coordination of this protein. The thermal effect results from a shift in the alkaline p*K* from 9.4 at 25 °C to 8.2 at 59 °C. The exchange rate from iron-bound to free methionine-80 at 59 °C is 1.8 s^{-1}, as measured by saturation transfer experiments. The free and bound methionine-80 ϵ-methyl groups in the ^1H spectrum are assigned as (1.87, 2.25) and −21.43, respectively; in the ^{13}C spectrum they are assigned as 15.6 and 12.8, respectively (all these values are in ppm from 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt).

The 695 nm band of ferric cytochrome *c* (Theorell & Akesson, 1941) arises from the promotion of a porphyrin electron to the d_{z^2} orbital of the low-spin iron (Eaton & Hochstrasser, 1967) and is associated with the ligation of the metal by a thioether sulfur (Shechter & Saludjian, 1967). The band disappears from the cytochrome *c* spectrum when the pH is moderately acid or alkaline (Theorell & Akesson, 1941; Davis et al., 1974), when the native ligand is replaced by exogenous ligands (Horecker & Kornberg, 1946), and when the protein is heated to moderate temperatures (Schejter & George, 1965). For the exogenous ligand binding and pH-induced changes, the loss of the band is accompanied by the displacement of the Met-80 sulfur from its iron liganding position, as demonstrated by chemical modification experiments (Schejter & Aviram, 1970) and by NMR studies (Wooten et al., 1981), respectively. This suggests that the effect of moderate heating on the intensity of the band may also be due to the rupture of the Fe−S bond. This hypothesis is also supported by the correlation between the midpoint temperatures of thermal titrations of cytochromes *c* of diverse species, and the p*K*'s of their alkaline ionizations (Osheroff et al., 1980). However, it has been argued, on the basis of NMR studies of cytochrome *c* at high temperatures, that the iron−sulfur bond may be still intact at 60 °C (Angstrom et al., 1982) and that the decrease in absorbance of the 695 nm band reflects a change in the geometry of the Met-80 side chain rather than a cleavage of the iron−sulfur bond. The present investigation was designed to solve this controversial question in the chemistry of cytochrome *c*, which is essential for understanding the role that the Fe−S bond plays in determining the structure and the thermodynamic stability of the protein (Margoliash & Schejter, 1995).

^{13}C NMR spectra of ferricytochrome *c* were reported by several authors (Wooten et al., 1981; Santos & Turner, 1992). The Met-80 ϵ-methyl resonance was expected to be shifted due to its direct bonding to the paramagnetic Fe^{III} center (Wooten et al., 1981). This was the reason that the peak at 13.7 ppm, that was observed at 30 °C, was only tentatively assigned to the Met-80 resonance (Santos & Turner, 1992). This peak was very broad and could not be observed at higher temperature (Santos & Turner, 1992). We have used cytochrome *c* in which the Met-65 and Met-80 groups were chemically labeled with ^{13}C, enabling us to monitor the Met-80 ^{13}C spectra with improved signal to noise ratio. Using this material, we have gathered full NMR evidence for the presence of both free and iron-bound Met-80 in neutral solutions of ferric cytochrome *c* at 60 °C, a temperature at which the protein is still folded (Privalov & Khetchinashvili, 1974). The free and bound Met-80 ϵ-methyl groups are assigned in both ^1H and ^{13}C spectra. We also calculated the exchange rate and apparent p*K* under this conditions and showed that the thermal equilibrium at neutral pH and the alkaline pH-induced equilibrium are essentially the same reaction.

MATERIALS AND METHODS

Preparative Procedures. Horse cytochrome *c* was prepared and purified from horse heart muscle according to Margoliash & Walassek (1967). Chemical enrichment with ^{13}C of the Met-80 and Met-65 methyl groups was already described (Schejter et al., 1978; Wooten et al., 1981).

Analytical Methods. For the NMR experiments, 5 ± 1 mM solutions of the ferric ^{13}C enriched protein were prepared

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in a deuterated solution of 0.1 M potassium phosphate buffer (99.9% D_2O). The pH was adjusted with DCl or NaOD; the pH values are given without correction for the deuterium isotope effect or the minor temperature drift and are designated as pH*.

The ^{13}C and proton NMR spectra were acquired on a Bruker ARX500 NMR spectrometer with a 5 mm QNP probe. All samples contained 3−5 mM TSP[1] as an internal reference ($\delta(^{13}C) = -1.7$ ppm, $\delta(^1H) = 0.0$ ppm). Saturation transfer measurements were carried out using 0.3 s preirradiation alternating between on- and off-resonance frequencies. Off-resonance frequencies were placed close to the on-resonance ones in order to avoid artifacts in the difference spectra (Satterlee & Moench, 1987).

