Assignment of ^{1}H and ^{13}C Hyperfine-Shifted Resonances for Tuna Ferricytochrome *c*

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ABSTRACT Tuna ferricytochrome *c* has been used to demonstrate the potential for completely assigning ^{1}H and ^{13}C strongly hyperfine-shifted resonances in metalloprotein paramagnetic centers. This was done by implementation of standard two-dimensional NMR experiments adapted to take advantage of the enhanced relaxation rates of strongly hyperfine-shifted nuclei. The results show that complete proton assignments of the heme and axial ligands can be achieved, and that assignments of several strongly shifted protons from amino acids located close to the heme can also be made. Virtually all proton-bearing heme ^{13}C resonances have been located, and additional ^{13}C resonances from heme vicinity amino acids are also identified. These results represent an improvement over previous proton resonance assignment efforts that were predicated on the knowledge of specific assignments in the diamagnetic protein and relied on magnetization transfer experiments in heterogeneous solutions composed of mixtures of diamagnetic ferrocytochrome *c* and paramagnetic ferricytochrome *c*. Even with that more complicated procedure, complete heme proton assignments for ferricytochrome *c* have never been demonstrated by a single laboratory. The results presented here were achieved using a more generally applicable strategy with a solution of the uniformly oxidized protein, thereby eliminating the requirement of fast electron self-exchange, which is a condition that is frequently not met.

INTRODUCTION

Paramagnetic metal sites of biological molecules are often studied because these sites are typically loci of macromolecular activity. From the NMR perspective these metal centers are inherent shift and relaxation agents with intrinsic properties that are advantageous for preferential detection of nuclei in the vicinity of the metal ion (La Mar, 1995; Berliner and Reuben, 1993; Bertini et al., 1993b; Bertini and Luchinat, 1986; Satterlee, 1986; La Mar et al., 1973). Despite this advantage there are a few impediments to effectively using hyperfine shifts. One of these is that hyperfine-shifted resonance positions for both ^{1}H and ^{13}C are not governed by the chemical shift correlations used diagnostically in initial assignment protocols for diamagnetic samples. Furthermore, in paramagnetic macromolecules, detection of nuclear resonances depends critically upon both nuclear properties and metal ion characteristics (Bertini and Luchinat, 1986; Satterlee, 1986; La Mar et al., 1973). Proton NMR spectroscopy has been most frequently used to study paramagnetic centers in biomolecules (for example, La Mar, 1995; Berliner and Reuben, 1993; Bertini et al., 1993b; de Ropp et al., 1991; Qin and La Mar, 1992; Holz et al., 1992; Bertini et al., 1992; Alam and Satterlee, 1995; Moore and Williams, 1984; Keller and Wuthrich, 1978; Santos and Turner, 1987; Feng et al., 1989, 1990; Gao et al., 1991), with fewer reports of other nuclei (Santos and Turner, 1986, 1992; Timkovich, 1991; Bertini et al., 1994a,b; Oh et al., 1990; Yamamoto et al., 1988).

A major difficulty is detecting strongly hyperfine-shifted resonances of nuclei close to paramagnetic centers. In the case of ^{1}H hyperfine-shifted resonances in magnetically anisotropic ferriheme proteins, fully one-half of the expected strongly shifted resonances lie under the 0–10 ppm region that is dominated by the intensities of the highly overlapping diamagnetic resonances. This "diamagnetic manifold" of resonances contains more than 98% of the total proton resonance intensity. As we demonstrate here, resonance overlap is less a complication for ^{13}C hyperfine-shifted resonances. Detectability problems arise not only from resonance overlap, but also from efficient nuclear relaxation due to the local hyperfine field of the ferriheme, which results in significant nuclear line broadening and real reduction in the "peak height/noise" ratio. For ligands directly attached to paramagnetic metal ions, ^{13}C resonances may also experience hyperfine shifts of several hundred ppm (Goff, 1981). Consequently, only partial assignments have been reported for the hyperfine-shifted heme carbons of several low-spin ferriheme proteins (Santos and Turner, 1986, 1992; Timkovich, 1991; Bertini et al., 1994a,b; Oh et al., 1990; Yamamoto et al., 1988).

Recent advances in adapting two-dimensional experiments to take advantage of paramagnetic relaxation enhancement for the purpose of differentially detecting hyperfine-shifted ^{1}H resonances of paramagnetic centers in the presence of vastly larger diamagnetic proton intensity have led to significant advances in ^{1}H assignment capability for many types of paramagnetic metalloproteins (La Mar et al., 1995; Luchinat and Piccioli, 1995). Virtually complete ^{1}H hyperfine resonance assignments have been reported for several heme proteins up to ~44 kDa in size (La Mar et al., 1995; Banci et al., 1991; de Ropp et al., 1991; Alam and Satterlee, 1994, 1995; Qin and La Mar, 1992).

