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Proton NMR Assignments of Heme Contacts and Catalytically Implicated Amino Acids in Cyanide-Ligated Cytochrome *c* Peroxidase Determined from One- and Two-Dimensional Nuclear Overhauser Effects†

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ABSTRACT: Proton NMR assignments of the heme pocket and catalytically relevant amino acid protons have been accomplished for cyanide-ligated yeast cytochrome *c* peroxidase. This form of the protein, while not enzymatically active itself, is the best model available (that displays a resolvable proton NMR spectrum) for the six-coordinate low-spin active intermediates, compounds I and II. The assignments were made with a combination of one- and two-dimensional nuclear Overhauser effect methods and demonstrate the utility of NOESY experiments for paramagnetic proteins of relatively large size (M_r 34 000). Assignments of both isotope exchangeable and nonexchangeable proton resonances were obtained by using enzyme preparations in both 90% H_2O/10% D_2O and, separately, in 99.9% D_2O solvent systems. Complete resonance assignments have been achieved for the proximal histidine, His-175, and His-52, which is a member of the catalytic triad on the distal side of the heme. In addition, partial assignments are reported for Trp-51 and Arg-48, catalytically important residues, both on the distal side. Aside from His-175, partial assignments for amino acids on the proximal side of the heme are proposed for the alanines at primary sequence positions 174 and 176 and for Thr-180 and Leu-232.

Cytochrome *c* peroxidase (EC 1.11.1.5, CcP) is a 34-kDa ferriheme enzyme that reduces hydrogen peroxide using cytochrome *c* (Kraut, 1981; Poulos & Finzel, 1984). Mechanisms for its function have been proposed that involve acid-base catalysis and charge stabilization by a catalytically active triad of amino acids: Trp-51, His-52, and Arg-48 (Poulos & Kraut, 1980; Kraut, 1981). These amino acids lie close to the heme and the ligand-binding site on the distal side of the heme (Poulos & Kraut, 1980; Finzel et al., 1984; Edwards et al.,

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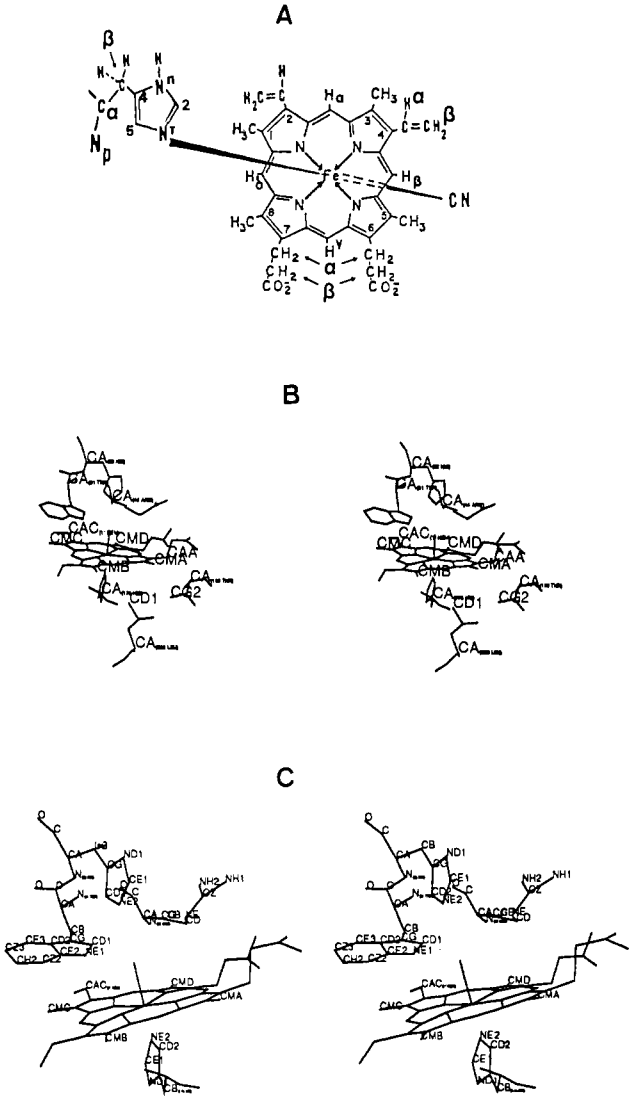

FIGURE 1: Labeling scheme used in this work for the CcP heme (A) and the structures of the heme pocket of CcP showing important amino acids (B and C). In B and C, amino acids α, β, γ, etc., positions are labeled by the english alphabet letter A, B, G, etc., and the heme pyrrole rings are labeled A, B, C, and D, such that the 8-CH_3 = CMA, 1-CH_3 = CMB, 3-CH_3 = CMC, and 5-CH_3 = CMD, etc.

1987; Figure 1). Also implicated in stabilizing the active enzyme intermediate (CcP compound I) that is formed initially upon hydrogen peroxide interaction with CcP are several amino acids on the proximal side of the heme, neighboring His-175, the proximal heme ligand. These include Asp-235, which directly interacts with His-175 (Poulos & Finzel, 1984; Mauro et al., 1989; Satterlee et al., 1990), Trp-191, Met-230, and Met-231 (Edwards et al., 1987; Erman et al., 1989).

Most of the evidence contributing to the current picture of the CcP mechanism has come from a series of elegant solid-state structural studies (Poulos & Kraut, 1980; Kraut, 1981; Finzel et al., 1984; Edwards et al., 1987) and recent work with CcP mutants (Erman et al., 1989; Fishel et al., 1987; Goodin et al., 1986, 1987; Mauro et al., 1988, 1989; Miller at al., 1988, 1990; Satterlee et al., 1990, Smulevich et al., 1988). Direct and conclusive solution-state structural results are, comparatively, lacking for CcP. One reason for this is that NMR assignments for CcP are not extensive (Satterlee et al., 1983, 1987). However, detailed analyses for proton hyperfine shifts have been carried out for the related horseradish peroxidase (HRP) (Thanabal et al., 1987a,b, 1988, and references therein).

