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Proton Homonuclear Correlated Spectroscopy as an Assignment Tool for Hyperfine-Shifted Resonances in Medium-Sized Paramagnetic Proteins: Cyanide-Ligated Yeast Cytochrome *c* Peroxidase as an Example[†]

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ABSTRACT: Two types of homonuclear proton COSY experiments are shown to be useful in making resonance assignments in cyanide-ligated cytochrome *c* peroxidase, a 34 kDa paramagnetic heme protein. Both magnitude COSY and phase-sensitive COSY experiments provide spectra useful for making proton assignments to resonances of strongly relaxed hyperfine-shifted protons. This initial investigation demonstrates that COSY experiments combined with NOESY experiments are feasible for hyperfine-shifted protons of paramagnetic proteins larger than metmyoglobins and ferricytochromes *c*, for which the nuclear spin-lattice relaxation times are in the range 70-300 ms. Taken together, COSY and NOESY experiments, although not yet widely applied to paramagnetic metalloproteins, provide a reliable protocol for accurately assigning hyperfine-shifted resonances that are part of a metalloenzyme's active site. Specific examples of expected proton homonuclear COSY connectivities that were not observed in these experiments are presented, and utilization of COSY with respect to the proton resonance line widths and apparent nuclear relaxation times is discussed. The COSY experiments presented here provide valuable verification of previously proposed hyperfine resonance assignments for cyanide-ligated cytochrome *c* peroxidase, which were made by using NOESY experiments alone, and in several instances expand these assignments to additional protons in particular amino acid spin systems.

Application of two-dimensional NMR methods to paramagnetic metalloproteins has lagged behind its use in studies of diamagnetic molecules (Wuthrich, 1986). One reason for this may have been a perception that the comparatively short T_1's[1] and T_2's of nuclei demonstrating hyperfine-shifted resonances in paramagnetic molecules would severely compromise coherence transfer in COSY-type experiments and dipolar cross-relaxation in NOESY experiments. Recently, however, homonuclear bond-correlated spectroscopy and NOESY experiments have been explicitly demonstrated to be valuable for assigning rapidly relaxing hyperfine-shifted proton resonances in paramagnetic heme proteins (Yamamoto et al., 1989; Emerson et al., 1990; Busse et al., 1990; Yu et al., 1990; Gao et al., 1990; Satterlee & Erman, 1991).

The significance of these successes resides in the fact that for metalloproteins the active site frequently incorporates a paramagnetic metal ion. This results in strongly shifted, rapidly relaxed, broad resonances for protons in the active site, which are frequently the protons of primary interest in NMR studies. Despite the importance of those protons in biochemical studies, hyperfine resonance assignment methods have been slow to develop. This is possibly due to the observation that paramagnetism-induced effects on the NMR spectrum of active site protons compromise both direct elucidation of spin-spin coupling constants and commonly used chemical shift correlations, both of which are normally employed as starting bases for making assignments in diamagnetic molecules.

Literature reports show that whereas 2D bond-correlated spectroscopy has been utilized before in making assignments in small paramagnetic proteins, in most cases the assigned protons were typically main-chain and side-chain protons that were far removed from the paramagnetic center and therefore not extensively influenced by paramagnetic effects (Veitch et al., 1988; Feng et al., 1989; Feng & Englander, 1990; Gao et al., 1990; Veitch & Williams, 1990). In a few instances COSY experiments have been specifically used to assign hy-

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[1] Abbreviations: CcPCN, cyanide-ligated cytochrome *c* peroxidase; CcP, native resting state cytochrome *c* peroxidase; HRP, native resting state horseradish peroxidase; HRPCN, cyanide-ligated horseradish peroxidase; 1D, one dimensional; 2D, two dimensional; T_1, nuclear spin-lattice relaxation time; T_1^{app}, apparent nuclear spin-lattice relaxation time; T_2, nuclear spin-spin relaxation time; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; COSY, two-dimensional correlated spectroscopy; DQFCOSY, double-quantum filtered two-dimensional correlated spectroscopy; MCOSY, magnitude two-dimensional correlated spectroscopy; RCT, relayed coherence transfer.