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In view of these results in comparatively larger proteins, we have wondered why completely assigning strongly shifted ^{1}H hyperfine resonances in the smaller-sized ferricytochrome *c* has been so involved (Keller and Wuthrich, 1978; Santos and Turner, 1986, 1987; Feng et al., 1989, 1990; Gao et al., 1990, 1991). Although there have been many attempts to make such ferricytochrome *c* assignments, no single set of experiments has ever completely located the heme proton resonances, much less identified the hyperfine-shifted resonances of amino acids located adjacent to the paramagnetic heme. Even the most complete set of ferricytochrome *c* heme proton assignments presented to date (Feng et al., 1990) has not located two of the heme meso-proton resonances. That work employed a method that has been historically (Keller and Wuthrich, 1978) used in making hyperfine ^{1}H resonance assignments in ferricytochrome *c*. It requires prior knowledge of ^{1}H assignments in the diamagnetic ferrocytochrome *c* and then uses magnetization transfer measurements in solutions containing ferrocytochrome *c*/ferricytochrome *c* mixtures. Others have attempted direct ^{1}H hyperfine resonance assignments in homogeneous solutions of ferricytochrome *c*, but even the most extensive set of these has been incomplete (Santos and Turner, 1986; Feng et al., 1989; Gao et al., 1990, 1991). As noted above, the assignment of carbon hyperfine-shifted resonances in ferriheme proteins is similarly incomplete (Santos and Turner, 1992; Timkovich, 1991).

It would clearly be advantageous to studies on paramagnetic metalloproteins that have no stable diamagnetic state to demonstrate that essentially total ^{1}H and ^{13}C hyperfine resonance assignments could be made from appropriate paramagnetic protein forms. This also eliminates the requirement that subject proteins undergo rapid electron self-exchange, a condition mandated by the method that uses mixtures of ferro- and ferricytochrome *c*, but one frequently not satisfied by many metalloproteins. Here we show that the complications of the previous studies can be avoided using homonuclear and heteronuclear two-dimensional NMR experiments to completely assign the strongly hyperfine-shifted ^{1}H resonances in homogeneous solutions of tuna ferricytochrome *c* and to assign virtually all of the ^{13}C hyperfine-shifted resonances. Success depended upon implementation of experiments in ways designed to take advantage of the enhanced relaxation rates of strongly hyperfine-shifted resonances.

We have employed tuna ferricytochrome *c* in this work for a variety of reasons. First, it is an important reference component in our continuing studies of the cytochrome *c* peroxidase:cytochrome *c* electron transfer complexes, and the experimental results derived here will be important for interpreting future studies of these complexes. Second, it is a readily available, typical member of the *c*-type cytochromes. Third, it is stable in the ferriheme form. Fourth, both diamagnetic and paramagnetic forms of the protein have been well characterized by proton NMR (Williams et al., 1985; Moore and Williams, 1980), yet there are few reported assignments of hyperfine-shifted resonances for the heme and neighbor amino acids, making it an excellent candidate both for testing these experiment capabilities and for producing new data.

EXPERIMENTAL

Tuna cytochrome *c* was purchased from Sigma and used without further purification. Cytochrome *c* was dissolved in a 50 mM potassium phosphate/10 mM potassium nitrate buffer (pH 6.8, uncorrected). Potassium ferricyanide was added to ensure 100% oxidation. The excess potassium ferricyanide was removed by applying the sample to a 1–×8 Dowex anion exchange column. Samples for proton NMR spectroscopy were 2–4 mM in protein concentration, and the sample used for natural abundance ^{13}C/^{1}H HMQC was 10 mM in protein concentration. All samples were exchanged into 50 mM potassium phosphate/10 mM potassium nitrate D_2O buffer (99.9 atom% D; Isotec) by cycles of dilution/concentration in an Amicon pressure ultrafiltration cell. Sample pH was adjusted as necessary with NaOD (Isotec) and DCl (Isotec). A standardized Fisher combination electrode and Fisher Accumet 925 pH meter were used for this purpose.

Because of limits on instrument access and instrument malfunctions, our NMR experiments had to be completed at institutions other than Washington State University (WSU). The work described here was carried out on the following instruments located at WSU, Pennsylvania State University (PSU), and the National Magnetic Resonance Facility at Madison (NMRFAM): a Bruker AMX 300 (WSU) operating at the nominal proton frequency of 300 MHz; a Varian VXR500s (WSU); a Bruker AMX2 (PSU); and a Bruker DMX500 (NMRFAM), all operating at the nominal proton frequency of 500 MHz (NMRFAM); and a Bruker DMX 750 (NMRFAM) operating at the nominal proton frequency of 750 MHz. The DEFT-NOESY (Kao and Hruby, 1986) experiment employed a DEFT (Becker et al., 1975) pulse sequence in place of the first pulse in the standard NOESY sequence:

$$\pi/2\text{-RD-}\pi\text{-RD-}\pi/2\text{-}t1\text{-}\pi/2\text{-}\tau_{mix}\text{-}\pi/2\text{-acq},$$

where RD is the relaxation delay interval, which, in these experiments, ranged between 1.0 ms and 500 ms, and τ_{mix} is the mixing period during which the nuclear Overhauser effect (NOE) develops. Typically this experiment was implemented using a 10-μs $\pi/2$ proton pulse, a relaxation delay of 150 ms, and a total recycle rate of 3 s^{-1}. CLEAN-TOCSY (Griesinger et al., 1988) experiments were carried out using a 10-μs $\pi/2$ proton pulse and a DIPSI-2rc (Cavanaugh and Rance, 1992) spin-locking field. CLEAN-TOCSY spectra were acquired with isotropic mixing times ranging from 10 to 25 ms.

All homonuclear 2D data were acquired with 2048 hypercomplex data points in the f2 dimension, 512–600 t1 increments, and 128–256 scans per block. All Varian spectra acquired utilized the hypercomplex acquisition method of States et al. (1992), and Bruker data utilized the acquisition method of TPPI. Data were processed using either VNMR (Varian Associates) or FELIX (BIOSYM) software running either on a Sun SparcStation ELC or a Silicon Graphics Indigo2. DEFT-NOESY data sets were processed using a combination of sinebell and Gaussian apodization functions. CLEAN-TOCSY experiments were processed with a sinebell apodization function. All 2-D matrix sizes were 2048 × 2048. Residual H_2O was assigned a chemical shift of 4.76 ppm relative to DSS at 25°C.

