# Binding of Horseradish, Lignin, and Manganese Peroxidases to Their Respective Substrates†

Lucia Banci,‡ Ivano Bertini,*,‡ Tiziano Bini,‡ Ming Tien,§ and Paola Turano‡

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

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ABSTRACT: The present study utilizes $^1$H NMR spectroscopy to characterize the binding of substrate to heme active site of three different peroxidases, horseradish peroxidase, lignin peroxidase, and manganese peroxidase. Information has been obtained on the site of p-cresol binding to the active-site cavity of the cyanide derivative of horseradish peroxidase. This information was obtained by relaxation enhancements of the substrate protons and connectivities between the latter and heme 8-$CH_3$ and a Phe residue. Manganese(II) is shown to bind to ferri-manganese peroxidase and its cyanide derivative in a specific site with a high-affinity constant ($10^4$ $M^{-1}$). Manganese(II) binding exhibits a slow exchange rate with respect to the difference in $T_2^{-1}$ of the affected signals in the manganese(II)-containing and manganese(II)-free species. Manganese(II) affects the line width of certain heme methyl resonances and of certain one-proton intensity signals in manganese peroxidase and its cyanide derivative. The behavior of MnP toward manganese(II) is compared to that of the closely related peroxidase, lignin peroxidase (LiP), with its native substrate veratryl alcohol. LiP does not have a specific binding site for manganese(II).

Peroxidases are ubiquitous proteins which catalyze the hydrogen peroxide-dependent one-electron oxidation of a large variety of substrates. They are heme-containing proteins with resting state iron(III). Their reaction with $H_2O_2$ results in the oxidation of the heme active site by two electrons generating an intermediate referred to as compound I. Compound I returns to resting enzyme by oxidizing two substrate molecules via one electron undergoing a single-electron oxidized state referred to as compound II. Much of what is known about peroxidases comes from studies on horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CcP). HRP oxidizes a large number of aromatic molecules (Saunders et al., 1964; Dunford & Stillman, 1976; Ator & Ortiz de Montellano, 1987; Halliwell & De Rycker, 1978; DePillis et al., 1990; Harris et al., 1991; Miller et al., 1992), whereas CcP is more specific in oxidizing cytochrome *c* (Dunford & Stillman, 1976; Poulos, 1988; Mauro et al., 1989).

Recently, two fungal peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), have been characterized by $^1$H NMR (Banci et al., 1991b, 1992; de Ropp, 1991a). LiP and MnP are involved in the degradation of the aromatic polymer lignin (Hammel & Moen, 1991; Wariishi et al., 1991) and are produced by the fungus *Phanerochaete chrysosporium* (Kirk & Farrel, 1987; Gold et al., 1989; DePillis et al., 1990; Harris et al., 1991; Miller et al., 1992). These two enzymes are characterized by their ability to oxidize highly recalcitrant substrates. LiP oxidizes nonphenolic aromatic substrates, whereas MnP oxidizes divalent manganese. Degradation of lignin by LiP and MnP has been proposed to involve low molecular weight redox mediators (Sarkanen & Ludwig, 1971; Crawford, 1981; Gold et al., 1984, 1989; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985; Reganathan et al., 1985; Bushwell & Odier, 1987; Kirk & Farrel, 1987; Leisola et al., 1987). In the case of MnP, manganese(II) is the proposed mediator which is essential for activity. Manganese(II) is oxidized to manganese(III) by compound I and compound II. Manganese(III), which has a high redox potential, performs the one-electron oxidation of the lignin polymer (Walters & Littler, 1965; Glenn & Gold, 1985; Leisola et al., 1985; Pasczynski et al., 1986; Wariishi et al., 1989a,b).

In the case of LiP, it has been proposed that veratryl alcohol (VA) acts as a redox mediator (Harvey et al., 1986). VA, also produced by the fungus, is a substrate for LiP. The product of its one-electron oxidation, the aryl cation radical, has been proposed to be a diffusable species capable of oxidizing other organic substrates such as lignin (Harvey et al., 1986).

The X-ray structure is known only for CcP (Poulos & Kraut, 1980), whereas for HRP some partial models have been built (Welinder, 1985; Welinder & Norskov-Lauritsen, 1986). These studies suggest similarities in the active-site cavity for the two proteins. The X-ray structure of LiP is under refinement (Poulos, personal communication); its active site shows close similarities with that of CcP.