In this work, we confirm and extend solution ^1H NMR assignments for CcPCN, which is, like the oxidized intermediates, a six-coordinate low-spin form of the enzyme. Our results come from a combination of one-dimensional (1D) and two-dimensional (2D) proton nuclear Overhauser effect (NOE) measurements that have resulted in assignments of protons belonging to several catalytically important active site residues of CcPCN. Although not an active enzyme form, CcPCN, like HRPCN, has proven to be an important analogue for the active low-spin oxidized enzyme intermediates for which ^1H NMR spectroscopy has so far proven to be useless as a result of large hyperfine resonance line widths (Satterlee & Erman, 1981). Critical to the success of the 2D work presented here and to the ultimate extension of these NOESY experiments with CcPCN are results, presented herein, that the previously published 1D NOE connectivities are observed in the NOESY spectra. The 2D work presented here and the 1D work presented before (Satterlee et al., 1987) are shown to be completely consistent. This gives confidence to the new proton assignments determined by NOESY experiments alone.

We have identified isotope-exchangeable protons belonging to Trp-51 and His-52. It is these protons along with those of Arg-48 that are believed to participate in hydrogen peroxide decomposition. In addition, essentially complete assignments for the His-175 spin system have been accomplished. Assignments of selected protons from Ala-174, Ala 176, Leu-232, and Thr-180 are supported by these data, and assignments of two heme meso protons are proposed. Both Leu-232 and Thr-180 are adjacent to the proposed (Edwards et al., 1987; Erman, et al., 1989; Mauro et al., 1990) Trp-191 residence site of the compound I free radical. The results presented here indicate that, although two-dimensional NMR methods have not yet been widely applied to paramagnetic proteins of this size, consistent sets of interpretable data can be successfully obtained.

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; Satterlee et al., 1987). All preparations of the enzyme conformed to the purity criteria previously described (Vitello et al., 1990). Enzyme samples for NMR spectroscopy were 1.5–2.7 mM, in either 0.2 M KNO_3 or 0.17 M KNO_3/0.03 M potassium phosphate. The solution pH was measured with a Beckman meter and a calibrated Fisher combination electrode. For samples in D_2O (99.9%, Isotec or MSD Isotopes), the uncorrected pH meter reading is reported as pD. Several samples were made during the four-year duration of this work, and, whereas care was taken to insure minimal deviation of the pH of a single sample during the course of NOE experiments, the overall range of pD (uncorrected meter reading) extended from 6.6 to 7.1.

NMR experiments were performed on a variety of spectrometers, including a Varian Unity 500 and a GE NT500, both operating nominally at 500 MHz, a JEOL GX 400 operating at 400 MHz, a GE NT360 operating at 361 MHz, and a Bruker AC250 operating at 250 MHz. Both phase-sensitive (with States-Haberkorn or TPPI methods) and absolute-value data were obtained by using standard library programs that were supplied with each spectrometer. Mixing times varied from experiment to experiment, ranging from 30 to 100 ms. One-dimensional NOE difference spectra were obtained as previously described (Satterlee et al., 1987) with irradiation times that varied between 15 and 100 ms. Data processing and spectral analyses included use of data that was both

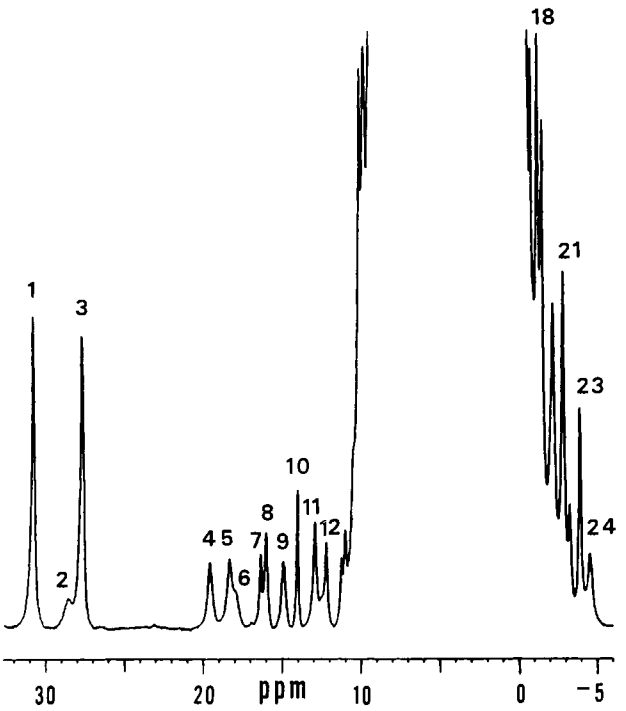

FIGURE 2: 500-MHz ^{1}H NMR spectrum of CcPCN in 90% H_2O/10% D_2O and 0.17 M KNO_3/0.03 M potassium phosphate, pD 6.6, at 23 °C. Resonances are numbered for identification purposes, starting with the highest frequency heme 3-methyl resonances at 1. Exchangeable resonances seen in this spectrum correspond to 2, 6, 7, 12, 13, and 14.

symmetrized and not symmetrized. Typical NOESY experimental parameters were 2K or 4K data points in t_2 with 128 to 512 t_1 increments and with appropriate zero filling in both dimensions prior to Fourier transformation so that 2D matrices ranged from 1K × 1K to 4K × 4K. These large data sets were required due to the large hyperfine shift dispersion exhibited by this paramagnetic protein.

