PROTON HYPERFINE RESONANCE ASSIGNMENTS USING THE NUCLEAR OVERHAUSER EFFECT FOR FERRIC FORMS OF HORSE AND TUNA CYTOCHROME *c*

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ABSTRACT Proton hyperfine resonance assignments for cytochromes *c* from several species are currently being successfully pursued by several laboratories. These efforts focus mostly on the ferrous forms. In contrast to that work, we have pursued assignments of the proton hyperfine shifted resonances for horse and tuna ferricytochromes *c*. Our results indicate that assignments are nearly identical in those two proteins. Using the pre-steady state nuclear Overhauser effect, several additional assignments have been made for the tuna protein, whereas for the horse protein, the following protons have been assigned: heme 7, αCH_2; heme 7, βCH_2; histidine 18, βCH_2 and αCH; and the methionine 80, βCH_2.

INTRODUCTION

Cytochromes *c* have historically attracted much attention from biological nuclear magnetic resonance (NMR) spectroscopists, probably as a consequence of their small size, stability, ease of isolation, and ubiquity in nature (1–25). Much of that work has focused on making proton resonance assignments in the reduced, or ferrous forms in which the heme iron ion is diamagnetic. For ferrous cytochrome *c*, nearly all protons exhibit shifts in the −3 to 10 ppm region. In cases where specific attention was given to the ferric form, in which the heme ion is paramagnetic and proton resonances span the range of −40 to 40 ppm, only a few of the hyperfine shifted resonances were assigned by nuclear Overhauser effect (NOE) methods and these only in the tuna protein (25). The hyperfine-shifted resonances occur as a consequence of the heme-centered paramagnetism and, due to heme-centered magnetic anisotropy, may result in sizeable proton shifts not only for the heme substituents and axial iron ligands, but for nearby amino acids as well (26, 27).

In the ferric form of several cytochromes, direct proton resonance assignments have been made for the heme methyl protons (10, 12, 22). The ϵ-CH_3 and γ-CH_2 resonances of the methionine, which serves as one axial ligand to the heme iron ion have, in most instances, been assigned (8, 10–13, 22–24). Recently, in tuna ferric cytochrome *c*, the β-CH_2 and α-CH proton resonances of the histidine (His-18) which also serves as a heme iron ligand, and the 7-position heme propionate protons (Fig. 1) have been assigned (25). All of these resonances are hyperfine shifted, lie outside the 0–10-ppm shift region in which the bulk of the cytochrome *c* protons lie, and exhibit temperature dependent shifts.

With the major emphasis on proton assignments focused in the 0–10-ppm region (1–24), we have emphasized direct assignment of the hyperfine resonances in several ferric cytochromes *c* as an aid to our studies of the physiologically relevant molecular redox complex which these cytochromes *c* form with yeast cytochrome *c* peroxidase (29–33). We have documented specific shifts in the hyperfine resonances of horse cytochrome *c* as a consequence of complex formation with cytochrome *c* peroxidase (CCP) established covalently (32) and noncovalently (33). The situation is such that, even at field strengths of 8.5 T, in the 1:1 complex of cytochrome *c* with CCP (complex molecular weight ≃ 46,000), the 0–10 ppm is extremely crowded with overlapping resonances of the two proteins. There is so little resolution that this region is not directly informative, whereas resolution outside this region (hyperfine shifts) remains, even in the 1:1 complex of horse ferric cytochrome *c* with CCP (32, 33). In horse ferric cytochrome *c* only 9 of the clearly resolved 18 hyperfine-shifted proton resonances are unambiguously assigned. As an aid to ongoing studies of the CCP-cytochrome *c* complex, this work presents the results of hyperfine proton resonance assignments in horse ferricytochrome *c* using the proton nuclear Overhauser technique.

MATERIALS AND METHODS

Horse cytochrome *c* (type VI) was purchased from Sigma Chemical Co., St. Louis, MO. Initially in our laboratory the protein was treated with fifty mole percent potassium ferricyanide (Mallinckrodt Inc., St. Louis, MO) in order to convert it entirely to the oxidized (ferric) form. This was done at 4°C and was followed by column chromatography (Dowex 1-X8; Bio-Rad Laboratories, Richmond, CA) and finally 20 h of dialysis against three changes of 5 liters each of triply deionized water (Barnstead

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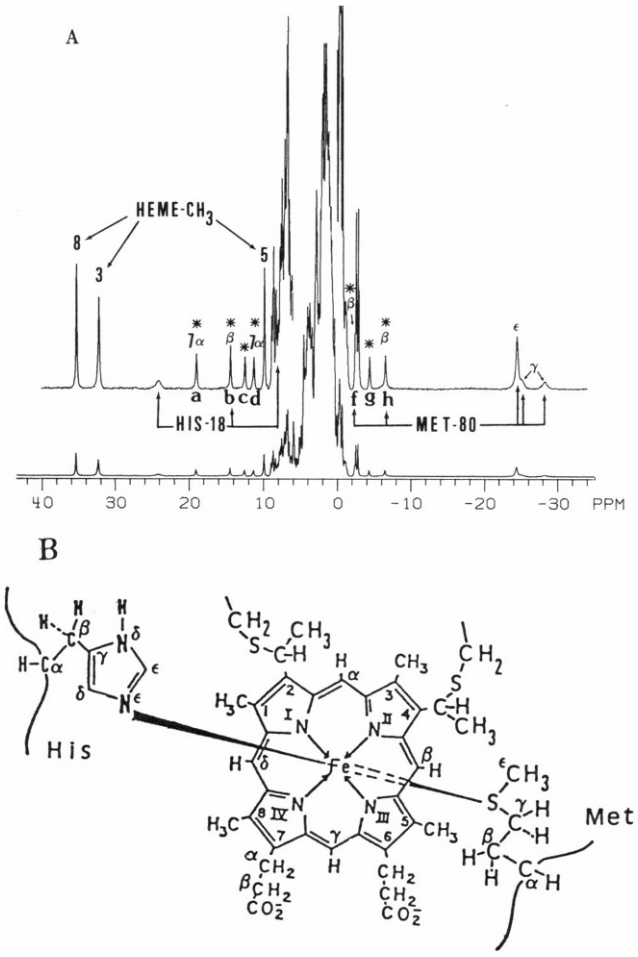