In the present study, we utilize $^1$H NMR spectroscopy to characterize the binding of manganese(II) to MnP. For comparative purposes, we also characterize the binding of *p*-cresol to HRP. Most of these studies are performed on the cyanide derivatives of the respective peroxidases. Characterization of the cyanide derivatives is advantageous in that they yield much more resolved NMR spectra relative to the high-spin forms (Bertini & Luchinat, 1986). This is due to the larger dipolar shifts, originating from a larger magnetic anisotropy, and to longer nuclear relaxation times, caused by the faster electron relaxation rate of low-spin iron(III) (Bertini & Luchinat, 1986; Banci et al., 1991a; Banci, 1993). The $^1$H NMR spectra of the investigated peroxidases have been assigned through 1D NOE, NOESY, and COSY experiments (Thanabal et al., 1987a,b, 1988; de Ropp et al., 1991a,b; Banci et al., 1991b, 1992). We have also attempted to investigate the interaction of VA with LiP, but $^1$H NMR spectroscopy has not provided any definite answer.

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‡ University of Florence.
§ Pennsylvania State University.

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## MATERIALS AND METHODS

*Preparation of the Samples.* Horseradish peroxidase was obtained from the Sigma Chemical Co. as a salt-free lyophilized powder (Sigma Type VI). The protein is known to be predominantly isoenzyme C and as such was used without further purification. NMR samples contained 2 mM HRP in $^2H_2O$/10 mM phosphate buffer at pH 7.0 (uncorrected for isotope effects). In NOE experiments where the substrate signals were saturated and in NOESY experiments acquired on the full spectral width, the HRP-containing NMR sample contained 58 mM *p*-cresol. The sample used for 2D experiments to characterize the aromatic region and for the 1D NOE spectra, where the 8-$CH_3$ signal was saturated, contained a lower concentration of *p*-cresol (6 mM). A lower concentration of *p*-cresol was used to avoid covering of the protein aromatic signals by the intense *p*-cresol resonances.

LiP and MnP protein were prepared as previously reported (Tien & Myer, 1990; Bonnarme & Jeffries, 1990; Kirk et al., 1990). MnP samples were about 0.5 mM, in 0.1 M phosphate buffer at pH 6.5. MnP was titrated with increasing concentrations of $MnSO_4$. NMR titration of VA with LiP was performed on a 10 mM sample of VA in 0.1 M phosphate buffer at pH 6.5 until a final concentration of LiP of 0.8 mM was reached.

*Spectroscopic Measurements.* $^1H$ NMR experiments on the HRP–$CN^-$ adduct were recorded on a Bruker AMX600 spectrometer. One-dimensional (1D) NOE difference spectra were collected as described (Banci et al., 1989), with saturation times for the *p*-cresol and 8-$CH_3$ resonances of 80 and 50 ms, respectively. NOESY experiments over a 70 ppm shift range were recorded at 301 and 311 K with mixing time of 50 ms and relaxation delay of 340 ms. The 2D maps were collected with 1K real data points in the *F*2 dimension and with 512 experiments in the *F*1 dimension. The data were processed using sine-squared bell window functions shifted 45° or 30° and zero-filled to obtain 1K × 1K real data points. NOESY experiments over a 20 ppm shift range were recorded at 301 and 318 K with a mixing time of 100 ms and recycling time of 340 ms. The spectra were acquired with 800 experiments collected with 1K real data points in the *F*2 dimension. The data were processed with 45° and 30° shifted sine-squared bell window functions in the *F*2 and *F*1 dimensions, respectively, and zero-filled to 1K × 1K. The MLEV-17 mixing scheme was used in the TOCSY experiments recorded at 318 K with a spin-lock duration of 39 ms and a recycling time of 340 ms. Eight hundred spectra were acquired with 1K data points in the *F*2 dimension. The data were processed with a 45° shifted sine-squared bell window function in both dimensions and zero-filled to 1K × 1K real data points. Phase-sensitive TOCSY and NOESY experiments employed the time-proportional phase incrementation method (Macura et al., 1982; Marion & Wüthrich, 1983).

The titrations of MnP–$CN^-$ and MnP with manganese(II) were performed at 600 MHz with a Bruker AMX600 spectrometer and at 200 MHz with a Bruker MSL200 spectrometer. The titration of VA with LiP was performed at 200 MHz with a Bruker MSL200 spectrometer.

The EPR spectra were collected at room temperature with a Bruker ER200 spectrometer operating at 9.5 GHz.

