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2D NMR of paramagnetic metalloenzymes: Cyanide-inhibited horseradish peroxidase

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SUMMARY

Two-dimensional (2D) proton NMR correlation spectroscopy, COSY, and nuclear Overhauser spectroscopy, NOESY, have been used to explore the applicability of these methods for the moderately large (42 KDa), paramagnetic cyanide-inhibited derivative of horseradish peroxidase, HRP-CN. The target resonances are those in the active site of HRP-CN which experience substantial hyperfine shifts and paramagnetic relaxation. The magnitude COSY experiment was found to yield cross peaks for all known spin-coupled heme substituents, as well as for the majority of non-heme hyperfine shifted protons, in spite of line widths of the order of ~100 Hz. Moreover, the rapid relaxation of the hyperfine-shifted resonances allows the extremely rapid collection of useful 2D NMR data sets without the loss of information. For the heme, the combination of COSY cross peaks for the vinyl and propionate substituents, and NOESY cross peaks among these substituent protons and heme methyls, allows assignment of heme resonances without recourse to deuterium labeling of the heme. A seven-proton coupled spin system was identified in the upfield region that is consistent with originating from the proposed catalytic Arg^{38} residue in the distal heme pocket, with orientation relative to the heme similar to that found in cytochrome *c* peroxidase. The upfield hyperfine-shifted methyl group in the substrate binding pocket previously proposed to arise from Leu^{237} is shown to arise instead from an as yet unidentified Ile. NOESY spectra collected at very short (3 ms) and intermediate (20 ms) mixing times indicate that build-up curves can be obtained that should yield estimates of distances in the heme cavity. It is concluded that 2D NMR studies should be able to provide the heme assignments, aid in identifying the catalytic residues, and provide information on the spatial disposition of such residues in the active site for cyanide complexes of a number of intermediate to large paramagnetic heme peroxidases, as well as for other paramagnetic metalloenzymes with line widths of ~100 Hz. Moreover, paramagnetic-induced hyperfine shifts and linewidths to ~100 Hz need not interfere with the complete solution structure determination of a small paramagnetic protein solely on the basis of 2D NMR data.

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INTRODUCTION

Modern 2D NMR methods are now capable of providing detailed solution structures for small diamagnetic proteins (Wüthrich, 1986). The two essential steps in this venture involve the assignment of spin systems via bond correlation experiments such as DQF-COSY (Edwards and Bax, 1986; Muller et al., 1986) or TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985), and then sequentially arranging the spin systems and detecting non-bonded inter-residue interactions via NOESY spectra (Macura et al., 1981). The presence of a paramagnetic ion at the active site of even a small protein, no matter how favorable the electronic relaxation time of the metal spin(s) (La Mar et al., 1973; Bertini and Luchinat, 1986), inevitably leads to line broadening for residues near the active site that can obscure spin coupling and undermine the development of NOEs (Dugad et al., 1990a). As even a diamagnetic protein becomes large (i.e., >20 kDa), the increased line widths make detection of COSY peaks problematical unless there is local mobility (Fairbrother et al., 1989; Oswald et al., 1989), and the larger number of protons present resolution problems. There are, however, numerous classes of interesting and important metalloenzymes which are both large by current 2D NMR standards, and paramagnetic in all functional states. Many of these systems exhibit reasonably well-resolved ^{1}H NMR spectra (La Mar, 1979; Bertini and Luchinat, 1986; Satterlee, 1986), and any interpretation of these spectra demands that the signals of potential interest be unambiguously identified by unique spin connectivity patterns, and that the relative spatial disposition of the key residues be determinable.

The class of proteins that we are interested in are the heme peroxidases which catalyze the reduction of a variety of organic and inorganic substrates at the expense of H_2O_2 (Dunford and Stillman, 1976). The resting state of these enzymes is readily inhibited by CN^- binding at the iron which affords a low-spin, ferric ground state characterized by well-resolved ^{1}H NMR spectra and highly informative hyperfine shifts. Horseradish peroxidase, HRP, is a 42-kDa glycoprotein that exhibits some sequence homology (Takio et al., 1980; Welinder, 1985) to crystallographically characterized cytochrome *c* peroxidase, C*c*P (Poulos and Kraut, 1980; Finzel et al., 1984), and extensive isotope labeling (La Mar et al., 1980; de Ropp et al., 1984) and 1D ^{1}H NOE experiments (Thanabal et al., 1987a,b, 1988) on HRP–CN have been interpreted on the basis of computer modeling (Sakurada et al., 1986) to show that HRP possesses the same catalytic residues in the active site as C*c*P; these residues are illustrated in Fig. 1. However, 1D NOE data were only shown to be consistent with, and not proof for, the proposed amino acids, and the identification depended on heme assignments determinable only upon isotope labeling of the removable prosthetic group. The unambiguous identity of amino acid signals, as well as the assignment of all heme resonances in the absence of isotope labeling, could be determined by 2D NMR methods if the appropriate cross peaks in such a paramagnetic system are detectable. A single 2D NMR study of HRP-CN (Veitch and Williams, 1990) did not address the hyperfine shifted resonances. The consensus expectation that the hyperfine-shifted peaks exhibit lines too broad (60–140 Hz) to allow detection of coherence has apparently deterred a serious test of whether coherence is detectable.

Abbreviations: NMR, nuclear magnetic resonance; 2D, two dimensional; COSY, 2D correlation spectroscopy; MCOSY, magnitude COSY; DQF-COSY, double-quantum-filtered COSY; PCOSY, 2D purged COSY; NOE, nuclear Overhauser effect; NOESY, 2D nuclear Overhauser spectroscopy; TOCSY, 2D total correlation spectroscopy; HRP, horseradish peroxidase; C*c*P, cytochrome *c* peroxidase; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

While the previous success of 1D NOEs (Thanabal et al., 1986, 1987a,b, 1988) bode well for the detection of cross peaks in NOESY spectra of HRP-CN, such NOESY cross peaks can be interpreted meaningfully only if the spin systems participating in the dipolar interactions are first identified by COSY experiments.

We target, in this report, the assignment of the hyperfine-shifted resonances of HRP–CN in ^2H_2O using solely 2D NMR methods. We have shown previously that this is possible in a small paramagnetic protein with much narrower lines, the isoelectronic low-spin, cyano complex of ferric myoglobin (16 kDa), metMbCN (Emerson and La Mar, 1990; Yu et al., 1990). The resolved resonances of interest in HRP–CN are 60–140 Hz broad, and exhibit T_1s in the range of 15–150 ms, and hence HRP–CN provides a more severe test than metMbCN for detecting spin connectivity. A qualitative assessment of the various spin-connectivity experiments with metMbCN (Yu et al., 1990) has indicated that, for the hyperfine-shifted and broadened resonances, DQF-COSY was the least useful technique because the DQF discriminated against broad lines, and the T_2s were too short to allow cross peaks to develop in the required mixing time in TOCSY experiments. In contrast, the normal magnitude COSY (MCOSY) experiment was shown to allow detection of all expected cross peaks. Although MCOSY yields a broad diagonal (Bax, 1982), our focus here on hyperfine-shifted resonances, which generally yield cross peaks well off the diagonal, minimizes this disadvantage. We report herein on a 2D NMR study of HRP-CN that addresses the following questions: Can spin connectivity among the strongly hyperfine-shifted and relaxed resonances be detected? Is it possible to assign the contact-shifted heme resonances solely on the basis of 2D NMR methods? Can the hyperfine-shifted heme pocket amino acid residues be assigned solely on the basis of spin-connectivity patterns? Lastly, do the present assignments agree with those proposed earlier on the basis of less-direct 1D NMR methods (Thanabal et al., 1986, 1987a,b)?

