X-ray Absorption Spectroscopy Comparison of the Active Site Structures of *Phanerochaete chrysosporium* Lignin Peroxidase Isoenzymes H2, H3, H4, H5, H8, and H10[†]

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Received March 20, 1995; Revised Manuscript Received May 25, 1995[⊗]

ABSTRACT: The iron heme and its immediate environment can provide information that is pivotal to our understanding of the structural and mechanistic features that confer unusual properties to the heme peroxidases. X-ray absorption spectroscopy (XAS), which is ideally suited for the investigation of the local environment and electronic structure of the heme iron of hemeproteins, has been used to characterize a variety of lignin peroxidase and manganese-dependent peroxidase isoenzymes produced by the white rot fungus *Phanerochaete chrysosporium*. The data suggest no differences within the error in the first coordination shell of iron for the isoenzymes H2, H3, H4, H5, H8, and H10 examined in this study. The pyrrole nitrogens are at a distance of 2.05 ± 0.015 Å, and the proximal histidine nitrogens are at 1.93 ± 0.02 Å, while the sixth ligands are located at 2.17 ± 0.03 Å. Significant differences are observed in higher coordination shells which may be related to conformational differences in the heme.

In addition to the ligninolytic activity of the *Phanerochaete chrysosporium* extracellular peroxidases (PcP),[1] the heme peroxidases are responsible for a wide range of biological functions. These include antibacterial activity in mammals (lactoperoxidase; Reiter & Perraudin, 1991), antibiotic production in fungi (chloroperoxidase; Hager et al., 1966; Hultquist & Morrison, 1963; Morris & Hager, 1966), and lignification of plant cell walls (numerous plant peroxidases; Halliwell, 1978; Harkin & Obst, 1973; Kersten & Kirk, 1987).

Typically, the peroxidase catalytic cycle begins with the reaction of resting enzyme (ferric, Fe^{3+}) with hydrogen peroxide (H_2O_2) to yield the ferryl iron (Fe^{4+}) porphyrin cation radical intermediate, compound I, which is two oxidizing equivalents above the resting state. Compound I is reduced by one electron via a reducing substrate (such as veratryl alcohol in the LiP reaction) to yield the iron-oxo intermediate, compound II. The electron quenches the porphyrin cation radical leaving ferryl iron (Fe^{4+}). Finally, a second-single electron reduction returns the enzyme to resting state (Yamazaki, 1974).

In response to limiting carbon or nitrogen limitation, *P. chrysosporium* produces a number of extracellular enzymes collectively referred to as the lignin degradation (ligninase) system (Jeffries et al., 1981; Keyser et al., 1978). The ligninase system is comprised of a family of lignin peroxidase (LiP) isoenzymes, manganese-dependent peroxidases (MnP), and an H_2O_2-generating system such as glyoxal oxidase. In this paper, we refer to the MnP and LiP isozymes separately but will refer to the entire collection of extracellular hemeproteins as the ligninase system.

Farrell et al. (1989) have shown that at least 10 hemeproteins are present in the extracellular culture fluid of ligninolytic cultures of *P. chrysosporium*. These heme proteins have been designated H1 to H10 signifying the order in which they elute from anion exchange columns during fast protein liquid chromatography. Six of these (H1, 2, 6, 7, 8, 10) exhibit significant veratryl alcohol oxidation ability and were consequently grouped together as the LiPs. Of the remaining four, at least three demonstrated manganese-dependent vanillylacetone oxidizing ability and were therefore grouped as the MnP (Gold et al., 1989). In other studies, Leisola et al. (1987) have presented evidence suggesting that up to 21 proteins may be present in the extracellular fluid of ligninolytic cultures of *P. chrysosporium*. The PcP contain a single ferric protoporphyrin IX per molecule (Gold et al., 1984; Tien & Kirk, 1984). The electronic absorption spectrum of the resting (ferric) ligninases is typical of most heme peroxidases, with a Soret maximum ~408−410 nm and visible absorption bands at ~504 and ~636 nm. The isozymes are N- and probably O-glycosylated. The molecular masses, determined by SDS−PAGE, range from 38 to 43 kDa, and the proteins exhibit isoelectric points between 3.3 and 4.7. The amino acid sequences of several peroxidase isoenzymes for *P. chrysosporium* and other fungi have been deduced, both from cDNA clones and genomic isolates (Black & Reddy, 1991; Kimura et al., 1991; Ritch & Gold, 1992, and references therein). There is significant homology between the different protein sequences.

Piontek et al. (1993) determined the X-ray crystal structure of one of the PcP isozymes, which is confirmed by others (Edwards et al., 1993; Poulos et al., 1993). The overall fold of LiP is remarkably similar to that of cytochrome *c*

[†] This work was supported by NIH Grant Numbers ES-04922 and RR-01633.