## RESULTS

*Binding of p-Cresol to HRP.* Measuring the change in the $T_1$ values has been an useful technique for characterizing the binding of aromatic substrates to the high-spin form of HRP

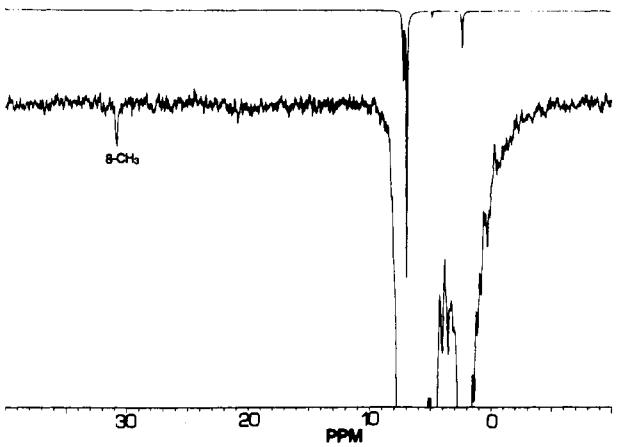
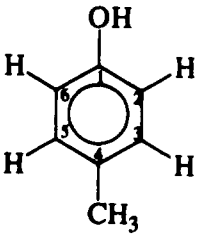



FIGURE 1: NOE difference spectrum at 600 MHz and 301 K of the *p*-cresol/HRP–$CN^-$ complex in $D_2O$. The signal of the substrate at 6.9 ppm (ring protons 2H/6H) has been saturated. The lower part shows the same spectrum expanded 512 times.

Chart I




(Burns et al., 1975; Leigh et al., 1975; Schejter et al. 1976; Morishima & Ogawa, 1979; Sakurada et al., 1986; Casella et al., 1991). The results indicate that the substrate binds close to the heme ring but not to the metal ion. The $T_1$ values are not significantly different for the various protons of the aromatic molecule, and the signals of the aromatic ring protons in equivalent positions are degenerate. The latter finding indicates that the aromatic ring exchanges rapidly between the free and the bound forms. The average distances of the protons of the bound substrate molecule from the iron ion are in the range 8.5–11 Å.

In an attempt to obtain detailed structural information on substrate binding, 1D NOE as well as 2D NOESY and COSY experiments were performed on the complex of *p*-cresol (Chart I) with the cyanide derivative of HRP. Morishima and Ogawa (1979) have shown that *p*-cresol binds to the cyanide derivative of HRP. An advantageous property of this derivative is that the spectra are better resolved and the paramagnetically shifted signals are largely assigned (Thanabal et al., 1987a,b, 1988; de Ropp et al., 1991).

By titrating HRP–$CN^-$ with *p*-cresol, we confirmed the shifts of the heme proton signals (Morishima & Ogawa, 1979). We also observed shifts in some signals in the aromatic region, around 7.5–8.5 ppm, and in the aliphatic region, around 1.8–3.0 ppm. The three signals of *p*-cresol also experience some shifts upon binding to HRP.

Saturation of each of the substrate proton signals has been performed: a NOE effect is observed between signal 2H/6H of *p*-cresol and 8-$CH_3$ of the heme (Figure 1A). This is the only connectivity between the substrate and the hyperfine shifted signals of HRP.

NOESY experiments performed both on the substrate-free and substrate-bound cyanide adducts of HRP confirm the connectivity betwee 8-$CH_3$ and 2H/6H of *p*-cresol (Figure 2, cross peak S). In the NOESY maps, the 8-$CH_3$ signal shows several cross peaks with signals in the aromatic and the

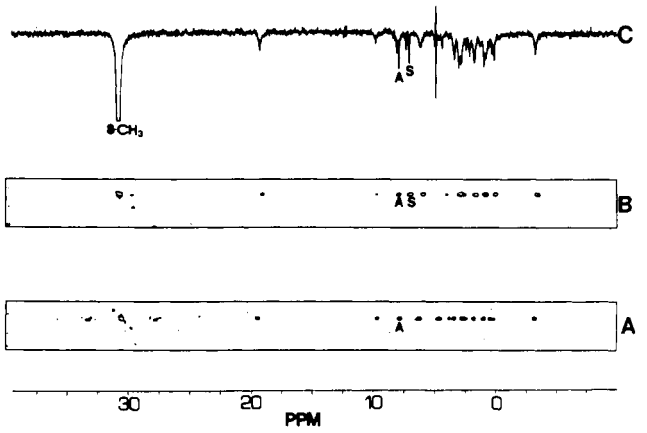



FIGURE 2: 8-$CH_3$ connectivities region of the 600-MHz and 301 K NOESY map with a 50-ms mixing time of HRP–$CN^-$ in the absence (A) and in the presence (B) of *p*-cresol in $D_2O$, pH 7.0 at 301 K. (C) NOE difference spectrum saturating the heme 8-$CH_3$ signal. The experimental conditions are the same as in panel B.