The exchange rate was measured using an inversion transfer sequence $90°_{sel}-t-180°_{nsel}$ (Forsen & Hoffman, 1963; Rudin & Sauter, 1992) with changing lengths of intervals. The results were fitted to the expression given by Rudin and Sauter (1992) using the nonlinear least mean squares Levenberg−Marquardt method. The HMQC experiment (Bax et al., 1983) was carried out using 2048 by 256 resolution with a τ of 3.6 ms according to the $^{13}C-^1H$ J coupling. The data were processed using zero filling to 512 for $F1$. A sine filter function was applied on both axes.

RESULTS

^1H NMR Studies. Figure 1a shows the 500-MHz proton NMR spectrum of horse ferricytochrome *c* at pH* = 7.5 and 59 °C. In the spectrum, the signals of interest that are obtained also at low temperature are designated as (N). The assignments are according to Santos and Turner (1987, 1992). The new peaks, appearing only at high temperatures, are similar to those appearing at alkaline pH and are designated as (B). Their assignments are given according to Hong and Dixon (1989). At a temperature of 59 °C, resonances (B) could be detected at pH* values as low as 6.7, but their intensities grew upon increasing pH (see Table 1). In order to identify the exchanging peaks of the heme methyls and to confirm their identity with those found by Hong and Dixon (1989) at alkaline pH, a saturation transfer experiment was carried out (Figure 1b−d). Panels b and c (Figure 1) show the NMR spectrum obtained upon selective saturation of the heme methyls 3 and 8 in their (N) form: each methyl group is exchanging with two different (B) forms (Hong & Dixon, 1989), resulting in two different peaks in the spectrum. Figure 1d gives the selective saturation of one of the (B) forms of heme methyl-5, resulting in the appearance of the heme methyl-5 (N) form. This experiment establishes the identity between the high temperature conformation at neutral pH and that of the alkaline pH at room temperature. We used the same technique in order to find the resonance of the Met-80 ϵ-methyl in the "alkaline" conformation. If in this conformation the Met-80 side chain is no longer acting as the iron ligand, its resonance should most probably appear in the crowded diamagnetic part of the spectrum (0−10 ppm) (Wooten et al., 1981). Indeed, upon selective saturation of the Met-80 ϵ-methyl, the difference spectrum (Figure 1e) revealed two peaks at 1.87 and 2.25 ppm, assigned as the Met-80 ϵ-methyl in the (B) form, where it is not attached to the heme iron.

[1] Abbreviations: TSP, 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt; HMQC, heteronuclear multiple-quantum coherence.

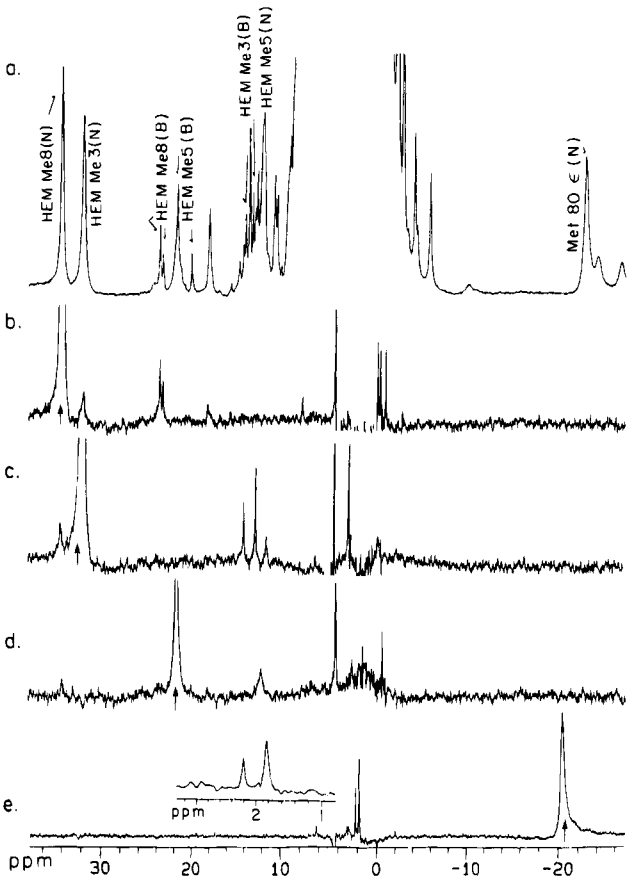

FIGURE 1: (a) The 500-MHz proton NMR spectrum of 5 mM horse ferricytochrome *c* at 59 °C and pH* = 7.5. Chemical shifts are given in ppm from TSP. (See text for (N), (B) designation.) Traces b−e are a set of saturation transfer experiments that were carried out at the same conditions as (a). Given here are the differences between spectra obtained with preirradiation at the arrow position and those obtained with off-resonance irradiation.

