NMR Investigation of Isotopically Labeled Cyanide Derivatives of Lignin Peroxidase and Manganese Peroxidase†

Lucia Banci,‡ Ivano Bertini,*,‡ I-Ching Kuan,§ Ming Tien,§ Paola Turano,‡ and Alejandro J. Vila‡

Department of Chemistry, University of Florence, 50121 Florence, Italy, and Department of Molecular and Cell Biology, Pennsylvania State University, University Park, Pennsylvania 16802

Received June 1, 1993; Revised Manuscript Received September 23, 1993•

ABSTRACT: The ^{1}H NMR spectroscopy was used to study lignin peroxidase (LiP) and manganese peroxidase (MnP) containing deuterated histidines. LiP and MnP were obtained from a histidine auxotroph of the fungus *Phanerochaete chrysosporium* grown in the presence of deuterated histidines. The derivatives with deuterated histidines have allowed a firm assignment of the protons of the distal and proximal histidines. We have also found that the LiP from this strain exhibits different orientations of the 2-vinyl group compared to the LiP from the strain previously studied. Mobility of the group has also been detected, thus explaining the apparent inconsistency between X-ray solid-state and NMR solution data. The ^{15}N shift values of ^{15}N-enriched CN^- in the cyanide derivatives of LiP and MnP have also been measured. The shift patterns, both for ^{15}N and for the proximal histidine protons of several peroxidases, are consistent with predominant contact shift contributions which reflect the bond strength of the metal–axial ligand. Finally, our results confirm a correlation between shift values of ^{15}N and those of proximal histidine protons and the Fe^{3+}/Fe^{2+} redox potentials.

Peroxidases are ubiquitous enzymes which use hydrogen peroxide for the oxidation of a variety of substrates (Dunford, 1982; Kirk & Farrell, 1987, 1990; Dawson, 1988; Bosshard et al., 1991; Tien, 1987; Tien & Kirk, 1983). They contain high-spin iron(III) bound to a protoporphyrin IX moiety. The iron is five-coordinated, the fifth ligand being a histidine referred to as the "proximal" histidine. On the other side of the heme plane is another histidine, referred to as the "distal" histidine. The distal histidine is involved in the reaction of the enzyme with H_2O_2.

A large degree of structural homology is observed among peroxidases such as horseradish peroxidase (HRP), cytochrome *c* peroxidase (CcP), lignin peroxidase (LiP), and manganese peroxidase (MnP). These peroxidases have molecular weight ranging from 34 000 to 46 000. The X-ray structure is available for CcP (Poulos & Kraut, 1980; Finzel et al., 1984) and LiP (Edwards et al., 1993; Piontek et al., 1993; Poulos et al., 1993). Local information on the residues around the iron can be obtained through NMR spectroscopy (La Mar et al., 1973; Bertini & Luchinat, 1986; Bertini et al., 1993b). The cyanide derivative, which contains a six-coordinated, low-spin iron(III), is particularly suitable for obtaining structural information on the active site through NMR. The low-spin iron(III) is characterized by fast electron relaxation times (Banci et al., 1991a), thus inducing relatively sharp NMR lines (Thanabal et al., 1987; Satterlee & Erman, 1991; Satterlee et al., 1991; Banci et al., 1991b,c, 1992; de Ropp et al., 1991a,b).

An empirical correlation has been found between the redox potential of the Fe^{3+}/Fe^{2+} pair in the above peroxidases and in myoglobin and the shift values of the protons of the proximal histidine in the cyanide derivatives (Banci et al., 1991c). Such shifts are determined by both pseudocontact and contact contributions, the former depending on the geometric coordinates of the protons within the magnetic susceptibility tensor, and the latter depending on the strength of the iron–nitrogen coordination bond and then on the mechanisms of the spin density transfer (La Mar & Walker, 1979).

We report here an NMR investigation on the cyanide derivatives of two isoenzymes of LiP and MnP, in which the histidines have been selectively deuterated in all but the β positions. This characterization has allowed us to make firm assignments of the protons of the proximal and distal histidines. The assignment was previously based on dipolar connectivities (Banci et al., 1991c, 1992; de Ropp et al., 1991a), which indicate which signals belong to protons close in space. Scalar connectivities, based on chemical bonds, have never been observed between the protons of histidines sensing paramagnetic metal ions.

Furthermore, we have measured in LiP and MnP the chemical shifts of ^{15}N of the iron-bound cyanide, using ^{15}N-enriched CN^-. The whole body of information sheds further light on the relation between electronic and geometric structures.

EXPERIMENTAL PROCEDURES

LiP and MnP were obtained from two different strains. In our previous studies, these isoenzymes were purified from an overproducing strain of *Phanerochalte chrysosporium*, PS-BL-1 (Orth et al., 1991; Tien & Myer, 1990). This particular strain is derived from wild-type BKM-F-1767 (in the text it is referred to as BKM). In the present study we also used a histidine auxotroph of *P. chrysosporium* strain ME 446. Growth conditions were identical for both strains with the exception that histidine (either deuterated or nondeuterated) was added to a final concentration of 50 μM for the ME 446 histidine auxotroph. The cultures were grown at 39 °C, as previously described (Orth et al., 1991). The strain ME 446 does not grow in the absence of added histidine, and neither does it revert, indicating that it is very stable. Extracellular fluid from 5 days of cultures was harvested and separated using an FPLC Mono Q column (Kirk et al., 1985). Peaks

† A.J.V. thanks the International Centre for Genetic Engineering and Biotechnology (UNIDO) for a fellowship, as well as Foundation Antorchas for further support.

‡ University of Florence.

§ Pennsylvania State University.

• Abstract published in *Advance ACS Abstracts,* November 15, 1993.