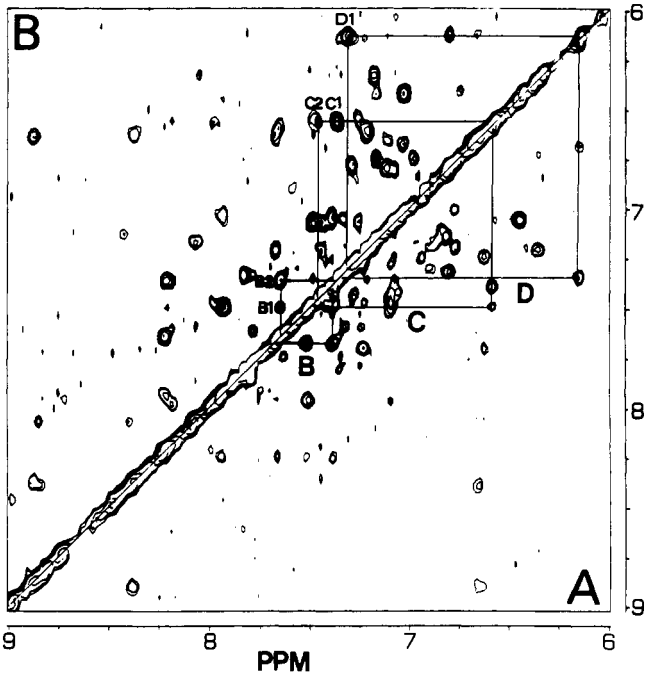



FIGURE 3: Split-diagonal representation of the aromatic region of the 600-MHz and 318 K TOCSY (A) and NOESY (B) maps of HRP–$CN^-$ in $D_2O$, 0.01 M phosphate buffer, pH 7.0.

aliphatic region of the spectrum (Figure 2A,B). NOESY and TOCSY maps in the diamagnetic region of the spectrum were obtained to assign these signals and to find other connectivities between the protein and the substrate resonances.

The TOCSY map is quite rich in connectivities, especially in the aromatic region. Figure 3A shows the aromatic region in the absence of substrate, while Figure 4A shows the map in the presence of substrate. In both maps, the patterns for several phenylalanines can be recognized. In Figure 3A cross peaks B1 and B2 label the side-chain signals of a Phe, called Phe B. Analogously, cross peaks C1 and C2 label Phe C. Signals at 7.30 and 6.12 ppm, connected by the cross peak D1, are assigned to the side-chain protons of a Phe residue (referred to as Phe D). This is in agreement with the assignment reported by Williams (Veitch & Williams, 1990; Veitch et al., 1992) for both the high- and low-spin cyanide species.

Upon addition of substrate, the degeneracy of some signals is removed. The TOCSY patterns for Phe B, Phe C, and Phe D remain and do not experience appreciable change in shift.

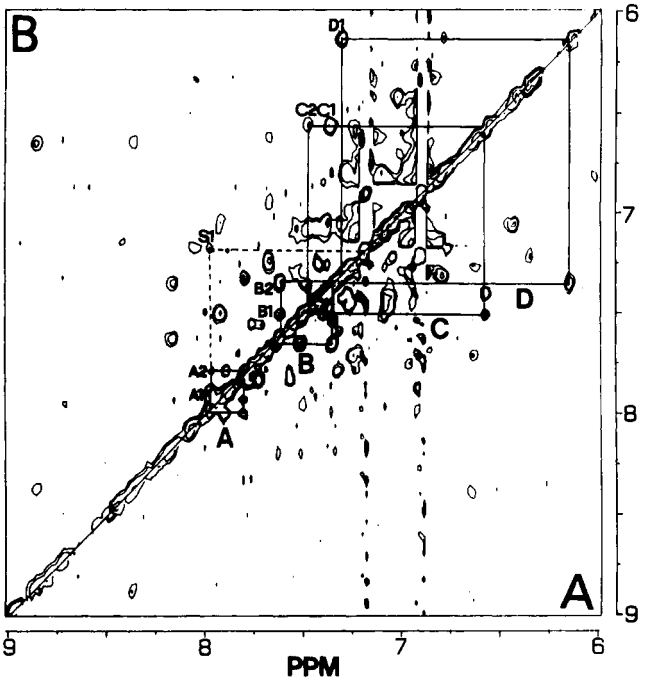



FIGURE 4: Split-diagonal representation of the aromatic region of the 600-MHz and 318 K TOCSY (A) and NOESY (B) maps of the adduct of HRP–$CN^-$ with *p*-cresol in $D_2O$, 0.01 M phosphate buffer, pH 7.0.