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perfine-shifted proton resonances, but until now these have been limited to *smaller paramagnetic proteins* like ferricytochromes *c* (~12 kDa) (Feng et al., 1989; Busse et al., 1990; Gao et al., 1990) and cyanide-ligated metmyoglobin (~16 kDa) (Emerson & La Mar, 1990; Yu et al., 1990; Yamamato et al., 1989). Also valuable would be information about the feasibility of using COSY-type experiments for the purpose of establishing bond-correlated connectivities to define *J*-coupled spin networks involving hyperfine-shifted proton resonances in paramagnetic proteins as large as CcPCN (~34 kDa). In order to provide this information and to expand proton resonance assignments, we have carried out a set of proton homonuclear COSY-type experiments on CcPCN, a paramagnetic heme protein that is 2–3 times larger than either myoglobin or cytochrome *c*.

The heme protein that is the subject of this study, cytochrome *c* peroxidase from *Saccharomyces cerevisiae* (EC 1.11.1.5), is prototypical of the heme peroxidases. By NMR standards it is relatively large (~34 kDa), paramagnetic in both its resting state, where the heme ferric ion is high spin, and in its cyanide-inhibited form, where the heme ferric ion is low spin. On the basis of kinetics studies, its inferred physiological role is to catalyze the hydrogen peroxide oxidation of reduced cytochrome *c*. Its role is apparently that of a cytotoxic protective agent. Because it is a relatively abundant soluble globular protein CcP has become the focus of physical and protein engineering studies designed to characterize aspects of its behavior related to its participation in electron transfer reactions with reduced cytochromes *c* (cf. Waldmeyer et al., 1982; Finzel et al., 1984; Erman et al., 1987, 1989; Fishel et al., 1987; Goodin et al., 1987; Northrup et al., 1988; Sivaraja et al., 1988; Satterlee et al., 1990).

Materials and Methods

Cytochrome *c* peroxidase was isolated and purified as previously described (Vitello et al., 1990). It was prepared for NMR spectroscopy as previously described (Satterlee & Erman, 1980, 1983). All preparations of the enzyme conformed to the purity criteria previously described (Vitello et al., 1990). The CcP concentration was 1.7 mM, in 99.9% D_2O (Isotec), 0.10 M KNO_3, and 0.005 M perdeuterated potassium phosphate (both Fisher), pH 7.0. The perdeuterated potassium phosphate salts were created as stock samples by individually weighing out 250 g each of KH_2PO_4 and K_2HPO_4 and individually dissolving them in excess 99.9% D_2O (MSD Isotopes), followed by lyophilization to dryness. This process was repeated twice prior to storing the dried salts sealed under argon (Matheson). The solution pH was measured with a calibrated Beckman meter and combination electrode.

Initial one-dimensional experiments, such as shown in Figure 1, and all two-dimensional NMR measurements were carried out on a Varian VXR/Unity-500 spectrometer, operating at 499.843 MHz. The following two-dimensional experiments were performed: conventional COSY employing magnitude (i.e., absolute value) processing (Bax et al., 1981; Martin & Zektzer, 1988) (MCOSY); phase-sensitive COSY (Marion & Wuthrich, 1983; Martin & Zektzer, 1988) with phase-sensitive processing; DQFCOSY (Rance & Wright, 1986; Muller et al., 1986; Martin & Zektzer, 1988) with phase-sensitive processing; and RCT, single relayed coherence transfer spectroscopy (Eich et al., 1982; Wagner, 1983). A typical experiment was set up at 27 °C to collect 4K data points over a 20.8-kHz bandwidth in t_2 with 512 increments in t_1 and a 20.8-kHz bandwidth. The minimal dwell time in dimension 1 was typically 48 μs. The typical 90° pulse width was 7 μs, with a 0.5-s combined acquisition plus relaxation delay time. Data sets were processed on a Sun SPARCStation-1 using Varian software (VNMR) to either 2K × 2K or 4K × 4K data points in the final two-dimensional spectrum. Digital filtering in both dimensions was optimized interactively and typically either a combination of line broadening plus pseudoecho was used, or phase-shifted Gaussian weighting was used in both dimensions. Both symmetrized and unsymmetrized data were analyzed. Symmetrized phase-sensitive COSY and MCOSY data presented in the form of contour plots are shown in Figures 2–4. Observed shifts in all spectra were referenced to the residual HDO resonance, which was assigned a value of 4.73 ppm. Residual HDO peak suppression was achieved by direct irradiation during the relaxation delay. Where necessary, quadrature detection in the t_1 dimension was implemented by the States–Haberkorn–Reuben method (States et al., 1982).