0006-2960/93/0432-13483$04.00/0

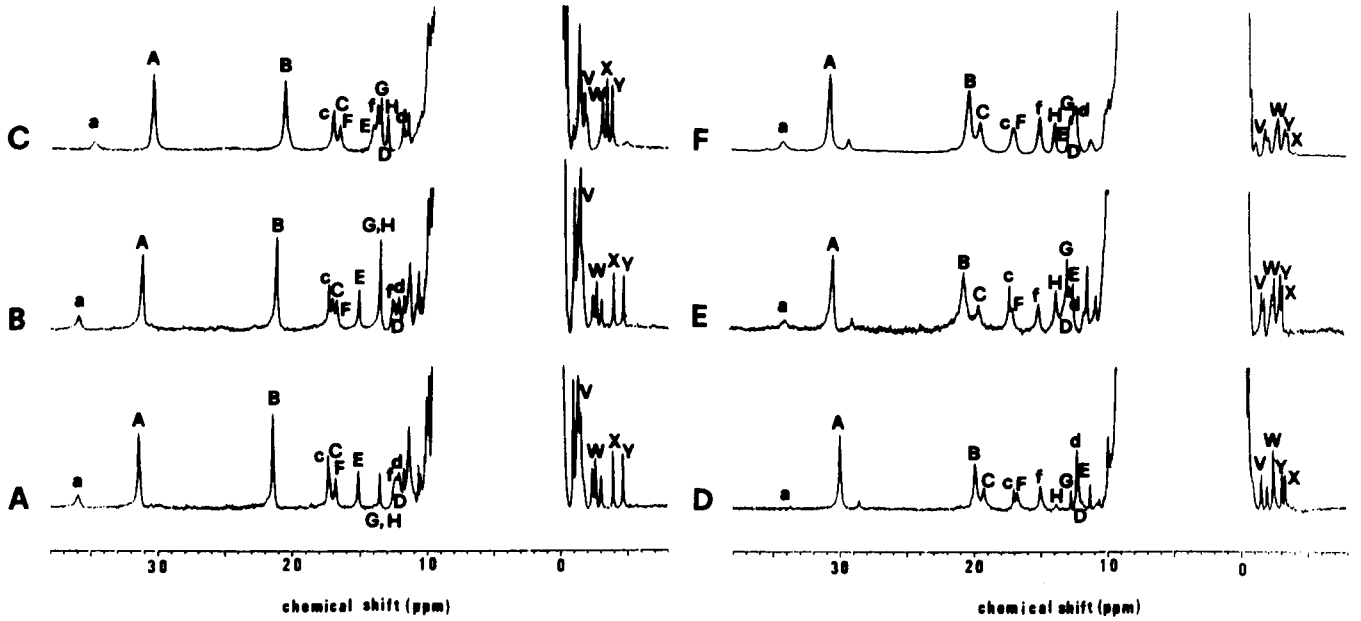


FIGURE 1: 600-MHz ^{1}H NMR spectra of the cyanide adducts of (A) LiP (strain ME 446) with histidine residues deuterated at Hε1, Hδ2, and Hα positions; (B) LiP (strain ME 446); (C) LiP isoenzyme H1 (strain BKM); (D) MnP (strain ME 446) with histidine residues deuterated at Hε1, Hδ2, and Hα positions; (E) MnP (strain ME 446); and (F) MnP isoenzyme H4 (strain BKM). Spectra A–E were recorded at 301 K, spectrum F at 298 K. All the spectra were obtained in H_2O solution. Note that in spectrum D, the intensities of signals a and c are considerably reduced due to saturation transfer effect from the solvent signal.

containing isoenzymes H3 (pI = 4.9), H4 (pI = 4.5), and H5 (pI = 4.2) were combined and dialyzed against 50 mM sodium succinate, pH 4.5. These isoenzymes were further purified by the procedure of Glenn and Gold (1985). The enzymes were subjected to chromatography on an 18-cm × 3-cm (~100-mL volume) blue agarose column (Cibracon Blue 3GA type 3000-L, Sigma) and eluted with 400 mL of a 0–0.4 M NaCl gradient in 50 mM sodium succinate, pH 4.5. Isoenzyme H4 was then further purified by flat-bed preparative isoelectric focusing as described previously (Pease & Tien, 1992).

LiP isoenzyme H1 (pI 4.7) was purified by Mono Q (Kirk et al., 1985) followed by preparative isoelectric focusing (Pease & Tien, 1992).

Histidine was selectively deuteriated on Hδ2, Hε1, and Hα; the complete deuteration was determined through NMR spectroscopy.

The ^{1}H NMR spectra of the cyanide adducts of MnP and LiP in 0.1 M phosphate buffer, pH 6.5, were recorded with Bruker MSL 200 and AMX 600 spectrometers. The identification of the two fast-relaxing, nonexchangeable protons of the proximal histidine ring was obtained by using a super WEFT pulse sequence (Inubushi & Becker, 1983) with recycle delay of 22 ms and τ values of 12 ms. The nuclear Overhauser effect (NOE) difference spectra were collected as previously described (Banci et al., 1989).

NOESY spectra (Macura et al., 1982) at 600 MHz were recorded using presaturation to eliminate the water signal. Phase-sensitive NOESY spectra were recorded with a recycle delay of 300 ms and a mixing time of 15 ms to avoid spin diffusion effects using the TPPI method (Marion & Wüthrich, 1982). A total of 512 experiments were collected with 1K data points in the F2 dimension. The data were multiplied in both dimensions by a sine-squared bell window function with a phase shift of 45°. Several different types of processing have been performed to enhance the intensity of cross peaks connecting signals of different line widths.

The ^{15}N spectra were recorded on a Bruker AMX 600 spectrometer with a modification of a pulse sequence designed to reduce the effects of the dead time (Belton et al., 1985). The spectra were collected with a recycle delay of 100 ms and number of scans ranging from 320 000 to 640 000.