In addition another Phe residue is detected, referred to as Phe A and identified by cross peaks labeled A1 and A2 in the map of Figure 4A.

The NOESY maps for the same region for both the substrate-free and the substrate-bound derivatives are shown in Figures 3B and 4B, respectively. The comparison of two maps allows the detection of a dipolar connectivity between the signal 3H/5H of *p*-cresol and a signal of Phe A (cross peak S1 in Figure 4B). By considering that the heme 8-$CH_3$ signal is dipolarly connected with the signal 2H/6H of *p*-cresol, we can conclude that the substrate molecule is located between the heme edge bearing the 8-$CH_3$ group and the aromatic ring of a Phe residue of the protein.

The 8-$CH_3$ signal also shows a NOESY cross peak with a protein signal in the aromatic region (labeled A in Figure 2). Due to the low resolution in the 2D maps recorded on a 70 ppm range (full spectrum), we cannot easily identify the signal dipolarly coupled with the 8-$CH_3$ in the aromatic region. Therefore, we performed 1D NOE spectra by saturating the 8-$CH_3$ signal. This allowed us to carefully measure the shift of this signal and to find that it corresponds to a signal of the Phe A residue.

Due to the lack of the crystal structure of HRP and to its size, we cannot assign Phe A. However, based on the modeling to CcP, two Phe residues can be predicted to be reasonably close to the 8-$CH_3$ heme side of HRP. The side chains of the corresponding CcP residues are too far from the 8-$CH_3$ group to provide NOE with the latter group. This would suggest that some structural rearrangements occur in the HRP structure with respect to that of CcP. Molecular dynamics calculations are in progress in this laboratory on this protein, which will hopefully help in the identification of this residue.

*Binding of VA to LiP.* We studied the binding of VA to LiP with similar techniques. By titrating both the high-spin form and the low-spin cyanide derivative of LiP with VA, no shift on the protein proton signals was detected. Similarly, no effect on shift values and relaxation rates of the VA signals is observed when the latter is titrated with LiP in the pH

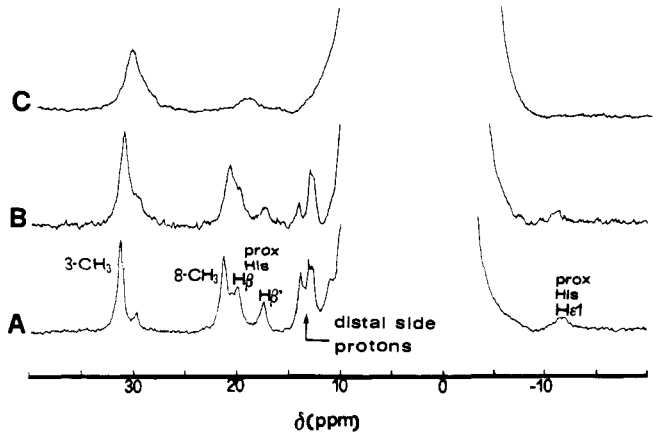



FIGURE 5: $^1H$ NMR spectra (200 MHz) of (A) MnP–$CN^-$ and (B) MnP–$CN^-$ in the presence of 0.4 equiv of manganese(II) and (C) MnP–$CN^-$ in the presence of 1 equiv of manganese(II). The spectra were recorded at 300 K in $^2H_2O$ solution, 0.1 M phosphate buffer, pH 6.5.

range 3.5–6.5 and in the temperature range 291–311 K. This suggests no direct binding of VA to the heme iron. The observed behavior can be explained either by no interaction or by strong interaction of VA with the protein as a whole. In the latter case the exchange would be slow on the NMR time scale and the proton signals of the mediator bound to LiP would fall under the diamagnetic envelope of the protein. The possibility of strong interaction is more consistent with the kinetic data. Indeed, LiP is characterized by a low $K_m$ value ($\simeq$75 $\mu$M) for VA (Tien et al., 1986).