Measurements of nonselective spin–lattice relaxation times were carried out on a Nicolet NT500 spectrometer operating at 500.087 MHz, at 25 °C with the standard inversion–recovery (180°–τ–90°) pulse sequence where the 180° pulse was composite. Calibrated 90° pulse widths were typically 10 μs. Inversion–recovery experiments were reproduced several times with the transmitter frequency relocated for each set of data acquired so that all resonances throughout the entire 30 to −10 ppm hyperfine-shift range were within 5000 Hz of the transmitter for at least one experiment. Inversion–recovery data were analyzed by using either the resident Nicolet software on a 1280 computer or Cricket Graph (Cricket Software, Malvern PA) running on a Macintosh SE computer.

Resonance line widths at half-maximum peak intensity were estimated in two ways. Digital data were analyzed with the NMR software line-fit program supplied with the spectrometer, or expanded spectral plots were simply measured by hand. For this, free induction decays were processed without apodization.

Results and Discussion

Figure 1 shows the 500-MHz proton spectrum of CcPCN in D_2O with the assigned resonances labeled. This is a reference figure for the COSY spectra shown in Figures 2–4, and the assignments are those proposed on the basis of proton 1D NOE (Satterlee et al., 1987), NOESY (Satterlee & Erman, 1991), and COSY-type experiments presented here. It is clear from those previous studies that NOESY is a 2D assignment technique applicable even to protons with extremely short T_1's (i.e., as low as 1 ms) even in comparatively large (34–42 kDa) paramagnetic proteins such as CcP, HRP, CcPCN, and HRPCN [G. N. La Mar (1991) private communication and Satterlee and Erman (1991)]. What has been less clear so far is whether bond-correlated 2D NMR experiments would be useful in making extensive proton assignments in these same proteins. A primary concern has been the effect of extremely short nuclear relaxation times (300–30 msec) on coherence transfer.

Figures 2–4 reveal that valuable hyperfine resonance assignment data can indeed be obtained from COSY-type experiments and provide some insights as to which experiments are most valuable for detecting cross-peaks involving rapidly relaxing nuclei. Figure 2A presents a projection, Figure 2B presents a phase-sensitive COSY contour plot, and Figure 2C presents an MCOSY contour plot of a major portion of the proton NMR spectrum of CcPCN. This figure excludes the two resolved heme methyls at higher frequency (Heme 3-CH_3 and 8-CH_3 in Figure 1) and the broad His-175 resonance at −22 ppm. None of these protons are geminally or vicinally *J*-coupled to other protons and therefore are not expected to