In general, spectra obtained in 90% H_2O/10% D_2O solutions were taken without suppressing the water resonance, for the reasons described under Results and Discussion. Where necessary, these spectra were acquired with either a Redfield-type composite 21412 observe pulse or a 1331 observe pulse. These methods are similar to those used for horseradish peroxidase and have been described in detail (Thanabal et al., 1988). Observed proton shifts are reported to three significant figures, the residual HDO resonance is used as an internal reference, and it is assigned a shift of 4.70 ppm. Variable-temperature observed proton shift data were collected between 4 and 34 °C and are graphed in Figure 6. Intercepts resulting from extrapolation of these data to $1/T = 0$ are reported in the text along with the statistical deviation derived from a linear least-squares analysis.

The CcP structure was examined on a Silicon Graphics 4D workstation using either MIDAS (UCSF) or Quanta (POLYGEN) to visualize the refined coordinates (Finzel et al., 1984) supplied by the Brookhaven Protein Data Bank (Bernstein et al., 1977). The numbering and labeling of amino acids in Table I and Figure 1A conforms to recommended IUB-IUPAC standards [(1985) *J. Biol. Chem. 260*, 14–42], which, however, introduces differences in notation compared to previous peroxidase work (Satterlee et al., 1983, 1987; Thanabal et al., 1987a,b, 1988) and compared to standard labels utilized in the MIDAS (Figure 1B,C) and POLYGEN graphics software.

Results and Discussion

The work presented here involves proton spectra taken in aqueous solutions consisting of two different isotope ratios. Solutions in which the solvent composition was 90% H_2O/10% D_2O were used to observe deuterium/protium exchangeable resonances, and solutions made in 99.9% D_2O were used for experiments whose focus was not the exchangeable resonances. For conciseness, and since CcPCN proton spectra in 99.9% D_2O have been published (Satterlee et al., 1983, 1987), we present only the proton spectrum taken in the 90% H_2O/10% D_2O solvent system (Figure 2) as the basis for our assignment analysis. The upfield proton hyperfine shift region (0 to −25 ppm) is identical with that observed when the enzyme is in 99.9% D_2O (Satterlee et al., 1983, 1987). The downfield hyperfine shift region contains all of the nonexchangeable resonances (resonances 1, 3, 4, 5, and 8–10 in Figure 2) for which assignments were previously made in D_2O solutions by using one-dimensional NOE spectroscopy (Table I) (Satterlee et al., 1987). In addition, several resonances whose intensities are sensitive to the isotopic composition of the solvent, exchangeable resonances 2, 6, 7, and 11–14, are also visible in Figure 2. In 99.9% D_2O solutions, all of these exchangeable resonances except resonance 11 disappear from the ^{1}H NMR spectrum due to rapid equilibration with the bulk solvent. Several of these exchangeable resonances were previously

Table I: Proton NMR Assignments for CcPCN from NOESY and 1D NOE Data[a]

peak no.[b]	assignment	observed shift[c]
	heme	
1	3-CH_3	30.6[d]
3	8-CH_3	27.7[d]
5	7α-CH_2	18.4,[d] 6.24[d]
19	7β-CH_2	−1.52[d]
8	4α-CH	16.0[d]
23	4β-CH (cis)	−3.99[d]
20	4β-CH (trans)	−2.19[d]
	δ-meso	5.31
	β-meso	5.84
	Arg-48	
19, 24	δ-CH_2[e]	−1.52, −4.66
22, 23	β-CH_2[e]	−3.28, −3.99
	Trp-51	
6	1NH	18.0
	His-52	
7	N_πH	16.4
10	2CH	14.0
2	N_τH	28.6
	5CH	9.60
	Ala-174	
	α-CH	2.65
	β-CH_3	0.30
	N_PH	10.0
	His-175	
4, 9	β-CH_2	19.5,[d] 14.9[d]
	C_αH	8.48
	N_PH	12.9[d]
	2CH	−22.0[d]
	Ala-176	
	N_PH	6.99
	Thr-180	
	γ-CH_3	2.80[d]
	Leu-232	
18	δ-CH_3	−1.24
21	δ-CH_3'	−2.78

[a] 22 °C, 0.17 M KNO_3, 0.03 M potassium phosphate, pD 6.8. [b] Corresponding to Figure 2. [c] In ppm, relative to the residual HDO or H_2O assigned to 4.70 ppm. [d] NOESY assignments from this work that confirm previous 1D NOE assignments (Satterlee et al., 1987). [e] Specific methylene assignments not confirmed.

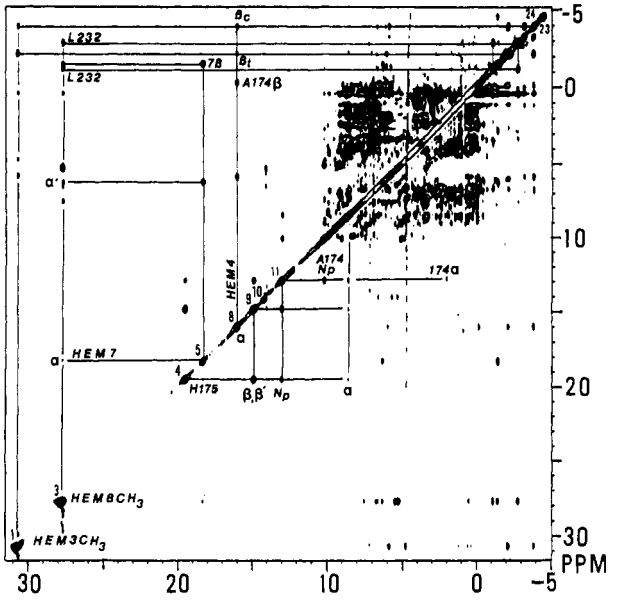

FIGURE 3: 500-MHz phase-sensitive ^{1}H NOESY spectrum of CcPCN in 99.9% D_2O; other conditions are identical with those in Figure 2. The diagonal peaks are numbered to correspond with Figure 2, and the label HEM refers to heme peaks.

detected, their pH dependence was measured, and they were tentatively assigned (Satterlee & Erman, 1983). Resonance 11 has the unique property of being deuterium exchangeable only in the apoprotein (Satterlee & Erman, 1983). In holo-CcPCN, it exhibits no evidence of deuterium exchange for up to six weeks when kept in a variety of environments between 25 and 4 °C. On the basis of this behavior, resonance 11 was previously assigned (Satterlee et al., 1987) to the peptide NH (N_pH) of His-175, the proximal histidine (Figure 1). An analogous resonance displaying similar shift and properties in HRPCN was also assigned to the proximal histidine peptide proton (Thanabal et al., 1988).