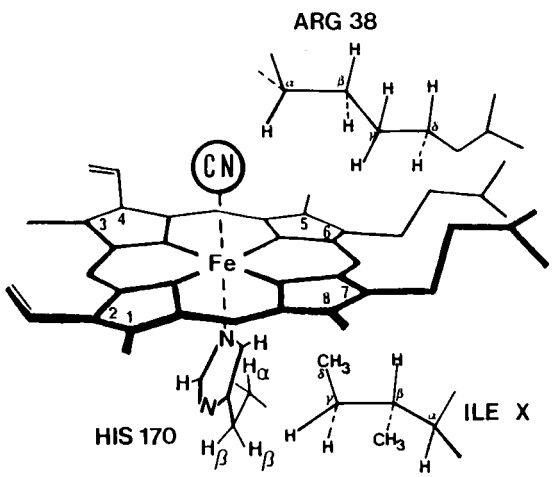

Fig. 1. Schematic representation of the heme pocket of horseradish peroxidase showing the proximal His[170], the distal Arg[38], and residue X, previously proposed as Leu[237], and now revised as an unidentified Ile X. The numbering scheme for heme substituents is also shown.

MATERIALS AND METHODS

Protein

Horseradish peroxidase type VI was purchased from Sigma as a lyophilized salt-free powder and used directly without further purification; the enzyme is >90% isozyme C.

NMR samples

3 mM solutions of HRP-CN were prepared in 99.9% ^2H_2O by addition of excess potassium cyanide to the protein solution. Solution pH, uncorrected for the isotope effect, was adjusted to 7.0 with dilute ^2HCl or NaO^2H. NMR samples of ca. 0.5 ml (for 5 mm NMR tubes) or ca. 2.0 ml (for 10 mm NMR tubes) were utilized.

NMR data collection

Data were obtained on a General Electric-NMR Ω-500 spectrometer operating at 11.75 Tesla. Magnitude COSY (MCOSY) spectra (Bax et al., 1981; Bax, 1982) were collected using 256 blocks in t_1 with 384 scans per block, and phase-sensitive NOESY spectra (Macura et al., 1981) were collected using the hypercomplex method (States et al., 1982) with 256 complex points in t_1 with 160 scans per block. Double-quantum filtered COSY, DQF-COSY, spectra (Piantini et al., 1982; Shaka and Freeman, 1983) were also collected using the hypercomplex method. For all 2D datasets, 1024 complex points in t_2 were collected over a band width of 31.25 KHz with a sampling rate of 32 μs using the Ω 16-bit, 250 KHz analog-to-digital converter. The residual solvent line was in all cases saturated during the predelay with selective decoupler irradiation. All pulse sequences utilized here employed single 90° (as opposed to composite 90°) pulses to maximize the

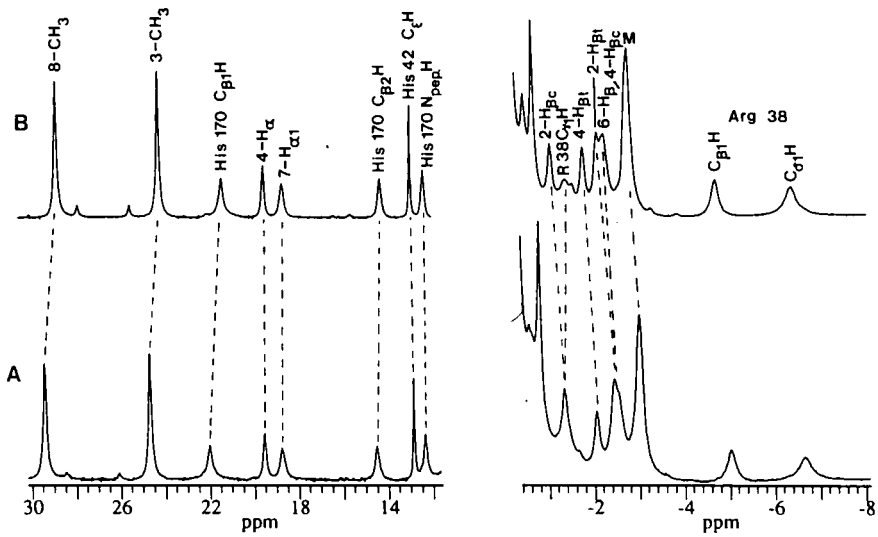

Fig. 2. The 500 MHz ^1H NMR trace of HRP–CN in ^2H_2O, pH 7.0 at (A), 40°C and (B), 50°C; the resolved resonances whose proposed assignments are confirmed by the present 2D studies are given; the unassigned upfield methyl peak previously attributed to Leu^{237} is labeled M.

band width of excitation. MCOSY and NOESY data were collected as a function of repetition rate over the range 0.6 to 11 scans s^{-1} by varying the predelay, including correcting for the 20–30 ms of 'recycle delay' inherent to the GE-NMR Ω spectrometer software as a function of pulse sequence. For phase-sensitive NOESY data, the delay between pulse and acquisition of the first data point (phase-delay) was adjusted to minimize the first-order phase correction in t_2. In all experiments, 32 dummy scans were used prior to collecting data. The majority of the data presented here were collected using a 10 mm sample tube; for these experiments, the GE Ω-500 spectrometer was equipped with a 10 mm ^1H probe from Cryomagnet Systems, Inc.

TABLE I
^1H NMR SPECTRAL PROPERTIES OF SELECTED HRP-CN RESONANCES

Resonance	Shift[a]	Line width (Hz)[b]	T_1 (ms)[c]
Heme			
8-CH_3	28.40	67	59
3-CH_3	23.83	63	82
4-H_α	19.13	66	141
4-$H_{\beta c}$	−2.82	n.r.[d]	–
4-$H_{\beta t}$	−1.86	~60	–
2-H_α	5.50	n.r.	–
2-$H_{\beta c}$	−1.13	~60	–
2-$H_{\beta t}$	−2.18	n.r.	–
His[170]			
$C_{\beta 1}H$	21.01	112	56
$C_{\beta 2}H$	13.90	85	55
$C_\alpha H$	9.34	n.r.	–
NpH	11.98	73	145
Arg[38]			
$C_\alpha H$	5.73	n.r.	–
$C_{\beta 1}H$	−4.78	84	108
$C_{\beta 2}H$	−0.61	n.r.	–
$C_{\gamma 1}H$	−1.45	~140	15
$C_{\gamma 2}H$	0.59	n.r.	–
$C_{\delta 1}H$	−6.44	135	20
$C_{\delta 2}H$	0.93	n.r.	–
Ile X			
$C_\delta H_3$ (M)	−2.91	45	85
$C_{\gamma 1}H$	0.18	n.r.	–
$C_{\gamma 2}H$	1.14	n.r.	–

[a]Shifts in ppm from DSS at 50°C in ^2H_2O, pH 7.0.
[b]Line width in Hz at 50°C in ^2H_2O, pH 7.0.
[c]T_1 in ms, at 55°C in ^2H_2O, pH 7.0.
[d]Not resolved.