RESULTS

To incorporate deuterated histidine into LiP and MnP, we utilized a strain of *P. chrysosporium* deficient in histidine biosynthesis and supplemented the medium with either deuterated histidine or normal histidine. This strain was derived from strain ME 446.

Figure 1 shows the ^{1}H NMR spectra, recorded at 300 K, of the cyanide derivatives of LiP and MnP from different sources. The ^{1}H NMR spectra of the cyanide derivatives of LiP and MnP from ME 446 are shown in Figure 1B and E, respectively. The LiP and MnP which we previously characterized (Banci et al. 1991c, 1992) were isolated from strain BKM. The spectra of these proteins are shown for comparison in Figure 1C and F, respectively. In MnP, the correlation of signals between the two proteins from different strains is rather straightforward. In the case of LiP, the correlation between the two 1D spectra cannot be performed due to the crowded 15/10-ppm and 0/−5-ppm regions. These signals experience shift variations as large as 1 ppm. The correlation between the 1D spectra in the case of LiP–CN^- was consequently obtained through 2D NOESY spectra (see later).

In Figure 1A and D, the spectra of the proteins with deuterated histidines are reported for the cyanide derivatives of both LiP and MnP. To resolve signals H and G, which are degenerate in spectra A and D of Figure 1, we recorded the NMR spectra at a different temperature (290 K, Figure 2), at which they are enough resolved to estimate the signal intensity. The broad fast-relaxing signals Z and H′ have been detected by recording the NMR spectra with very fast repetition rates (Figure 3), which lead to the disappearance of the slower relaxing signals. Signal Z in LiP–CN^- and MnP–CN^-, which has been assigned as Hε1 of the proximal histidine, is reduced by 30%, whereas most of the other signals belonging to histidines disappear completely. This can be explained by

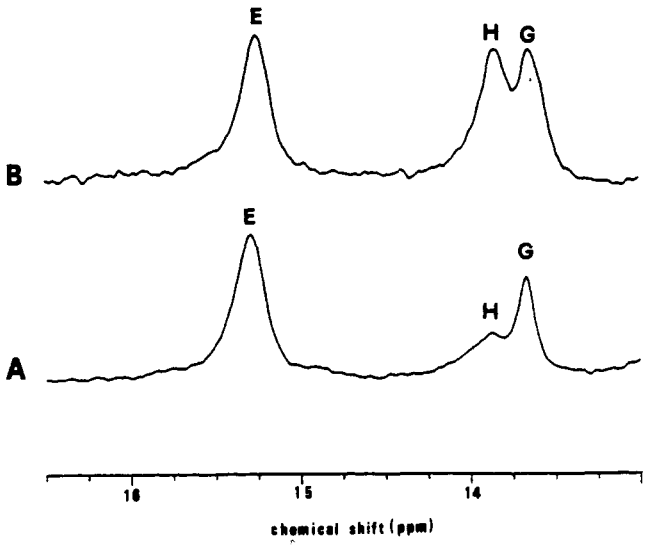


FIGURE 2: Enlargement of the 600-MHz ^{1}H NMR spectra of LiP (strain ME 446) recorded at 290 K to resolve signals H and G. Panel A shows the spectrum of the protein with histidine residues deuterated at positions Hϵ1, Hδ2, and Hα. Panel B shows the spectrum of the same protein with nondeuterated histidine. The decrease in intensity of signal G is 30–35%, whereas that of signal H is about 80%, as it results from signal integration.

the partial deuterium/proton exchange occurring during the growth (at 39 °C) of microorganisms and the purification of the enzyme. It is well known that protons in Hϵ1 position of the histidine ring can exchange with the bulk solvent (Cass et al., 1979). This consideration has allowed us to assign signal G, which is also reduced by 30%, as Hϵ1 of the distal histidine. In the spectrum of LiP–CN$^-$ shown in Figure 1B, signal G is degenerate with signal H, but it is resolved at 290 K (Figure 2). Signal H also belongs to a histidine because it is not present in the deuterated sample and is assigned as Hδ2 of the distal histidine, because it is dipolarly connected to the exchangeable signal a, which in turn is assigned as Hϵ2 of the same residue. In the case of MnP–CN$^-$, comparison of spectra shown in Figure 1D and E leads to the assignment of signals H and G to Hδ2 and Hϵ1, respectively, of the distal histidine.

In both LiP–CN$^-$ and MnP–CN$^-$, a COSY cross peak had been observed between signals H and K (around 6.5 ppm) (Banci et al. 1991c, 1992). This connectivity has been detected also in NOESY maps. We recently discovered that in paramagnetic compounds with high molecular weight, as in the present systems, COSY experiments reveal dipolar connectivities (Bertini et al., 1993a). Therefore, signal *K* can be attributed to a proton close to signal H and is at the moment unassigned. HRP–CN$^-$ and CcP–CN$^-$ do not show such connectivity (de Ropp et al., 1991b; Banci et al., 1991b; Satterlee & Erman, 1991; Satterlee et al., 1991).

The other two ring protons of the proximal histidine are readily identified as in the previous studies (Banci et al. 1991c, 1992). Hδ2 (signal H′), which is fast relaxing due to its close position to iron, is detected in the 1D spectrum recorded with very fast repetition rates (Figure 3). Hϵ2 (signal f) is located by detecting an NOE upon saturation of signal Z.

The NOESY spectrum of LiP–CN$^-$ in H_2O at 300 K is shown in Figure 4. The samples are quite dilute because the amount of LiP produced from strain ME 446 is very low. NOESY cross peaks can be observed between signals with relatively long T_1s, for which the NOE effect is relatively large. For the signals characterized by short T_1s, 1D NOE experiments are more sensitive, as they can be optimized for the signals of interest (Banci et al., 1993).