FIGURE 1 Proton NMR spectrum at 500 MHz (*A*) of 2.8 mM horse ferricytochrome *c* in D_2O, no salt, 23°C, pD = 6.9, 361 MHz. Single proton resonances are labeled *a–h*. Assignments either previously made, or made in this work, are shown above and below the expanded spectrum. The 7-position refers to the heme propionic acid group. (*B*) The heme and axial iron coordination sphere in cytochromes *c*. The heme and axial ligands histidine-18 and methionine-80 are labeled for reference to the text discussion.

Co., Div. of Sybron, Boston, MA). Control experiments to those described below in which the protein was used directly from the bottle showed no primary quantitative differences to the more elaborately prepared protein despite the presence of up to 10% ferrous form in the former. After dialysis, the protein was lyophilized. Proton NMR and UV-visible spectra taken after each individual stage in this process showed no differences to one another. Only changes associated with converting the mixture of ferrous and ferric proteins completely to the ferric form were detectable when comparing visible spectra of our final lyophilized preparation with the directly used Sigma protein.

Proton NMR spectra (39) were recorded on several samples, weighed out from lyophilized powder and dissolved in D_2O (100%; Merck, Sharp, and Dohme Div., West Point, PA). The solutions were 0.01 M in KNO_3 and pD was adjusted to 6.5–6.9 (meter reading) with DCl and NaOD (both from MSD Isotopes, Montreal). A pH 70 meter (Beckman Instruments Inc., Fullerton, CA) and a combination electrode (Fisher Scientific Co., Pittsburgh, PA) were used for this purpose. Spectra were recorded at either 361 MHz (University of New Mexico) using a GE360 spectrometer or 500 MHz (University of California, Davis) using an NT-500 spectrometer that employed active temperature control at several temperatures between 19°C and 40°C. Observed shifts are referenced internally at the residual HDO peak which was assigned a value of 4.63 ppm. Nuclear Overhauser effects (NOE) were observed by using the proton decoupler to irradiate the desired resonance. The method we have employed here is similar to that used for tuna cytochrome *c* (25) and the cyanide-ligated form of horse radish peroxidase (HRP-CN) (34). Power levels of <100 mW were used. Studies of the establishment of the NOE for pairs of protons were carried out by varying the length of time that the decoupler pulse was applied. For each on-resonance irradiation time, an off-resonance reference spectrum with the same decoupler pulse length was accumulated. NOEs are presented in the text on a per-proton basis. Spectra are presented in the "difference" mode obtained by subtraction of off-resonance irradiation from the on-resonance irradiation spectra. The off-resonance placement of the decoupler was always close to the on-resonance irradiation in order to minimize off-resonance anomalies, and the quality of the difference spectra indicate the success in this approach.

RESULTS AND DISCUSSION

Horse Cytochrome *c*

Fig. 1 shows the 500-MHz proton NMR spectrum of a typical horse ferricytochrome *c* solution (*A*) and the structure of heme *c* and its axial ligands as it appears in the protein (*B*). The hyperfine resonances previously assigned are labeled above each resonance, but without an asterisk. Those specific assignments resulting from this work are marked with an asterisk in addition to being labeled. Table I presents the shifts of these resonances as well as the assignments which have been made in this study.

For paramagnetic heme proteins, it has been shown by

TABLE I
OBSERVED PROTON SHIFTS* AND ASSIGNMENTS FOR IRON LIGANDS IN HORSE AND TUNA FERRICYTOCHROME *c*

Resonance assignment	Horse	Tuna	References	
			Horse	Tuna
Heme				
8-CH_3	35.22	35.29	10, 12, 41	
3-CH_3	32.17	32.42	10, 12, 41	
7-αCH_2	18.99	19.66	This work	25
	11.20	10.41		
5-CH_3	10.11	10.04	10, 12, 41	
2-βCH_3	−2.62	−2.58	10, 12, 41	
4-βCH_3	3.0	3.1	10, 12, 41	This work
Histidine-18				
$\epsilon_1 H$	24.30	23.19	10, 12	25
βCH_2	14.30	14.08	This work	25
	8.72	8.60		
αCH	8.90	8.79	This work	25
Methionine-80				
ϵCH_3	−24.50	−24.27	10, 12, 41	
γCH_2	−25.3	(−24.2)	10, 12, 41	
	−28.4	−28.4		
βCH_2	−2.30	−1.70	This work	This work
	−6.60	−6.04		

*Shifts in ppm referenced to HDO = 4.63 ppm at 23°C; tuna 5.0 mM, pD = 6.9, horse 2.8 mM, pD = 6.95, both in 10 mM KNO_3, minus signs indicate upfield shifts.

