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Oxidation–Reduction Potentials and Ionization States of Extracellular Peroxidases from the Lignin-Degrading Fungus *Phanerochaete chrysosporium*†

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ABSTRACT: The oxidation–reduction potentials of lignin peroxidase isozymes H1, H2, H8, and H10 as well as the Mn-dependent peroxidase isozymes H3 and H4 are reported. The potentiometric titrations involving the ferrous and ferric states of the enzyme had Nernst plots indicating single-electron transfer. The E_{m7} values of lignin peroxidase isozymes H1, H2, H8, and H10 are −142, −135, −137, and −127 mV versus standard hydrogen electrode, respectively. The E_{m7} values for the Mn-dependent peroxidase isozymes H3 and H4 are −88 and −93 mV versus standard hydrogen electrode, respectively. The midpoint potential of H1, H8, and H4 remained unchanged in the presence of their respective substrates, veratryl alcohol and Mn(II). The midpoint potential between the ferric and ferrous forms of isozymes H1 and H4 exhibited a pH-dependent change between pH 3.5 and pH 6.5. These results indicate that the reductive half-reaction of the enzymes is the following: ferric peroxidase + $1e^-$ + H^+ → ferrous peroxidase. Above pH 6.5, the effect of pH on the midpoint potential is diminished and indicates that an ionization with an apparent pK_a equal to approximately 6.6–6.7 occurs in the reduced form of the enzymes. A heme-linked ionization group in the ferrous form of the enzymes was confirmed by studying the effect of pH on the absorption spectra of isozymes H1 and H4. These spectrophotometric pH titration experiments confirmed the electrochemical results indicating pK_a values of 6.59 and 6.69 for reduced isozymes H1 and H4, respectively. These results indicate the presence of a heme-linked ionization of an amino acid in the reduced form of the lignin peroxidase isozymes similar to that of other plant peroxidases.

The oxidation–reduction potentials of the ferric/ferrous couple of the lignin peroxidase (ligninase) isozymes and the Mn-dependent peroxidase isozymes of *Phanerochaete chrysosporium* were determined in the present study. These two classes of isozymes are the predominant enzymes produced by the white-rot fungus *P. chrysosporium* and are thought to impart this wood-destroying fungus the ability to degrade lignin (Tien & Kirk, 1984; Glenn & Gold, 1985; Paszczynski et al., 1986). Both types of isozymes are heme-containing glycoprotein peroxidases. The heme iron in the native protein is in the high-spin, pentacoordinate ferric state with a histidine residue coordinated as the fifth ligand (Tien & Kirk, 1984; Kuila et al., 1985; Andersson et al., 1987). The primary structures deduced from sequencing the cDNA encoding for the lignin peroxidases (Tien & Tu, 1987a,b; de Boer et al., 1987) and Mn-dependent peroxidases (Pribnow et al., 1989; Pease et al., 1989) reveal homology of active-site residues with other peroxidases.

The lignin and the Mn-dependent peroxidases show different substrate specificity. The lignin peroxidase isozymes oxidize not only lignin and lignin model compounds (Tien & Kirk, 1983; Glenn et al., 1983; Higuchi, 1985) but also a large number of other organic compounds (Kamaya & Higuchi, 1984; Kersten et al., 1985; Hammel et al., 1986; Kirk et al., 1986). The Mn-dependent peroxidase isozymes oxidize a number of phenolic compounds and dyes as well as several amines (Kuwahara et al., 1984; Glenn & Gold, 1985; Paszczynski et al., 1986). Like other peroxidases, both the lignin and Mn-dependent peroxidases utilize H_2O_2 as the oxidant to achieve the higher oxidation state of compound I, which is 2 oxidation equivalents above the native enzyme (Chance, 1952). Compound I returns to native enzyme by sequential one-electron oxidations of aromatic substrates by way of the one-electron oxidized intermediate compound II (Nakamura et al., 1985; Andrawis et al., 1988). Lignin peroxidases utilize aromatic substrates whereas the Mn-de-