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[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: XAS, X-ray absorption spectroscopy; H_2O_2, hydrogen peroxide; LiP, lignin peroxidase; MnP, manganese dependent peroxidase; PcP, *P. chrysosporium* peroxidase; H2, H3, H4, H5, H8, and H10, isozymes of PcP; CcP, cytochrome *c* peroxidase; FPLC, fast protein liquid chromatography; FeTPP, bis(imidazole)-(5,10,15,20-tetraphenylporphinato) iron III chloride; Fe^{2+}/Fe^{3+} acac, ferrous/ferric acetylacetonate; LFIR, ligand field indicator region; HRP, horseradish peroxidase; LPO, lactoperoxidase.

0006-2960/95/0434-13176$09.00/0

peroxidase (CcP) despite the low level (<20%) of amino acid similarity between the two proteins. LiP has an additional 49 terminal residues that lie along the surface of the protein.

Although the LiP isozymes exhibit reactions that appear typical of other heme containing peroxidases, they are different in several respects. Notably, the ability to oxidase compounds of high redox potential such as lignin, its model compounds, and a variety of xenobiotics (Gold et al., 1989; Kirk & Farrell, 1987; Schoemaker, 1990; Tien, 1987) and the extremely low pH optimum (Farhangrazi et al., 1992; Rengenathen et al., 1987; Tien et al., 1986).

Under our culture conditions, H2 is the predominant isozyme produced (Tuisel et al., 1990); our initial studies therefore focused on this protein. This isozyme is probably identical to the H2 reported by Farrell et al. (1989). This paper reports XAS on the structure of the heme active site of a number of ligninase enzymes. Included in this work is H8, which is the focus of most other studies. The work compares the active heme site structure of the isoenzymes and complements other structural and functional studies on this enzyme system.

EXPERIMENTAL PROCEDURES

Protein Purification. PcP isoenzymes were purified according to Tuisel et al. (1990) from 150 mL cultures grown in 500 mL Erlenmeyer flasks for 5 days. The extracellular fluid was treated using a freeze/thaw approach to remove mucilaginous material and concentrated prior to FPLC purification of the individual heme containing fractions. Chromatography was performed using either a Pharmacia Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) or a Baker Versa-ten column (J. T. Baker, Phillipsburg, NJ). Peak fractions were dialyzed extensively against water and stored at −20 °C. At this stage, the A_{408}/A_{280} ratio was determined for each preparation and was generally between 4.0 and 4.5.

Preparation of XAS Samples. Protein preparations were concentrated to volumes of approximately 1 mL by ultrafiltration using an Amicon 8010 cell and Amicon Diaflow membranes. If further concentration was necessary, we flowed dry nitrogen gas over the surface of the solution at 4 °C. XAS samples generally had protein concentrations of 1.5−2.0 mM. These were prepared in 100 mM phosphate buffer at pH 7.0 to maintain consistency with studies on other peroxidases in our laboratory and contained 40% ethylene glycol as a cryoprotectant (Chance et al., 1983).

Determination of Protein Integrity. Due to the destructive nature of X-radiation, it is necessary to monitor the protein viability during these studies. Following preparation of a concentrated sample, a small aliquot was removed and diluted with buffer to allow optical spectroscopic evaluation of the sample. The remainder of the sample was immediately loaded into a Plexiglas sample holder and frozen in liquid nitrogen. The quality of XAS samples was carefully monitored throughout the procedure. The veratryl alcohol oxidation activity of the various protein preparations was determined before the final concentration procedures. In addition to the optical spectroscopy described above, reflectance spectroscopy was used to examine directly the visible region of the sample at −100 °C prior to XAS data collection. Samples were stored in liquid nitrogen, and the sample was maintained in a cryostat at between −90 and −120 °C during data collection. Following data collection, samples were again stored in liquid nitrogen, and the reflectance spectroscopy was repeated before the sample was allowed to thaw. Aliquots of material were then diluted and used for optical absorption and activity analysis.

XAS Data Collection and Analysis. Data for the LiP and MnP were collected at the National Synchrotron Light Source Beamline X9A (Brookhaven National Laboratory, Upton, NY). The beamline monochromator was equipped with Si 111 crystals, and sample fluorescence was monitored using a 13-channel germanium detector. Some data were also collected at the Stanford Synchrotron Light Source beamline 2−1 (Stanford Synchrotron Light Source, Stanford, CA). Using Si 111 crystals, sample fluorescence was monitored with a phototube equipped with a phosphorescent screen (Khalid et al., 1986). The EXAFS experimental setup was that described previously (Powers et al., 1981). Data were collected under identical conditions for the model compounds bis(imidazole)-(5,10,15,20-tetraphenylporphinato) iron III chloride (FeTPP; Collins et al., 1972), ferrous acetylacetonate (Fe^{2+} acac), and ferric acetylacetonate (Fe^{3+} acac; Iball & Morgan, 1976), except that transmission mode was used.