NMR data processing

Datasets were processed on a Sun 3/260 work station using standard GE Ω software. MCOSY data were optimally processed (Bax, 1982) with an unshifted sine-bell-squared apodization in both dimensions to reduce the severe tailing of peaks from the intense diamagnetic envelope. MCOSY datasets were zero-filled twice in t_1 prior to Fourier transformation, and in most cases symmetrized subsequent to Fourier transformation in t_1. The effect of the period of the apodization function in t_2 was examined; periods of 25%, 50% and 100% (i.e. apodization over 256, 512, or 1024 complex points in t_2) were utilized. A period of 25% maximized signal-to-noise of cross peaks at the expense of resolution, while a period of 50% gave the best combination of sensitivity and resolution in the MCOSY spectrum. Hence apodization ove 512 points in t_2 is used for all MCOSY datasets presented here. Phase-sensitive NOESY spectra were processed with 30° shifted sine-bell-squared apodization in both dimensions, over 256 points in t_1 and 1024 points in t_2. The data set was baseline corrected with a fifth-order polynomial in f_2 and zero-filled twice prior to Fourier transformation in t_1. The optimum phasing in f_1 was obtained by use of a small zero-order correction only, though this leaves some phase error in peaks of large hyperfine shift in the final 2D dataset. Final 2D datasets were in all cases 1024×1024 points; although digital resolution is thus only ~ 30 Hz point^{-1}, this was adequate for observation of the hyperfine-shifted peaks of interest here.

RESULTS

COSY spectral parameters

The ^{1}H NMR trace of HRP-CN exhibits numerous overlaps among the upfield hyperfine-shifted resonances, as detailed previously (de Ropp et al., 1984; Thanabal et al., 1987a,b), and the traces at the two temperatures found optimal, 40°C and 50°C, are illustrated in Fig. 2. The previously proposed assignments are given for those peaks where the present report confirms the assignment; other resolved peaks are labeled by letters. The required increase in spectral width for HRP-CN by a factor of ~ 5 relative to diamagnetic proteins decreases the dwell-time by the same factor, and hence to achieve digitization comparable to diamagnetic proteins, impractically large datasets would have to be collected. However, while the protein contains slowly relaxing, relatively narrow signals that would require long acquisition times for proper encoding, our emphasis on broad and rapidly relaxing protons allows us to work with much shorter acquisition times. The hyperfine-shifted resonances of interest for HRP-CN exhibit peaks with line widths of 60–140 Hz, which translate to T_2s of 2.3 to 5 ms, and non-selective T_1s of 15–150 ms, as listed in Table 1. Therefore, the condition $T_2^{-1} > > J$ applies for all expected couplings and the slowest-decaying coherence signal is optimally detected at an acquisition time $2T_2 \leqslant 10$ ms for a COSY spectrum, using a pseudo-echo filter symmetrical about $t_1 = t_2 \sim T_2$ (Bax, 1982). This is achieved at the relatively low t_1 digitization of 256 blocks (acquisition time 8.4 ms in t_1), and the digitization in the t_1 domain is readily improved by zero-filling to 1024 points prior to Fourier transformation. In all cases, 256 $t_1 \times 1024$ t_2 points (acquisition time 33 ms in t_2) were collected as the extra t_2 points did not prolong the experiment.

A 3 mM HRP-CN sample in ^2H_2O in a 5 mm tube yielded a useful MCOSY map in ~ 60 h at 40°C at the 'normal' diamagnetic protein repetition time of 1.6 s; this was improved to 30 h using a 10-mm tube. Moreover, a significant decrease in the pulse sequence repetition time, from 1.6 s

to 0.50, 0.33, 0.20 and 0.09 s, revealed that intensities of cross peaks among hyperfine-shifted peaks were undiminished as the repetition time decreased because of the efficient relaxation of the peaks of interest (not shown). Thus informative MCOSY maps could be collected within 3 h, thereby allowing data collection to 55°C without sample degradation. Even with a reasonably