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pendent peroxidases are unique in utilizing Mn(II) as the reducing substrate. The catalytic cycle thus entails the oxidation of Mn(II) by compound I or/and compound II to yield Mn(III). Mn(III), in turn, mediates the oxidation of organic substrates (Glenn & Gold, 1985):

$$Mn(III) + RH \rightarrow Mn(II) + R^{\cdot} + H^{+} \quad (1)$$

Although mechanisticaly similar to other peroxidases, the Mn-dependent and the lignin peroxidase isozymes are distinct in their ability to oxidize substrates of high redox potential (Hammel et al., 1986; Kersten et al., 1985; Kamaya & Higuchi, 1984) and in their low pH optimum for activity (Tien et al., 1986; Andrawis et al., 1988). These properties suggest (i) a heme environment which is more electron deficient than most other peroxidases and (ii) differences in the active-site residues which control the pH dependency. The electrochemical properties of heme proteins have frequently been used to probe their active-site structure. As part of an overall goal to characterize the heme active site, the present study characterizes the oxidation–reduction potential of the extracellular peroxidases from *P. chrysosporium*. Our results demonstrate that the active site is more electron deficient than horseradish peroxidase (HRP)[1] and the presence of an ionizable group in the ferrous enzyme which is similar to other peroxidases.

Experimental Procedures

Materials. Methyl viologen and blue agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Safranin O, methylene blue, phenazine methosulfate, and indigo disulfonate were purchased from Kodak Chemical Co. (Rochester, NY). Lumiflavin 3-acetate was kindly supplied by Sandro Ghisla, University of Konstanz, Konstanz, FRG. Gold, silver, and platinum wires were purchased from Johnson Matthey, Inc. (Seabrook, NH). Argon gas was freed of trace dioxygen by passage over a Cu–Cr catalyst (Harshaw Cu 1164T, BASF, Cleveland, OH) at 150 °C. It was then H_2O-saturated by passage through distilled water containing reduced methyl viologen as an indicator for anaerobiosis. All other chemicals were reagent grade and used without further purification.

Enzyme Purification. Lignin peroxidase isozymes H1 (p*I* = 4.7), H2 (p*I* = 4.4), H8 (p*I* = 3.5), and H10 (p*I* = 3.3) [as designated by Kirk et al. (1985)] were isolated and purified from *P. chrysosporium* strain BKM-F-1767 as previously reported (Kirk et al., 1985). The Mn-dependent peroxidase isozymes were purified by modifying a method previously reported (Kuwahara et al., 1984). A strain (derived from strain BKM-F-1767; S. Myer and M. Tien, unpublished results) overproducing Mn-dependent peroxidase isozymes was used for production of the enzyme. Extracellular fluid from 5-day-old cultures was harvested and subjected to chromatography on Mono Q as described previously (Kirk et al., 1985). Protein peaks containing isozymes H3, H4, and H5 were pooled from numerous preparations and dialyzed against 50 mM sodium succinate, pH 4.5. These isozymes were further purified utilizing blue agarose chromatography as previously described (Glenn & Gold, 1985). Enzyme activity was measured by phenol red oxidation (Kuwahara et al., 1984). Protein preparations typically exhibited R_Z values of 5 for the Mn-dependent peroxidases (A_{406}/A_{280}) and 4 for the lignin peroxidases (A_{409}/A_{280}).

[1] Abbreviations: HRP, horseradish peroxidase; E_m, midpoint potential; E_{m7}, midpoint potential at pH 7; CCP, cytochrome *c* peroxidase; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Characterization of Enzymes. Enzyme concentrations were determined spectrophotometrically by using the extinction coefficients for the Soret band: 189 mM^{-1} cm^{-1} for H10, 168 mM^{-1} cm^{-1} for H8, 165 mM^{-1} cm^{-1} for H2, 169 mM^{-1} cm^{-1} for H1 (Farrell et al., 1989). The extinction coefficient for the Mn-dependent peroxidases was determined experimentally by using the pyridine hemochromogen method (Miller & Gennis, 1983).