Individual spectra were examined for the presence of aberrations, and several were rejected on the grounds of abrupt changes in signal or beam intensity. The remainder were averaged and analyzed by previously reported procedures (Chance et al., 1984; Lee et al., 1981). For samples that were examined at both synchrotron sources, the k^3-multiplied background subtracted data sets were added together, weighted according to estimates of the signal-to-noise ratio for each. The edge and ligand field indicator region (LFIR) of each averaged, background subtracted data set was examined. Although the edge region can provide important information about the scattering atom, the transitions in the heme iron are often too broad to permit rigorous comparisons. The LFIR region was extracted from these data according to the procedure of Chance et al. (1984, 1986a). While some phenomena, such as ruffling of the heme, can affect the results, there is generally a good correlation between the LFIR parameter and the displacement of iron from the mean heme plane, and the displacement from the heme plane can be calculated from the LFIR parameter by (LFIR − 0.52)/1.70 (Chance et al., 1986a). The EXAFS modulations were then normalized to one absorbing atom, multiplied by k^3 and plotted vs k. The different ligand shells were revealed by a Fourier transform of these modulations. The contribution from the first ligand shell was isolated by a Fourier filter, and the back-transformed data from $k = 0$ to 12 $Å^{-1}$ were used for further analysis. A two-atom fitting procedure (Lee et al., 1981) was used to fit filtered data with FeTPP as a model for Fe−N contributions and Fe^{2+} acac or Fe^{3+} acac as a model for the Fe−O contributions (Powers et al., 1981; Woolery et al., 1985). The numbers of scatterers were constrained to their known values, and 5:1 and 4:2 ratios were used to search parameter space for mathematically and chemically reasonable solutions for axial and iron to pyrrole nitrogen (Fe−N_p) distances. A three-atom constrained fitting procedure was then used as a consistency test to determine which solutions from the two-atom procedure when combined together were contained in the data. Note that this procedure

Table 1: Summary of XAS Results for Lignin and Manganese-Dependent Peroxidases H2, H3, H4, H5, H8, and H10

isozyme	LFIR (±0.05)	Fe−Ct (±0.5 Å)	Fe−N_p (±0.015 Å)	Fe−N_ϵ (±0.02 Å)	Fe−X (±0.03 Å)
H2[a]	1.55	0.6	2.055	1.93	2.17
H3	1.24	0.4	2.04	1.92	2.15
H4	1.28	0.45	2.05	1.95	2.18[b]
H5	1.32	0.5	2.05	1.94	2.16
H8	1.30	0.45	2.05	1.93	2.19
H10	1.38	0.5	2.05	1.92	2.15

[a] Sinclair et al. 1992. [b] Error ± 0.1 Å.

holds distances (r) and numbers (N) constant, so the total number of independent variables is the same as for the two-atom fitting procedure. In order to ensure that this combination was indeed a minimum, each r parameter was allowed to vary separately holding its corresponding N value and all other r and N values constant. If any r value changed more than the error found from the two-atom type fitting procedure, the solution was not considered acceptable. The full procedure is described by Chance and co-workers (Chance et al., 1983, 1984). Error estimation was performed as described by Powers and Kincaid (1989). Comparison of the fitting results required the sum of the residuals $\sum R^2 \geq \sum R^2_{min}(1 + 1/\phi_f) \sim \sum R^2_{min}$ (1.4) for two fits to be judged different. $\sum R^2_{min}$ is the minimum sum of residuals for a physically reasonable solution; the number of independent parameters in the fit, ϕ_f, is equal to $\phi_d - p$, where p is the number of variables in the fit and ϕ_d is the number of degrees of freedom in the filtered data. ϕ_d is estimated to be 8.4 by $2\Delta w \Delta k/\pi$, where Δw is the width of the filter window (full width at half-maximum = 1.1 Å) and Δk is the length of the data fit (0−12 Å^{-1}).

RESULTS

The native LiP and MnP exhibited optical spectra similar to those reported by other groups (Gold et al., 1984; Tien & Kirk, 1984). Minor variations in the position of the Soret band were observed (between ~408 and ~410 nm), and a minor spectral feature was sometimes observed at ~540 nm in preparations of H2, H3, H5, and H10. Following X-ray exposure, the optical spectra of the preparations were essentially the same; some minor changes at ~580 nm were noted in some preparations, suggesting possible contamination by an unidentified intermediate species, which comprised a very minor (<10%) component of the sample. Contamination at this level would not be expected to influence the XAS analysis.