Most of the connectivities observed in the map shown in Figure 4 are identical to those already observed in the map on LiP from strain BKM (Banci et al., 1991c). This allows us to compare the two spectra of LiP from the different strains (Table I). The cross peaks are assigned in the caption of Figure 4. There are some differences in the 2D spectra of LiP–CN$^-$ from the two different strains. A significant difference for the heme proton resonances is the different NOESY connectivities between the 3-CH_3 and the two vinyl groups. In the present LiP derivative (from ME 446), 4-Hβtrans experiences a more intense cross peak with 3-CH_3 than in LiP previously studied (from strain BKM) (Banci et al., 1991c). The connectivity between 3-CH_3 and 4-Hβcis, previously observed in LiP from strain BKM, is not observed in the present map. Furthermore, in the present sample we do not detect dipolar connectivities between the 3-CH_3 group and any proton signal of the 2-vinyl moiety. In the case of LiP from strain BKM, a strong connectivity is detected between 3-CH_3 and 2-Hβtrans.

To obtain more structural details on the substituents of this side of the heme ring, we performed 1D NOE experiments as a function of saturation time (truncated NOE). In these experiments, the NOE η_{ij} observed for a signal *i* upon saturation of a signal *j* is given by (Noggle & Schirmer, 1971; Neuhaus

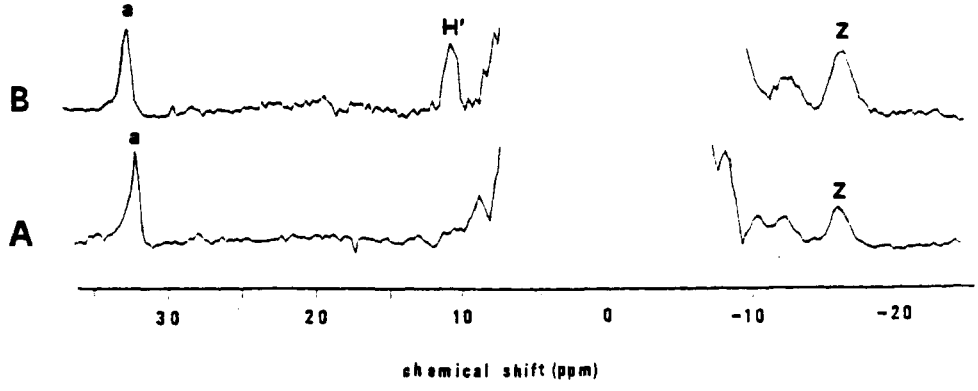


FIGURE 3: 200-MHz ^{1}H NMR spectra of LiP (strain ME 446). Panel A shows the spectrum of the protein with histidine residues deuterated at Hϵ1, Hδ2, and Hα positions. Panel B shows the spectrum of the same protein with nondeuterated histidine. The spectra were acquired with SUPERWEFT pulse sequence with recycle delay of 22 ms and τ values of 12 ms. Only the signals with T_1 values lower than 10 ms are still detected in the spectrum, which appears of low quality owing to the extreme recording conditions. By signal integration, signals Z and H′ of the proximal histidine are found to be reduced by 35–40% and by ≈100%, within the error of the present spectra, respectively.

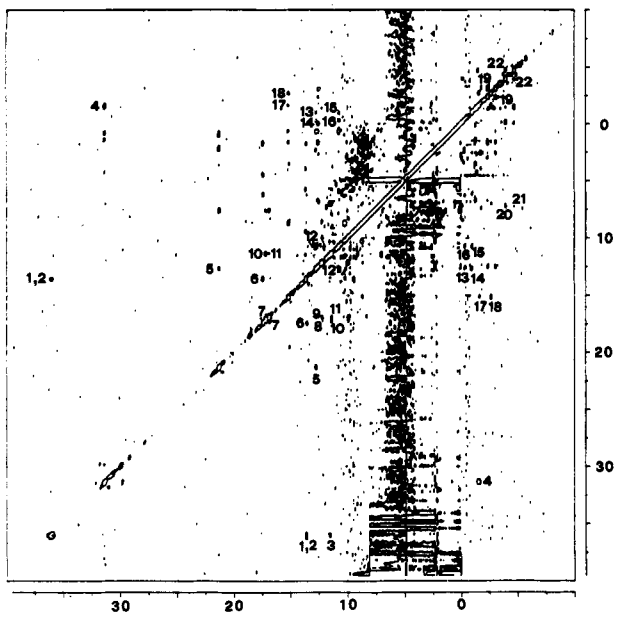


FIGURE 4: 600-MHz ^{1}H NMR NOESY spectrum of LiP–CN$^-$ from strain ME 446, recorded in H_2O solution at 301 K. Cross peak assignments: (1) Hε2 dist His, Hδ2 dist His; (2) Hε2 dist His, Hε1 dist His; (3) Hε2 dist His, not assigned proton of the distal side; (4) 3-CH3, 4-Hα; (5) 8-CH3, 7-Hα; (6) Hδ1 dist His, Hε1 dist His; (7) Hβ prox His, Hβ′ prox His; (8) Hβ prox His, Hδ1 prox His; (9) Hβ′ prox His, Hδ1 prox His; (10) Hβ prox His, NHp prox His; (11) Hβ′ prox His, NHp prox His; (12) 7-Hα, 7-Hα′; (13) 7-Hα, 7-Hβ; (14) 7-Hα, 7Hβ′; (15) 7-Hα′, 7-Hβ; (16) 7Hα′, 7-Hβ′; (17) 4-Hα, 4-Hβtrans; (18) 4-Hα, 4-Hβcis; (19) 4-Hβtrans, 4-Hβcis; (20) 2-Hα, 2-Hβcis; (21) 2-Hα, 2-Hβtrans; (22) 2-Hβcis, 2-Hβtrans.