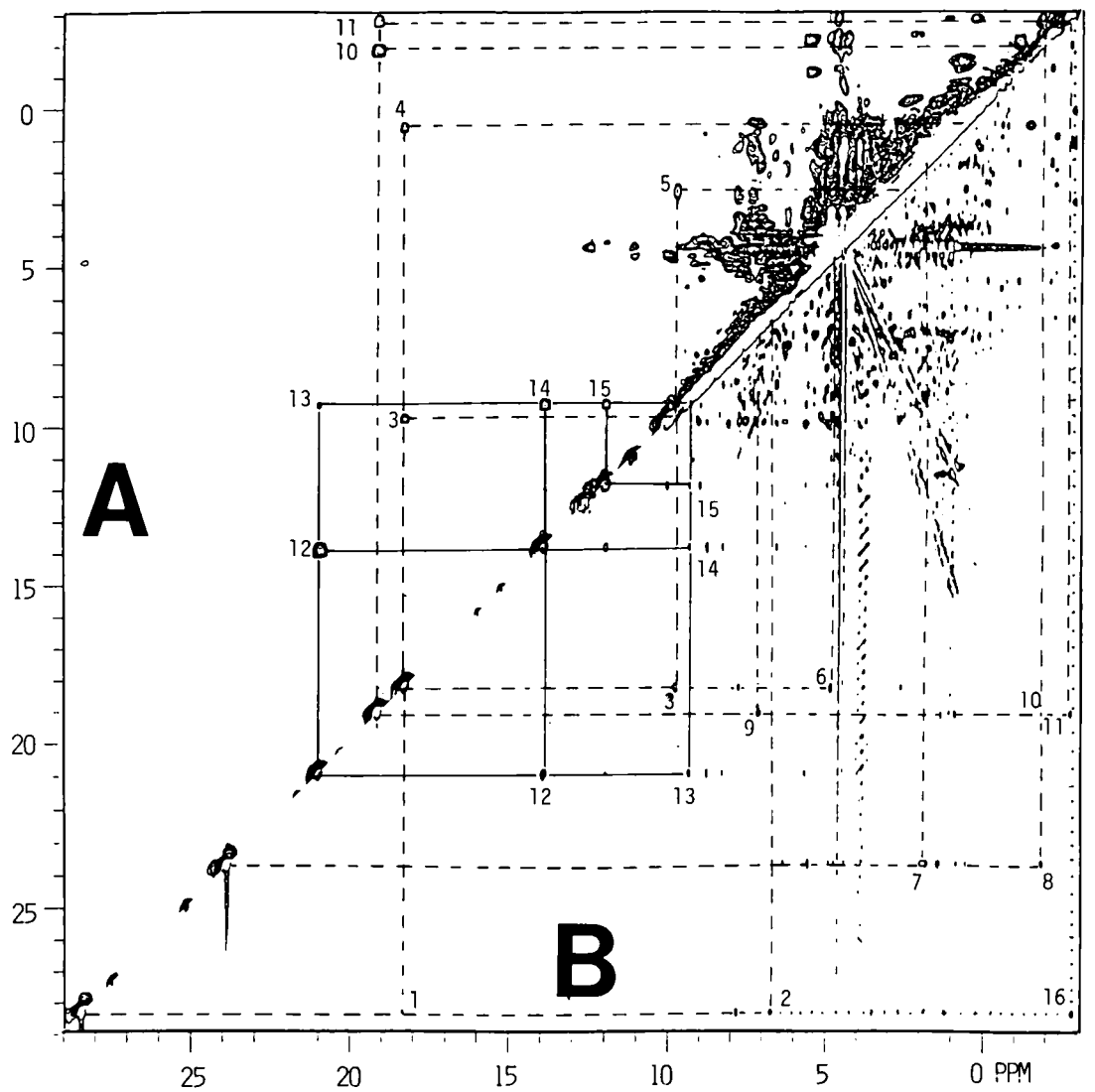

Fig. 3. A split-diagonal representation of the 29 to −3 ppm region of the MCOSY (A) and NOESY (B) map with a 20 ms mixing time of HRP-CN in ^2H_2O, pH 7.0 at 50°C. Both datasets are collected at a repetition rate of 5 s^{-1}. The intra-heme connectivities are indicated by dashed lines, the intra-amino acid residue peaks are indicated by solid lines, and amino acid-residue/heme connectivities are given in dotted lines. The cross peaks of interest are labeled with numbers according to: Intra-heme cross peaks; 1 (8-CH_3:7-$H_{\alpha 1}$), 2 (8-CH_3:δ-meso-H), 3 (7-$H_{\alpha 1}$:7-$H_{\alpha 2}$), 4 (7-$H_{\alpha 1}$:7-$H_{\beta 1}$), 5 (7-$H_{\alpha 2}$:7-$H_{\beta 2}$), 6 (7-$H_{\alpha 1}$:γ-meso-H), 7 (3-CH_3:α-meso-H); 8 (3-CH_3:4-$H_{\beta t}$), 9 (4-H_α:β-meso-H), 10 (4-H_α:4-$H_{\beta t}$), 11 (4-H_α:4-$H_{\beta c}$); Intra-His[170] cross peaks; 12 ($C_{\beta 1}$H:$C_{\beta 2}$H), 13 ($C_{\beta 1}$H:C_αH); 14 ($C_{\beta 2}$H:C_αH), 15 (C_αH:N_{pep}.H), and Ile X cross peak, 16 (8-CH_3:$C_\delta H_3$).

long recycle time for the experiment, i.e., 1.6 s, the intense narrow resonances within the diamagnetic envelope still yielded very serious t_1 noise under all processing conditions. This t_1 noise, however, was effectively deleted by symmetrization of the square dataset, thereby yielding a clear set of cross peaks that can be established to arise from coherence (see below).

The lowfield portion of the optimal MCOSY map of HRP-CN at a repetition rate of $5\,s^{-1}$ at 50°C is given in Fig. 3A, and the crowded upfield portion of the same map is expanded in Fig. 4. All cross peaks detected in Figs. 3A and 4 at 50°C are also detected at 40°C (not shown). The higher temperature map is analyzed because accidental degeneracies complicate interpretation at 40°C. In order to minimize the broad tails of the diagonal peaks characteristic of the phase-

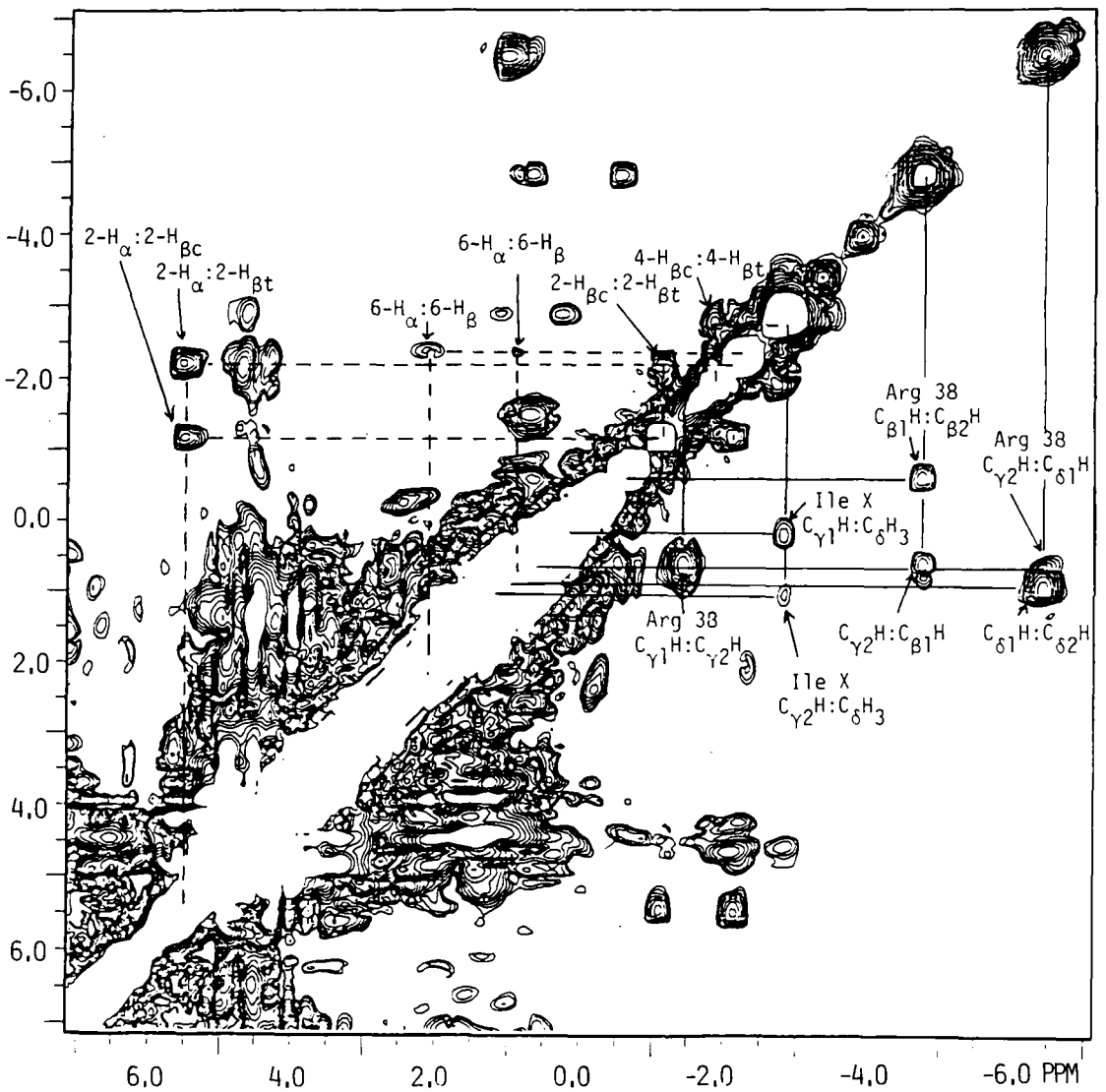

Fig. 4. Expanded upfield portion (7 to −7 ppm) of the MCOSY map of HRP-CN in ^2H_2O, pH 7.0, 50°C. Connectivities are indicated as described in Fig. 3.

twisted lineshape of magnitude COSY maps (Bax. 982), only unshifted sine-bell or sine-bell-squared window functions were used; the latter were found more advantageous.