LaMar et al. (34) that there are two useful limits in which one might carry out NOE measurements. Noggle and Schirmer (35) give the applicable equation for a pair of dipole-coupled nuclei of identical magnetogyric ratio, γ, as

$$\eta_1(t) = [\sigma_{1,2}/\rho_1]\,[1 - \exp(-\rho_1 t)], \qquad (1)$$

where $\eta_1(t)$ is the time dependent enhancement, $\sigma_{1,2}$ represents the cross relaxation rate between nuclei 1 and 2, ρ_1 is the intrinsic (i.e., selective) relaxation time of nucleus 1, and t is the time over which a given nucleus is irradiated. For long irradiation times, this equation becomes Eq. 2, a function of both $\sigma_{1,2}$ and ρ_1.

$$\eta_1\,(t = \infty) = \sigma_{1,2}/\rho_1 \qquad (2)$$

$$\eta_1(t) = \sigma_{1,2}t. \qquad (3)$$

This limit is the so-called steady state NOE. For short irradiation times, t, Eq. 1 takes the form of Eq. 3. The NOE is seen to depend only on $\sigma_{1,2}$, the cross relaxation rate between the two nuclei. This is the pre–steady state NOE. As shown previously (34) the magnitude of pre–steady state nuclear Overhauser enhancements for two interacting protons depends upon the inverse of the length of an internuclear vector connecting the two. Nearest neighbor nuclei display larger relative NOEs than do those that are more distant (34, 35).

Development of this method has come from work on HRP-CN (34). In that case, it was shown that the NOE magnitude followed an exponential buildup, with spin diffusion as a possible complicating factor at longer irradiation times ($t > 100$ ms). To assure that these experiments were carried out in the pre–steady state regime, so that primary NOEs to nearest neighbors were observed, the buildup of the NOE for three geminal proton pairs was followed as a function of irradiation time of one of the protons in the pair. This was carried out at both 361 and 500 MHz and allows for application of Eqs. 1–3. The data are plotted in Fig. 2 for one of these pairs (heme 7αH pair) and shows that for the choice of irradiation times employed here (30 and 100 ms) the steady state regime with accompanying spin diffusion has not been reached at 11.7 tesla.

Fig. 3 shows the degree to which the hyperfine resonances were suppressed by irradiation in these experiments. Data are presented here only for irradiation of some of the downfield hyperfine resonances. This figure illustrates that negative NOEs are the rule for these proteins (34) by comparing, for example, the relative intensity of peak d when peak a is irradiated (Fig. 3 B) versus when peak d is not irradiated (Fig. 3 C; peak b irradiated). This indicates that peaks a and d are a dipole-coupled pair which exhibit a primary NOE.

These types of data are more readily visualized in difference spectra presented in Figs. 4–9. In each case the

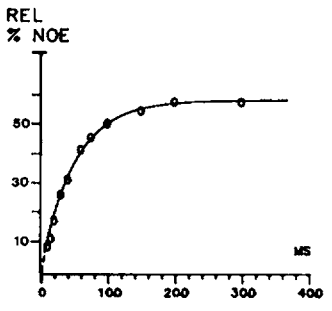

FIGURE 2 Time dependent negative NOE buildup for the heme 7-αCH_2 geminal protons. This is the increase in magnitude of the negative NOE of resonance a as a function of the time that its partner, d, is irradiated. This experiment was carried out at 500 MHz on a 2.8-mM horse cytochrome c solution in D_2O at 32°C, pD = 6.8, 10 mM KNO_3.

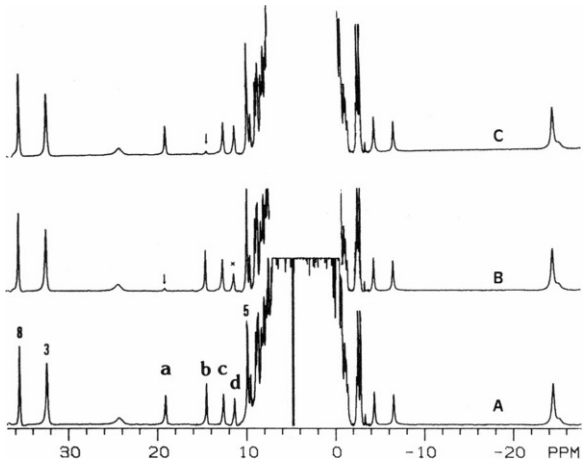

FIGURE 3 (A) 361 MHz proton NMR spectrum of 2.8 mM horse ferricytochrome c in D_2O, 23°C, pD = 6.8, 10 mM KNO_3. (B and C) Same as A but decoupler irradiation at arrows (duration = 100 ms). Note the change in peak d intensity in B.

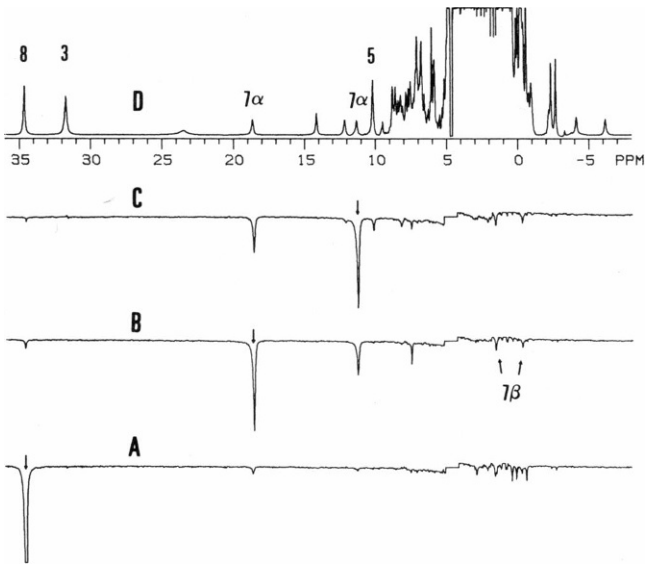

FIGURE 4 (D) 500 MHz proton NMR spectrum of horse ferricytochrome c solution as in Fig. 3 caption but at 30°C. (A–C) NOE difference spectra. (A) Irradiation at the heme 8-CH_3 showing small NOE to resonances a (18–19 ppm) and d (11–12 ppm). (B) Irradiation of resonance a, showing NOE to d. (C) Irradiation of resonance d showing reciprocal NOE to a. Note small NOEs upfield in both B and C indicated as 7β. The decoupler pulse was of 30 ms duration.

spectrum with off-resonance irradiation is subtracted from that with on-resonance irradiation. The irradiation position is indicated by an arrow in each figure. Several primary NOEs, establishing nearest neighbor connectivities, are apparent in these figures.