& Williamson, 1989):

$$\eta_{ij} = k\tau_c/r_{ij}^6\rho_i \quad (1)$$

where k contains physical constants, τ_c is the reorientational correlation time, r_{ij} is the distance between proton i and proton j, and ρ is the reciprocal of the selective T_1 of signal i. If a sufficient number of experiments are performed with different saturation times and the distance r_{ij} is known, ρ and τ_c are simultaneously obtained from eq 1. The analysis of the time dependence of NOE on 2-Hβcis upon saturation of 2-Hβtrans provides a τ_c of $(4.8 \pm 0.6) \times 10^{-9}$ s, which is shorter than that of the overall protein ($\tau_c = 2.0 \times 10^{-8}$ s). This indicates mobility of the 2-vinyl moiety. This behavior is not present in the LiP protein previously studied from strain BKM (Banci et al., 1991c). However, it has been observed in MnP from strain BKM (Banci et al., 1992). From the 3-CH_3 signal, a small NOE is detected also on 2-Hβtrans. This indicates that, despite the internal mobility, the 2-vinyl group is mainly in the conformation shown as **I** in Chart I. Quantitative estimation of NOE within the 4-vinyl protons is not possible, as all the signals of this residue are not resolved from overlapping signals. However, analysis using truncated NOE at different irradiation times on the 4-Hβtrans with saturation of the 3-CH_3 signal provides simultaneously determination of ρ and the product $\tau_c r_{ij}^{-6}$. By taking the τ_c value of the entire protein tumbling, a reasonable distance between 3-CH_3 and 4-Hβtrans is obtained, which is consistent with the 4-vinyl group having the conformation shown in Chart I. If a shorter τ_c is assumed, a unreasonably short 3-CH_3/4-Hβtrans distance is obtained. This experiment shows that the 4-vinyl group does not have mobility with a τ_c shorter than molecular tumbling.

^{15}N NMR Spectra. The ^{15}N spectra of LiP–C^{15}N$^-$ and MnP–C^{15}N$^-$ from strain BKM at concentrations of ca. 3 mM reveal signals at 608 and 639 ppm, with a signal-to-noise ratio of 5:1. The line widths are 500 and 400 Hz, respectively. Such shift values are comparable with that of HRP–C^{15}N$^-$ (Behere et al., 1985), but they are largely different from that of Mb–C^{15}N$^-$ (Morishima & Inubushi, 1977, 1978; Morishima et al., 1977) (see Table II).

Table I: Chemical Shift Values at 301 K for Paramagnetically-Shifted Resonances for the Cyanide Derivatives of MnP and LiP[a]

	shift (ppm)				
signal	LiP–CN$^-$ (ME446)	LiP–CN$^-$ [b] (BKM)	MnP–CN$^-$ [c] (ME446)	MnP–CN$^-$ [d] (BKM)	assignment
a	35.9	35.2	33.8	34.2	Hε2 dist. His
A	31.2	31.0	30.3	30.7	3-CH_3
B	21.2	20.4	20.5	20.4	8-CH_3
c	17.3	17.0	17.1	17.0	Hδ1 dist. His
C	17.1	17.4	19.4	19.5	Hβ prox. His
d	11.7	11.6	12.3	12.4	NH_p prox. His
D	12.5	13.0	12.6	12.5	7-Hα
E	15.1	14.2	12.6	12.7	4-Hα
f	12.6	14.0	14.9	15.1	Hδ1 prox. His
F	16.8	17.0	16.9	16.9	Hβ′ prox. His
G	13.5	13.6	12.8	12.8	Hε1 dist. His
H	13.5	13.1	13.6	13.9	Hδ2 dist. His
H′	14.7	13.3	20.2	20.3	Hδ2 prox. His
I	10.7	9.2		8.0	7-Hα′
J	7.2	8.5		8.6	2-Hα
V	−1.6	−2.0	−1.9	−1.8	4-$H\beta_{trans}$
W	−2.7	−3.2	−2.6	−2.8	4-$H\beta_{cis}$
X	−3.9	−3.6	−3.3	−3.4	2-$H\beta_{cis}$
Y	−4.7	−4.1	−3.1	−3.2	2-$H\beta_{trans}$
Z	−11.2	−9.0	−11.6	−11.8	Hε1 prox. His

[a] Lower case letter labeling corresponds to exchangeable protons. [b] Taken from Banci et al. (1991c). [c] Signal assignment is based on the analogy with the results obtained in Banci et al. (1992). [d] Chemical shift values at 298 K taken from Banci et al. (1992).

DISCUSSION

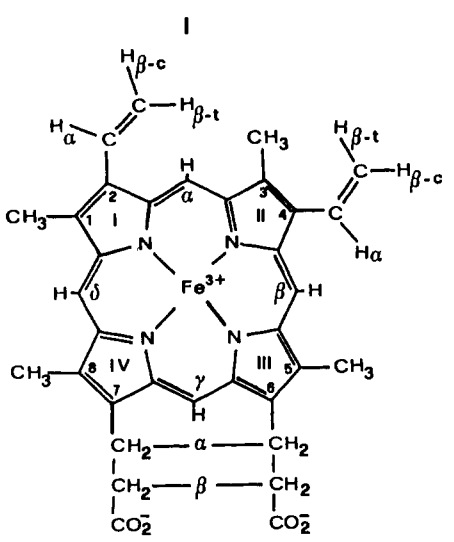
The present investigation using selectively deuterated proteins has permitted a firm assignment of the signals of the protons of both the proximal and the distal histidines. In all peroxidases, the Hε1 proton of the proximal histidine is shifted upfield, whereas the Hδ2 is shifted downfield with respect to their diamagnetic values (Thanabal et al., 1987; de Ropp et al., 1991a,b; Satterlee & Erman, 1991; Satterlee et al., 1991; Banci et al., 1991b,c, 1992). The extent of the upfield shift is very different within the series. In metMb–CN$^-$, Hε1 of the proximal histidine is shifted downfield and Hδ2 slightly upfield (Emerson & La Mar, 1990a).