It was possible to prepare the ferrous and ferrous-oxy forms of the preparations from the X-ray exposed material. The resultant spectra were not as good as those prepared from unexposed material, suggesting that a small portion of the sample had been damaged. Activity measurements indicated some loss of reactivity of the protein preparations.

LFIR parameters and the correlated iron displacements (Chance et al., 1986a) are shown in Table 1 and range from an iron displacement of 0.6 Å for H2 to 0.4 Å for H3. The EXAFS modulations from background-subtracted, k^3-multiplied data are plotted for the six isoenzymes in Figure 1. A multiple reflection from the Si 111 crystals results in a glitch at $k \sim 10.5$ Å^{-1}. To eliminate this artifact from further analysis, this region of the spectrum was deleted from data from both the protein samples and the model compounds

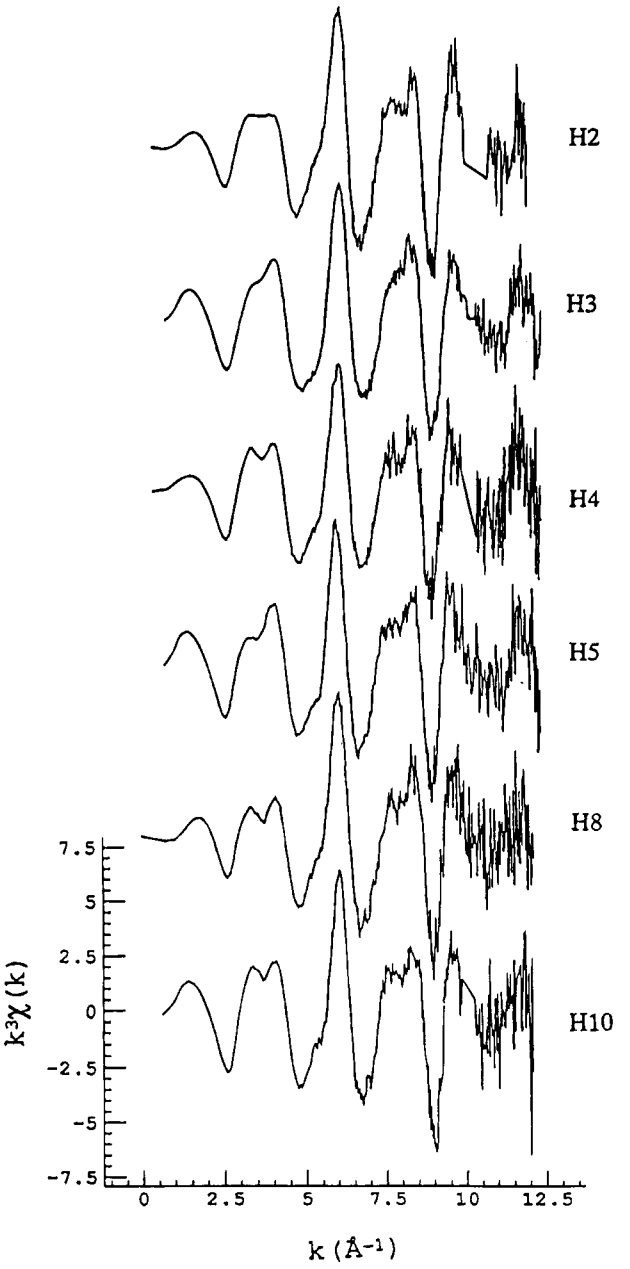

FIGURE 1: EXAFS modulations of lignin- and manganese-dependent peroxidase isozymes H2, H3, H4, H5, H8, and H10.

(Lee et al., 1981). The Fourier transforms of these data are shown in Figure 2. The first shell contributions to these data were Fourier filtered and back-transformed to provide amplitude and phase information used in the fitting analysis. Data for the three model compounds, collected under identical conditions, were treated in an identical manner. Back-transformed data from k = 0−12 Å^{-1} were used for further analysis; data from k = 3.5−12 Å^{-1} were also examined and gave identical results.