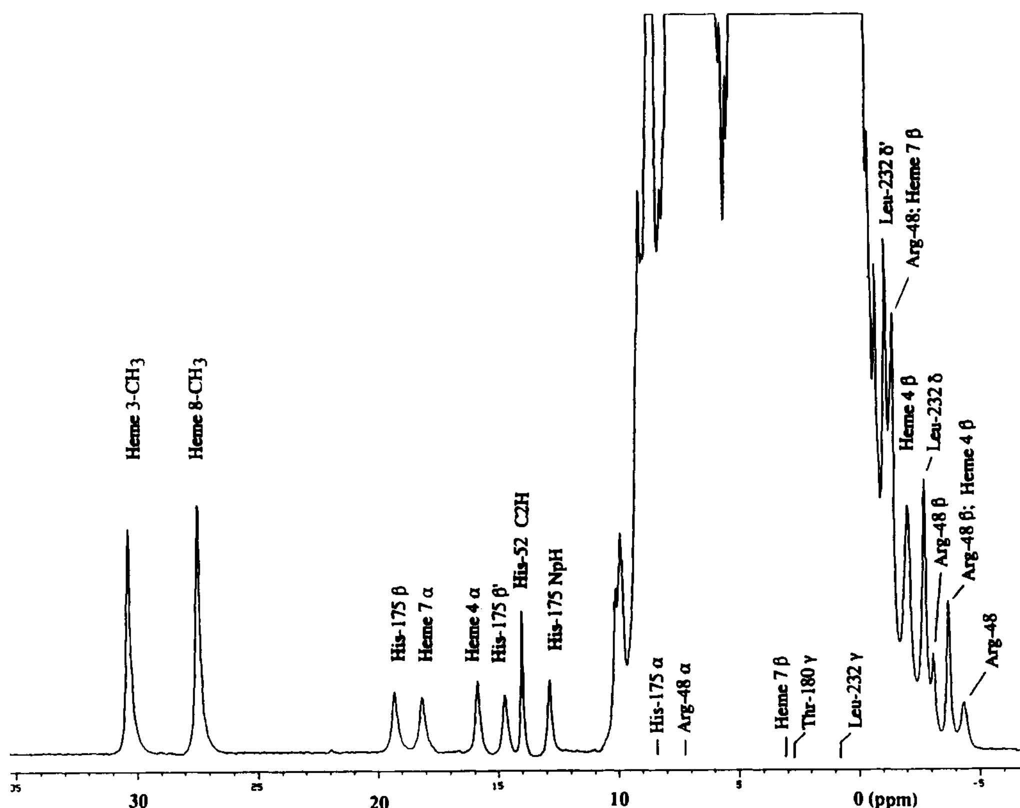

FIGURE 1: Proton NMR spectrum taken at 500 MHz of CcPCN in deuterated buffer/D_2O at 27 °C, pH' 7.0. The assignments are labeled above each resonance.

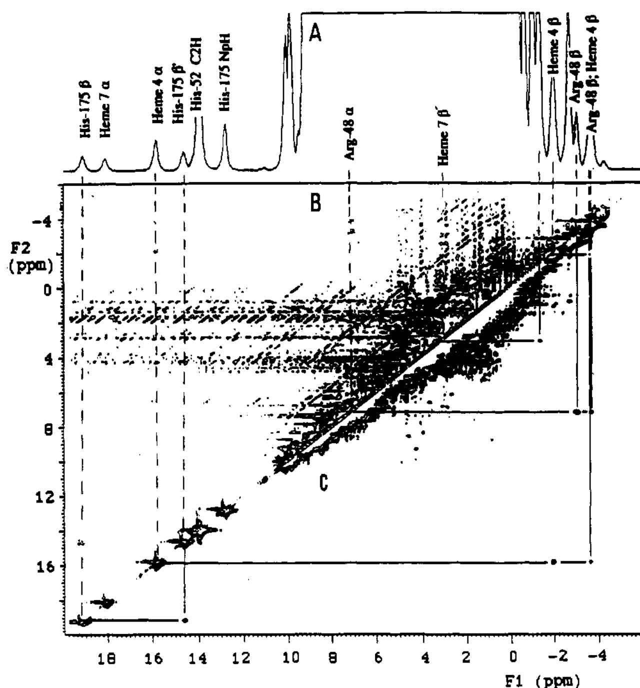

FIGURE 2: 500-MHz proton two-dimensional bond-correlated spectra for CcPCN. (A) Reference projection, (B) phase-sensitive COSY proton contour plot, and (C) MCOSY contour plot. The region shown is 20 to −6 ppm with spectra taken at 27 °C, pH' 7.0.

display COSY connectivities. The data presented in Figure 2 reveal that cross-peaks among highly shifted, rapidly relaxing, hyperfine proton resonances are readily observed. Connectivities among the hyperfine-shifted proton resonances that can be readily identified in Figure 2 include His-175 β,β'; heme 4-vinyl α,β_{cis}, α,β_{trans}, and β_{cis}, β_{trans}; heme 7-propionate β,β' (new assignment); Arg-48 β,α and β',α (new assignment).

Figure 3 presents a projection (panel A) and an MCOSY contour plot (panel B) of an expanded region of the proton spectrum of CcPCN. In this figure the MCOSY contour plot was generated from data that were processed with a higher fraction of pseudoecho weighting than the data shown in Figure 2C. As a result, additional cross-peaks may be identified, including Leu-232 δ,γ and δ',γ (new assignment) and Arg 48 β,β'. Figure 4A is an expansion of the MCOSY contour plot generated from the same data but processed to enhance the signal-to-noise ratio in order to confirm identification of the weak heme vinyl $4\beta_{cis},\beta_{trans}$ coupling.

In this work, COSY results have been used to substantially support hyperfine-shifted proton resonance assignments proposed previously from NOE experiments (Satterlee et al., 1987; Satterlee & Erman, 1991). Our interest in assigning these protons comes from their proximity to the heme group and rests in the fact that these are resolved protons of the enzyme's active site. For example, the proposed Leu-232 δ,δ'-methyl group assignments are supported in this work by their mutual scalar coupling to the γ-proton, at +0.08 ppm (new assignment, Figure 3). Identification of this scalar coupling pattern lends increased credibility to the Leu-232 assignments proposed from NOE connectivities alone since NOESY spectra did not reveal the γ-proton. The benefit of employing combined COSY and NOESY experiments directed to hyperfine-shifted proton resonances is further demonstrated by the following.