The hyperfine shift values of the protons of the proximal histidine are determined by both pseudocontact and contact contributions. The former depends on the values and the orientation of the magnetic susceptibility tensor and is given by (McConnell & Robertson, 1958; Kurland & McGarvey, 1970):

$$\delta_{dip} = -1/(3Nr^3)\,[\Delta\chi_{ax}\,(3\cos^2\theta - 1) - {}^3/_2\Delta\chi_{rh}(\sin^2\theta\cos 2\phi)] \quad (2)$$

where N is the Avogadro constant, $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ represent the axial and rhombic anisotropies of the χ tensor, r is the iron–atom distance, and θ and ϕ are the polar and azimuthal angles in the magnetic metal-centered coordinate system. The g values of the cyanide derivatives of some peroxidases (HRP, CcP, LiP) are almost identical with each other, thus indicating very similar values for the magnetic susceptibility tensor (Blumberg et al., 1968; Wittenberg et al., 1968).

Chart I



It is generally accepted (Emerson & La Mar, 1990b; Hori, 1971; Peisach et al., 1971; Shulman et al., 1971) that the z axis of the magnetic susceptibility tensor is determined by the orientation of the cyanide ion. The latter, in turn, is influenced by the position of the distal histidine, the Nδ2 atom of the latter residues being H-bonded to CN^-. In Mb and its mutants, the z axis is slightly tilted (14.5–16°) from the perpendicular to the heme plane (Emerson & La Mar, 1990b; Rajarathnam et al., 1992). Indeed, in Mb the CN^- ion is tilted from the perpendicular by 15°. On the other hand, in both CcP and LiP, the Nδ2 atom of the distal residue lies on the perpendicular to the heme plane, and the CN^- ion in the CcP–CN^- (Edwards & Poulos, 1990) structure is found to be essentially aligned along the heme perpendicular. Therefore, in peroxidases the z axis could be essentially perpendicular to the heme plane.

In CcP, both Hϵ1 and Hδ2 of the proximal histidine form an angle less than 54.8° perpendicular to the heme plane. Independent of the ϕ angle (which is determined by the orientation of the plane of the proximal histidine), the pseudocontact contribution is found positive (downfield) for both protons with the present anisotropy values. In eq 2, the experimental g anisotropy has been used instead of the χ values, thus introducing some error in the absolute values of the calculated pseudocontact shifts (Horrocks & Greenberg, 1973). The negative (upfield) shift of Hϵ1 of the proximal histidine can be reproduced only by large tilt angles of the z axis. The same angle variation of the z axis would determine a large change in the shift values of the Hδ2 (Emerson & La Mar, 1990b; Rajarathnam et al., 1992; our own calculations), which are not experimentally observed. Indeed the latter signal experiences a shift variation of only 10 ppm within the peroxidase series. The shift of the Hϵ1 of the proximal histidine in peroxidases is therefore negative (upfield) due to a large contact contribution. Indeed, in metMb–CN^- (Emerson & La Mar, 1990b) and in cytochrome c (Feng et al., 1990; Turner, 1993) the contact contribution is negative for both protons.

The position of the proximal histidine in CcP and LiP seems very similar (Poulos & Kraut, 1980; Finzel et al., 1984; Edwards et al., 1993; Poulos et al., 1993). Furthermore, the distal histidine has a similar geometry in the two proteins. This then yields a similar orientation of the magnetic susceptibility tensor in the series of peroxidases. Therefore, the differences in the proton shifts among peroxidases are mainly attributed to different contact rather than pseudocontact contributions. In this respect, the ^{15}N data are also informative (Table II). In the series of peroxidases, the ^{15}N shift of bound CN^- experiences an increase of about 60 ppm from HRP (Behere et al., 1985) to MnP. The ^{15}N shift values for CN^- bound to several heme proteins (Morishima & Inubushi, 1977, 1978; Behere et al., 1985; Shiro et al., 1989; Morishima et al., 1977) and hemin model complexes (Morishima & Inubushi, 1977, 1978; Aviles & Chang, 1992; Yamamoto et al. 1989) have been reported. The ^{15}N shift values depend on many factors; one of them is the strength of the axial ligand (Morishima & Inubushi, 1978; Golding et al. 1976). For example, the shift values for the imidazolate complexes are substantially smaller than those of the imidazole complexes (Shiro et al., 1989). Consequently, the lower ^{15}N shifts of peroxidases, compared with those of globins, could tentatively be attributed to the anionic nature of their proximal histidine imidazole. Furthermore, the smaller the ^{15}N shift, the stronger the iron–imidazole nitrogen bond and the larger the Hϵ1 and the Hδ2 shifts of the proximal histidine protons. The strength of the iron–imidazole bond is correlated to the strength of the H-bond of the proximal histidine (Nanthakumar & Goff, 1989; La Mar & de Ropp, 1982; Banci et al., 1991b,c, 1992; Christianson, 1991; Smulevich, 1993). The patterns of the shifts of ^{15}N of cyanide and of the protons of the proximal histidine are therefore quite consistent with each other. The larger the histidinate character, the larger the

Table II: Comparison of the ^{15}N Chemical Shift Values for the Bound Cyanide, the ^1H Shift Values for the Hϵ1 of the Proximal Histidine, and the Corresponding Redox Potential Values for the Couple Fe^{3+}/Fe^{2+} in the Cyanide Derivatives of Several Heme Proteins

protein	^{15}N shift (ppm)[a]	^1H shift (ppm)[b]	$E°(Fe^{3+}/Fe^{2+})$ (mV)[c]
HRP	576	−29.9	−278
CcP	587	−20.6	−194
LiP	608	−9.0	−142
MnP	639	−11.8	−93
Mb	930	19.2	+200