Figure 3 is the full NOESY spectrum of CcPCN in 99.9% D_2O. It shows a significant number of cross peaks between the well-resolved hyperfine shifted resonances (peaks 1–24) and peaks lying in the diamagnetic region (which also includes some hyperfine shifted resonances), as well as cross peaks between the hyperfine shifted resonances themselves. A spectrum like this is significant for several reasons: (i) It illustrates and confirms that it is possible to carry out 2D NMR on paramagnetic proteins as large as CcP (34 kDa). (ii) It reinforces and extends previous assignments made by 1D NOE methods (Satterlee et al., 1987). (iii) It reveals that several connectivities exist between hyperfine shifted protons, which belong either to iron ion ligands or to neighboring active site amino acids and amino acids further removed from the active site, thereby providing connectivities from the well-resolved hyperfine resonances to the main polypeptide chain.

Some of the assignments derived from NOESY experiments are labeled in Figures 3 and 4. Figure 4 shows expanded regions of NOESY spectra and will be discussed in detail later. Assignments are collected in Table I.

Figure 3 clearly shows consistent connectivity patterns involving the following groups that were detected by 1D NOE (Satterlee et al., 1987): the heme 8-CH_3 and 7-propionic acid; the heme 3-CH_3 and 4-vinyl; and the His-175 β-CH_2 and α-CH, and the peptide NH. This confirms and reinforces our previous work and the current use of NOESY experiments for CcPCN. It also provides a basis for interpreting the additional connectivities seen in the NOESY spectra. Some of these became apparent through detailed analysis of expanded regions

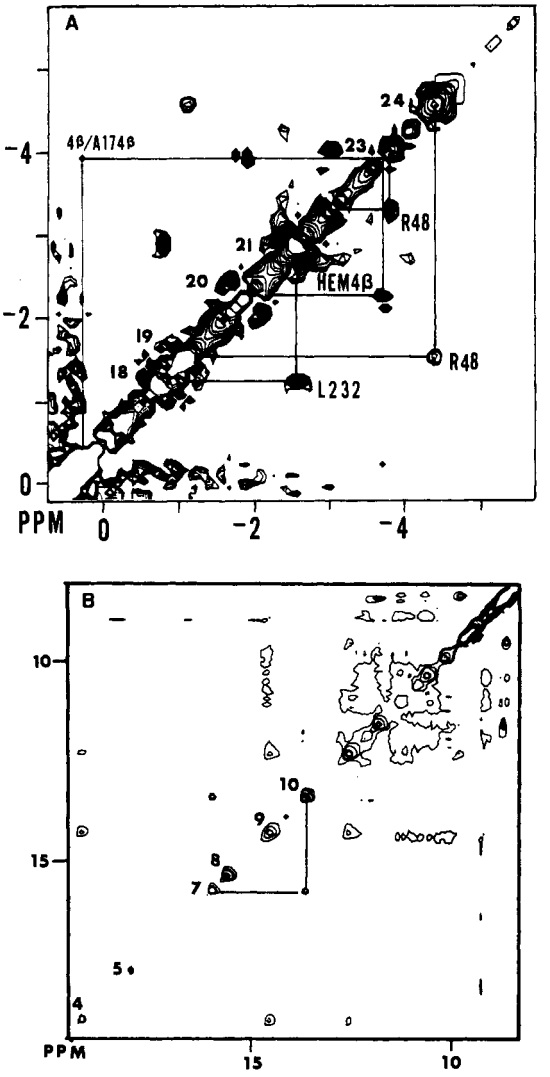

FIGURE 4: (A) Expanded upfield region of the 500-MHz phase-sensitive NOESY spectrum of CcPCN in 99.9% D_2O; other conditions are identical with those in Figure 2. (B) Expanded downfield hyperfine shift region of a 400-MHz phase-sensitive ^{1}H NOESY spectrum of CcPCN in 90% H_2O/10% D_2O solution with conditions identical with those in Figure 5. In both spectra, the diagonal peaks are numbered corresponding to Figure 2, and HEM refers to heme resonances.

of the NOESY spectra near 10 ppm (not shown) and 0 ppm (Figure 4A).

Further specific assignments are derived from 1D and 2D NOE experiments in combination with the published crystal structure (Poulos & Kraut, 1980; Finzel et al., 1984). Several specific amino acid assignments will be presented later; however, delineation of the catalytically relevant proximal and distal hydrogen-bond networks in CcPCN is of particular interest. On the proximal side of the heme, this involves His-175 and Asp-235 (Figure 1; Poulos & Finzel, 1984; Mauro et al., 1989; Satterlee et al., 1990). On the distal side of the heme, where H_2O_2 binds and is decomposed, it involves the catalytic group consisting of Trp-51, His-52, and Arg-48 (Poulos & Kraut, 1980; Kraut, 1981; Finzel et al., 1984) (Figure 1). For both the proximal and distal networks, the hydrogen-bonded protons belong to nitrogen or oxygen atoms in the static structure and consequently are potentially isotope exchangeable. On the basis of experience with other heme proteins, they are expected to be susceptible to rapid solvent exchange, being fully visible only in proton spectra taken in H_2O solutions, as demonstrated in Figure 2 in comparison to previous NMR work on CcPCN in D_2O (Satterlee et al., 1983, 1987, 1990).