Spectroelectrochemical Measurements. All absorption spectra were obtained with a Perkin-Elmer Lambda 4B UV/vis spectrophotometer. Redox potentiometric titrations at pH 7.0 were carried out as described by Stankovich (1980) in an anaerobic spectroelectrochemical cell. The potential during the titrations was controlled with a Model 371 Potentiostat-Galvanostat from Princeton Applied Research, Inc. All potentials are reported versus the standard hydrogen electrode. The working electrode was a gold coiled wire. The auxiliary electrode was a silver–silver chloride electrode where the silver wire was contained in a fritted compartment which was filled with 0.1 M KCl solution. The reference electrode was a silver–silver chloride electrode whose potential was checked before and after each experiment against standard ferro–ferricyanide (O'Reilly, 1973). The enzyme concentration was typically between 7 and 10 μM during the potentiometric titrations. All potentiometric titrations were carried out in the presence of mediator dyes: 100 μM methyl viologen and a mixture of lumiflavin 3-acetate, indigo disulfonate, phenazine methosulfate, and methylene blue at a concentration of 1 μM each. Spectral changes and the potential readings were recorded when changes in potential were less than 0.1 mV/min, which typically required 10–50 min for chemical titrations (with singly reduced methyl viologen, see below) and 50–100 min for electrochemical titrations. The buffers were at a constant ionic strength of 0.1 and are described in the figure legends. For pH experiments conducted below pH 7.0, the reduction of protons by the gold electrode became more kinetically favorable. Consequently, the singly reduced methyl viologen was titrated into the spectroelectrochemical cell from a 50-μL Hamilton gas-tight syringe in the absence of the auxiliary electrode. The pK_a values from the electrochemical studies were determined by computer fitting the E_m and pH values to the equation:

$$E_m = E_m(\text{min}) + 0.059 \log [(10^{-pK_a} + 10^{-pH})/(10^{-pK_a})]$$

where E_m(min) is equal to the midpoint potential for the basic form of the enzyme. The correlation coefficient for the fit for isozymes H1 and H4 was equal to or better than 0.996.

The singly reduced methyl viologen was generated by reducing methyl viologen with hydrogen using palladium black as a catalyst in an unbuffered solution at pH 11. After reduction, the methyl viologen solution was filtered, evacuated, and flushed with oxygen-free argon gas. All experiments were performed at 25 °C.

Spectrophotometric pH Titrations. The effect of pH on the absorption spectra of the reduced enzymes was studied with an anaerobic spectrophotometric cell containing two side arms. The enzyme solution (~50 μM) in 5 mM sodium phosphate buffer, pH 7, was placed in the cell with 50 μL of concentrated buffer in one of the side arms. The concentrated buffers are as described in the figure legends and were varied in concentration to maintain an ionic strength of 0.8. The cell was alternately evacuated and purged with argon. A few crystals of sodium dithionite were added. After reduction (as determined by no further absorbance changes at 560 nm), the absorption spectrum of the enzyme solution was recorded. The concentrated buffer was then added from the side arm of the

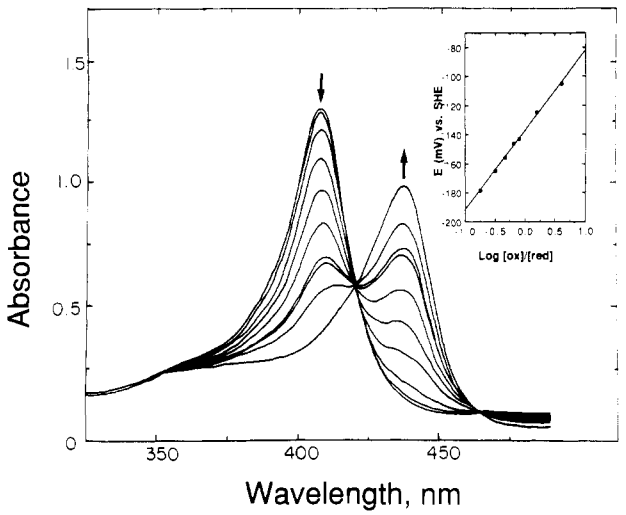

FIGURE 1: Spectral changes of lignin peroxidase isozyme H2 during potentiometric titration. The absorption spectrum of the ferric enzyme shows an absorption maximum at 409 nm. Addition of charge converts the enzyme to the ferrous state which shows an absorption maximum at 437 nm. Direction of spectral change is indicated by arrows. The concentration of lignin peroxidases is 7.7 μM in 40 mM sodium phosphate, pH 7.0. The inset shows the Nernst plot. See Experimental Procedures for details.

cell to adjust the pH, and the spectrum was recorded. The change in the absorbance at 560 nm was then plotted versus pH. The pK_a values were determined by computer fitting the data to the equation:

$$\Delta A_{560nm} = \Delta A_{560nm}(max)10^{-pH}/(10^{-pH} + 10^{-pK_a})$$

The correlation coefficient of the fit for isozymes H1 and H4 was equal to or better than 0.996.