Table 2a−f shows the best two-atom type fits and the consistency test results for the six isozymes studied. While the data and results for H2 have been reported by Sinclair et al. (1992), they are repeated here for comparison with the other isoenzymes. The 4:1 amplitude ratio for H2 showed four ligands at an average distance of 2.06 Å and one at 1.92 Å (Table 2a). The 5:1 amplitude ratio fits suggested axial ligands at 1.92 and 2.16 Å, with a very similar $\sum R^2$ for both the 4:1 and 5:1 fits. However, the three-atom

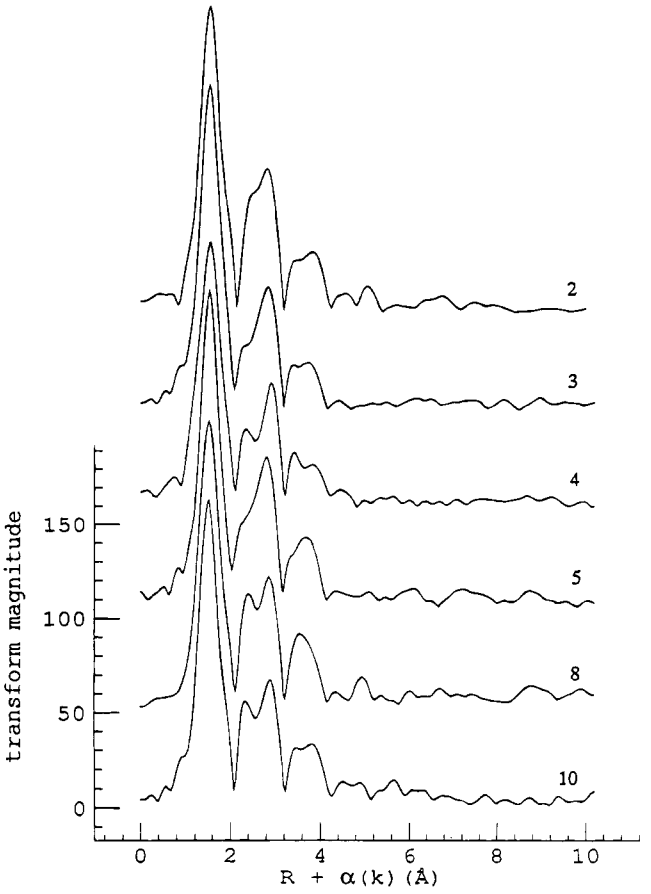

FIGURE 2: Fourier transforms of the EXAFS modulations shown in Figure 1 for lignin- and manganese-dependent peroxidase isozymes H2, H3, H4, H5, H8, and H10.

consistency test using six ligands gave a significantly improved fit over the 4:1 fit since ΣR^2 decreased by more than a factor of 2. The H2 data can therefore be explained by four pyrrole nitrogen ligands at 2.055 (± 0.015) Å, a fifth (proximal) at 1.93 (± 0.02) Å, and a sixth (distal) at 2.17 (± 0.03) Å. H3, H4, H5, H8, and H10 all give significantly better results with a 5:1 amplitude ratio than with a 4:1 amplitude ratio in the two-atom type fitting procedures. This suggests that the isozymes are all six coordinate at −100 °C. Similar fitting procedures allow determination of the Fe−ligand distances for the other isozymes. For Fe−N_p (±0.015), Fe−N_ϵ (±0.02), and Fe−distal (±0.03), respectively, the distances are as follows: H3, 2.04, 1.92, 2.15; H4, 2.05, 1.95, 2.18; H5, 2.05, 1.94, 2.16; H8, 2.05, 1.93, 2.19; and H10, 2.05, 1.92, 2.15 (Table 1 and Figure 3). Some differences in the outer shell contributions were noted for the different isoenzymes (Figures 1 and 2). While it is often difficult to rigorously analyze or even model these contributions, they may represent similar conformation of the heme in some isozymes. Their possible significance is discussed in the following section.

DISCUSSION

The small differences observed in the optical spectra of the different isoenzymes are probably explained by differences in culture conditions and inconsistencies in the purification procedure. Leisola et al. (1987) describe differences in various ligninase preparations due to inactivation of minor components and incomplete separation of the isoenzymes. Tien et al. (1986) also refer to preparations of

Table 2: Fitting Parameters for the Six Lignin and Manganese-Dependent Peroxidases Used in This Study