The ^{13}C/^{1}H HMQC experiments were acquired on a Bruker AMX 300 utilizing the GARP sequence for ^{13}C decoupling, repetition rates of 1–10 s^{-1} in consecutive experiments, and a delay (1/2 *J*) of 3.9 ms (Bax et al., 1983). A total of 1024 scans were taken for each of the 300 *t*1 increments collected. The ^{13}C dimension of the HMQC experiment was referenced to external TMS, which was assigned a value of 0.0 ppm.

RESULTS AND DISCUSSION

The heme *c* structure and modified Fischer (IUPAC, 1988) labeling scheme employed in this work are shown in Fig. 1.

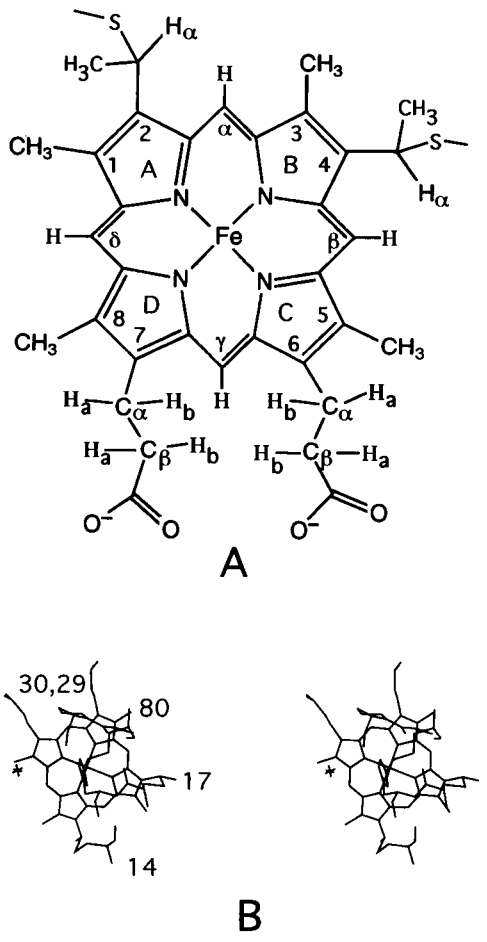

FIGURE 1 (*A*) Heme *c* showing the Fischer labeling system used in this work. (*B*) Stereo view of the heme vicinity of tuna cytochrome *c* derived from the Protein Data Bank coordinate file 3CYT, showing coordinating ligands His 18 and Met 80, as well as nearby amino acids Cys 14, Cys 17, Gly 29, and Pro 30.

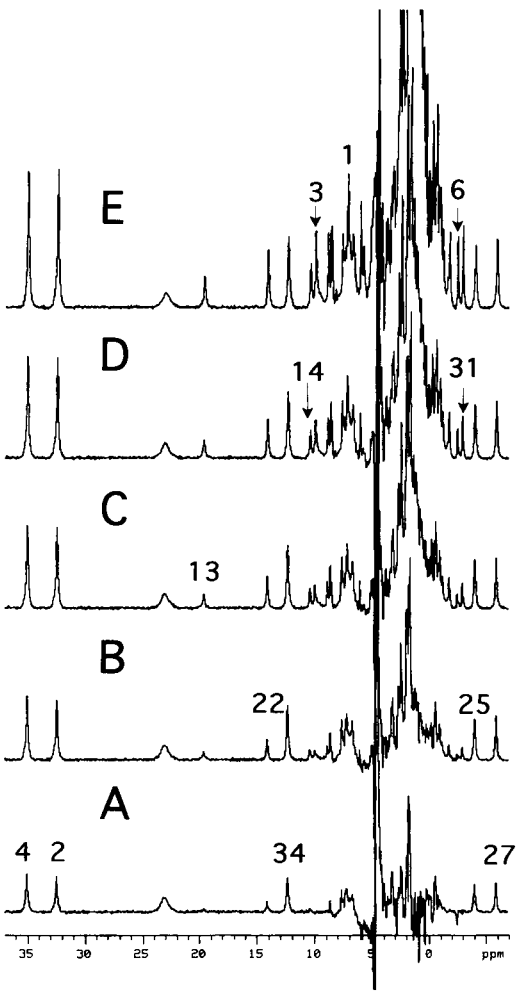

FIGURE 2 500-MHz 1D DEFT spectra of tuna ferricytochrome *c* taken with a repetition rate or 3 s^{-1} and relaxation delay (RD) times of (*A*) 30 ms, (*B*) 50 ms, (*C*) 70 ms, (*D*) 90 ms, and (*E*) 140 ms. The numbers above the resonances correspond to the assignments listed in Table 1. Solution conditions: D_2O, pH 6.8, 25°C.

This figure also provides an edited stereo view based on the crystal structure of the protein (Takano and Dickerson, 1981; Bernstein et al., 1977), showing the heme vicinity with inclusion of amino acids for which hyperfine resonance assignments have been made in this work.