*Binding of Manganese(II) to MnP.* Electron paramagnetic resonance (EPR) and NMR spectroscopy were utilized to characterize the complex of MnP and manganese(II). The enzyme is purified with some manganese(II) bound (Tien, unpublished). To free the sample of manganese(II), we have treated the enzyme solution with EDTA. We have successfully removed manganese down to a content lower than 5% of the protein concentration.

At room temperature, the manganese(II) aquaion shows a well-resolved EPR signal, whereas when the manganese(II) ion is bound to a slow rotating protein the EPR resonance is broadened beyond detection. By titrating a $2 \times 10^{-3}$ M solution of manganese(II) with MnP, the EPR signal of the free ion decreases in intensity. When the MnP:manganese(II) ratio is $\simeq$1:9, the EPR signal is below 3% of the original signal. This indicates that the protein has several binding sites.

According to the NMR spectra, LiP, produced by the same fungus, has an active site similar to that of MnP. (Banci et al., 1991b, 1992). However, LiP does not use manganese(II) as a mediator of the enzymatic reaction. The EPR titration of a manganese(II) solution with LiP shows behavior similar to the titration with MnP, although quantitatively slightly different. This suggests that these binding sites (but at least one in the case of MnP, see later) are common to both proteins and probably are not relevant for the enzymatic activity. It is possible that the glycosylated part of the protein binds divalent cations, such as manganese(II).

To further characterize the interaction of MnP with manganese(II), we performed NMR studies on both the $CN^-$, low-spin, and the high-spin forms of MnP. The $^1H$ NMR spectrum of MnP–$CN^-$ is shown in Figure 5A. The signals of the 8-$CH_3$ and the 3-$CH_3$ of the heme moiety and some of the proximal histidine are labeled. The 1-$CH_3$ and 5-$CH_3$ signals fall under the intense diamagnetic protein signals.

By titrating MnP–$CN^-$ with manganese(II), a broadening of paramagnetically shifted NMR signals is clearly evident. The extent of broadening differs with each signal. In particular, addition of manganese(II) to MnP–$CN^-$ causes a decrease in the intensity of the 8-$CH_3$ signal and a broadening of the 3-$CH_3$ signal (Figure 5). The spectra of the MnP–$CN^-$ derivative in the absence and in the presence of 1 equiv of manganese(II) are shown in Figure 5, panels A and C, respectively. The extent of signal broadening is directly correlated to the manganese(II)/MnP–$CN^-$ ratio, and it is complete upon addition of just 1 equiv of manganese(II). We attribute this observation to the presence of one high-affinity manganese(II) binding site close to the heme. The disappearance of the 8-$CH_3$ resonance, the disappearance of the distal side proton signals near 12 ppm, and the broadening of the other hyperfine shifted signals can be explained by a slow exchange on the NMR time scale between manganese(II)-bound and manganese(II)-free enzyme. The line shape of the 3-$CH_3$ signal at various manganese(II)/MnP–$CN^-$ ratios can be simulated by two resonances with the same shift: one of MnP–$CN^-$ with $\Delta\nu$ = 100 Hz and the other of the manganese(II)/MnP–$Cn^-$ derivative with $\Delta\nu$ = 230 Hz. The disappearance of the 8-$CH_3$ resonance in the manganese(II) complex is also consistent with the presence of two resonances with the same shift, the one in the manganese(II) complex being broadened beyond detection. Analysis of the intensity of the 8-$CH_3$ signal at increasing amounts of manganese(II) indicates that the effect is due to a single equivalent of manganese and provides an estimate of the affinity constant. This constant is about $10^4$ $M^{-1}$.

The broadening of the paramagnetic resonances is due to the dipolar coupling between the manganese(II) ion ($S = {}^5/_2$), which has a long electron relaxation time, and the nearby nuclei. We can therefore suggest that manganese(II) approaches the heme in a position near the 8-$CH_3$ and the distal side protons. The proximal histidine $\beta$-$CH_2$ signals are broadened by the interaction with manganese(II), as it occurs for the 3-$CH_3$ signal, and may collapse in a single signal at about 18 ppm. The contribution to the line width due to the presence of manganese(II) can be analyzed through the following equation for the electron–nucleus coupling (Solomon, 1955; Gueron, 1975; Vega & Fiat, 1976; Bertini & Luchinat, 1986; Banci et al., 1991a):

$$T_{2M^{-1}} = \frac{1}{15}\left[\frac{\mu_0}{4\pi}\right]^2 \frac{\gamma_N^2 g^2 \mu_B^2 S(S+1)}{r^6} \left[\frac{13\tau_c}{1+\omega_S^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + 4\tau_c\right] + \frac{1}{5}\left[\frac{\mu_0}{4\pi}\right]^2 \frac{g^4\mu_B^2\omega_I^2 S^2(S+1)^2}{(3KT)^2 r^6}\left[4\tau_r + \frac{3\tau_r}{1+\omega_I^2\tau_r^2}\right] \quad (1)$$