Heme COSY peaks

For the heme resonances previously assigned by isotope labeling (La Mar et al., 1980; de Ropp

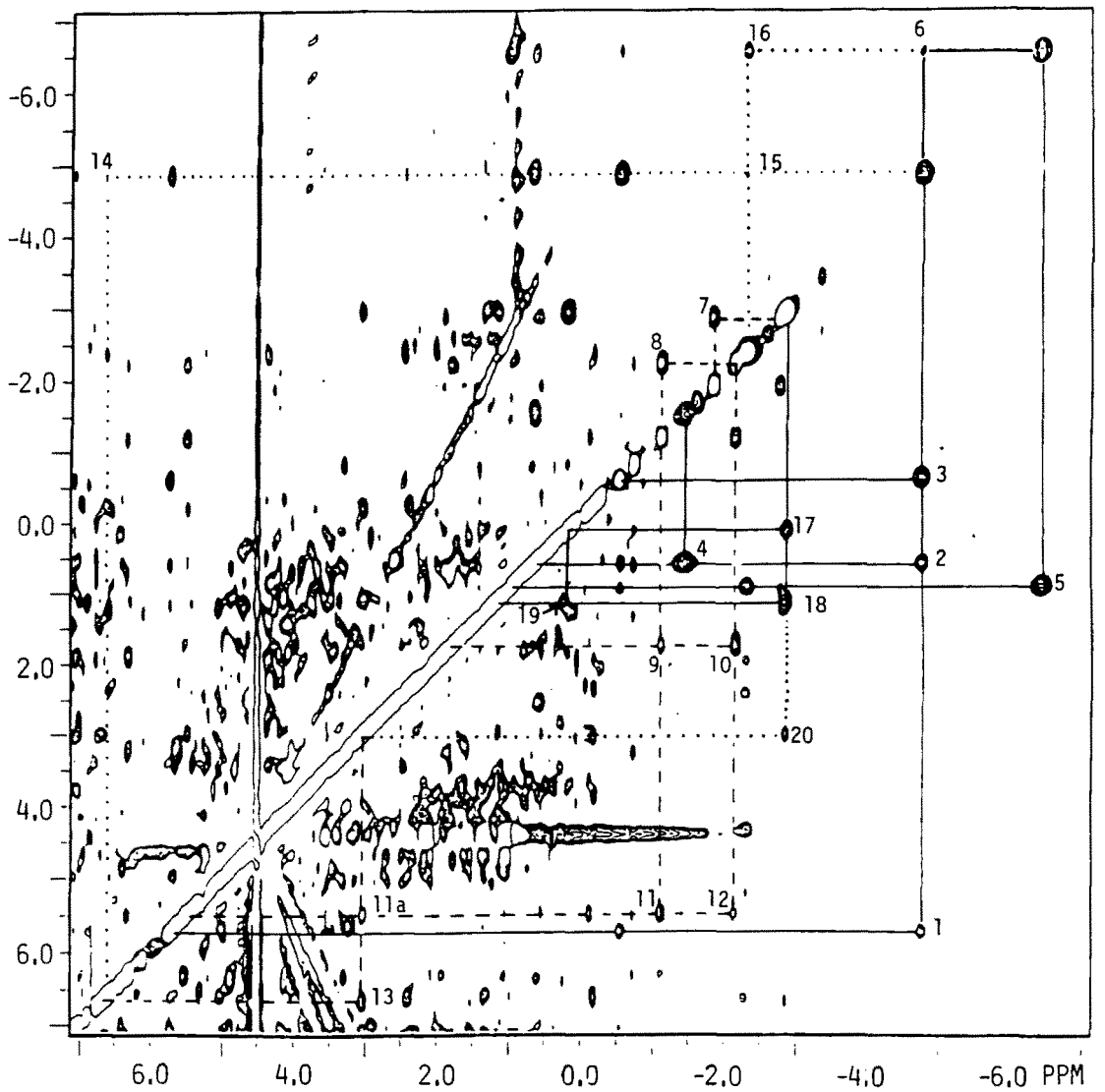

Fig. 5. Expanded upfield portion (7 to −7 ppm) of the NOESY map of HRP-CN in ^2H_2O, pH 7.0,50°C collected at a mixing time of 20 ms. Connectivities are indicated as described in Fig. 3. The cross peaks of interest are labeled with numbers according to: Intra-Arg[38] peaks; 1 ($C_\alpha H$:$C_{\beta 1}H$), 2 ($C_{\beta 1}H$:$C_{\gamma 2}H$), 3 ($C_{\beta 1}H$:$C_{\beta 2}H$), 4 ($C_{\gamma 1}H$:$C_{\gamma 2}H$); 5 ($C_{\delta 1}H$:$C_{\delta 2}H$), 6 ($C_{\beta 1}H$:$C_{\delta 1}H$); Intra-heme peaks, 7 (4-$H_{\beta c}$:4-$H_{\beta t}$), 8 (2-$H_{\beta c}$:2-$H_{\beta t}$), 9 (2-$H_{\beta c}$:α-meso-H), 10 (2-$H_{\beta t}$:α-meso-H), 11 (2-$H_{\beta c}$:2-H_α), 11a (1-CH_3:2-H_α), 12 (2-$H_{\beta t}$:2-H_α), 13 (1-CH_3:δ-meso-H); heme-Arg[38] cross peaks, 14 (5-CH_3:$C_{\beta 1}H$), 15 (6-$H_{\beta s}$:$C_{\beta 1}H$), 16 (6-$H_{\beta s}$:$C_{\delta 1}H$); Intra-Ile X cross peaks, 17 ($C_\delta H_3$:$C_{\gamma 1}H$), 18 ($C_\delta H_3$:$C_{\gamma 2}H$), 19 ($C_{\gamma 1}H$:$C_{\gamma 2}H$) and heme-Ile X cross peak 20 (1-CH_3:$C_\delta H_3$).