N^b	r (Å)	$\Delta\sigma^2$ (Å^2)^c	ΔE_o (eV)^d	ΣR^2
		(a) H2[a]		
5 (4)	2.06	2.8×10^{-3}	−0.4	2.7 (3.7)
1	1.92	5.4×10^{-3}	8.0	
5	2.02	1.8×10^{-3}	0.8	5.0
1	2.16	7.5×10^{-3}	4.8	
4	2.055[e]	2.9×10^{-3}	3.6	1.0
1	1.93[f]	7.0×10^{-3}	−1.7	
1	2.17[g]	-1.5×10^{-3}	−1.5	
		(b) H3		
5 (4)	2.04	2.4×10^{-3}	0.4	4.7(6.7)
1	1.92	6.9×10^{-3}	1.6	
5 (4)	2.00	4.1×10^{-3}	1.0	5.3(7.0)
1	2.14	7.3×10^{-3}	−0.1	
4	2.04[e]	6.3×10^{-3}	−0.1	3.6
1	1.92[f]	8.9×10^{-3}	2.8	
1	2.15[g]	-6.5×10^{-3}	−1.7	
		(c) H4		
5	2.01	4.0×10^{-3}	0	4.2
1	2.13	8.3×10^{-3}	4.6	
5	2.05	3.3×10^{-3}	0.4	4.0
1	1.95	7.6×10^{-3}	−1.8	
4	2.06	4.7×10^{-3}	−0.5	3.6
1	1.94	8.4×10^{-3}	3.5	
4	2.05[e]	5.5×10^{-3}	0.1	0.9
1	1.95[f]	9.1×10^{-3}	−0.5	
1	2.18[g]	-1.2×10^{-2}	−3.1	
		(d) H5		
5 (4)	2.06	3.4×10^{-3}	−0.2	4.3(8.3)
1	1.95	8.8×10^{-3}	1.3	
5 (4)	2.01	4.5×10^{-3}	0.5	4.9(9.2)
1	2.16	8.4×10^{-3}	−1.5	
4	2.05[e]	5.8×10^{-3}	−0.2	3.8
1	1.94[f]	9.8×10^{-3}	1.1	
1	2.16[g]	3.0×10^{-3}	−1.8	
		(e) H8		
5	2.04	1.8×10^{-3}	−0.6	4.5
1	1.96	4.1×10^{-3}	5.5	
4	2.06	4.8×10^{-3}	−0.7	5.7
1	1.93	7.9×10^{-3}	5.7	
5	2.02	2.6×10^{-3}	0	4.5
1	2.13	1.1×10^{-4}	−1.9	
4	2.05[e]	6.9×10^{-3}	−0.5	3.0
1	1.93[f]	8.9×10^{-3}	1.1	
1	2.19[g]	4.8×10^{-6}	−3.0	
		(f) H10		
5	2.06	4.3×10^{-4}	0	7.0
1	1.93	2.2×10^{-3}	3.1	
4	2.07	2.7×10^{-3}	−0.2	10.2
1	1.93	5.4×10^{-3}	3.5	
5	2.02	-9.6×10^{-4}	0.7	7.0
1	2.12	3.3×10^{-3}	−0.8	
4	2.05[e]	2.3×10^{-3}	0.1	4.5
1	1.92[f]	4.4×10^{-3}	3.2	
1	2.15[g]	3.2×10^{-3}	−1.2	

[a] Sinclair et al. (1992). [b] Held fixed at integer values. [c] $\Delta\sigma^2$ (model-enzyme) ± 45%. [d] ΔE_o (model-enzyme) ± 2.5eV. [e] Fe−N ± 0.015 Å. [f] Fe−N ± 0.02 Å. [g] Fe−O ± 0.03 Å.

H8 containing varying amounts of inactive enzyme. The minor differences in this work are probably the result of similar phenomena and, due to their small contribution to the sample, are not likely to significantly influence the XAS results. The optical spectroscopy used to monitor changes

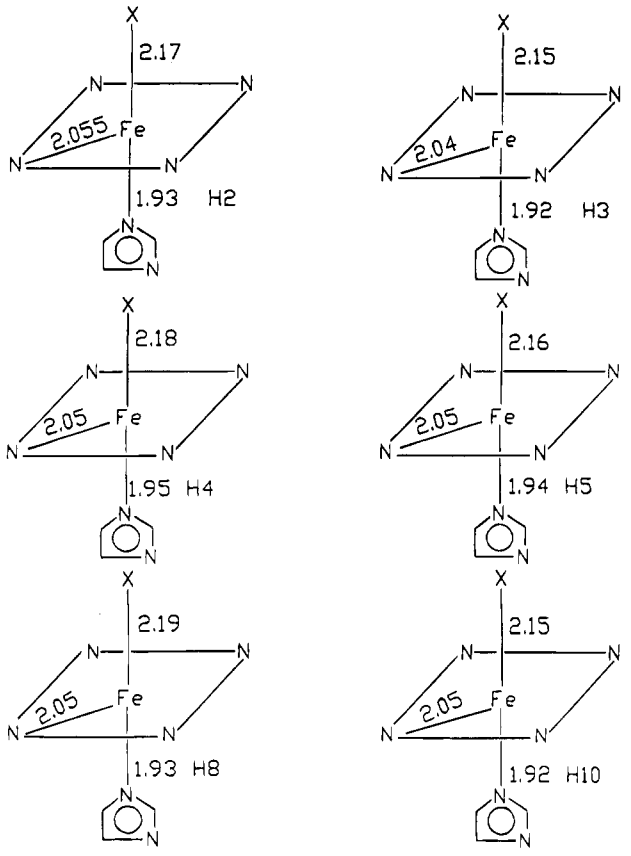

FIGURE 3: Pictorial representation of the XAS results for the heme active sites of lignin- and manganese-dependent peroxidases H2, H3, H4, H5, H8, and H10. Errors are listed in Table 1.

in the samples during X-ray exposure suggests that small portions of the preparations were damaged during the procedure. However, the extent of the damage is small, and no time-dependent changes in the XAS spectra could be detected. Following XAS data collection, the preparation of ferrous derivatives and activity assays confirmed minimal damage to the samples.