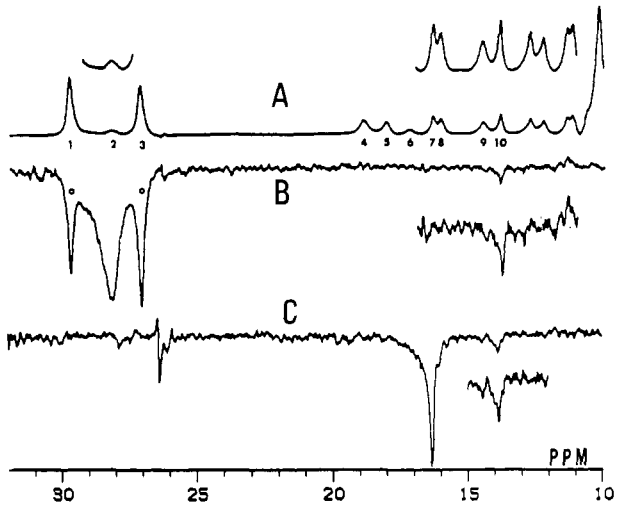

FIGURE 5: One-dimensional NOE difference experiments at 361 MHz on CcPCN in 90% H_2O/10% D_2O and 0.2 M KNO_3, pD 6.8, at 25 °C: (A) Absorption spectrum, no irradiation; peaks are numbered as in Figure 2, and insets are vertical expansions. (B) Peak 2 is irradiated, showing connectivity to peak 10; decoupler spill over to peaks 1 and 3 is noted by circles. (C) Peak 7 is irradiated, showing connectivity to peak 10. In D_2O no connectivity between peak 10 and other hyperfine shifted resonances is found. The feature at ~27 ppm is the carrier position.

Further assignments of exchangeable resonances were carried out with proton 1D NOE difference spectroscopy, as shown in Figure 5. Trace A in Figure 5 represents the proton absorption spectrum. Traces B and C are NOE difference spectra showing the 1D NOE connectivities between resonances 2 and 10 and between resonances 7 and 10. Reciprocal NOEs were detected (not shown). The connectivity between resonances 7 and 10 is confirmed in the proton NOESY spectrum shown in Figure 4B. Obtaining resolved NOESY spectra in 90% H_2O solutions when water is not suppressed (as in Figure 4B) is challenging for a paramagnetic protein whose concentration is limited. Water suppression was avoided in these experiments for the reason that rapid saturation transfer occurs between the solvent and these exchangeable resonances, which results in resonance intensity loss. We were only successful in detecting the resonance 7/10 NOESY connectivity and not the resonance 2/10 NOESY connectivity, apparently because resonance 2 is so broad (hence less intense) that the cross peak was not detected above the noise level in our spectra.

The combined results from the 1D and 2D NOE experiments (Figures 4B and 5) show that exchangeable peaks 2 and 7 exhibit identical NOEs (~4% each) to peak 10, which has previously been assigned to His-52 (Satterlee et al., 1987). Irradiation of peak 2 also produces a weak NOE (~2%) to a resonance at approximately 9.60 ppm, whereas resonance 7 shows no such connectivity (not shown).

This general pattern of NOE connectivities is characteristic of a protonated histidine ring, which we propose as the distal histidine of the catalytic site residue, His-52. The NOE data reported here are virtually identical with the pattern found for the analogous His-42 of HRP (Thanabal et al., 1988). These results reinforce our previous assignment of peak 10 to the His-52 ring 2CH (formerly, and in the MIDAS structures in Figure 1B,C labeled $C_\epsilon H$). The fact that two exchangeable protons display NOEs to resonance 10 confirms its assignment and indicates that His-52, like His-42 in HRP, is protonated at both ring nitrogens. The second NOE connectivity (at 9.60 ppm) displayed by peak 2 confirms peak 2 as the His-52 ring $N_\tau H$ (formerly, and in graphics software, labeled $N_\epsilon H$). On this basis, peak 7 must be the $N_\pi H$ (formerly labeled $N_\delta H$). The resonance at 9.6 ppm is therefore the 5CH (formerly $C_\delta H$).

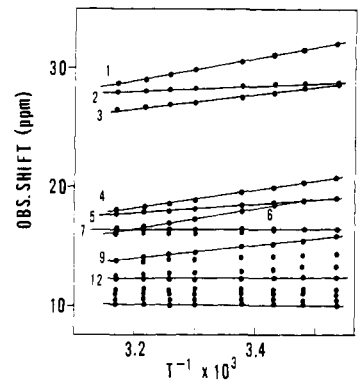

FIGURE 6: Curie plot for downfield hyperfine proton resonances of CcPCN variable-temperature NMR data at 361 MHz. The lines drawn through points are linear least-squares representations and serve both to guide the viewer and for calculation of extrapolated intercepts. Lines through some sets of points are omitted for clarity.