Definitive assignments to the heme substituents of pyrrole IV (Fig. 1 *B*) are shown in Fig. 4. This figure contains data taken with a 30-ms irradiation time at 500 MHz, although data taken at 361 MHz and 100- or 30-ms irradiation times are qualitatively identical. Fig. 4 *A* shows that irradiating the heme 8-CH_3 resonance produces a 2.3% NOE to resonance *a* and a 1% effect on resonance *d*. Other effects can also be seen in the aliphatic region (−1–3 ppm) which must be due in part to the proximity of leucines 32, 35, 64 (carbons within 2.9–4.9 Å of the 8-CH_3). Fig. 4, *B* and *C* show that resonances *a* and *d* show reciprocal primary effects to each other with NOEs of 34% and secondary NOEs to the heme 8-CH_3. Thus, peaks *a* and *d* are a geminal pair due to the large magnitude of their NOE. Their NOE to the 8-CH_3 assigns them as the two 7α protons. Peaks *a* and *d* also show secondary NOEs of 8–10% each to two resonances, at −0.15 and 1.7 ppm, which are most likely assignable to the two diastereotopic 7-β propionic protons (Figs. 1 *B* and 4 *B*). This is in accord with the assignment of β propionic acid protons upfield in other low spin ferriheme proteins (26, 27, 38). Except for the β CH_2 protons, these assignments are consistent with assignments in tuna ferricytochrome *c* (25). The β proton assignments were not reported for tuna cytochrome *c* (25). In addition, resonance *a* displays a strong NOE (24%) to a resonance at 7.6 ppm, which probably comes from a neighboring amino acid proton. Likely candidates for this resonance may be identified by the observed shift, comparison to the tuna and horse protein sequences, and the published tuna structure as shown in Fig. 10 (36, 37). Tyrosine-67 and tryptophan-59 emerge from this comparison as potential sources for this NOE. Although Trp-59 has been specifically identified as the source of this NOE based on the structure of reduced tuna cytochrome *c* (25), we note that the crystal structure of the oxidized protein shows that the Tyr-67 Cδ to heme 8-CH_3 carbon-carbon distance is only 4.51 Å, implying that protons would be closer than 3.0 Å (see Fig. 1 *B*) and that Tyr-67 is a viable candidate for this assignment.

Other NOE difference spectra obtained when downfield hyperfine resonances are irradiated are shown in Fig. 5. In Fig. 5 *A* the heme 3-CH_3 displays NOEs of 11% to a resonance at 3.0 ppm and 6% to a resonance at 6.1 ppm. The 6.1 ppm resonance may be due to phenylalanine-82 protons, based on a previous resonance assignment in tuna (9), observation of a similar effect in tuna (see Fig. 8), the tuna-horse sequence homology (40), and the structure in Fig. 10, which shows the close proximity of Phe-82 and the heme 3-CH_3 in tuna cytochrome *c*. The 3.0 ppm NOE is probably due to another of the heme substituents which are nearest neighbors to the heme 3-CH_3 on pyrrole II, namely

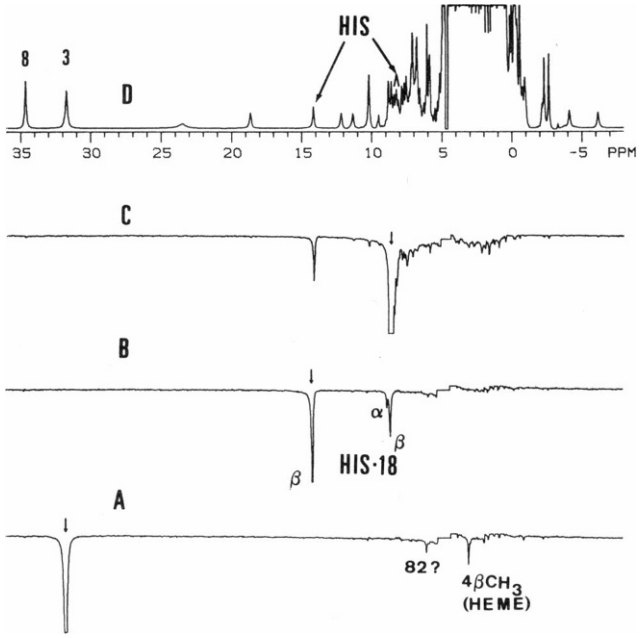

FIGURE 5 (*D*) Spectrum as described in Fig. 4 caption. (*A*) Irradiation of heme 3-CH_3 showing specific NOEs to resonances at 6.1 ppm (Phe-82?) and 3.0 ppm (heme 4-βCH_3?). (*B*) Irradiation of resonance *b* (14–15 ppm) showing NOE to protons at 8.8 ppm. (*C*) Reciprocal NOE caused by irradiation at 8.8 ppm confirms connectivity. Decoupler pulse duration = 100 ms.

the α-CH or β-CH_3 of the 4-position substituent group which forms the thioether bridge (Fig. 1 *B*). Judging by the size of the NOE, assignment to the 4−βCH_3 group is preferred and this is consistent with the assignment made by Keller and Wüthrich (10, 12).