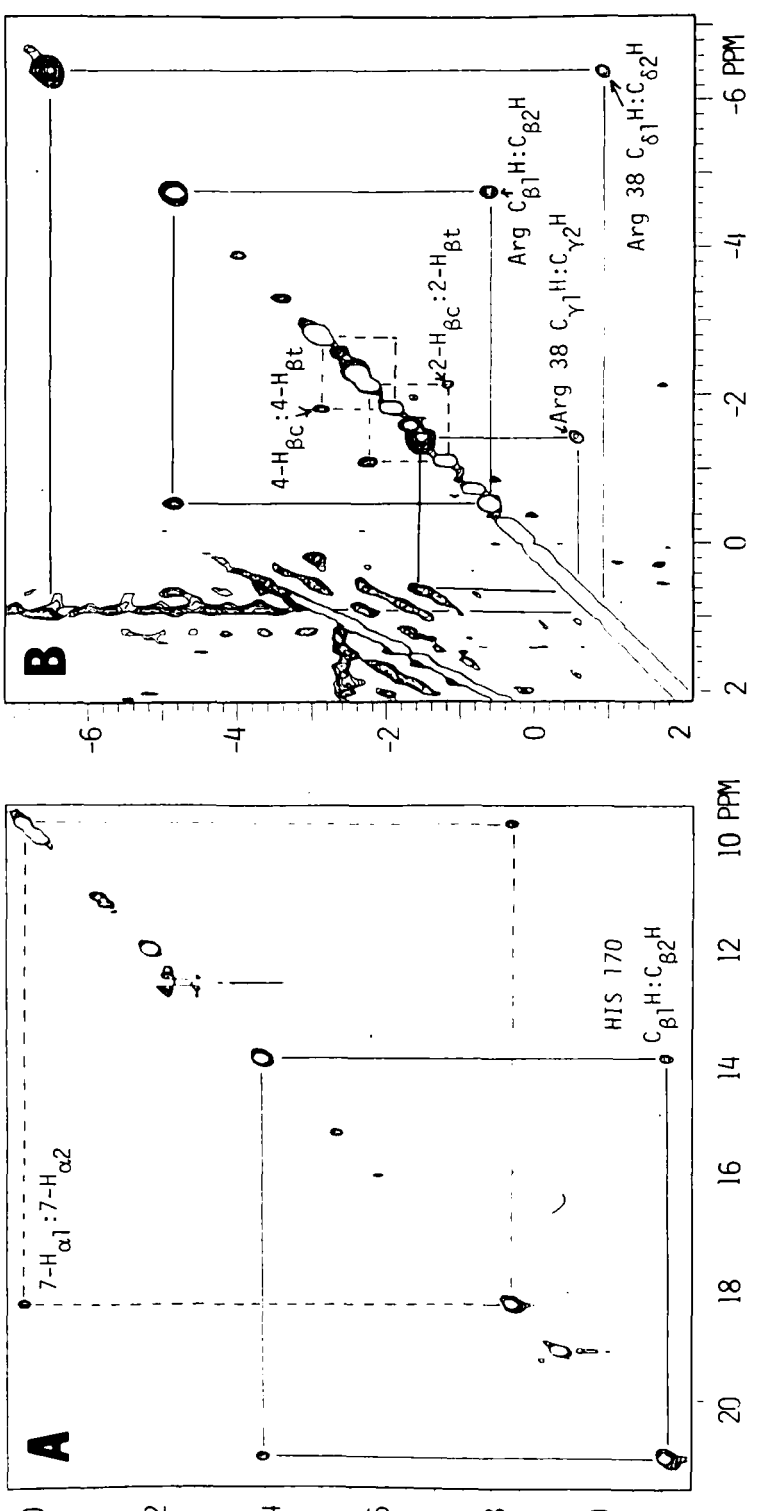

Fig. 6. The lowfield, 21 to 10 ppm (A), and upfield portion, 2 to −8 ppm (B), of the NOESY map of HRP-CN in ^2H_2O, pH 7.0 at 50°C collected with a mixing time of 3 ms, at a repetition rate of 5 s^{-1}. Note that only the known geminal pairs of methylene protons yield detectable cross peaks for His^{170} $C_\beta H_2$, 7-propionate $C_\alpha H_2$, and the two vinyl $C_\beta H_2$s; the only other detected cross peaks are those for the $C_\beta H_2$, $C_\gamma H_2$ and $C_\delta H_2$ of Arg^{38}.

et al., 1984), we detect cross peaks not only for the resolved 4-vinyl system (Fig. 3A), but also the 2-vinyl group for which only the H_{β}s are resolved (Fig. 4). The larger H_{α}:$H_{\beta t}$ ($\sim$16 Hz) than H_{α}:$H_{\beta c}$ ($\sim$10 Hz) coupling (Scheer and Katz, 1975) is manifested in the greater intensity for the former cross peak for both vinyls. Moreover, both vinyl $H_{\beta c}$:$H_{\beta t}$ cross peaks are seen in spite of the small ($\sim$2 Hz) coupling (Fig. 4). The assigned 7-propionate H_{α} at 18.3 ppm exhibits relatively strong coupling to the unresolved 7-H_{α} at 9.7 ppm, and each of those peaks exhibits a further cross peak to one of the 7-propionate H_{β}s (Fig. 3A). The essentially degenerate pair of 6-propionate H_{β} resonances at -2.3 ppm yield two cross peaks that identify the two 6-H_{α}s at 2.1 and 1.0 ppm (Fig. 4). Hence all expected spin connectivities for heme substituents are observed.

Amino acid COSY cross peaks

With the location of all expected COSY cross peaks for the four heme substituents, all remaining hyperfine-shifted cross peaks must arise from amino acid residues in the heme pocket. Inspection of Figs. 3A and 4 reveals that each resolved non-heme resonance exhibits at least one COSY cross peak, with several showing multiple cross peaks, with the exception of the very narrow peak at 12.6 ppm. The network of non-heme-coupled spins includes a set of four signals solely in the low-field window with shifts 21.0, 13.9, 12.0 and 9.3 ppm (mapped in solid lines in Fig. 3A). Two networks of cross peaks are detected upfield (Fig. 4). One such system involves the three resolved broad single-proton resonances, and includes at least three, and likely four, additional unresolved signals, as marked in dashed lines in Fig. 4. The third network includes the resolved methyl peak M at -2.9 ppm which exhibits two cross peaks (solid lines in Fig. 4) to unresolved protons.

NOESY spectra

NOESY maps were generated at 1.6, 0.5, 0.2 and 0.1 s repetition times; again, the faster repetition rate had no deleterious effect on cross-peak intensity involving any of the hyperfine-shifted resonances, and the fastest rate yielded a complete dataset in $\sim$3 h. The low-field portion of the NOESY map of HRP–CN at 50°C collected at a repetition rate of $5\,s^{-1}$ is illustrated in Fig. 3B for a mixing time of 20 ms, and the crowded upfield section is expanded in Fig. 5. The asymmetry in intensity of the two cross peaks for a number of pairs of resonances is due to the expected suppression of the cross peaks (Neuhaus and Williamson, 1989) at the f_1 frequency of the slower-relaxing proton under the rapid pulse repetition rates employed. Cross sections through the resolved resonances yield slices (not shown) with similar NOE pattern to those reported previously based upon 1D steady-state NOEs (Thanabal et al., 1987a,b). The dashed lines trace the pattern around the heme. Intra-residue NOESY cross peaks are detected parallel to the COSY cross peak for the non-heme resonances, as shown in solid lines in Figs. 3B and 5. NOESY cross peaks between amino acid residues and the heme are shown as dotted lines. The 21 to 10 and $+2$ to -8 ppm diagonal sections of the NOESY map of HRP–CN at 50°C collected at a mixing time of 3 ms are illustrated in A and B, respectively, of Fig. 6. It is clear that the 3-ms mixing time already allows the clear detection of very strongly dipolar coupled protons, i.e., the known geminal protons of His^{170} $C_{\beta}H_2$, the 7-propionate $C_{\alpha}H_2$ (Fig. 6A), and the 2- and 4-vinyl $C_{\beta}H_2$s (Fig. 6B).