Table 1: Apparent pK for the Alkaline Isomerization of Ferricytochrome *c* at 59 °C

pH*	[(B)]/[(N)]	apparent pK
7.20	0.09 ± 0.01	8.2 ± 0.1
7.50	0.25 ± 0.02	8.1 ± 0.1
7.65	0.27 ± 0.02	8.2 ± 0.1

The exchange rate under the described conditions was measured using an inversion transfer experiment (Rudin & Sauter, 1992) on the heme methyl-8 (N) and (B) forms (Figure 2). The observed k_f was 1.8 ± 0.2 s^{-1}, and both (B) peaks exchanged at the same rate with the (N) peak (Figure 2), as described by Hong and Dixon (1989). The exchange rate measured by the inversion transfer was confirmed by a steady-state saturation transfer experiment (Satterlee & Moench, 1987) which gave an observed k_f of 2.1 ± 0.3 s^{-1}.

In order to calculate the apparent pK, we used the fact that the spectrum revealed both the (N) and the (B) forms. Thus, using integration of the NMR peaks and the measured pH* for the overall process:

$$(N) \rightleftarrows (B) + H^+ \quad (1)$$

where (B) is the form lacking the Fe−S(Met-80) bond, we get:

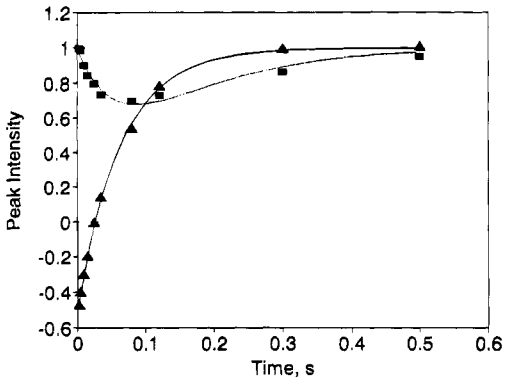

FIGURE 2: Inversion transfer experimental results (symbols) and fitted curves (lines). The experiment was carried out at pH* = 7.5 and 59 °C on the heme methyl-8 (N) and (B) forms. The (N) form signal was inverted, and the measured intensities of the (N) form (▲) and the (B) form (■) signals are given as a function of the recovery time, τ. Both inversion transfer curves were fitted using the same set of parameters: $T_1(N) = 6.3$ ms; $T_1(B) = 9.2$ ms; $k_f = 1.8\ s^{-1}$; the ratio [B]/[N] = 0.26 was calculated from the relative spectral integrals.

$$K_{apparent} = [(B)][H^+]/[(N)] \quad (2)$$

The apparent pK was calculated for several pH* values, and the results are given in Table 1.

^{13}C NMR. The ^{13}C spectra of ^{13}C-labeled Met-65 and Met-80 ferricytochrome *c* at 10 and 59 °C are given in Figure 3, showing the appearance of a new peak near the Met-65 resonance at 59 °C. We assigned this resonance to the free form of Met-80, following the same assignment for the alkaline form (Wooten et al., 1981). The use of labeled Met-80 enabled us to assign the bound Met-80 ^{13}C with certainty, using selective decoupling of the Met-80 ϵ-methyl protons. At 59 °C, bound Met-80 ϵ-methyl ^{13}C was observed at 12.8 ppm. This value is upfield to the value tentatively assigned at 30 °C by Santos & Turner (1992) and confirmed by us to be 13.7 ppm.

2D $^1H-^{13}C$ Correlation Experiment. The correlation between the proton and ^{13}C NMR results was observed in a HMQC experiment (Bax et al., 1983), shown in Figure 4. The sample contained residual ferrous cytochrome *c*, resulting in a Met-65 (ferrous) resonance at $\delta(^{13}C) = 15.3$ ppm and $\delta(^1H) = 2.13$ ppm, as previously assigned by Wooten et al. (1981), Santos and Turner (1992), and Gao et al. (1990). Bound Met-80 resonances in either the ferric or the ferrous form of the protein were not detected using HMQC due to the short relaxation times of the iron-bound ligand (Redfield & Gupta, 1971; Santos & Turner, 1986). The complete assignments summarizing all the collected data for both bound and free ^{13}C and protons are given in Table 2.