Fig. 5 *B* shows that irradiating resonance *b* produces a strong NOE to a peak at 8.72 ppm (35%) with a shoulder at 8.90 ppm which yields an 8% NOE. This three-resonance pattern, compared with similar experiments on tuna ferricytochrome *c* (25), and the absence of demonstrated NOEs to other heme substituents, suggests that the resonances at 14.4 ppm (*b*) and 8.72 ppm are the geminal β-CH_2 protons of histidine-18 (Fig. 1 *B*). The peak that exhibits the smaller NOE and lies at 8.90 ppm at this temperature is probably the His-18 α-CH resonance. This is similar to the assignments in tuna cytochrome *c* (25), and the reciprocal NOE (Fig. 5 *C*) confirms the connectivity.

Resonance *c* does not display significant primary NOEs for irradiation times up to 150 ms at either 361 or 500 mHz. The largest NOE detectable is to a resonance at 7.0 ppm (7%), indicating that no geminal partner exists for resonance *c*.

The result of these experiments, so far, is the firm, specific assignment of all downfield hyperfine resonances except for peak *c*. The results determined here for horse ferricytochrome *c* are very similar to those found for tuna ferricytochrome *c* (25, see below) although the resonance positions are different for corresponding protons in each

protein. These results and a comparison with the tuna protein are presented in Table I.

The upfield hyperfine shift region contains only three resolved single proton resonances at 361 MHz that have been unassigned in both horse and tuna cytochrome *c*. For the horse protein, these are labeled *f, g, h* in Fig. 1 *A*. The results of NOE experiments for these three protons are shown in Fig. 6. Fig. 6 *B* shows an NOE experiment that establishes the geminal partners *f, h* on the basis of a 55% NOE. Similarly, Fig. 6 *A* establishes the geminal partner of resonance *g* at −0.81 ppm, which exhibits a 57% NOE.

Further specific assignment of the *f-h* pair is shown in Fig. 7. Fig. 7 *A* shows that irradiation principally of the Met-80 (Fig. 1 *B*) ϵ-CH_3 produces no significant NOE to peaks *f, g,* or *h*. However, when the Met-80 γ-CH resonance (which slightly overlaps ϵ-CH_3) is irradiated (Fig. 7 *B*) a strong NOE is observed to both resonances *f* and *h*. This establishes connectivity between resonances *f, h,* and one of the methionine γ protons, but not to the methionine S-CH_3 group. Such a pattern suggests that resonances *f* and *h* may be assigned to the methionine β-CH_2 protons. Further evidence for this assignment is presented below on the basis of experiments on the tuna protein, its sequence homology with horse cytochrome *c*, and its published crystal structure (Figs. 1 and 10). Resonance *g* and its geminal partner remain unassigned, although experiments with the tuna protein suggest that, by comparison, they may belong to a geminal methylene pair of lysine-79 which is identical in both horse and tuna proteins.

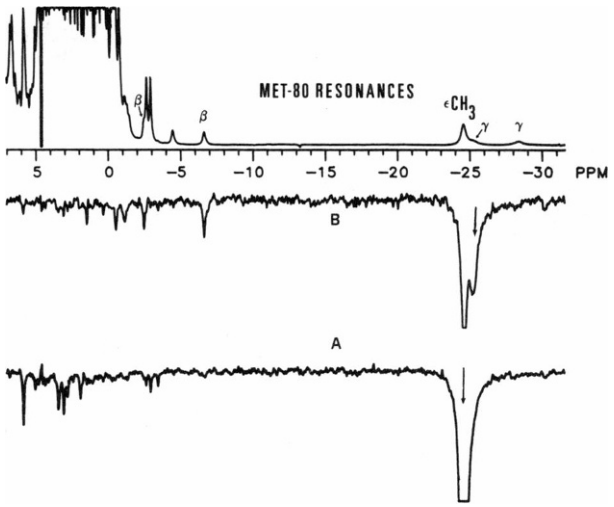

FIGURE 7 For the protein solution described in the Fig. 6 caption, at 361 MHz: (*A*) irradiation of the Met-80 ϵ-CH_3 group; (*B*) irradiation of the Met-80 γ proton that overlaps the methyl group. In *B*, partial irradiation of the ϵ-CH_3 group occurs due to decoupler spillover. Assignments of the Met-80 resonances are shown above the absorption spectrum (*top*). Decoupler pulse duration = 100 ms.

Tuna Cytochrome *c*

Several downfield hyperfine resonance assignments have been made in tuna ferricytochrome *c* using a related technique, the steady state nuclear Overhauser effect (25). The 7α-CH_2 resonances and the histidine-18 αCH$\beta$$CH_2$ group identified in that work are similar to those resonances identified above for the horse protein. We have repeated experiments on those resonances in the pre–steady state regime and obtained identical results.

Additional experiments were carried out on tuna ferricytochrome *c* for comparison with horse and because the

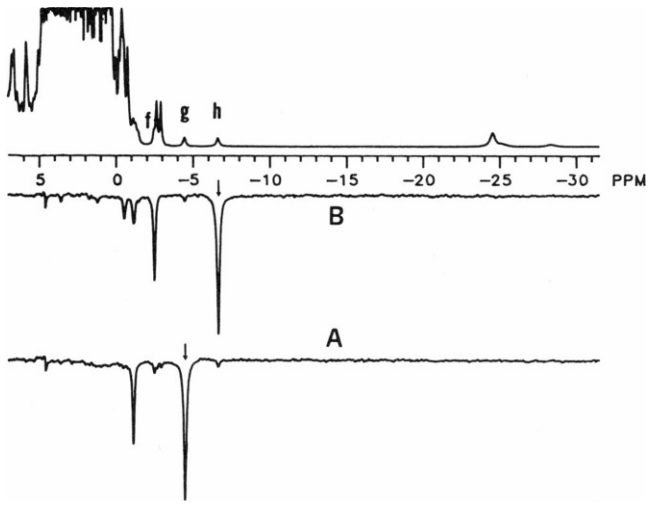

FIGURE 6 Upfield proton assignments for a 2.8-mM horse ferricytochrome *c* solution in D_2O, pD = 6.9, 10 mM KNO_3, 23°C at 361 MHz. (*A*) Irradiation at peak *g* identifies its geminal partner at 0–1 ppm. (*B*) Irradiation of resonance *h* showing its connectivity via NOE to its geminal partner peak *f*. Decoupler pulse duration = 30 ms.