Whereas in NOESY and heme reconstitution studies only one resonance of the heme 7β geminal pair was identified, its spin-coupled geminal partner (new assignment) is identified in Figures 2 and 3 at +3.03 ppm. Also, the proposed Arg-48 β-methylene geminal proton pair are supported in this work by identification of their mutual coupling to the α-proton (new assignment) at +7.18 ppm (Figure 2). Even the expected, weak heme 4-vinyl $\beta_{cis},\beta_{trans}$ proton coupling is observed (Figure 4A).

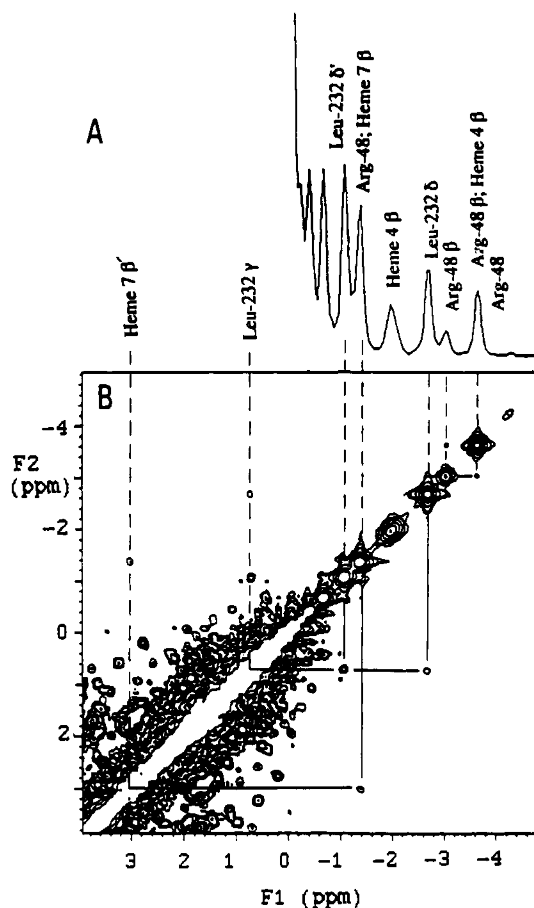

FIGURE 3: 500-MHz proton two-dimensional bond-correlated spectra for CcPCN. (A) Reference projection and (B) MCOSY proton contour plot spectrum of CcPCN. The region shown is 4 to −5 ppm, 27 °C, pH′ 7.0.

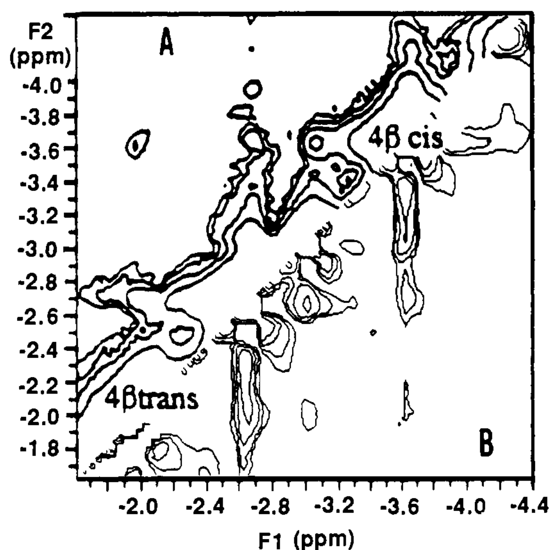

FIGURE 4: 500-MHz proton COSY spectra of CcPCN at 27 °C, pH′ 7.0. The expanded region shows the weak heme 4-β vinyl cross-peak. (A) MCOSY contour plot; (B) phase-sensitive COSY contour plot.