Results

Purification and Physical Properties of the Mn-Dependent Peroxidases. Three major Mn-dependent peroxidase isozymes were eluted from the blue agarose column with a linear NaCl gradient (0–0.4 M). The molecular weights of these isozymes, H3, H4, and H5, are approximately 45 000, with isozyme H5 being slightly larger as determined by SDS–PAGE. Isoelectric focusing confirmed the purity of isozymes H3 and H4; however, H5 contained a mixture of two isozymes. The p*I* values for the isozymes are 4.9 for isozyme H3, 4.5 for isozyme H4, and 4.1–4.3 for the two isozymes found in the H5 fraction.

The extinction coefficients for isozymes H3 and H4 were determined by the pyridine hemochromogen method. In 5 mM sodium succinate, pH 4.5, the extinction coefficient is 125 mM^{-1} cm^{-1} for isozyme H3 and 127 mM^{-1} cm^{-1} for isozyme H4.

Oxidation–Reduction Potential of the Lignin and Mn-Dependent Peroxidases. Figure 1 illustrates spectral changes observed in the Soret region of lignin peroxidase isozyme H2 between the ferric and ferrous states. Successive addition of electrical charge results in a decrease in the 409-nm absorbance with a concomitant increase in the 437-nm absorbance, indicative of formation of ferrous enzyme. An increase in the 558-nm absorbance was also observed for the reduced form of the enzyme. Six sharp isosbestic points at 365, 420, 467, 526, 608, and 658 nm are observed during the reductive titration. Similar spectra are observed in the Soret region for the ferric and ferrous forms of lignin peroxidase isozymes H1, H8, and H10 as well as for the Mn-dependent peroxidase isozymes H3 and H4.

The inset in Figure 1 shows a typical Nernst plot for the titration of lignin peroxidase isozyme H2 at pH 7. Similar plots were also obtained for all the other isozymes. The points are experimental data, and the line was calculated by a least-squares fitting. In all cases, the potentiometric titrations yielded Nernst plots with slopes of 59 mV, indicative of reversible single-electron transfer.

Table I: E_{m7} Values for Lignin Peroxidases, Mn-Dependent Peroxidases, and Several Heme Proteins

heme protein	E_{m7} (mV)	ref
HRP	−278	Makino et al. (1976); Yamada et al. (1975)
turnip peroxidase (P_1)	−223	Ricard et al. (1972)
CCP	−194	Conroy et al. (1978)
lignin peroxidase H1	−142	this work
chloroperoxidase	−140	Makino et al. (1976)
lignin peroxidase H8	−137	this work
lignin peroxidase H2	−135	this work
lignin peroxidase H10	−127	this work
turnip peroxidase (P_7)	−100	Ricard et al. (1972)
Mn-dependent peroxidase H4	−93	this work
Mn-dependent peroxidase H3	−88	this work
myeloperoxidase	+24	Ikeda-Saito & Prince (1985)
metmyoglobin	+46	Taylor & Morgan (1942)

Table II: Effect of Substrate on the E_m of Mn-Dependent and Lignin Peroxidases[a]

isozyme	E_m (mV) (−substrate)	E_m (mV)[b] (+substrate)
lignin peroxidase H1	+64	+64
Mn-dependent peroxidase H4	+78	+79
lignin peroxidase H8	+70	+67

[a] E_m measurements determined at 25 °C in sodium tartrate, pH 3.5. [b] Veratryl alcohol concentration was 0.5 mM for isozyme H1 and 0.75 mM for isozyme H8. The $MnSO_4$ concentration was 1.0 mM for isozyme H4.