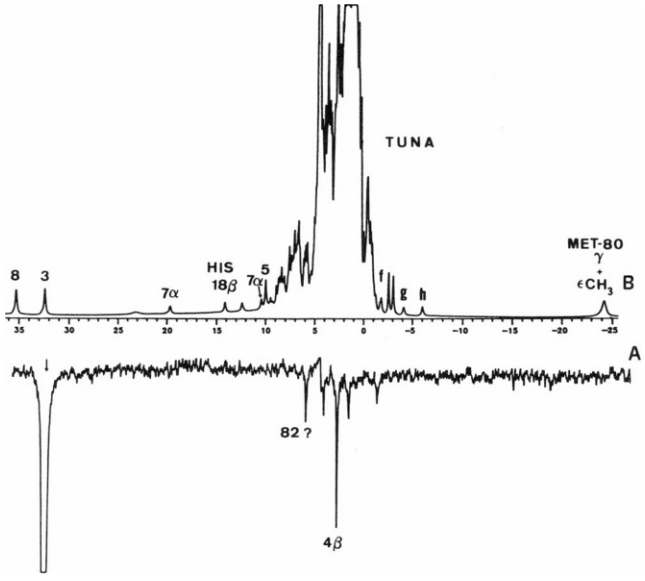

FIGURE 8 (*A*) Irradiation of the heme 3−CH_3 resonance in tuna ferricytochrome *c*, 5 mM, 10 mM KNO_3, 23°C, pD = 6.9. Assignments from reference 25 are indicated above the absorption spectrum. Note similarity in NOE pattern to that exhibited in Fig. 5 and similar assignments to phenylalanine 82 and the heme 4-$\beta$$CH_3$ group. Decoupler pulse = 30 ms.

available crystal structure is of the tuna protein (Fig. 10). Fig. 8 shows the NOEs observed for the remaining unreported downfield resonance (heme 3-CH_3). In Fig. 8 *A*, irradiation of the heme 3-CH_3 shows NOEs in a pattern nearly identical to that for horse ferricytochrome *c* (see above). By the same reasoning discussed earlier, the 6.1 ppm resonance is very likely to arise from protons of Phe-82, and the 2.85 ppm NOE is likely to be from the heme 4-βCH_3.

The assignment of the upfield geminal pairs of single protons is shown in Fig. 9. The same connectivity (*f, h* and *g, g'*) as in the horse protein is observed in Fig. 9, *B* and *C*. The assignment of the *f, h* pair to the Met-80 βCH_2 protons is seen in the connectivity illustrated in Fig. 9 *D*. This figure illustrates why a combination of data for the horse and tuna proteins is important. In horse cytochrome *c*, connectivity was observed between the Met-80 γCH_2 group and resonance *f* and *h*. Here, in the tuna protein, the γ-CH_2 proton most strongly coupled to the *f, h* pair lies underneath the ϵ-CH_3 group. As shown in Fig. 9 *D*, irradiation at −24.2 ppm simultaneously saturates the ϵ-CH_3 group and a γCH_2 proton. The NOE observed is less specific than in the horse protein but includes the *f, h* proton pair and not the *g', g* pair, indicating, as before, that these protons (*f, h*) also belong to the Met-80. Consequently, the *f, h* pair are assigned to the Met-80 β-CH_2 group. These assignments are mutually consistent for both proteins and consistent with the crystal structure (Fig. 10), which clearly indicates that the Met-80 βCH_2 protons are close enough to the heme group and in an orientation (over the heme plane) consistent with an upfield shift as a consequence of both heme ring current and paramagnetic dipolar effects. The Met-80 β-carbon to nearest heme pyrrole carbon distance is 3.21 Å.

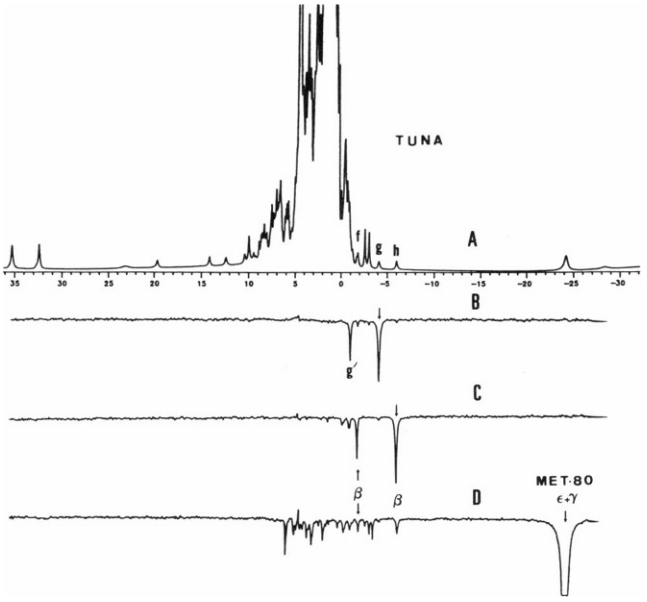

FIGURE 9 Assignment of the Met-80 β-CH_2 resonances: (*A*) absorption spectrum; (*B*) irradiation at peak *g* establishes its geminal partner, *g'*; (*C*) irradiation *h* establishes its geminal partner *f*. The *f, h* pair exhibits connectivity to the Met-80 γ and ϵ resonances in *C*. Note similarity to Fig. 7.