Enhanced paramagnetic relaxation occurs for magnetic nuclei located close to the paramagnetic center, in this case the ferriheme and several neighbor amino acids, and this is demonstrated for the proton resonances of tuna ferricytochrome *c* in Fig. 2. Fig. 2 displays a series of proton 1D-DEFT spectra at several different relaxation delay times (RD). This differential relaxation comprises the spectroscopic basis for differentially enhanced detection of the hyperfine-shifted ^1H resonances. Fig. 2 illustrates the fact that many fast-relaxing resonances appear as normal positive peaks at short RD times, when more slowly relaxing resonances have minimized intensity or remain inverted. It also demonstrates the increasing positive intensities that occur as the RD lengthens. By judicious choice of the RD one can minimize spectral intensity with more slowly relaxing diamagnetic nuclei, thereby effectively reducing the spectral dynamic range, while differentially detecting the faster relaxing, hyperfine-shifted resonances.

Critically important to achieving unambiguous assignments was our ability to detect NOE cross-peaks at very short mixing times (1–20 ms). As we now show, cross-peaks observed in this mixing time regime result from primary NOE's (Neuhaus and Williamson, 1989). Establishing the primary nature of NOE's is absolutely critical to the reliable interpretation of NOESY cross-peak patterns because only primary NOE's are related to internuclear distance. Discrimination between primary and relayed NOE's was achieved by systematic mixing time variations in repeated NOESY and DEFT-NOESY experiments.

Fig. 3 illustrates the NOE behavior of several typical heme ^1H hyperfine resonances measured in a set of normal NOESY experiments. These rise curves reveal several primary NOE's reflected by the extrapolated intercept of the

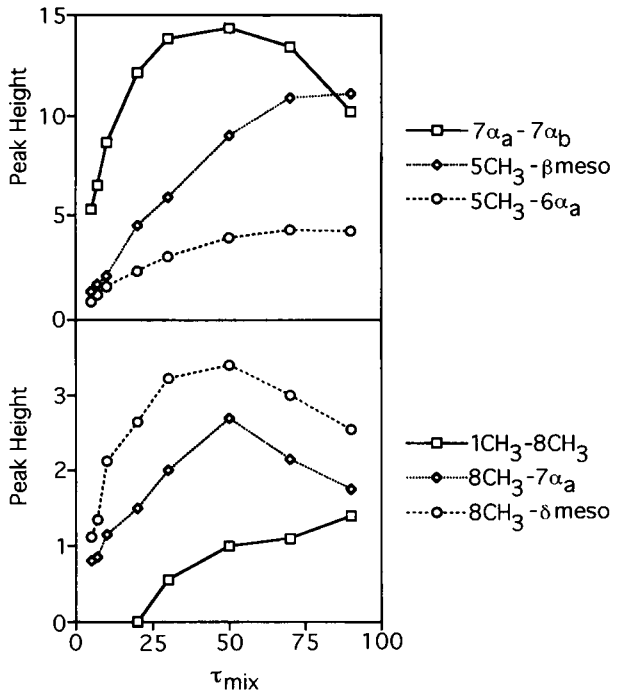

FIGURE 3 NOE rise curves for several of the heme cross-peaks in tuna ferricytochrome $c(D_2O$, pH 6.8, 25°C). The NOESY spectra were accumulated with a repetition rate of 2 s^{-1}, and mixing times (τ_{mix}) were varied from 3 ms up to 90 ms. Several of the cross-peaks can be seen in other figures, for example: $7\alpha H_b/7\alpha H_a$ (Fig. 4 *A* and labeled 14/13), $5CH_3/\beta$ meso (Figs. 4 and 6 3/18), $5CH_3/6\alpha H_a$ (Fig. 6 3/10), $8CH_3/7\alpha H_a$ (Fig. 4 *B* 4/13), and $8CH_3/\delta$ meso (Fig. 4 *B* 4/20).

curves at 0, 0 (τ_{mix}, intensity). One relayed (or secondary) NOE is also shown, for the cross-peak $1CH_3$-$8CH_3$, and is characterized by its extrapolated nonzero/zero intercept (~21 ms on the τ_{mix} axis). The data shown in Fig. 3 are typical of all the cross-peaks between heme hyperfine-shifted ^1H resonances and reveal that their individual NOE maxima are achieved in the range of 50–100 ms. This means that in most cases cross-peaks in spectra detected at mixing times as long as 25 ms can be interpreted as primary NOE's.

Table 1 reveals hyperfine-shifted proton assignments and identifies each assigned resonance with a label number. The label numbers are used throughout the subsequent discussion to identify cross-peaks in the 2D contour spectra shown in Figs. 4–6 in the following way. Each cross-peak is designated in the form *x/y*, where *x* refers to the Table 1 label for the resonance whose shift is given by extrapolating to the vertical axis (dimension 2), and *y* refers to the resonance whose shift is given by extrapolating to the horizontal axis (dimension 1).

Primary NOE's resulting from short mixing time 500-MHz DEFT-NOESY experiments are shown in Fig. 4, a split-diagonal contour plot of experiments conducted at 4 ms (Fig. 4 *A*) and 10 ms (Fig. 4 *B*) mixing times. For the shorter mixing time experiment the contour plot is dominated by cross-peaks from geminal partner protons. As

TABLE 1 ^1H and ^{13}C NMR resonance assignments for heme and adjacent amino acids of tuna ferricytochrome *c*