Demonstrating success in making COSY-type assignments for CcPCN is an important step in removing lingering ambiguities in NOE-based assignments due to the lack of specificity inherent in NOE results. NOE experiments indiscriminately detect near-neighbor nuclei, and in the case of highly-shifted protons, which do not conform to simple chemical shift correlations, even geminal partner protons may display widely different (i.e., not chemically correlated) ob-

Table I: Assignments, Line Widths, and Initial Relaxation Rate Parameters for Cyanide-Ligated Cytochrome *c* Peroxidase Hyperfine-Shifted Proton Resonances with Bond-Correlated Connectivities[a]

resonance	observed shift (ppm)	line width (Hz)	T_1^{app} (ms)
heme			
3-CH_3	30.1	65	134
8-CH_3	27.3	71	96
7α-CH_2	18.0	107	128
	6.00	106	
7β-CH_2	−1.35	(66)	
	3.03*		
4α	15.8	83	125
$4\beta_{cis}$	−3.64	61	(312)
$4\beta_{trans}$	−2.05	*b*	(235)
Arg-48			
δ-CH_2	−4.28	133	39
	−1.35	(66)	
β-CH_2	−3.64	61	(312)
	−3.01	66	(311)
α-CH*	7.18		
His-52			
C2H	14.0	27	281
His-175			
β-CH_2	19.1	87	77
	14.6	96	101
NpH	12.8	73	121
Leu-232			
δ-CH_3	−2.67	66	97
	−1.24	60	
γ-CH*	0.08		

[a] Observed shifts are reported to three significant figures, at 27 °C, pH′ 7.0. They differ slightly from previously reported shifts at lower temperatures, different pH values, and different referencing (Satterlee & Erman, 1991; Satterleee et al., 1987). Shifts are referenced to internal HDO at 4.73 ppm. Line widths are obtained from data Fourier transformed without apodization and are the full width at half-maximum height. An asterisk (*) indicates new assignment. Parentheses indicate estimates of line width or T_1^{app} for envelopes of multiple overlapping resonances containing the resonance of interest and are therefore poor approximations. [b] Resolution-enhanced spectra show this line to be a composite of three superimposed peaks of differing line width, with the narrowest ~58 Hz and the widest ~100 Hz.

served hyperfine shifts. Examples of this (shown in Figure 1 and Table I) are the His-175 geminal β-methylene pair (19.1 ppm/14.6 ppm) and the heme 7-propionate geminal α-methylene pair (18.0 ppm/6.00 ppm).

One of the most obvious conclusions from Figures 2 and 4 is that MCOSY spectra are of superior quality compared to the phase-sensitive experiments. There are several reasons for this. In the case of a large paramagnetic molecule such as CcP, both the resonance line width, which is a function of the nuclear T_2, and the spin–lattice relaxation time (T_1) can potentially compromise observation of bond-correlated connectivities in two-dimensional NMR experiments. Values for the unenhanced (i.e., "natural") line widths measured at the individual peak's half-maximum intensity reported in Table I range from 27 to 133 Hz. They are substantially larger line widths than normally found for protons in diamagnetic molecules, even in a protein this large. The source of larger line widths is the enhanced nuclear T_2 relaxation that results from rotationally modulated electron moment–nuclear moment coupling (Satterlee, 1986). Broader lines cause at least three complications for observing COSY cross-peak connectivities.

First, in phase-sensitive experiments, overlap of antiphase components produces cancellation within a cross-peak and can lead to severe reduction in cross-peak intensity. In the specific case of naturally broad resonance lines that are well separated from neighbor peaks, employing phase-sensitive experiments will not produce expected resolution improvements but will

lower the intensity of cross-peaks compared to the MCOSY experiment. This is illustrated in Figures 2 and 4. Figure 2 compares both types of bond-correlated spectra of CcPCN acquired as nearly identically as the two different experiments allow. Both the phase-sensitive COSY (Figure 2B) and MCOSY (Figure 2C) experiments are presented in symmetrized format. The phase-sensitive experiment processed in phase-sensitive mode (Figure 2B) reveals lower cross-peak intensity by virtue of being closer to the noise level of the f_1 and f_2 noise ridges compared to the MCOSY spectrum in Figure 2C where the cross-peak intensities are well above the noise. Similarly, Figure 4 compares the phase-sensitive (panel B) and magnitude (panel A) COSY spectra (both symmetrized) in detecting the heme vinyl $4\beta_{trans}/4\beta_{cis}$ coupling. This is a weak coupling, and its detection is obviously ambiguous in the phase sensitive spectrum (Figure 4B) but not so in the MCOSY spectrum (Figure 4A). For this cross-peak, at least two contour levels are shown in the MCOSY plot (Figure 4A) whereas in Figure 4B the single contour may actually be a noise ridge extension. The MCOSY experiment is more efficient, and we judge it superior for detecting cross-peaks involving broader hyperfine resonances even though many identical cross-peaks are obvious in both types of experiments (Figure 2).