The LFIR ratio varies from 1.55 (±0.05) in H2 to 1.24 (±0.05) in H3, corresponding to a range of displacements of the iron from the heme plane of 0.6−0.4 Å (Table 1). As discussed by Chance et al. (1984), if iron displacement from the plane defined by the pyrrole nitrogens were the only phenomena being observed, we would anticipate a direct relationship between the distance from the iron to the center of the heme plane (Fe−Ct) and the average Fe−N_p distance. While this trend is difficult to verify with the PcP proteins, due to the errors inherent in the calculation of Fe−Ct from the LFIR, there does appear to be some correlation. Deviations from a strict correspondence between the LFIR parameter and Fe−N_p distance might be explained by ruffling or twisting of the heme. This would change the Fe−N_p−C angle and consequently effect multiple scattering effects.

The distances found from the fitting procedures are presented in Figure 3 and Table 1. All isozymes studied appear to be six coordinate at the experimental temperature of −100 °C. This is in agreement with data presented by Kuila et al. (1985) for LiP, which suggests a pentacoordinate high spin form at 25 °C that changes to a hexacoordinate species when the temperature is lowered. The iron to proximal nitrogen (Fe−N_ϵ) and sixth ligand distances are identical within their error for the six isozymes. Chance et al. (1984, 1986b) noted a significant difference in the Fe−N_ϵ bond length in the active site of horseradish peroxidase (HRP) versus that in myoglobin. All intermediates of myoglobin have an iron to proximal nitrogen distance around 2.1 Å, while comparable distances in HRP were around 1.9 Å. Other work in our laboratory (Chang et al., 1992; Sinclair et al., 1992) and this current work add additional support to our argument (Chance 1984; Sinclair et al., 1992) that the length of the proximal bond distinguishes the peroxidases and the globins. The distal ligands observed in this work are also very similar, both to each other and to other peroxidases. This suggests that the structure of the distal heme pocket is similar between the different ligninase isoenzymes and possibly in the plant family peroxidases in general. One further observation that is also significant concerns the rigidity of the sixth ligand. MnP H4 has a large Debye−Waller factor associated with the sixth ligand. In this protein, the sixth ligand is very mobile and probably has a three times larger error associated with it than indicated in Table 1 (~0.1 Å rather than 0.03 Å). It is tempting to speculate that this may be of relevance to the reaction of H4 with manganese, although if it were important, why are similar values not observed for H3 and H5?

There appears to be a significant discrepancy between our results and those presented in the crystal structure. In cytochrome *c* peroxidase, the XAS and crystallography are in good agreement (XAS Fe−N_ϵ 1.94 Å vs crystal structure Fe−N_ϵ 1.96 Å). In the different ligninase isoenzymes, however, we measure the Fe−N_ϵ distances between 1.92 and 1.95 Å (±0.02 Å). However, Piontek et al. (1993; 2.5 Å resolution crystal structure, $R = 0.203$) report Fe−N_ϵ of 2.28 Å, while Poulos et al. (1993; 2.0 Å resolution crystal structure, $R = 0.15$) report values between 2.05 and 2.19 Å with a deviation of 0.19 Å. It appears likely that, within the errors of the two procedures, the crystallographically determined Fe−N_ϵ is similar to that measured using XAS. We measure distally coordinated ligands (probably water) in all isozymes. These distal water molecules lie between 2.15 and 2.19 Å from the iron (±0.03 Å). Both crystal structures clearly reveal some electron density around 2.5 Å from the iron, which could correspond to this water. It has been previously observed that a change from five to six coordination occurs upon cooling (Kuila et al., 1985). Our measurements were performed at ~−100 °C, and it is therefore not surprising that we see a Fe−distal distance that is both shorter and more precisely defined than that seen in the crystal structure. This temperature difference may also contribute to the differences in Fe−N_ϵ distances.