Resonance	Label	^1H (25°C)	^{13}C (25°C)
$1CH_3$	1	7.12	−19.39
$3CH_3$	2	32.39*	−56.98
$5CH_3$	3	9.96*	−25.85
$8CH_3$	4	35.01*	−71.13
2CαH	5	−1.08	6.49
$2CH_3$	6	−2.39*	38.02
4CαH	7	2.05	−39.60
$4CH_3$	8	3.20*	84.11
$6\alpha H_b$	9	2.25	−2.81
$6\alpha H_a$	10	−1.66	−2.81
$6\beta H_a$	11	1.03	ND
$6\beta H_b$	12	2.52	ND
$7\alpha H_a$	13	19.60#	−35.96
$7\alpha H_b$	14	10.37#	−35.96
$7\beta H_a$	15	1.76	10.93
$7\beta H_b$	16	−0.16	10.93
αmeso	17	1.83	ND
βmeso	18	−0.49	17.80
γmeso	19	7.62	14.17
δmeso	20	2.58	ND
Cys 14 αH	21	4.29	ND
His 18 βH_a	22	14.07#	25.08
His 18 βH_b	23	8.56#	25.08
His 18 αH	24	8.87#	74.00
Gly 29 αH_a	25	−3.94	35.19
Gly 29 αH_b	26	−0.87	35.19
Pro 30 δH_a	27	−5.82	45.29
Pro 30 δH_b	28	−1.68	45.29
Pro 30 γH_a	29	−0.13	ND
Pro 30 γH_b	30	−0.75	ND
Leu 68 δCH_{3a}	31	−2.85§	16.99
Leu 68 δCH_{3b}	32	−0.70§	16.99
Leu 68 γH_a	33	0.61§	ND
Met 80 βH_a	34	12.30	3.25
Met 80 βH_b	35	7.13	3.25
Met 80 ε	36	−24.27*	14.27¶

*Satterlee and Moench (1987).
#Moore and Williams (1984).
§Williams et al. (1985).
¶Only observed at 35°C.
ND, No data.

identified in Fig. 4 *A*, these include (Fig. 1) the heme 6-propionate $6\alpha H_a/6\alpha H_b$ cross-peak {10/9}, heme 7-propionate $7\alpha H_b/7\alpha H_a$ cross-peak {14/13}, His18 $\beta H_b/\beta H_a$ cross-peak {23/22}, Gly29 $\alpha H_a/\alpha H_b$ cross-peak {25/26}, Pro30 $\delta H_a/\delta H_b$ cross-peak {27,28}, and the Met80 $\beta H_b/\beta H_a$ cross-peak {35/34}. At 10-ms mixing time additional primary NOE cross-peaks appear, such as the heme $7\alpha H_b/$heme γ-meso {14/19}, heme $7\alpha H_b/7\beta H_b$ {14/16}, heme 5-CH_3/heme β-meso {3/18}, heme $8CH_3$/heme δ-meso {4/20}, and heme $8CH_3$/heme $7\alpha H_a$ {4/13}.

Fig. 4 reveals the improved detectability of hyperfine-shifted resonances, particularly in the normally proton-dense 10 ppm to −2 ppm region, that can be achieved by taking advantage of the differential relaxation rates of these paramagnetically influenced resonances. We now describe how connectivities may be followed sequentially around the heme periphery.

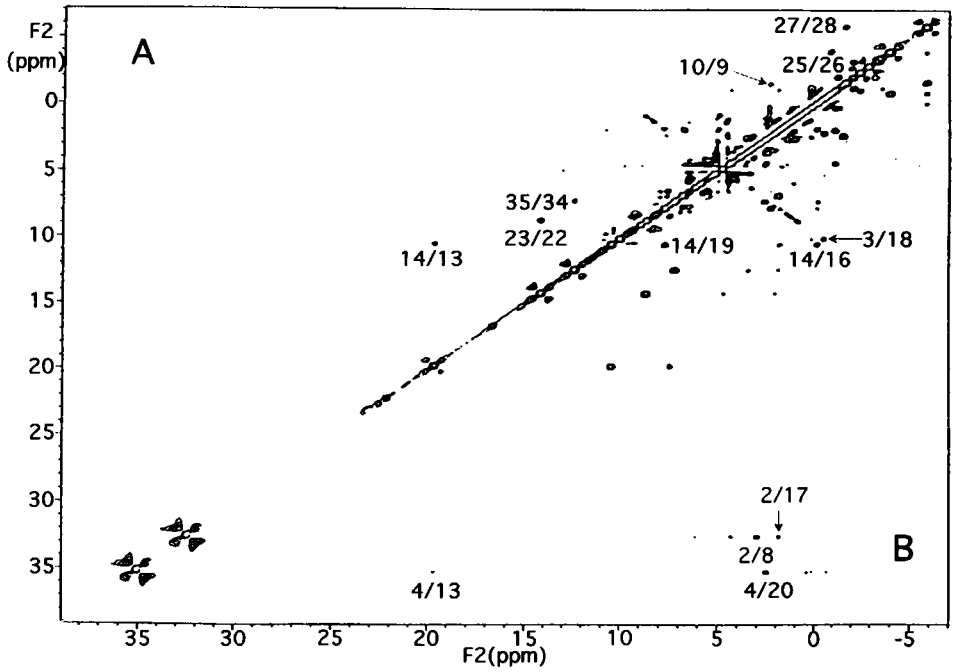

FIGURE 4 Split diagonal presentation of 500 MHz unsymmetrized ^{1}H DEFT-NOESY contour spectra of tuna ferricytochrome *c* collected at 25°C (D_2O, pH 6.8) with a repetition rate of 3 s^{-1} and a relaxation delay of 150 ms. (*A*) A 4-ms DEFT-NOESY spectrum showing connectivities between heme and amino acid geminal partners. (*B*) A 10-ms DEFT-NOESY spectrum showing longer range NOE connectivities.