Second, enhanced T_2 relaxation means more rapid loss of transverse phase coherence, thereby mitigating cross-peak intensities in comparison to nuclei with longer T_2 values. Third, broader resonances experience a real reduction in comparative peak heights, which compromises detection of the broader resonances. Figure 3B illustrates these points for the Arg-48 resonance at −4.28 ppm. Note how few contour levels this resonance shows on the diagonal relative to even other hyperfine resonances. This resonance exhibits the largest line width (133 Hz) and the shortest nuclear relaxation time (T_{1app} = 39 ms) and no cross-peaks. It was tentatively identified as one of the Arg 48 δ-methylene geminal pair of protons (Satterlee & Erman, 1990); however, the expected cross-peak to its presumed geminal partner at −1.35 ppm went undetected in these experiments. We did not measure T_2's for this molecule, but they will be at least as short as the true T_1 values (Farrar & Becker, 1971) and it may be this rapid transverse relaxation that compromises observation of the expected cross-peak.

In this respect our data, although not exhaustive, provide insight concerning the impact of nuclear relaxation on the ability to observe cross-peaks in 2D bond-correlated experiments. Comparatively short nuclear relaxation times are a consequence of proximity to the paramagnetic heme iron ion. We have carried out inversion–recovery experiments designed to quantitate spin-lattice relaxation times for the nuclei displaying resolved hyperfine shifts. The relaxation time values included in Table I are qualified by their notation "app", meaning "apparent". True T_1 values for protons in CcPCN are difficult to evaluate due to the presence of spin diffusion (Satterlee, 1986). The net effect of spin diffusion in this case is to produce inversion–recovery experiment results that cannot be fit to a single-exponential equation, as is commonly done (Farrar & Becker, 1971). Proton inversion–recovery data for CcPCN (Satterlee, 1986) as well as for other proteins (Kalk & Berendsen, 1976; Akasaka, 1983) reveal this phenomenon. It is readily apparent for CcPCN when proton inversion–recovery data are processed graphically. The result is obvious nonlinearity in graphs of ln $[(I_\infty - I_\tau)/2I_\infty]$ plotted against τ (Satterlee, 1986). The experimentally measured peak intensities in the inversion–recovery experiment are I_∞, the fully

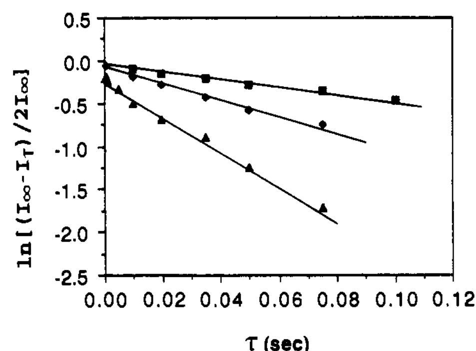

FIGURE 5: Graph of the initial recovery rate intensity functions for early delays (τ) in inversion–recovery measurements. Shown are data for the heme 8-methyl resonance (◆), His-52 C2H resonance (■), and the Arg-48 resonance at −4.28 ppm (▲). The lines drawn through the experimental points are best fits calculated from linear regression analysis.

relaxed equilibrium intensity for a resonance, and I_τ, the intensity of the line at a given τ, where τ is the interpulse delay time in the inversion–recovery pulse sequence (see Materials and Methods). For relaxation characterized by a single-exponential decay, graphs such as these should be linear with an intercept of 0 and a slope equal to $-1/T_1$ (Farrar & Becker, 1971).

We are currently in the process of fully characterizing field-dependent nuclear relaxation properties for CcPCN, but so far from our work and that of others (Kalk & Berendsen, 1976; Akasaka, 1983) it has become apparent that one way to characterize a proton's relative rate of return to equilibrium magnetization in this situation is from the slope of the initial linear portion of a semilog plot of inversion–recovery data (Figure 5), as described in the previous paragraph. For CcPCN, we have calculated apparent magnetization decay constants, which we call T_1^{app}, from the slopes of linear portions of the initial data points in semilog plots of inversion–recovery data. Examples of this data are shown in Figure 5 for the heme 8-methyl resonance (intermediate T_1^{app}), the His-52 C2H resonance (longest T_1^{app} for resolved hyperfine resonances), and the Arg-48 proton at −4.28 ppm, which has the shortest T_1^{app}. All slopes used in calculating the T_1^{app} values reported in Table I were calculated by using linear regression and had correlation coefficients of either 0.99 or 1.00.