Further support for these assignments comes from variable-temperature NMR studies that also provide criteria for additional assignments. The temperature dependence of the exchangeable resonances is shown in the Curie plot (Figure 6). Resonances 2 and 7 display relatively large shifts, far greater than expected for even the strongly hydrogen-bonding histidine $N_\pi H$ and $N_\tau H$. They also exhibit a small but reproducible temperature dependence characterized by non-Curie extrapolated intercepts (Figure 6; peak 2 intercept = 20.8 ± 2.8 ppm; peak 7 intercept = 16.3 ± 2.2 ppm). Over the 30 °C range of the variable-temperature experiments, peak 2 shifted 0.7 ppm, peak 7 shifted 0.6 ppm, and the nonexchangeable His-52 2CH (peak 10) shifted 0.6 ppm. This temperature dependence confirms that the heme-centered paramagnetism contributes to these shifts, indicating that this spin system must, indeed, lie close to the paramagnetic heme. The temperature coefficient of these two peaks is less than that of the corresponding protons in HRPCN (Thanabal et al., 1988). The actual cause of these differences between the two enzymes is likely to be a complicated balance of effects: (i) The relative structures of His-52 (CcPCN) and His-42 (HRPCN) may be different, with accompanying differences in dipolar shifts and ring-current induced shifts. (ii) The extent of hydrogen bonding between heme-coordinated CN and the histidines may be different in each protein. (iii) The extent of contact shift allowed by such hydrogen bonding and the precise spin density distribution in the heme molecular orbitals may be different in each protein. At this time it is impossible to precisely determine the cause of the differences in distal histidine resonances' temperature behavior between CcPCN and HRPCN.

These assignments for His-52 in CcPCN are also supported by similarity to the proton spectrum of HRPCN (Thanabal et al., 1987a,b, 1988) and the assignments of the corresponding His-42 spin system in that enzyme. In CcPCN, careful consideration of the heme pocket amino acid composition and structure, refined by Finzel et al. (1984), presents no other satisfactory histidine as a possible candidate for this assignment. Previous assignments (Satterlee et al., 1987) and the discussion presented below emphasize that heme-coordinated His-175 cannot display the resonance pattern detected here.

Assignments for resonances 2 and 7 account for two out of the three downfield hyperfine-shifted exchangeable resonances that display both substantial hyperfine shifts and temperature dependence. Another exchangeable resonance, 6, exhibits an

observed shift of 17.8 ppm (23 °C) and a very substantial temperature dependence (Figure 6), both of which are properties characteristic of a proton positioned close to the heme iron ion. No comparable resonance exists in the HRPCN spectrum (Thanabal et al., 1988). Despite the apparent high level of structural and sequence correspondence between the heme pockets of CcP and HRP that is emerging from modeling (Sakurada et al., 1986; Poulos & Finzel, 1984) and NMR (Thanabal et al., 1987a,b, 1988; Satterlee et al., 1987; this work), HRP does not possess an exact analogue to Trp-51 in CcP (Figure 1). Instead, HRP possesses a Phe-41, whose side chain cannot provide an exchangeable proton. On the basis of this comparison, the observed shifts and temperature dependence of peak 6, and the CcPCN crystal structure, it is proposed that peak 6 be assigned to the Trp-51 indole ring 1NH (formerly labeled $N_\epsilon H$).

The remaining exchangeable resonances, peaks 12–14, exhibit no significant temperature dependence (Figure 6), which indicates that they come from groups that are not situated close enough to the heme to substantially experience the paramagnetic influence of the heme iron ion. Further assignments of these protons are in progress, and here they will not be considered further. We proceed in our analysis by next presenting and discussing specific spin system assignments for His-175 (the proximal histidine), Arg-48 (one of the catalytic triad), and Leu-232, which is located physically adjacent to the residues proposed to be the radical site in CcP compound I (Trp-191, Met-230, and Met-231) (Edwards et al., 1987; Erman et al., 1989).

His-175 Assignments. One-dimensional NOE experiments and comparison with heme models produced assignments of five of the seven protons of the CcPCN proximal histidine (His-175, Figure 1) (Satterlee, et al., 1983, 1987). Those assignments are confirmed in the present work by the NOESY results, as demonstrated by the spectra presented in Figure 3. The His-175 connectivity pattern is clearly shown in these figures, revealing that both C_β protons display connectivities to the peptide proton (N_pH; 12.9 ppm) and the C_α proton (8.48 ppm). Judging from the $C_\alpha H$ and N_pH shifts, it is clear that the heme iron paramagnetic effect is significant at these two positions. The peptide NH also displays relatively strong NOESY cross peaks to resonances at 10.0, 6.99, and 2.65 ppm (Figure 3, but expansions not shown). These are connectivities that were not detected in the previous 1D data (Satterlee et al., 1987). The crystal structure shows that the His-175 peptide nitrogen lies 2.45 Å from Ala-174 C_α, 2.69 Å from Ala-174 peptide N, and 2.78 Å from Ala-176 peptide N. Considering the three-dimensional CcP crystal structure, including the mutual relative orientations of individual protons and the relative distances of specific protons from the heme, leads to the most likely assignments of these resonances: Ala-174 $C_\alpha H$ = 2.65 ppm, Ala-174 N_pH = 10.0 ppm, and Ala-176 N_pH = 6.99 ppm.

Observation of these connectivities (along with the proposed identities), when the enzyme is dissolved in 99.9% D_2O solution, suggests that the proximal side of the heme in CcPCN is inaccessible to the solvent. This property is typified by the behavior of the firmly assigned His-175 N_pH as discussed above (Satterlee & Erman, 1983). We note in this respect that it is the proximal side of the heme, involving Met-230, Met-231, and Trp-191 (a structural neighbor in contact with His-175), that seems to be the most likely site of residence for the CcP compound I free radical (Edwards et al., 1987; Erman et al., 1989). Perhaps the solvent-shielded environment of the heme proximal side helps stabilize this radical.

Two resonances so far remain unassigned for His-175 in CcPCN. One of these, the imidazole ring 5CH, remains unidentified despite this work. By comparison with the corresponding resonance's identification in HRPCN, it is expected to be very broad, as is the His-175 imidazole ring 2CH of both HRPCN (Thanabal et al., 1987a,b, 1988) and CcPCN (Satterlee et al., 1987, and references therein). The observed shift of the 2CH is at −22 ppm in wild-type CcPCN (Satterlee et al., 1983, 1987, 1990) and is not shown in Figure 2. The 5CH assignment in HRPCN was achieved through high-temperature NMR work, whereas CcPCN is less thermally stable, precluding such high-temperature experiments.