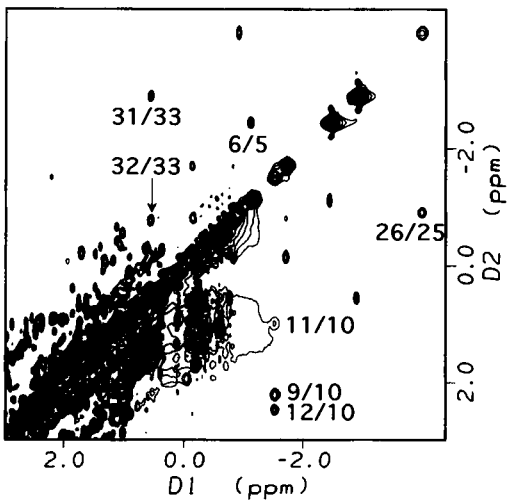

FIGURE 5 Partial contour plot of the 500 MHz ^{1}H CLEAN-TOCSY spectrum of tuna ferricytochrome *c* in D_2O, pH 6.8, 25°C, recorded with 20-ms mixing time. The spectrum shows the 6-propionate spin system, the 2-methine spin system, as well as partial spin systems of amino acids that lie spatially close to the heme such as Leu 68 and Gly 29. Table 1 gives specific assignments.

Proton assignments

As mentioned previously, the assignment procedure began with known hyperfine-shifted resonance assignments (Moench et al., 1991), which were expanded by NOESY, DEFT-NOESY, and CLEAN-TOCSY experiments to delineate and connect the proton spin systems of the heme peripheral substituents. These experiments also gave additional assignments for hyperfine-shifted proton resonances of amino acids near heme *c* (Table 1).

Identification of heme peripheral spin systems was achieved by CLEAN-TOCSY experiments at 500 MHz. The necessity of employing this type of experiment rather than standard COSY-type experiments derives from the recently identified cross-correlation phenomena termed relaxation allowed coherence transfer (Bertini et al., 1993a; Qin et al., 1993), which occurs in paramagnetic metalloproteins and renders standard COSY experiments useless for elucidating true bond correlations (La Mar et al., 1995; Luchinat and Piccioli, 1995). It has been shown that the CLEAN-TOCSY experiment allows bonafide spin-coupling networks to be detected in paramagnetic heme proteins (La Mar et al., 1995; Luchinat and Piccioli, 1995). Fig. 5 presents a CLEAN-TOCSY 2D contour plot showing the heme 6-propionate spin system {9/10, 11/10, 12/10}, the heme $2CH_3/2C\alpha H$ spin system {6/5}, and partial spin systems of nearby amino acid residues Gly 29 (26/25) and Leu 68 {31/33, 32/33} (Table 1).

NOESY and DEFT-NOESY studies that were then carried out over a range of mixing times between 1 ms and 60 ms, and at several temperatures between 0°C and 25°C, led to sequential connectivities around the heme periphery. Fig. 6, a 750 MHz DEFT-NOESY 2D contour plot (25-ms mixing time), is used to summarize our results.

The known $8CH_3/7\alpha H_a$ connectivity {4/13}, (Fig. 4 *B*; 15) leads to the stereospecific (Fig. 1) identification of the geminal partners $7\alpha H_b/7\alpha H_a$ (14/13, Fig. 4 *A*). The connectivities from $7\alpha H_b$ to both $7\beta H_a$ {14/15} and $7\beta H_b$ {14/16} are seen in Fig. 6, as is the $7\beta H_b/7\beta H_a$ cross-peak {16/15}. The closest protons to the heme Hγ-meso are the heme $7\alpha H_b$ and $6\alpha H_b$, and it is this mutual connectivity that is used to bridge the heme D and C pyrrole rings (Fig. 1) via the $7\alpha H_b$/Hγ-meso cross-peak (14/19, Figs. 4 *B* and 6) and

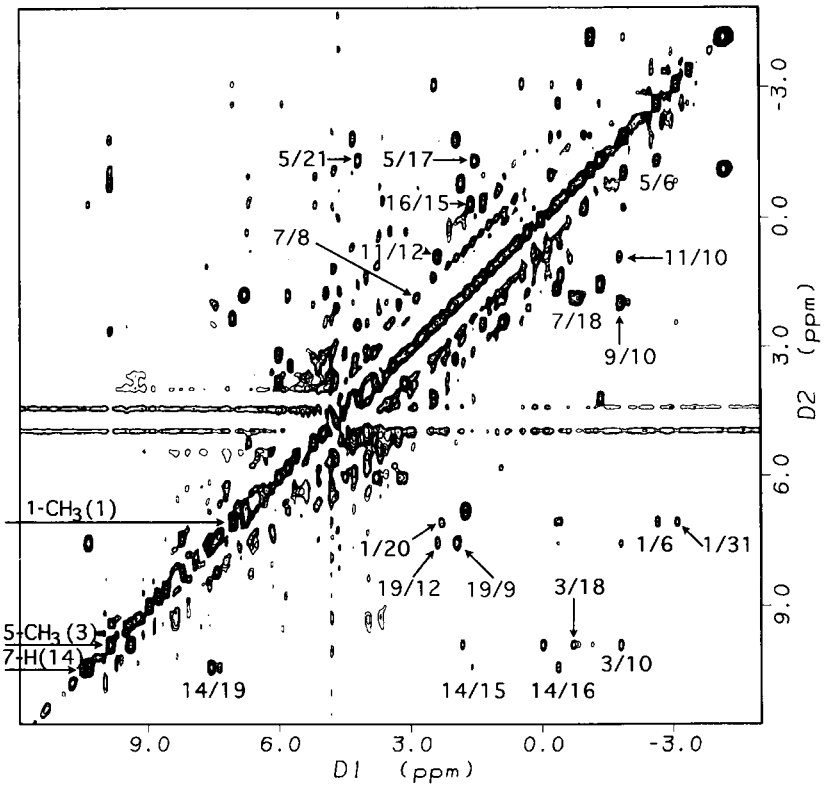

FIGURE 6 Partial contour plot of the ^{1}H 750 MHz DEFT-NOESY spectrum of tuna ferricytochrome *c* in D_2O, pH 6.8, 25°C, recorded with a 25-ms mixing time. This unsymmetrized spectrum summarizes most of the heme assignments. A description of the assignment procedure is presented in the text.