The E_{m7} values of the ferric–ferrous couple for the lignin and Mn-dependent peroxidase isozymes are summarized in Table I along with the potentials of several plant peroxidases and a mammalian peroxidase. The experimentally determined E_{m7} values for the lignin peroxidase isozymes H1, H2, H8, and H10 are very similar, near −130 mV (Table I). The E_{m7} values for the Mn-dependent peroxidase isozymes H3 and H4 are similar to each other (−88 and −95 mV, respectively), but as a class, they are more positive than the lignin peroxidases.

Effect of Substrate on the E_m. The E_m values for lignin peroxidase isozymes H1 and H8 and Mn-dependent peroxidase isozyme H4 are not affected by their respective substrates, veratryl alcohol or Mn(II) (Table II). The E_m values at pH 3.5 for lignin peroxidase isozymes H1 and H8 in the presence of saturating veratryl alcohol remained unchanged. The E_m value for the Mn-dependent isozyme H4 at pH 3.5 also remained unchanged in the presence of substrate Mn(II).

Effect of pH on the E_m. The pH dependence of the E_m of lignin peroxidase isozyme H1 and of the Mn-dependent peroxidase isozyme H4 is shown in Figure 2. The E_m for both isozymes increases linearly as the pH changes from pH 6.5 to 3.5. A relationship of 56 mV to 52 mV per pH unit is observed, which is in agreement with the theoretical value (59 mV/pH at 25 °C) for a one electron–one proton transfer process. From the E_m versus pH curves, an ionization with an apparent pK_a of approximately 6.70 for isozyme H1 and 6.63 for isozyme H4 was determined for the reduced form of the enzymes (Figure 2). At higher pH, the isozymes are coverted to a denatured species as observed by a decrease in absorbance in the Soret region. Thus, the electrochemical experiments were limited to pH values below 7.5.

Effect of pH on the Absorption Spectrum of Reduced Isozymes H1 and H4. The ionization in the ferrous form of

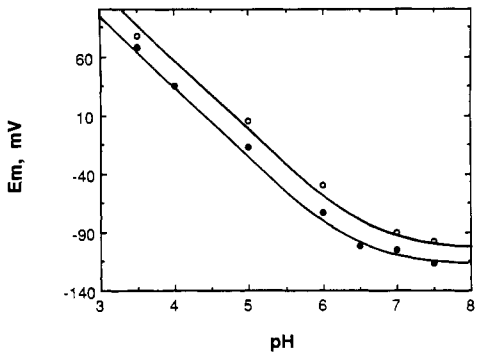

FIGURE 2: Effect of pH on the E_m of Mn-dependent and lignin peroxidases. The experimental conditions were the same for both peroxidases as described under Experimental Procedures. The measured potential expressed in millivolts relative to the standard hydrogen electrode is plotted as a function of pH for lignin peroxidase isozyme H1 (closed circles) and Mn-dependent peroxidase isozyme H4 (open circles). The experimental data were fitted to the equation described under Experimental Procedures for a single ionization. The buffers used were sodium phosphate for pH 7.5–5.5 and sodium tartrate for pH 5.5–3.5 and were of the same ionic strength.

the enzymes (H1 and H4) was confirmed by studying the effect of pH on the visible spectrum of the ferrous enzymes.[2] The spectral changes are small but measurable. The absorption spectra of isozyme H1 in pH 7.5 and pH 4.6 are shown in Figure 3, upper panel. The inset shows the change in 560-nm absorbance as a function of pH. An apparent pK_a of 6.59 is obtained from computer fitting, which is in close agreement with the pK_a of 6.70 calculated from the electrochemical experiments (Figure 2). Analogous results were obtained with Mn-dependent peroxidase isozyme H4 (Figure 3, lower panel). An apparent pK_a of 6.69 is obtained for isozyme H4 (inset of Figure 3), in close agreement with the value of 6.63 from the electrochemical results (Figure 2). The differences between these values and those obtained by potentiometric titrations are probably due to the differences in ionic strengths for the two experiments.