DISCUSSION

The NMR experiments described above establish the interdependence between the pH- and temperature-induced local conformational changes between states III and IV (Theorell & Akensson, 1941) of ferricytochrome *c*. The identical nature of these two processes was assumed to result from the temperature dependence of the slow conformation change that follows the ionization of the protein (Angstrom et al., 1982). As expected (Angstrom et al., 1982), the apparent pK decreases, from 9.5 to 25 °C (Davis et al., 1974; Hong & Dixon, 1989; Wooten et al., 1981) to 8.2 at 59 °C,

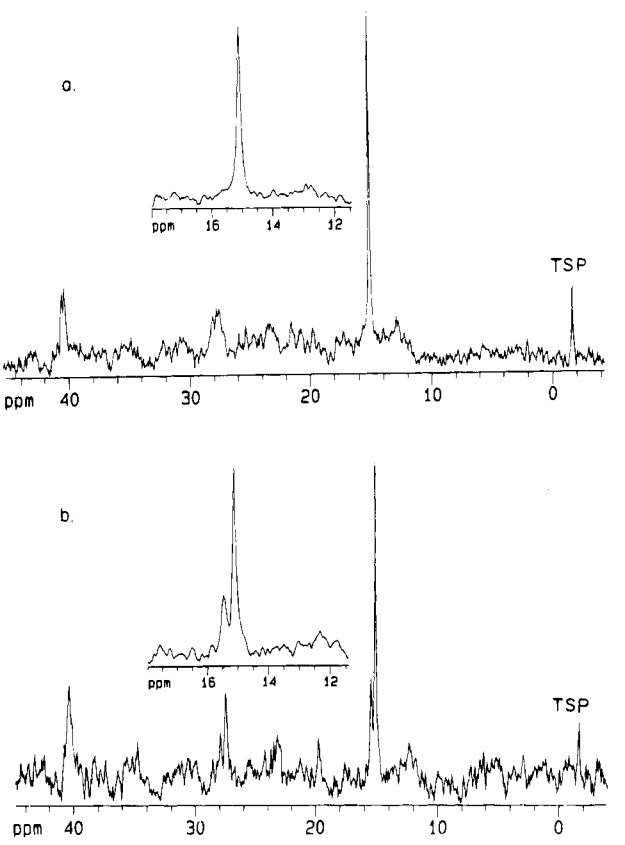

FIGURE 3: Part of the ^{13}C NMR spectra of 5 mM horse ferricytochrome *c* ^{13}C labeled at the ϵ-methyl of Met-65 and Met-80, at pH* 7.5 and (a) 10 °C, (b) 59 °C. Chemical shifts are given relative to TSP: $\delta(^{13}C) = -1.7$ ppm, $\delta(^1H) = 0.0$ ppm.

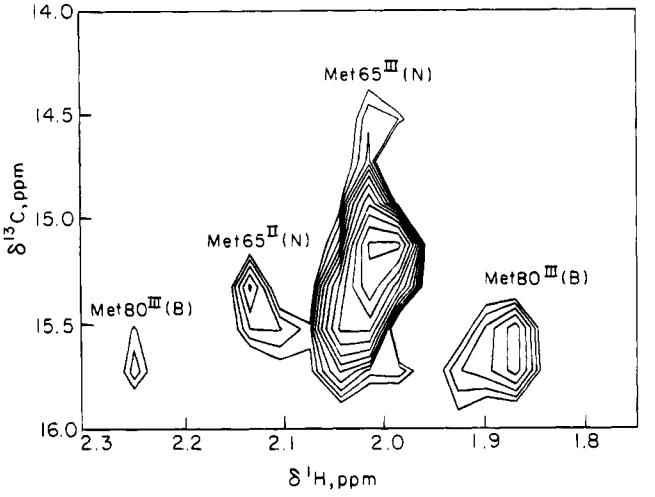

FIGURE 4: Section of a HMQC spectrum of horse cytochrome *c* ^{13}C labeled at the ϵ-methyl of Met-65 and Met-80, at pH* = 7.5 and 59 °C. Ferric and ferrous forms are designated III and II, respectively.