The remaining unassigned resonance for His-175 is the imidazole ring $N_\pi H$, which is expected to be isotope exchangeable by comparison to HRPCN and the behavior of heme-coordinated histidines in other heme proteins. For HRPCN, this resonance was identified by comparing 1D NOE spectra of HRPCN taken either in 99% D_2O or in a solvent mixture consisting of 90% H_2O/10% D_2O and observing an exchangeable resonance that appeared at 9.9 ppm in the latter solvent system but not in the former. That exchangeable resonance in HRPCN exhibited an NOE connectivity to each of the two resolved proximal histidine C_β proton resonances (Thanabal et al., 1988). On the basis of the NOEs, the isotope sensitivity, and the structural proximity of these sets of protons, the 9.9 ppm resonance was logically and consistently assigned as arising from the proximal histidine ring $N_\pi H$ in resting state HRP, with this proton being shuttled to an adjacent anionic acceptor in HRPCN.

For CcPCN, similar sets of experiments were carried out in an effort to identify the corresponding His-175 $N_\pi H$, and some of the results are presented in Figure 7. This figure shows the 500-MHz proton absorption spectrum and a 1D NOE difference spectrum resulting from His-175 $C_\beta H$ irradiation for CcPCN in a solution of 90% H_2O/10% D_2O. These results are identical with the NOE experiments carried out for CcPCN in 99.9% D_2O (Satterlee et al., 1987; see Figures 4 and 5 therein). Other experiments are not shown for succinctness, since in our hands there is no discernible isotope dependence in this type of 1D NOE data for CcPCN.

A series of 1D NOE experiments in which each of the His-175 $C_\beta H$ protons were separately irradiated, with irradiation times ranging between 15 and 100 ms in individual experiments, were carried out for CcPCN in the 90% H_2O/10% D_2O buffer system. Two NOESY experiments with mixing times of 30 and 100 ms were similarly performed. Those experiments were done in attempts to compensate for possibly anomalous cross-relaxation rates interfering with observation of a His-175 $C_\beta H$–$N_\pi H$ connectivity (data not shown). However, no solvent isotope composition dependence was observed.

We were unsuccessful in detecting an exchangeable resonance with connectivities to the His-175 C_β protons. While this is a negative result, it does suggest that in CcPCN His-175 is deprotonated, forming a proximal imidazolate ring. In fact, on the basis of the CcP structure (Finzel et al., 1987; Mauro et al., 1989), Asp-235 was shown to be in a suitable position to act as a hydrogen-bond acceptor for the His-175 $N_\pi H$. Recent NMR work with the CcP mutant D235N showed that His-175 resonances of CcPCN are dramatically affected by that point mutation (Satterlee et al., 1990). That work was interpreted as indicating *at least* strong hydrogen-bonding between Asp-235 and His-175 in the cyanide-ligated wild-type enzyme. In combination, the mutant CcP work and the results for CcPCN presented here strongly suggest that the state of

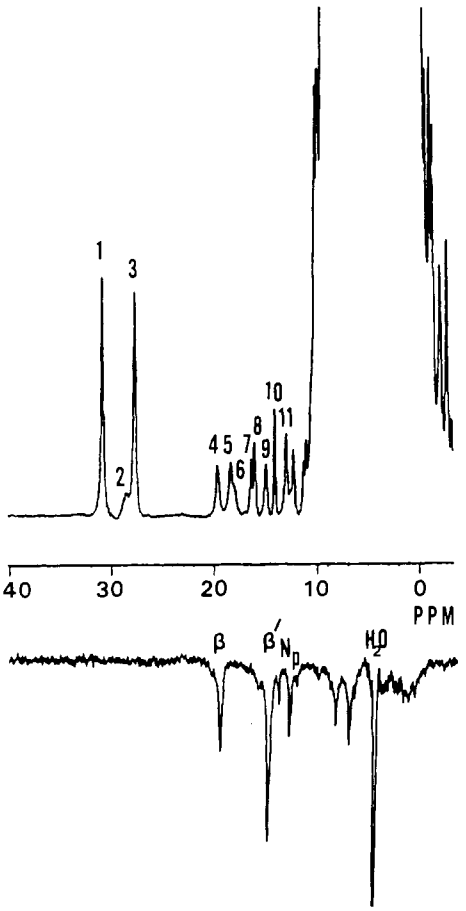

FIGURE 7: 500-MHz in 1D NOE experiment on CcPCN in 90% H_2O/10% D_2O other conditions identical with those in Figure 2. (A) Absorption spectrum and (B) NOE difference spectrum due to irradiation of one of the diastereotopic geminal β protons of His-175.

proximal hydrogen bonding is similar in CcpCN and HRPCN. There may be small structural differences, as evidenced by the lack of NOEs in CcPCN compared to those observed in HRPCN.

Arg-48 Assignments. A comparison of proton assignments for HRPCN (Thanabal et al., 1987a,b, 1988) and CcPCN (Satterlee et al., 1987) along with spectra presented here illustrates the similarity in shifts for corresponding amino acids in the two enzymes. In both, the proximal and distal histidine resonances lie downfield, along with the heme 8-CH_3, 3-CH_3, 4αH, and 7αH proton resonances. The heme vinyl 4βH and propionic acid 7βH protons all lie upfield. The similarity in the proton hyperfine resonance patterns of CcPCN and HRPCN indicates that the magnetic axes are oriented similarly in HRPCN and CcPCN, as described in detail for HRPCN (Thanabal et al., 1987a,b, 1988).