The T_1^{app} values reported in Table I should be interpreted as expressing relative initial magnetization recovery rates and not absolute values. The errors in these values result from the NMR experiment itself and peak intensity measurements, which together constitute possibly as much as a 15% variation in the actual T_1^{app} number reported.

Table I reveals a general correlation that resonances with narrower line widths have longer T_1^{app}'s (implying longer T_2's as well). This is illustrated by comparing the His-52 C2H, the heme methyls, and the Arg-48 resonance at −4.28 ppm. However, there are several specific exceptions, probably as a result of differences in paramagnetic relaxation enhancement due to different contributions from the contact, dipolar, and Curie-spin relaxation mechanisms for individual nuclei.

Aside from the missing cross-peak between the proposed Arg-48 δ,δ′ geminal methylene pair (−4.28 ppm/−1.35 ppm) discussed above, there is another specific example of an expected COSY cross-peak that is not unambiguously detected in any of our spectra. No cross-peak has so far been detected for the αCH_2 geminal methylene proton pair of the heme 7-propionic acid. The assignment of this pair of resonances (18.0 ppm/6.00 ppm) seems firm on the basis of NOE results (Satterlee et al., 1987; Satterlee & Erman, 1991), and we are

unable to explain the absence of this cross-peak. Whereas, the line widths of both of these protons are somewhat larger than other resolved hyperfine-shifted resonances (>100 Hz, Table I), the T_1^{app} of the partner at 18.0 ppm is comparatively long and should not be the reason for the absence of the cross-peak in the COSY spectra.

This work differs significantly from two-dimensional NMR studies on the related horseradish peroxidase, HRP, reported by Veitch and Williams (1990). In that work, NOESY and COSY connectivities of protons in various paramagnetic forms of HRP were detected and characterized by spin system type. That HRP work did not address assignments of proton hyperfine-shifted resonances, which are more time-demanding experiments. The HRP assignments that were made were localized to the aromatic region (6–9 ppm) and the methyl resonance region (−1 to +1 ppm) and resulted in valuable spin system classification. Perhaps most importantly, that work illustrated the potential for extending proton assignments into the diamagnetic region for these peroxidases. It differs from the work we present here in that we have addressed a different category of protons, namely, those that are hyperfine-shifted and that are much faster relaxing. Judging from our inversion–recovery data on CcPCN and the use of 200-ms NOESY mixing times for the HRP derivatives (Veitch & Williams, 1990), the aromatic and aliphatic protons of the HRP derivatives have much longer apparent relaxation times (ranging between 0.8 and 1.5 s) and are narrower than the hyperfine-shifted resonances that were the objects of the work presented here.

Conclusions

In summary, we have shown that certain types of bond-correlated homonuclear proton 2D NMR experiments can be valuable aids for making proton assignments of rapidly relaxing hyperfine shifted protons in relatively large paramagnetic metalloproteins. MCOSY is by far the most efficient experiment of those we attempted, and therefore in our opinion it is also the most valuable. MCOSY results in higher cross-peak sensitivity than phase-sensitive COSY experiments, which actually suffer from the broadness of the resonances. Application of MCOSY experiments to CcPCN has confirmed NOE-based assignments and provided new proton assignments.

We also carried out RCT experiments and DQFCOSY experiments. The RCT experiments suffer (in our hands at least) from the enhanced nuclear relaxation effects demonstrated by hyperfine-shifted protons and (apparently) loss of transverse magnetization during the relay periods, resulting in severe sensitivity problems. We have found the DQFCOSY experiment not useful for making hyperfine resonance assignments in CcPCN, for reasons previously discussed in detail (Yu et al., 1990). It is likely, however, that both of these latter two experiments will be useful for high-resolution assignments within the diamagnetic region. In fact, on the basis of our own experiments and those published by La Mar and co-workers (Yu et al., 1990) it is likely that experiments similar to both the RCT and DQFCOSY experiments can be utilized to filter out connectivities due to broader hyperfine-shifted resonances that occur in and near the 10 to −1 ppm region that could complicate interpretation of these crowded spectral regions.

Registry No. CcP, 9029-53-2.

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