The first term represents the dipolar contribution and the second the Curie contribution to the line width. $r$ is the metal–proton distance and the other symbols have their usual meaning. For the dipolar contribution, $\tau_c$ is determined by the shortest correlation time among the electron, the rotational, and the exchange correlation times. The latter is the longest, due to the slow exchange between the free and bound manganese(II). The rotational correlation time ($\tau_r$) depends on the size of the molecule and can be estimated from the Stokes–Einsten equation (Stokes, 1956; Einstein, 1956) to be $2 \times 10^{-8}$ s. The electron relaxation time ($\tau_s$) for manganese(II) is field dependent and increases with the magnetic field. At 600 MHz, it is much longer than $\tau_r$ and therefore $\tau_c$ is dominated by $\tau_r$. By taking $\tau_r = 2 \times 10^{-8}$ s, a distance between the manganese(II) ion and the protons of 3-$CH_3$ of 18 Å is

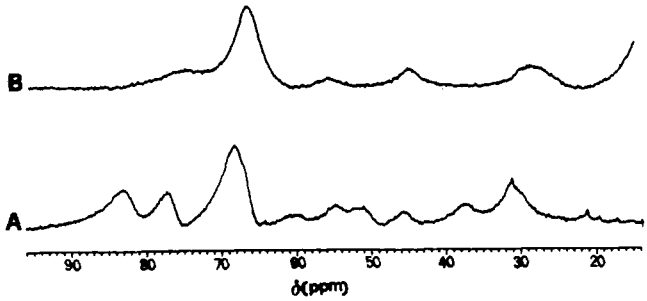



FIGURE 6: $^1$H NMR spectra (600 MHz) of (A) MnP and (B) MnP in the presence of 1 equiv of manganese(II). The spectra were recorded at 300 K in $^2H_2O$ solution, 0.1 M phosphate buffer, pH 6.5.

obtained. By also assuming a lower limit for the line width of 8-$CH_3$ in the manganese(II) complex, we can estimate an upper limit for the distance between manganese(II) and the protons of 8-$CH_3$. If we consider that a signal is not detectable if it has a line width larger than 5000 Hz and we apply eq 1 to the increase in line width induced by the binding of manganese(II), we estimate an upper limit of the manganese-(II)/8-$CH_3$ distance of 11 Å. Interestingly, manganese(II) does not cause any other shift. This can be accounted for on the basis of the $S = {}^5/_2$ nature of the of manganese(II) which provides negligible magnetic susceptibility anisotropy.

When the same NMR titration is performed on LiP, no effect is observed on any paramagnetically shifted signal. The data can be interpreted by assuming a specific binding site for manganese(II) in MnP and not in LiP. The binding of manganese(II) ion to MnP affects the line widths of the heme protons. Furthermore, there are several binding sites in both proteins for manganese(II) which are far from the heme. These are important results which establish some differences between MnP and LiP, relevant for the catalytic behavior.

The distances between manganese(II) and the heme protons in MnP could help in the location of the specific binding site of manganese(II) in the protein frame. By inspection of the structure of CcP and by comparing the sequences of CcP and MnP, it is possible to locate, at the right distances from 8-$CH_3$ and 3-$CH_3$, several aspartate residues (namely, Asp 179, Asp 182, Asp 84, and Asp 85). The Asp residues are negatively charged and could provide a binding site for manganese(II) in MnP.