Table 2: Assignments of the Met-80 ϵ-Methyl at pH* 7.5 and 59 °C (in ppm Relative to TSP)

^1H		^{13}C	
bound	free	bound	free
−21.43	1.87, 2.25	12.8	15.6

corroborating with explanation. The value of the apparent pK at 59 °C and those determined in an earlier study (Davis et al., 1974) fit very well ($r = 0.98$) the linear dependence

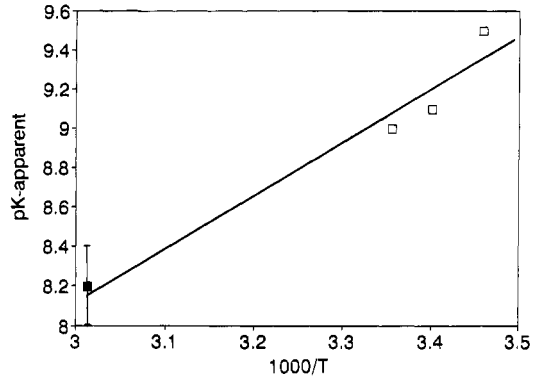

FIGURE 5: (□) A linear fit (van't Hoff plot) of the $pK_{apparent}$ vs $1/T$ of spectroscopic results based on the relative intensity of the 695 nm band of ferricytochrome *c* at various temperatures (Davis et al., 1974); (■) the NMR result found at 59 °C.

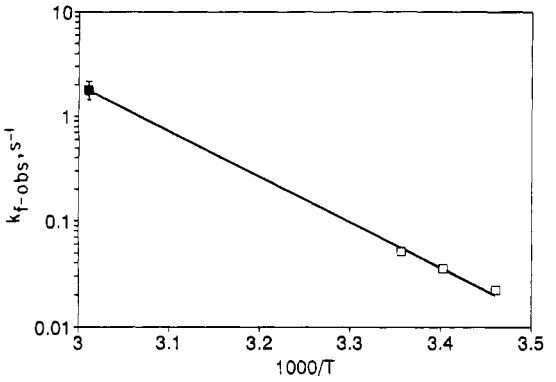

FIGURE 6: (□) A linear fit (Arrhenius plot) of the logarithm of the observed k_f vs 1/T of spectroscopic results of experiments of pH jump to alkaline values at various temperatures (Davis et al., 1974); (■) the NMR result found at 59 °C and pH* = 7.5. The observed k_f's for the pH jump experiments were calculated using the experimental results and a pH value of 7.5.

of the apparent p*K*'s with the reciprocal temperatures expected from a van't Hoff plot of the observed equilibrium constants (Figure 5).

The slow nature of the conformation change makes it the rate determining step in the kinetics of the alkaline isomerization, which explains the fact that both alkaline forms are exchanging with the native form at the same rate as shown by Hong and Dixon (1989) at alkaline pH, and by us at neutral pH.

Our rate measurements yield $k_{f\text{-}obs}$, which is the observed rate, k_{obs}, of the pH jump to alkaline values described by Davis and co-workers (Davis et al., 1974). The rate measured in our NMR experiment at pH* 7.5 and 59 °C fits well (r = 0.99) the Arrhenius plot (Figure 6) of the k_{obs} obtained in the earlier experiments (Davis et al., 1974). As the rate constant increases with temperature and pH (Davis et al., 1974), the difference detected between the rate constant determined here, 1.8 s^{-1}, and that of Hong and Dixon (1989), 4.0 s^{-1}, arises from the fact that their measurements were carried out at lower temperature (27 °C) and higher pH (pH = 8.5−10).

We have been able to measure the positions of both the ^1H and ^{13}C signals of the ferric cytochrome *c*, state IV. The proton signal was assigned using the saturation transfer with the bound Met-80, and the ^{13}C signal was obtained from the specifically labeled protein. Finally, the HMQC experiment gave us an additional confirmation of these independent experiments by showing the correlation between the corresponding proton and ^{13}C signals.

The appearance of the proton peaks in the diamagnetic region of the spectrum, near the Met-65 resonance at 2.1 ppm (Santos & Turner, 1992), strongly suggests that the displaced Met-80 side chain lies away from the iron and in the diamagnetic region of the protein, since Met-65 is known to be far from the paramagnetic heme environment. In this regard, it is pertinent to recall that studies of artificial cytochrome *c* mutants (Schejter et al., 1992) have suggested that the Met-80 methyl group is displaced by alkalinization to the region containing the internal water, W-1 (Takano & Dickerson, 1981). Using the assignments presented above, future ^1H 2D ROESY and NOESY experiments can be used to assess the validity of this hypothesis.

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