the Hγ-meso/6αH$_b$ cross-peak (19/9, Fig. 6). Connectivities to the rest of the 6-protons {9/10, 11/10, and 12/10} are obvious from Figs. 5 and 6. The cross-peak {3/10} from the known heme 5CH$_3$ resonance (9.6 ppm) to the 6αH$_a$ resonance completes pyrrole C stereospecific assignments.

The 5CH$_3$/Hβ-meso cross-peak {3/18} can be seen in the 10-ms mixing time DEFT-NOESY shown in Fig. 4 *B* at 10.02/−0.55 ppm (also in Fig. 6), and this supplies a connectivity path to pyrrole B via the 4CαH/Hβ-meso cross-peak (7/18, Fig. 6). The 4CαH/4CH$_3$ cross-peak {7/8} can be seen in Fig. 6. A cross-peak (2/8, Fig. 4 *B*) from the previously assigned 3CH$_3$ to the 4CH$_3$ completes the pyrrole B assignments. Stereochemical information is also derived from the 3CH$_3$/4CH$_3$ connectivity, apparent at short mixing times, in combination with the absence of 3CH$_3$/4CαH connectivity at short mixing times. Together these two results indicate that the two methyl groups are oriented near each other, whereas the 4CαH is positioned further away from the 3CH$_3$. Consistent with this is the 4CαH/Hβ-meso connectivity, indicating the proximity of these latter two protons. Connectivities between the 3CH$_3$/Hα-meso (2/17, Fig. 4 *B*) and 2CαH/Hα-meso (5/17, Fig. 6) link pyrrole A and pyrrole B. Within pyrrole A the 2αH/2CH$_3$ connectivity is given by cross-peak {5/6} in Figs. 5 and 6. Cross-peak {1/6} (Fig. 6) completes the connectivity for pyrrole A, showing proximity of the heme 2CH$_3$ to the heme 1CH$_3$. Multiple NOE experiments reveal that the stereochemistry of the covalent heme linkage at position 2 on pyrrole A is similar to that at position 4 on heme B. Cross-peaks between the heme 1-CH$_3$/Hδ-meso (1/20, Fig. 6) and 8CH$_3$/Hδ-meso (4/20, Fig. 4 *B*) connect pyrroles A and D and finish the complete heme proton assignments given in Table 1. The proton assignments presented here agree well with previously reported assignments for corresponding resonances in horse (Feng et al., 1989) ferricytochrome *c* and (C102T) yeast iso-1 (Gao et al., 1990, 1991) ferricytochrome *c*. The stereospecific assignments for the heme 2-, 4-, 6-, and 7-position substituents agree with the corresponding side-chain conformations shown in the crystal structure (Takano and Dickerson, 1981; Bernstein et al., 1977).

Carbon assignments

Although ^{13}C NMR studies on paramagnetic porphyrin complexes were reported in 1981 (Goff, 1981), interest in ^{13}C/^{1}H heteronuclear correlated spectroscopy applied to paramagnetic centers in proteins has been recent and quite limited. Nevertheless, demonstrations both with heme (Santos and Turner, 1986, 1992; Timkovich, 1991; Yamamoto et al., 1988) and nonheme (Bertini et al., 1994a,b; Oh et al., 1990) proteins have established the potential for deriving more extensive assignments and hence greater information about a protein's molecular structure.

Our approach has been to employ natural abundance ^{13}C/^{1}H HMQC experiments implemented with rapid recycle rates (1–18 s^{-1}) to differentially suppress magnetizations

from the more slowly relaxing, diamagnetic nuclei. The results are shown in Fig. 7 and Table 1. The ^{13}C assignments shown in this figure follow directly from the ^{1}H assignments described above. It is clear from this figure that one can obtain a significant number of $^{13}C/^{1}H$ correlations for resonances that are strongly influenced by the heme paramagnetism. A significant feature of this work is that we have assigned all of the proton-bearing carbons of the heme, except for the 6Cβ, and the Cα-meso and Cγ-meso. These three carbon resonances occur in regions of high ^{13}C resonance density and we have been unsuccessful, so far, in unambiguously locating them.

Importantly, we were able to detect the $^{13}C/^{1}H$ correlation of the Met80 εCH_3 group (no. 36; Fig. 7 and Table 1), which, although previously assigned by 1D decoupling methods (Santos and Turner, 1992), has apparently not been detected before this in 2D correlation experiments. This is probably attributable to efficient paramagnetic relaxation of both ^{13}C and ^{1}H for this methyl group via the contact mechanism, because the Met80 εC is separated from the paramagnetic heme ferric ion by only two bonds (Fig. 1). The εH's are, similarly, only three bonds thus removed with a resonance that demonstrates a large upfield shift (Table 1), a comparatively large linewidth (110 Hz at half-maximum height at 300 MHz), and an associated T_2 of 2.9 ms at 35°C. Although this correlation produces a very weak cross-peak, it is nonetheless unambiguous and emphasizes the fact that one can detect heteronuclear correlations to protons with fairly large linewidths.