DISCUSSION

Detection of coherence

Because of the phase-twisted lineshape of the diagonal peak in a magnitude COSY display, the star-shaped signals yield ridges in both t_1 and t_2 dimensions, and these ridges can extend considerably even for optimal window functions (Bax, 1982). Hence, a careful scrutiny was made of all cross peaks on a contour map to ascertain that they did not originate from the apparent cross peaks that result from the intersection of two ridges. All cross peaks discussed herein exhibited intensities 5–50 times greater than that which would arise from the sum of the ridges, and hence do not reflect artifacts. Moreover, cross peaks are absent for the two most intense resolved residues, the low-field heme methyls at 28.4 and 23.8 ppm (Fig. 3A). All cross peaks moved with temperature in a manner as prescribed by the known temperature dependence (near Curie) of the component resonances, as reported earlier (de Ropp et al., 1984; Thanabal et al., 1987a,b). For the heme, the cross-peak intensities correlate with the expected spin-coupling patterns for the known substituents (Scheer and Katz, 1975). The cross peaks detected for non-heme resonances include several pairs of peaks 80–140 Hz wide (Table 1), at least some of which involve spin-coupling between vicinal rather than geminal partners (see below). As found earlier in our 2D studies of the smaller metMbCN system (Yu et al., 1990), a DQF-COSY map suppresses cross peaks from broadened peaks, and a comparable DQF-COSY map of HRP-CN to the MCOSY maps in Figs. 3A and 4 yielded only the vinyl H_α:H_β cross peaks for any of the hyperfine-shifted lines (not shown).

It is now clear that very useful COSY and NOESY maps can be obtained for a large paramagnetic protein such as HRP-CN, with its inherently low sensitivity due to the greater line widths and weaker cross peaks compared to smaller diamagnetic systems. Moreover, high-quality 2D NMR spectra can be collected in remarkably reasonable times ($\sim$3–5 h) in a 10 mm tube by taking advantage of the rapid repetition rates allowed by the efficiently relaxed resonances near the active site. Thus, an MCOSY map collected at 11 s^{-1} has not only retained all the cross peaks observed at a more conventional 0.6 s^{-1} cycle time, but the partial steady-state saturation of the slowly relaxing diamagnetic envelope appears to suppress somewhat the broad diagonal, thereby slightly improving the resolution near the diamagnetic envelope. It is likely that phase-sensitive COSY (Marion and Wüthrich, 1983) or PCOSY (Marion and Bax, 1988), which exhibit similarly intense cross peaks for broad lines, but allow detection of peaks quite close to the diagonal (Yu et al., 1990), will reveal additional cross peaks involving relaxed and shifted resonances closer to the diagonal.

NOESY spectra

Less surprisingly, the NOESY maps allow detection in reasonable time of all cross peaks involving hyperfine-shifted/relaxed peaks observed previously by 1D NOEs (Thanabal, 1986, 1987a,b, 1988), as well as many not accessible by these methods. Again, adequate sensitivity within reasonable acquisition times is achieved by increasing the repetition rates to 1–4 times the longest T_1 of the hyperfine-shifted signals and using the 10 mm sample tube. The NOESY maps at mixing times of 3 ms (Fig. 6) and 20 ms (Figs. 3B, 5) indicate that detailed cross-peak build-up curves can be obtained that should yield distance estimates. The wide range of T_1s for the resonances in the active site, 20–150 ms, with the fact that the cross peaks have maximum intensity at

$\tau_m \sim T_1$ (Neuhaus and Williamson, 1989), will dictate the need for a larger number of mixing times for obtaining distance estimates than for a diamagnetic system. Theoretical considerations of external (paramagnetic) relaxation have already shown (Duben and Hutton, 1990) that the initial slope of the cross-peak intensity versus τ_m, as in a diamagnetic system, should yield the cross-relaxation rate and, with the overall molecular reorientation time, an estimate of distance.

Heme assignments

The NOESY data, together with the heme COSY cross peaks, allow the assignment of all heme substituent signals. The heme dipolar connectivities for HRP-CN are indicated in dashed lines in Figs. 3B and 5. The two vinyl assignments and the NOEs of one of the heme methyls at 23.8 ppm to H_βs of both vinyls (seen only in NOESY slices; not shown) uniquely assign the 3-CH_3, and dictate that the 2-vinyl has a *trans* and the 4-vinyl a *cis* orientation, as previously proposed (Thanabal et al., 1986). The 1-CH_3 at 3.0 ppm is uniquely identified by a common NOE to one of the vinyl H_αs (the 2-vinyl), and the low-field methyl at 28.4 ppm, which identifies the latter as the

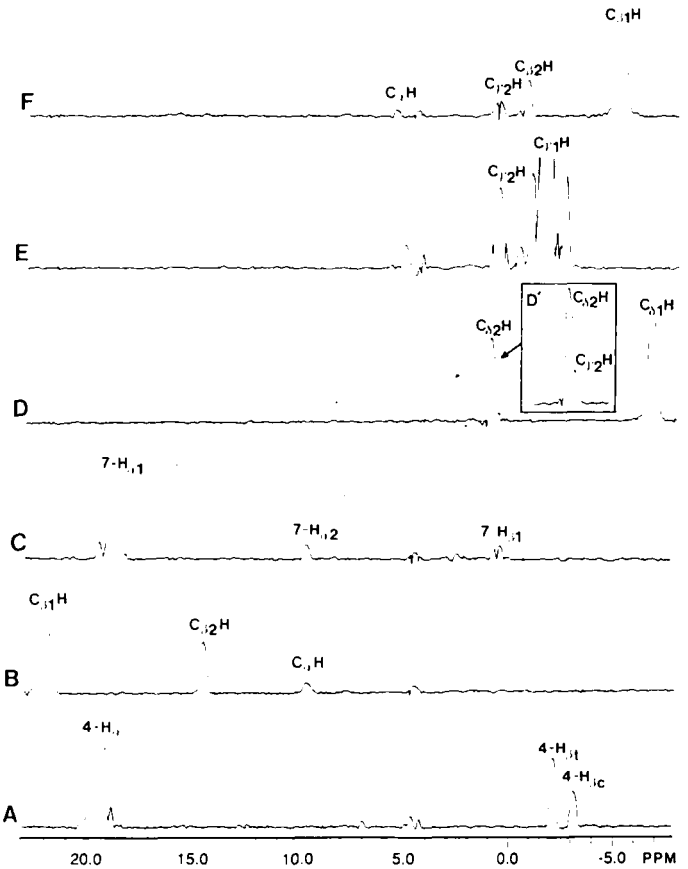

Fig. 7. Slices of the MCOSY map of HRP-CN in ^2H_2O, pH 7.0, at 40°C, showing the relative intensities of cross peaks. The slices are parallel to the f_2 axis through: (A), heme 4-vinyl H_α; (B), His^{170} $C_{\beta 1}H$; (C), heme 7-propionate $C_{\alpha 1}H$; (D), Arg^{38} $C_{\delta 1}H$; the cross peak from $C_{\delta 1}H$ to $C_{\gamma 2}H$ is better resolved at 50°C as shown in the inset D'. (E), Arg^{38} $C_{\gamma 1}H$. The 4-vinyl $H_{\beta 1}$, nearly degenerate with $C_{\gamma 1}H$, is responsible for the additional unlabeled peaks. (F), Arg^{38} $C_{\beta 1}H$. The cross peaks are labeled as in Figs. 4 and 5. (The peak at 4.6 ppm present in most slices is residual solvent.).