Electrochemical studies on the Fe^{2+}/Fe^{3+} couple of PcP (Millis et al., 1989) indicated that the heme environment of PcP was more electron deficient than other peroxidases. This led us to suggest (Sinclair et al., 1992) that a relatively electron-deficient heme could be generated by a redistribution of heme electrons or by an expansion of the heme itself. Therefore, a relatively expanded heme, as indicated by a larger Fe−N_p distance or a greater LFIR parameter, could have mechanistic significance. Our previous argument (Sinclair et al., 1992) was based on the observation that LiP H2 has a far more expanded heme than HRP, and that LiP is also able to oxidize numerous recalcitrant xenobiotics, many of which have extremely high redox potentials and are consequently resistant to degradation by HRP. The new

structural data presented here allow us to examine this hypothesis in more detail.

Kersten et al. (1990) investigated the ability of LiP H8 to degrade a series of methoxybenzene compounds. The results were compared with similar data for HRP. Popp and Kirk (1991) extended the study to include MnP (pI 4.7). Both MnP and HRP were able to oxidize all compounds in the series up to 1, 2, 6, trimethoxybenzene, which has a half-wave potential ($E_{1/2}$) of 1.12 V. (The value of the half wave potential, $E_{1/2}$, is usually close to the value of the redox potential, E_o', under standard conditions.) Mn^{3+} chelates exhibited similar redox activities to MnP. LiP H8, however, was able to oxidize all compounds up to 1,3,5-trimethoxybenzene, $E_{1/2} = 1.49$ V. Our previous hypothesis would lead to the prediction that H8 should have a more expanded heme than H4 or HRP. The measured Fe−N_p distances for H8 and H4 are identical (2.05 Å) and slightly longer than that in HRP (2.04 Å). The Fe−Ct distance in HRP is identical to that in H8 (0.5 Å), marginally larger than the value for H4 (0.45 Å). It should be noted that all of these values are within the respective errors from each other. It seems, therefore, that either the relative expansion of the heme does not correlate with the redox potential of the protein or that structural differences that might be important to function are masked by errors in the measurements.

Farhangrazi et al. (1992) showed that H2 and H8 have similar reactivities toward halide; both are able to oxidize bromide while neither catalyze the oxidation of chloride. H2 does not exhibit a significantly more expanded heme than H8, and it is possible that reactivity studies using compounds with smaller changes in redox potential than the difference between Br^- and Cl^- will reveal additional reactivity differences to support or refute the hypothesis.

deRopp et al. (1991) presented ^1H NMR studies of LiP at 25 °C. The resonances for His177 (the proximal ligand to the iron) show significantly reduced hydrogen bond strength for the imidazole of LiP when compared to HRP. The weaker hydrogen bond in LiP is also observed in resonance Raman studies (Andersson et al., 1985). Andrawis et al. (1988) suggest that the weaker hydrogen bond might provide an explanation of both the higher oxidation potential and the reduced stability of compound I in LiP. A weaker hydrogen bond at N_δ might be expected to withdraw less electron density from the imidazole ring, which in turn could result in a longer Fe−N_ϵ bond. We have used a similar argument (Sinclair et al., 1992) to explain the differences between globin and peroxidase Fe−N_ϵ length. Both H8 and H2 have a marginally longer Fe−N_ϵ bond than HRP (1.93 vs 1.90 Å). The activity of H4 is very similar to HRP as reported by Popp and Kirk (1990). However, H4, does not fit the trend. As the redox activity of H4 is similar to the activity of Mn^{3+} chelates, there may be additional phenomena, such as Mn being used as a mediator, that bias the apparent H4 redox activity. Consequently, Fe−N_ϵ distance could still provide an indicator of the redox potential of a peroxidase.

Studies on lactoperoxidase (LPO; Chang et al., 1992) reveal a longer Fe−N_ϵ distance than HRP, H2, or H8, suggesting its redox activity could be higher. Our reactivity studies do not allow us to discriminate between H8, H2, and LPO, so additional data are needed to examine this possibility.

We have investigated the heme−iron structure of six LiP and MnP isoenzymes and shown that all share structural features typical of peroxidases. There are differences in the second and third shell atoms, which are revealed by the Fourier transforms in Figure 2. The second shell data suggest that H4, H8, and H10 form a group, H3 and H5 form a second group, and H2 is different from either. These differences can be attributed to outer shells in the heme and the proximal histidine ligand. Such differences have been observed in heme model compound studies and may be due to differences in the heme conformation such as buckling, ruffling, or twisting of the heme. These differences in heme conformation also effect the LFIR parameter which roughly follows this sequence. In both cases, H2 is different from the other isoenzymes.

ACKNOWLEDGMENT

We thank Dr. Syed Khalid for invaluable assistance at NSLS beamline X9. We also thank Michael Chen and Bing He for technical assistance with XAS data collection.

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BI950619R