[a] References: HRP, Behere et al. (1985); CcP, unpublished results of this lab; LiP, MnP, present work; Mb, Morishima et al. (1977), Morishima and Inubushi (1977), Morishima and Inubushi (1978). It should be noted that in Mb the proximal His ligand is less histidinate in character. [b] References: HRP, Thanabal et al. (1987); CcP, Banci et al. (1991b); LiP, Banci et al. (1991c); MnP, Banci et al. (1992); Mb, Emerson and La Mar (199a). [c] References: HRP, Makino et al. (1976); Yamada et al. (1975); CcP, Conroy et al. (1978); LiP, MnP, Millis et al. (1989); Mb, Taylor and Morgan (1942).

hyperfine shift (in absolute value) of the ring protons of the proximal histidine and smaller the shifts on ^{15}N.

In the case of MnP–CN^-, the observed shift values of Hε1 and Hδ2 protons of the proximal histidine are a few ppm higher than expected on the basis of a linear dependence of the redox potentials, although qualitatively they fit into the scheme (Banci et al. 1992). In this case, the discrepancy could be accounted for by assuming a small tilt of the z axis. The latter axis should move away from the heme normal in a direction perpendicular to that defined by the proximal histidine plane. Indeed, it can be calculated from eq 2 that this movement results in an increase of the pseudocontact contribution for both protons.

The pattern of the shift values of the β-CH_2 protons of the proximal histidine within the series of peroxidases (see Table I) is indicative of a variation of the contact contribution in the different proteins. The pseudocontact contribution is calculated to be almost identical for the two protons for a large range of orientations of the χ tensor.

A final comment concerns the mobility of the vinyl group. Mobility for the side chains of some heme substituents has been observed in several myoglobins (Ramaprasad et al., 1984; Yamamoto et al., 1991) and in MnP from strain BKM (Banci et al., 1992). It is important to note that the structure of LiP shows the 2-vinyl in the conformation depicted as **II** in Chart I (Edwards et al., 1993), whereas in solution its conformation is as shown in **I** (Banci et al., 1991c). In LiP obtained from strain ME 446 we observe a reorientational correlation time for the 2-vinyl shorter than the rotation correlation time τ_c. We can then conclude that NMR provides a dynamical picture of this group, sometimes the mobility being faster than the molecular tumbling. In the crystalline form (Edwards et al., 1993), only one orientation is frozen out, which may not be the predominant one in solution.

ACKNOWLEDGMENT

Thanks are expressed to Prof. Claudio Luchinat for the helpful discussions.

REFERENCES

Aviles, G., & Chang, C. K. (1992) *J. Chem. Soc., Chem. Commun.* 31.

Banci, L., Bertini, I., Luchinat, C., Piccioli, M., Scozzafava, A., & Turano, P. (1989) *Inorg. Chem. 28*, 4650.

Banci, L., Bertini, I., & Luchinat, C. (1991a) *Nuclear and Electron Relaxation. The Magnetic Nucleus-Unpaired Electron Coupling in Solution*, VCH, Weinheim.

Banci, L., Bertini, I., Turano, P., Ferrer, J. C., & Mauk, A. G. (1991b) *Inorg. Chem. 30*, 4510.

Banci, L., Bertini, I., Turano, P., Tien, M., & Kirk, T. K. (1991c) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6956.

Banci, L., Bertini, I., Pease, E., Tien, M., & Turano, P. (1992) *Biochemistry 31*, 10009.

Banci, L., Bertini, I., & Luchinat, C. (1993) in *Methods in Enzymology* (James, T. L., & Oppenhaimer, N., Eds.) Academic Press, Inc., Orlando, FL.

Behere, D. V., Gonzalez-Vergara, E., & Goff, H. M. (1985) *Biochim. Biophys. Acta 832*, 319.

Belton, P. S., Cox, I. J., & Harris, R. K. (1985) *J. Chem. Soc., Faraday Trans. 2 81*, 63.

Bertini, I., & Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin/Cummings, Menlo Park, CA.

Bertini, I., Luchinat, C., & Tarchi, D. (1993a) *Chem. Phys. Lett. 203*, 445.

Bertini, I., Turano, P., & Vila, A. J. (1993b) *Chem. Rev.* (in press).

Blumberg, W. E., Peisach, J., Wittenberg, B. A., & Wittenberg, J. B. (1968) *J. Biol. Chem. 243*, 1854.

Bosshard, H. R., Anni, H., & Yonetani, T. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) pp 51–84, CRC Press, Boca Raton, FL.

Cass, A. E. G., Hill, H. A. O., Bannister, J. V., Bannister, W. H., Hasemann, V., & Johansen, J. T. (1979) *Biochem. J. 183*, 127.

Christianson, D. W. (1991) *Adv. Protein Chem. 42*, 281.

Conroy, C. W., Tyma, P., Daum, P. H., & Erman, J. E. (1978) *Biochim. Biophys. Acta 537*, 62.

Dawson, J. H. (1988) *Science 240*, 433.

de Ropp, J. S., La Mar, G. N., Wariishi, H., & Gold, M. H. (1991a) *J. Biol. Chem. 266*, 15001.

de Ropp, J. S., Yu, L. P., & La Mar, G. N. (1991b) *J. Biomol. NMR 1*, 175.

Dunford, H. B. (1982) *Adv. Inorg. Biochem. 4*, 41.

Edwards, S. L., & Poulos, T. L. (1990) *J. Biol. Chem. 265*, 2588.

Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H., & Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 750.

Emerson, S. D., & La Mar, G. N. (1990a) *Biochemistry 29*, 1545.

Emerson, S. D., & La Mar, G. N. (1990b) *Biochemistry 29*, 1556.