FIGURE 10 Stereoview of the heme vicinity of tuna cytochrome *c*, using the National Protein Data Bank coordinates and the MIDAS program.

The results of this work can now be applied to an interpretation of the shifts in horse cytochrome *c* hyperfine resonances caused by noncovalent complex formation with yeast cytochrome *c* peroxidase (33). In the downfield shift region, complex formation causes changes in the heme 8-CH_3, 3-CH_3, and 7-αCH_2 resonances. Upfield, the unassigned resonance, *g*, exhibits a significant shift, whereas the protons of Met-80 exhibit only very small changes. Because downfield there is little change exhibited by the His-18 resonances it appears that, fundamentally, there is little change in the unpaired spin density distribution at the heme site along the (His-Fe-Met) electronic *Z*-axis as a consequence of complex formation.

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REFERENCES

1. Williams, G., N. J. Clayden, G. R. Moore, and R. J. P. Williams. 1985. Comparison of the solution and crystal structures of mitochondrial cytochrome c. Analysis of paramagnetic shifts in the NMR spectrum of ferricytochrome c. *J. Mol. Biol.* 183:447–460.
2. Moore, G. R., M. N. Robinson, G. Williams, and R. J. P. Williams. 1985. Solution structure of mitochondrial cytochrome c. II. ^1H nuclear magnetic resonance of ferrocytochrome c. *J. Mol. Biol.* 183:429–446.
3. Williams, G., G. R. Moore, R. Porteous, M. N. Robinson, N. Soffe, and R. J. P. Williams. 1985. Solution structure of mitochondrial cytochrome c. I. ^1H nuclear magnetic resonance of ferricytochrome c. *J. Mol. Biol.* 183:409–429.
4. Wand, A. J., H. Roder, and S. W. Englander. 1986. Two-dimensional ^1H NMR studies of cytochrome c: hydrogen exchange in the N-terminal helix. *Biochemistry.* 25:1107–1114.
5. Wand, A. J., and S. W. Englander. 1986. Two-dimensional ^1H NMR studies of cytochrome c: assignment of the N-terminal helix. *Biochemistry.* 25:1100–1106.
6. Moore, G.R., Z. X. Huang, C. G. S. Eley, H. A. Barker, G. Williams,

M. N. Robinson, and R. J. P. Williams. 1982. Electron transfer in biology: the function of cytochrome *c*. *Faraday Discuss. Chem. Soc.* 74:311–329.