8-CH_3 as well. The NOESY cross peak between 8-CH_3 and the propionate H_α peak at 18.3 ppm, identifies the 7-propionate group and leaves the upfield H_β signals at -2.30 ppm as arising from the 6-propionate. An NOE from the 4-vinyl H_α peak at 19.1 ppm reveals the location of the 5-CH_3 (observed only in NOESY slice; not shown). All four heme meso protons are located by NOESY cross peaks from adjacent heme substituents (Fig. 3B); hence, the combination of COSY and NOESY data allows the assignment of all 22 heme resonances.

Amino acid assignments

The cross peak for the His^{170} $C_\beta Hs$ at 21.0 and 13.9 ppm confirms the earlier definitive assignment based on saturation transfer from the uniquely low-field contact-shifted pair of non-heme resonances in resting state HRP. The additional cross peaks (solid lines in Figs. 3A and 3B) to one non-labile proton peak at 9.3 ppm, and to the very slowly exchanging labile proton at 12.0 ppm, locate the remaining non-ring resonances of the ligand bound to the iron.

The broad (90–140 Hz), single-proton resonances at -6.4, -4.8 and -1.4 ppm, and their relatively strongly dipolar coupled partners, as detected by 1D NOE (Thanabal et al., 1987b), were proposed to originate from Arg^{38} on the basis of consistency of the 1D NOE pattern among those resonances and structural homology to *Cc*P (Finzel et al., 1984; Welinder, 1985). These dipolar couplings are clearly seen in the NOESY map in Fig. 5 (solid lines). The present MCOSY cross peaks for these three resolved resonances (Fig. 4) clearly show that each of them is spin-coupled to the proposed geminal partner (Thanabal et al., 1987b), and the significant cross-peak intensity (as seen more clearly in the slices in Fig. 7D, 7E) of the peaks at -6.4 and -1.4 ppm is suggestive of large spin-coupling, thereby supporting the geminal origin of these pairs. The third of these protons at -4.8 ppm yields a cross peak considerably weaker (as seen in the COSY slice in Fig. 7F) than for the other two resonances. That the resonances at -4.8 and -0.6 ppm are geminal protons is supported by NOESY data; at $\tau_m = 3$ ms, only geminal protons exhibit detectable cross peaks, as observed for the 7-propionate $H_\alpha s$ and His^{170} $C_\beta Hs$ (Fig. 6A), 2-vinyl and 4-vinyl $C_\beta Hs$, and the peaks labeled $C_\delta Hs$ and $C_\gamma Hs$ for Arg^{38} (Fig. 6B). Since a similar cross peak for $C_\beta Hs$ is also observed, this indicates that it reflects a similarly large cross-relaxation rate, and hence originates from a geminal pair, $C_\beta H_2$. The weaker cross peaks between the $C_{\delta 1}H$ and $C_{\gamma 2}H$ (better detected in the COSY slice through $C_{\delta 1}H$ at 50°C in Fig. 7D'), between $C_{\beta 1}H$ and $C_{\gamma 2}H$ (Fig. 7F), and from $C_{\beta 1}H$ to $C_\alpha H$ (Fig. 7F) identify a spin-connected $CH–CH_2–CH_2–CH_2$ fragment which must originate from an Arg, Lys, or Pro; the sequence homology to *Cc*P dictates that it be Arg^{38} (Sakurada et al., 1986). Thus, the MCOSY cross peaks are detected for both the large geminal and weaker vicinal spin-couplings, in spite of the ~ 100 Hz line widths. The NOESY cross peaks from the Arg^{38} resonances to the 6-propionate H_β and 5-CH_3 peaks (dotted lines in Fig. 5) confirm that Arg^{38} is situated over the same pyrrole as found in the X-ray crystal structure of *Cc*P (Finzel et al., 1984).

The expected spin-coupling of 12–15 Hz between geminal protons of Arg^{38} makes the likelihood for detecting weak COSY cross peaks for ~ 100 Hz line widths reasonable in the presence of sufficient sensitivity. The MCOSY slices through six resonances which have geminal partners are shown in Fig. 7. The observed substantial intensity for many of the cross peaks is surprising, as is the variable intensity of the cross peaks among the various geminal proton sets with rather similar line width. The MCOSY cross peaks evolve according to (Bax, 1982; Ernst et al., 1987): $\alpha = \sin(\pi J t_1) e^{-t_1/T_2}$. Thus, the cross peak in the limit that $T_2^{-1} >> J$, should increase with J

for fixed T_2, and increase with T_2 (reciprocal line width) for fixed J. However, the cross-peak intensities for some of the Arg^{38} geminal methylene pairs are larger than for the 4-vinyl H_α:$H_{\beta t}$ COSY cross peak, in spite of the fact that the latter cross peak likely involves both a larger spin-coupling constant ($\sim$16 Hz) and clearly narrower lines (70, 60 Hz) than, for example, Arg^{38} $C_\delta H_2$ (expected J $\sim$12–15 Hz, line widths $\sim$100, 130 Hz). It is clear that additional data are needed to provide a more quantitative understanding of the COSY experiment in such a system.

The upfield hyperfine-shifted methyl peak M has been shown to yield 1D NOEs from the 8–CH_3 (Thanabal et al., 1987b), as confirmed by the NOESY map in Fig. 3B (dotted lines), and the sensitivity of the chemical shift for M upon substrate binding has suggested it may reside in the substrate-binding pocket. The proximity of the binding pocket to the heme 8-CH_3 environment was established by suicide inhibitor studies of HRP with phenylhydrazine (Ator and Ortiz de Montellano, 1987), and was confirmed by observing transferred NOEs to the substrate from both 8-CH_3 and 7-CH_2 (Sakurada et al., 1986; Thanabal et al., 1987b). The methyl signal M was originally proposed to arise from Leu^{237} on the basis of computer modeling (Sakurada et al., 1986). The present MCOSY map, in fact, reveals two cross peaks to methyl M, eliminating Leu^{237} as the candidate. Conversely, the cross peaks from methyl M (the third cross peak near M is from the 4-$H_{\beta c}$ under M) to two chemical shifts indicates an Ile δ-CH_3 spin-coupled to the two C_γHs, which we label Ile X. An inspection of the HRP sequence does not reveal an obvious candidate based on the computer modeling (Sakurada et al., 1986). Further analysis of the NOESY map, together with phase-sensitive COSY data, may shed light on the sequence origin of the Ile X.

CONCLUSIONS

The present studies reveal not only that the heme assignments can be inferred solely from 2D NMR data for low-spin complexes of heme peroxidases, thereby eliminating the need for isotope labeling, but also show that it is possible to establish the presence and identity of key catalytic residues in the heme pocket of a reasonably large enzyme such as HRP–CN. It is expected that detailed further studies should shed considerably more light on the heme pocket of the cyanide complexes, not only of HRP, but of a variety of other heme peroxidases which have been shown to yield similarly well-resolved hyperfine-shifted ^1H NMR spectra (Goff et al., 1985a,b; Thanabal and La Mar, 1989; Dugad et al., 1990b). The present successful detection of the spin-connectivities via MCOSY for the residues closest to the metal center, together with the observation of NOESY cross peaks at different mixing times to establish build-up curves, reinforces our earlier proposal that paramagnetism need not be an insurmountable barrier to the complete solution-structure determination of paramagnetic metalloenzymes solely on the basis of NMR data.

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