Everse, J., Everse, K. E., Grisham, M. B., Eds. (1990) *Peroxidases in Chemistry and Biology, Vols. 1 and 2*, CRC Press, Boca Raton, FL.

Feng, Y., Roder, H., & Englander, S. W. (1990) *Biochemistry 29*, 3494.

Finzel, B. C., Poulos, T. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 13027.

Glenn, J. K., & Gold, M. H. (1985) *Arch. Biochem. Biophys. 242*, 329.

Golding, R. M., Pascual, R. O., & Vrbancich, J. (1976) *J. Mol. Phys. 31*, 731.

Hori, H. (1971) *Biochim. Biophys. Acta 251*, 227.

Horrocks, W. D., Jr., & Greenberg, E. S. (1973) *Biochim. Biophys. Acta 322*, 382.

Inubushi, T., & Becker, E. D. (1983) *J. Magn. Reson. 51*, 128.

Kirk, T. K., & Farrell, R. L. (1987) *Annu. Rev. Microbiol. 41*, 465.

Kirk, T. K., Croan, S., Tien, M., Murtagh, K. E., & Farrell, R. L. (1985) *Enz. Microb. Technol. 8*, 27.

Kurland, R. J., & McGarvey, B. R. (1970) *J. Magn. Reson. 2*, 286.

La Mar, G. N., & Walker, F. A. (1979) in *The Porphyrins* (Dolphin, D., Ed.) Vol. 6, pp 61–157.

La Mar, G. N., & de Ropp, J. S. (1982) *J. Am. Chem. Soc. 104*, 5203.

La Mar, G. N., Horrocks, W. D., Jr., Holm, R. H., Eds. (1973) *NMR of Paramagnetic Molecules*, Academic Press, New York.

Macura, S., Wüthrich, K., & Ernst, R. R. (1982) *J. Magn. Reson. 47*, 351.

Makino, R., Chiang, R., & Hager, L. P. (1976) *Biochemistry 15*, 4748.

Marion, D., & Wüthrich, K. (1982) *Biochem. Biophys. Res. Commun. 113*, 967.

McConnell, H. M., & Robertson, R. E. (1958) *J. Chem. Phys. 29*, 1361.

Millis, C. D., Cai, D., Stankovic, M. T., & Tien, M. (1989) *Biochemistry 28*, 8484.

Morishima, I., & Inubushi, T. (1977) *J. Chem. Soc., Chem. Commun.* 616.

Morishima, I., & Inubushi, T. (1978) *J. Am. Chem. Soc. 100*, 3568.

Morishima, I., Inubushi, T., Neya, S., Ogawa, S., & Yonezawa, T. (1977) *Biochem. Biophys. Res. Commun. 78*, 739.

Nanthakumar, A., & Goff, H. M. (1989) *Inorg. Chem. 28*, 4559.

Neuhaus, D., & Williamson, M. (1989) *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH, New York.

Noggle, J. H., & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect*, Academic Press, New York.
Orth, A. B., Denny, M., & Tien, M. (1991) *Appl. Environ. Microbiol. 57*, 2591.
Pease, E. A. & Tien, M. (1992) *J. Bacteriol. 174*, 3532.
Peisach, J., Blumberg, W., & Wyluda, B. J. (1971) *Proc. Eur. Biophys. Congr., 1*, 109.
Piontek, K., Glumoff, T., & Winterhalter, K. (1993) *FEBS Lett. 315*, 119.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199.
Poulos, T. L., Edwards, S. L., Wariishi, H., & Gold, M. H. (1993) *J. Biol. Chem. 268*, 4429.
Rajarathnam, K., La Mar, G. N., Chiu, M. L., & Sligar, S. G. (1992) *J. Am. Chem. Soc. 114*, 9048.
Ramaprasad, S., Johnson, R. D., & La Mar, G. N. (1984) *J. Am. Chem. Soc. 106*, 3632.
Satterlee, J. D., & Erman, J. E. (1991) *Biochemistry 30*, 4398.
Satterlee, J. D., Russell, D. J., & Erman, J. E. (1991) *Biochemistry 30*, 9072.
Shiro, Y., Iizuka, T., Makino, R., Ishimura, Y., & Morishima, I. (1989) *J. Am. Chem. Soc. 111*, 7707.
Shulman, R. G., Glamur, S. H., & Karplus, M. (1971) *J. Mol. Biol. 57*, 93.
Smulevich, G. (1993) in *Biomolecular Spectroscopy, Part A, Vol 20, Advances in Spectroscopy* (Clark, R. J. H., & Hester, R. E., Eds.) pp 163–193, J. Wiley and Sons, London.
Taylor, J. F., & Morgan, V. E. (1942) *J. Biol. Chem. 144*, 15.
Thanabal, V., de Ropp, J. S., & La Mar, G. N. (1987) *J. Am. Chem. Soc. 109*, 7516.
Tien, M. (1987) *CRC Crit. Rev. Biochem. 15*, 141.
Tien, M., & Kirk, T. K. (1983) *Science 221*, 661.
Tien, M., & Myer, S. B. (1990) *Appl. Environ. Microbiol. 56*, 2540.
Turner, D. L. (1993) *Eur. J. Biochem. 211*, 563.
Wittenberg, B. A., Kampa, L., Wittenberg, J. G., Blumberg, W. E., & Peisach, J. (1968) *J. Biol. Chem. 243*, 1863.
Yamada, H., Makino, N., & Yamazaki, M. (1975) *Arch. Biochem. Biophys. 169*, 344.
Yamamoto, Y., Nanai, N., Inoue, Y., & Chujo, R. (1989) *J. Chem. Soc., Chem. Commun.* 1419.
Yamamoto, Y., Chujo, R., & Suzuki, T. (1991) *Eur. J. Biochem. 198*, 285.