As a result of this, we searched for the CcPCN Arg-48 resonances in the upfield spectral region between 0 and −6 ppm, where the corresponding Arg-38 resonances were found in HRPCN. The upfield region of the proton 1D absorption spectrum for CcpCN (Figure 2) displays two single proton resonances (peaks 22 and 24) analogous to two Arg-38 single proton resonances similarly displayed in HRPCN. In CcPCN, one of these is resolved from its neighbors well enough so that careful 1D NOE difference experiments produced a six-resonance connectivity pattern (not shown) quite similar to the pattern observed for the similar experiment carried out on HRPCN (Thanabal et al., 1988). This result indicates that each of the methylenes in the Arg-48 side chain are diastereotopic in this environment and that peak 24 and peak 19 form one set of geminal partners in the Arg-48 spin system in CcPCN. On the basis of this result, additional partners were assigned from the 500-MHz NOESY experiments.

The upfield region of the NOESY spectrum is shown in Figure 4A, where it can be seen that peaks 24/19 and 22/23 form clearly visible connectivities characteristic of geminal pairs. All four of these resonances appeared in the 1D NOE difference spectrum described above and are accordingly assigned to Arg-48. Resonance 23 is, therefore, a composite peak, consisting both of an Arg-48 single proton resonance and the heme vinyl 4β cis resonance. Judging from its intensity (Figure 2), resonance 19 is also a composite, at least consisting of an Arg-48 single proton resonance and a heme propionic acid 7βH resonance. The result is assignment of four of the six nonexchangeable geminal partner protons in the Arg-48 side chain. There are several possibilities for assigning the remaining geminal pair, but an unambiguous assignment is not possible at this time. The upfield spectral region is too crowded with overlapping resonances for a satisfactory application of selective 1D NOE experiments. An unambiguous answer should emerge from further higher resolution NOESY experiments currently in progress.

Identification of Arg-48 resonances upfield, in positions similar to those found for Arg-38 in HRPCN, is additional evidence that CcPCN and HRPCN have similar magnetic environments.

Leu-232 Assignments. Comparative resonance integration of resonances in the upfield one-dimensional spectral region (not shown) relative to the firmly assigned heme 3- and 8-methyl resonances (peaks 1 and 3 in Figure 2) indicated that peaks 18 and 21 have intensities of at least three protons each. Considering the CcP structure, the fact that these two methyl groups exhibit strong NOE connectivity (Figure 4), and similar resonances in HRPCN, these two peaks are assigned to the two δ-methyl groups in Leu-232. Additional support for this assignment comes from the connectivity between peak 18 and the heme 8-CH_3 that can be seen in Figure 3. In the crystal structure, the Leu-232 $C_{\delta 1}$ and $C_{\delta 2}$ lie 4.56 and 4.18 Å, respectively, from the heme 8-CH_3, close enough for proton NOEs to be detected between these groups.

Other Inferred Assignments. The NOESY data presented in Figures 3 and 4 contain several major unlabeled cross peaks involving the hyperfine shifted heme protons. One particularly intense cross peak to the heme 8-methyl group (peak 3) corresponds to a proton resonance at 5.31 ppm. On the basis of the cross-peak intensity, the specific connectivity, the parent resonance shift position, assignments in HRPCN, and the structural closeness of the heme 8-methyl and δ-meso protons (C–C distance = 3.02 Å), it is proposed that the heme δ-meso proton be assigned a shift of 5.31 ppm. An intense cross peak to the heme 4α vinyl resonance with similar characteristics corresponds to a resonance at 5.84 ppm. By the same argument just presented (4αC–meso C distance = 3.11 Å), it is proposed that the heme β-meso proton be assigned a shift of 5.84 ppm. In Figures 3 and 4, a weak cross peak is observed that involves the heme 4β proton and a peak with a shift of 0.30 ppm. From the crystal structure and the traceability of this proton to the Ala-174 α proton (see Figure 3), the Ala-174 β-methyl group is proposed to have a shift of 0.30 ppm.

SUMMARY

The work presented here represents a significant advance in proton resonance assignments for catalytically relevant amino acids of the heme pocket in CcPCN. Importantly, it establishes the fact that His-52 can be viewed as a protonated imidazolium ion in the low-spin six-coordinate cyano form of

CcP, which is an important aspect of the proposed CcP hydrogen peroxide decomposition mechanism.

Our work shows that, with NOESY data, crystal structure parameters, and comparisons with one-dimensional NOE data for CcPCN, connectivities can be established between the well-resolved proton hyperfine resonances and the main part of the enzyme's polypeptide chain, which will be useful in expanding these assignments. Fundamental to these additional assignments found in the NOESY spectra presented here is the fact that NOESY data reproduce the 1D NOE assignments, giving confidence in the 2D approach for large paramagnetic proteins such as CcPCN. It also reveals that there are both similarities and differences between CcPCN and HRPCN. The general similarities in their hyperfine shift patterns indicate similar orientations of the heme-centered magnetic axes for HRPCN and CcPCN. Such a situation implies that the general structures of the HRP and CcP heme pockets must be comparable.

This report illustrates the usefulness of NOESY spectroscopy for making assignments in such a large paramagnetic protein. It demonstrates that NOESY and 1D NOE connectivities are identical, despite the different ways in which the two experiments are carried out. Our experiences have taught that both techniques are useful, although in some applications, such as for selective detection or assignment of broad and/or exchangeable resonances, the 1D method is more efficient. Finally, it is apparent from this work that, contrary to predictions that NOESY spectroscopy would be fruitless in a paramagnetic protein of this size due to the extremely short proton spin-lattice relaxation times, useful data can be obtained.

Registry No. Heme, 14875-96-8; Arg, 74-79-3; Trp, 73-22-3; His, 71-00-1; Ala, 56-41-7; Thr, 72-19-5; Leu, 61-90-5.

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