7. Wand, A. J., and S. W. Englander. 1985. Two-dimensional ^{1}H NMR studies of cytochrome c. *Biochemistry.* 24:5290–5294.
8. Keller, R. M., and K. Wüthrich. 1981. ^{1}H NMR studies of structural homologies between the heme environments in horse cytochrome c and in cytochrome c-552 from *Euglena gracilis*. *Biochim. Biophys. Acta.* 668:307–320.
9. Boswell, A. P., G. R. Moore, R. J. P. Williams, J. C. W. Chien, and L. C. Dickenson. 1980. Nuclear magnetic resonance studies of the phenylalanine residues of eukaryotic cytochrome c. *J. Inorg. Biochem.* 13:347–352.
10. Keller, R. M., and K. Wüthrich. 1978. Assignment of the heme c resonances in the 360 MHz ^{1}H NMR spectra of cytochrome c. *Biochim. Biophys. Acta.* 533:195–208.
11. Senn, H., A. Eugster, and K. Wüthrich. 1983. Determination of the coordination geometry at the heme iron in three cytochromes c from *Saccharomyces cerevisiae* and from *Candida krusei* based on individual ^{1}H NMR assignments for heme c and the axially coordinated amino acids. *Biochim. Biophys. Acta.* 743:58–68.
12. Keller, R. M., and K. Wüthrich. 1981. Multiple irradiation ^{1}H NMR experiments with hemeproteins. *In* Biological Magnetic Resonance. L. J. Berliner and I. Reuben, editors. Plenum Publishing Corp., New York. 3:1–52.
13. Senn, H., R. M. Keller, and K. Wüthrich. 1980. Different chirality of the axial methionine in homologous cytochromes c determined by ^{1}H NMR and CD spectroscopy. *Biochem. Biophys. Res. Commun.* 92:1362–1369.
14. Keller, R. M., A. Schejter, and K. Wüthrich. 1980. ^{1}H NMR studies of the coordination geometry at the heme iron and the electronic structure of the heme group in cytochrome c-552 from *Euglena gracilis*. *Biochim. Biophys. Acta.* 626:15–22.
15. Moore, G. R., and R. J. P. Williams. 1980. Nuclear-magnetic-resonance studies of eukaryotic cytochrome c. Assignment of resonances of aromatic amino acids. *Eur. J. Biochem.* 103:493–502.
16. Moore, G. R., and R. J. P. Williams. 1980. Nuclear-magnetic-resonance studies of eukaryotic cytochrome c. Assignment of resonances of aliphatic amino acids. *Eur. J. Biochem.* 103:503–512.
17. Moore, G. R., and R. J. P. Williams. 1980. Nuclear-magnetic-resonance studies of ferrocytochrome c. pH and temperature dependence. *Eur. J. Biochem.* 103:513–521.
18. Moore, G. R., and R. J. P. Williams. 1980. The solution structures of tuna and horse cytochromes c. *Eur. J. Biochem.* 103:533–541.
19. Perkins, S. J. 1980. Ring current models for the heme ring in cytochrome c. *J. Magn. Res.* 38:297–312.
20. Eley, C. G. S., G. R. Moore, R. J. P. Williams, W. Neupert, P. J. Boon, H. H. K. Brinkhoff, R. J. F. Nivard, and G. I. Tesser. 1982. Structural role of the tyrosine residues of cytochrome c. *Biochem. J.* 205:153–165.
21. Moore, G. R., and R. J. P. Williams. 1980. The stability of ferricytochrome c temperature dependence of its NMR spectrum. *Eur. J. Biochem.* 103:523–532.
22. McDonald, C. C., and W. D. Phillips. 1973. Proton magnetic resonance studies of horse cytochrome c. *Biochemistry.* 12:3170–3180.
23. Senn, H., and K. Wüthrich. 1983. A new spatial structure for the axial methionine observed in cytochrome c from *Pseudomonas mendocina*. Correlations with the electronic structure of heme c. *Biochim. Biophys. Acta.* 747:16–22.
24. Senn, H., and K. Wüthrich. 1983. Individual ^{1}H-NMR assignments for the heme groups and the axially bound amino acids and determination of the coordination geometry at the heme iron in a mixture of two isocytochromes c-551 from *Rhodopseudomonas gelatinosa*. *Biochim. Biophys. Acta.* 743:69–74.
25. Moore, G. R., and G. Williams. 1984. Assignment of ^{1}H-NMR resonances of the heme and axial histidine ligand of mitochondrial cytochrome c. *Biochim. Biophys. Acta.* 788:147–150.
26. Satterlee, J. D. 1986. NMR spectroscopy of paramagnetic heme proteins. *In* Annual Reports on NMR Spectroscopy. G. Webb, editor. Academic Press, Inc., London. 17:79–178.
27. LaMar, G. N. 1979. Model compounds as aids in interpreting NMR spectra of hemeproteins. *In* Biological Applications of Magnetic Resonance. R. G. Shulman, editor. Academic Press, Inc., New York. 305–343.
28. Poulos, T. L., and J. Kraut. 1980. A hypothetical model of the cytochrome c peroxidase · cytochrome c electron transfer complex. *J. Biol. Chem.* 255:10322–10330.
29. Kang, D. S., and J. E. Erman. 1982. The cytochrome c peroxidase-catalyzed oxidation of ferrocytochrome c by hydrogen peroxide. Steady state kinetic mechanism. *J. Biol. Chem.* 257:12775–12779.
30. Kang, C. H., S. Ferguson-Miller, and E. Margoliash. 1977. Steady state kinetics and binding of eukaryotic cytochromes c with yeast cytochrome c peroxidase. *J. Biol. Chem.* 252:919–926.
31. Erman, J. E., K. L. Kim, L. B. Vitello, S. J. Moench, and J. D. Satterlee. 1987. A covalent complex between horse heart cytochrome c and yeast cytochrome c peroxidase: kinetic properties. *Biochim. Biophys. Acta.* 911:1–10.
32. Moench, S. J., J. D. Satterlee, and J. E. Erman. 1987. Proton NMR and electrophoretic studies of the covalent complex formed by crosslinking yeast cytochrome c peroxidase and horse cytochrome c using a water soluble carbodiimide. *Biochemistry.* In press.
33. Satterlee, J. D., S. J. Moench, and J. E. Erman. 1987. A proton NMR study of the non-covalent complex of horse cytochrome c and yeast cytochrome c peroxidase and its comparison with other interacting protein complexes. *Biochim. Biophys. Acta.* 912:87–97.
34. Thanabal, V., J. S. de Ropp, and G. N. LaMar. 1987. ^{1}H NMR study of the electronic and molecular structure of the heme cavity in horseradish peroxidase. Complete heme resonance assignments based on saturation transfer and nuclear Overhauser effects. *J. Am. Chem. Soc.* 109:265–272.
35. Noggle, J. H., and R. E. Schirmer. 1971. The Nuclear Overhauser Effect. Academic Press, Inc., New York. 113–119.
36. Swanson, R., B. L., Trus, N. Mandel, G. Mandel, O. Kallai, and R. E. Dickerson. 1977. Tuna cytochrome c at 2.0Å resolution. I. Ferricytochrome c structure analysis. *J. Biol. Chem.* 252:759–775.
37. Takano, T., B. L. Trus, N. Mandel, G. Mandel, O. Kallai, R. Swanson, and R. E. Dickerson. 1977. Tuna cytochrome c at 2.0Å resolution. II. Ferrocytochrome c structure analysis. *J. Biol. Chem.* 252:776–785.
38. Satterlee, J. D., J. E. Erman, G. N. LaMar, K. M. Smith, and K. C. Langry. 1983. Assignment of hyperfine-shifted resonances in low-spin forms of cytochrome c peroxidase by reconstitutions with deuterated hemins. *J. Am. Chem. Soc.* 105:2099–2104.
39. Satterlee, J. D., and J. E. Erman. 1980. Proton magnetic resonance studies of cytochrome c peroxidase: pD dependence of the isotropically shifted resonances. *Arch. Biochem. Biophys.* 202:608–616.
40. Nicholls, P. 1974. Cytochrome c binding to enzymes and membranes. *Biochim. Biophys. Acta.* 346:261–310.
41. Keller, R. M., and K. Wüthrich. 1978. Evolutionary change of heme c electronic structure: ferricytochrome *c*-551 from *Pseudomonas aeruginosa* and horse heart cytochrome c. *Biochem. Biophys. Res. Commun.* 83:1132–1136.