In Figure 6 the NMR spectrum of the manganese(II) complex with the high-spin form of MnP is shown (panel B) and compared to that of the free form of MnP (panel A). The NMR spectrum of the latter form shows, paramagnetically shifted, the signals of the four heme methyls and of the $\alpha$ protons of the heme vinyl and propionate side chains, as well as of the $\beta$-$CH_2$ protons of the proximal histidine. These signals have not been further assigned. However, more information on some heme methyl signals can be obtained upon titration of the protein with manganese(II). Upon addition of manganese(II), two methyl signals disappear. In agreement with the data on the low-spin form, one of the two disappearing methyl signals can be attributed to the 8-$CH_3$. We may also suggest that the other disappearing $CH_3$ signal belongs to the 1-$CH_3$, which is on the same side of the heme as the 8-$CH_3$. Three one-proton intensity signals also disappear upon titration with manganese(II), at 58.8, 51, and 37.4 ppm, which presumably belong to the $\alpha$-protons of the vinyl and propionate side chains close to the above methyls. This picture is overall consistent, as the side of the heme bearing 1- and 8-$CH_3$ has three protons in the $\alpha$ position. The broad and overlapping signals of the spectrum containing high-spin iron(III) do not allow further analysis. These NMR results are consistent with the work of Harris et al. (1991), which suggests that manganese(II) binds to the protein on the heme side of the meso proton between position 1 and 8.

*Effect of Oxalate on Binding of Manganese(II) to MnP.* During the catalytic cycle, manganese(II) is oxidized by the protein to manganese(III), which then oxidizes the substrate. It has been shown that chelation of manganese(III) by organic acids stabilizes such an oxidation state (Glenn & Gold, 1985; Pasczynski et al., 1986; Wariishi et al., 1988, 1898a). The complex formation may facilitate the release of the metal from the protein. A typical organic acid which has been shown to activate the enzyme is oxalate. We have investigated the interaction of manganese(II)/MnP in the presence of oxalate. Oxalate has little effect on the NMR titration of MnP and MnP–$CN^-$ with manganese(II). This can be due to the high affinity of manganese(II) for the protein, which turns out to be larger than that for oxalate.

*Binding of Calcium(II) to MnP.* When dialysis to remove manganese(II) from MnP is performed with high EDTA concentrations ($\geq$10 times the concentration of the protein), the resulting samples are characterized by NMR spectra slightly different from those of MnP treated with EDTA under mild conditions. In the case of the cyanide adduct the shift variations are not larger than 1 ppm. By addition of 1 equiv of calcium(II) we obtain again the "normal" spectrum. This may be taken as a direct proof that MnP contains at least 1 equiv of calcium(II) which can be removed from the protein by exhaustive dialysis with EDTA. The absence of this cation influences the conformational properties of the active site, as it is reflected in the shift differences. Indeed, the presence of calcium ions has been suggested as a stability component in peroxidases (Shiro et al., 1986). In the case of HRP, calcium-(II)-dependent conformational properties of the active site have been also demonstrated by NMR (Ogawa et al., 1979; Morishima et al., 1986). Addition of calcium(II) to the MnP–$CN^-$/manganese(II) complex does not produce any variation in the $^1$H NMR spectrum. Furthermore, by addition of 1 equiv of manganese(II) to MnP–$CN^-$ reconstituted with calcium(II) we obtain the typical spectrum of the manganese-(II) complex. These data indicate that the binding sites for calcium(II) and manganese(II) are different and that there is no competition between them.

## CONCLUDING REMARKS

Peroxidases perform two one-electron oxidation reactions when oxidized by $H_2O_2$. HRP oxidizes small substrates which form complexes with the protein and bind near the heme periphery. In the present study, we have detected some dipolar connectivities of the substrate *p*-cresol with the heme 8-$CH_3$ and with a phenylalanine inside the so-called active-site cavity. The substrates enter in a position which has now been better identified and will be still better identified as soon as the X-ray structure or molecular dynamics calculations will be available. The picture is consistent with the first proposal of Ortiz de Montellano and with their elegant experiments on suicide inhibitors of the enzyme (Ator & Ortiz de Montellano, 1987), which have been shown to bind at the $\delta$-meso position. The available data on aromatic acids are also consistent with this model (La Mar et al., 1992).

Our work here on MnP shows that manganese(II) does not bind at the heme periphery. However, the binding site can be located on the side of the 8-$CH_3$ heme edge. This is consistent with the work of Ortiz de Montellano showing that the heme is modified at the $\delta$ meso position by suicide inhibitors (Harris et al., 1991).

The present research has further improved the characterization of the binding site of substrates to HRP through 2D NMR experiments. It has also provided the first evidence of the interaction of the manganese(II) with MnP through the analysis of the line broadening of the $^1H$ NMR signals. Further clarification and characterization of substrate binding will soon be available through the X-ray crystallographic data of these important fungal peroxidases.

## NOTE ADDED IN PROOF

During the final steps of the processing of this paper, two reports on the X-ray structure characterization of LiP appeared (Poulos et al., 1993; Piontek et al., 1993).

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