We did not explicitly assign the His18 C_2 and C_4 ring protons, although these have been assigned for horse ferricytochrome *c* using 1D NOE difference spectroscopy (Santos and Turner, 1987). These two protons both display broad resonances with linewidths of $\geq$250 Hz (half-maximum intensity) at 300 MHz (35°C), corresponding to T_2 of 1.3 ms. For example, the His18C_4H resonance is shown at ~23 ppm in Fig. 2, and we have not yet identified cross-peaks to either of these protons in either homonuclear or heteronuclear 2D experiments. Such a short ^{1}H T_2 obviously compromises the detection of dipolar coupling and coherence transfer in these types of experiments, indicating that there is a practical limit in this ^{1}H T_2 region for implementing this approach.

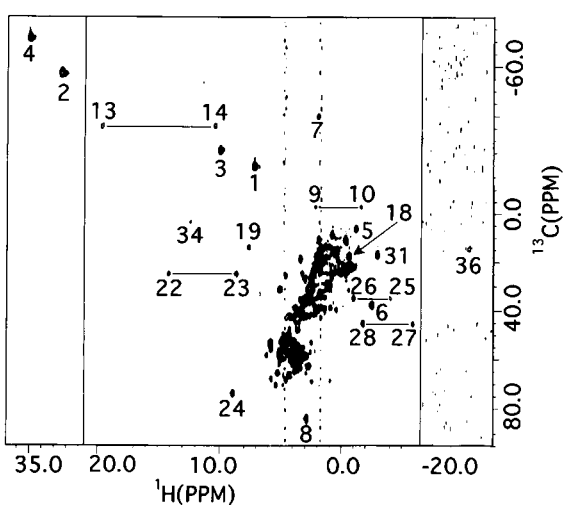

FIGURE 7 Partial contour plot of the 300-MHz $^{1}H/^{13}C$ HMQC spectrum of tuna ferricytochrome *c* in D_2O, pH 6.8, 25°C. The figure is divided into three different spectral regions. The low-frequency part of the spectrum corresponding to cross-peak 36 was obtained at 35°C.

SUMMARY

Although tuna ferricytochrome *c* is a comparatively small protein by NMR standards, until now complete proton hyperfine resonance assignments have only been achieved through magnetization transfer experiments conducted on mixtures of oxidized and reduced protein. No ^{13}C hyperfine shift assignments have been reported for tuna ferricytochrome *c*, although some ^{13}C hyperfine shift assignments have been reported for other small cytochromes (Santos and Turner, 1986; Yamamoto et al., 1988; Timkovich, 1991). Even though 14 of the 36 proton resonances shown in Table 1 have been previously assigned, the uniqueness of this work is that we show that it is possible to use 2D NMR experiments to achieve complete heme ^{1}H hyperfine-shifted resonance assignments, to easily assign all but three of the proton-bearing ferriheme ^{13}C resonances and to make several ^{13}C and ^{1}H hyperfine-shifted resonance assignments for heme site amino acids. Fifty newly assigned hyperfine resonances (^{1}H and ^{13}C) are thus identified for tuna ferricytochrome *c*. For corresponding resonances our results compare favorably with the more limited ^{13}C assignments reported for horse ferricytochrome *c* (Santos and Turner, 1986, 1992) and ferricytochrome c_{551} (Timkovich, 1991).

In the course of this work we have relied on ^{1}H data taken at 750 MHz. Paramagnetic proteins have not yet been extensively studied at this high field. In fact, the Curie spin relaxation mechanism (Gueron, 1975), which enhances T_2 relaxation and causes lines to broaden as the square of B_o, engenders questions concerning the appropriateness of studying paramagnetic molecules at such high fields. Some of our data at 17.6 T are presented here (Figs. 6 and 8). In Fig. 8 we show comparisons of identically processed ferricytochrome *c* spectra taken on the same sample at 500 MHz and 750 MHz. One can see that there is a slight gain in shift dispersion at the higher field, indicating that Curie spin relaxation does not compromise studies of ferriheme (S = 1/2) proteins up to ~12.5 kDa in size.

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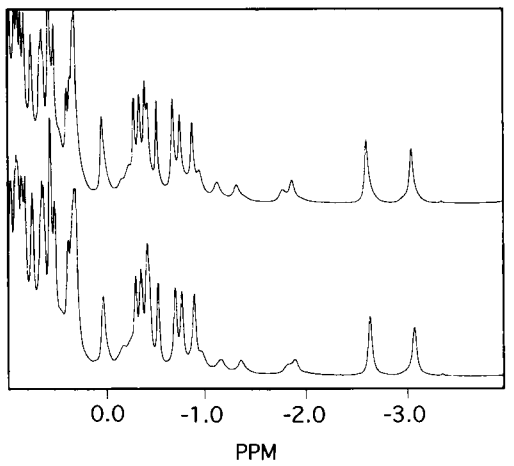

FIGURE 8 Low-frequency (high-field) portion of the proton 1D spectrum for tuna ferricytochrome *c* in D_2O, pH 6.8, 25°C. (*bottom*) One-dimensional spectrum collected at 500 MHz with a recycle rate of 2 s^{-1}. (*top*) The same spectral region as *A*, but collected with identical conditions at 750 MHz.

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