Discussion

In order to probe the environment of the heme active site of the lignin and Mn-dependent peroxidases, we have characterized the electrochemical and spectral properties of the enzymes as a function of pH. When compared to other peroxidases [excluding myeloperoxidase which contains a chlorin as a prosthetic group (Ikeda-Saito et al., 1984; Sibbett & Hurst, 1984; Ikeda-Saito & Prince, 1985) and chloroperoxidase which contains a thiolate ligand (Cramer et al., 1978)], the E_{m7} values of the lignin and Mn-dependent peroxidases are more positive. The higher E_{m7} values for the ferric/ferrous couple are consistent with the heme active site of these fungal peroxidases being somewhat more electron deficient. This is in keeping with the ability of these enzymes to oxidize substrates of higher oxidation–reduction potential. These results would suggest a compound I or II intermediate more electron deficient and consequently of higher oxidation–reduction potential. The oxidation–reduction potentials of HRP compounds I and II have been measured to be both approximately 0.95 V (Hayashi & Yamazaki, 1979). The oxidation–reduction potential of lignin peroxidase compound I, estimated from the oxidation–reduction potential of its substrates, indicates a potential much higher than that of HRP (Hammel et al., 1986). The oxidation–reduction potential of Mn-dependent

[2] The pH-dependent spectral changes in the reduced enzyme were first observed in collaborative studies by Chris Bull.

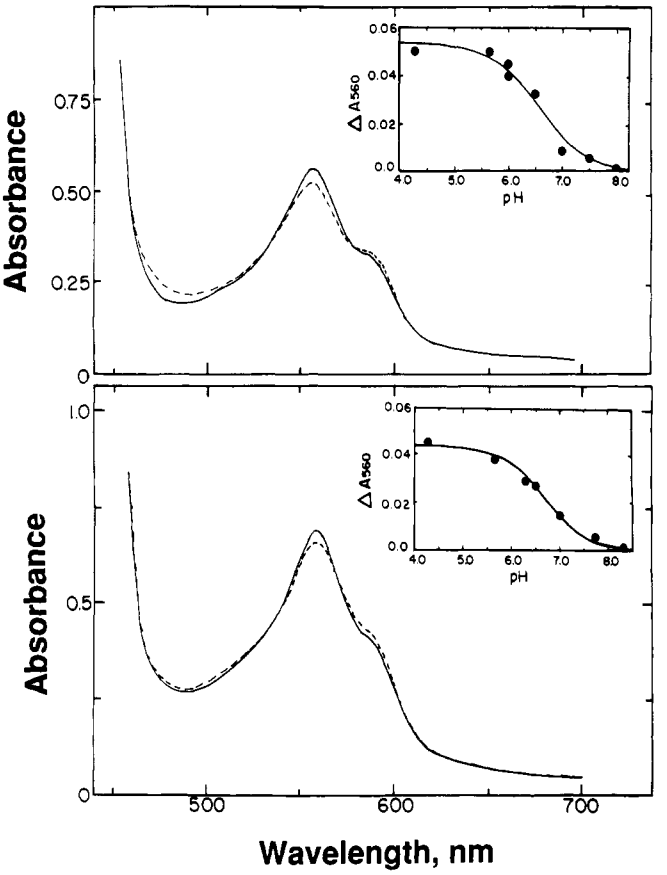

FIGURE 3: Effect of pH on the absorption spectrum in the visible region of ferrous lignin peroxidase isozyme H1(upper panel) and Mn-dependent peroxidase isozyme H4 (lower panel). The upper panel shows the absorption spectrum of ferrous isozyme H1 at pH 7.0 (solid line) and pH 4.6 (dashed line). The inset plots the absorbance change at 560 nm as a function of pH. The line is drawn from a computer fit as described under Experimental Procedures. The lower panel shows the absorption spectrum of isozyme H4 at pH 7.0 (solid line) and pH 5.2 (dashed line). The inset plots the absorbance change at 560 nm as a function of pH. The line is drawn from a computer fit as described under Experimental Procedures. The buffers used were sodium phosphate for pH 7.5–5.6 and sodium tartrate for pH 5.5–4.0 as described under Experimental Procedures.

peroxidase compound I is also estimated to be high; the oxidation–reduction potential of Mn(III) is 1.5 V.

The pH-dependent electrochemical and spectral changes indicate the presence of an ionization in the ferrous enzymes. Oxidation-linked ionizations have been characterized in other peroxidases. Theorell (1947) noted the existence of such an ionization for HRP. This was later confirmed by Harbury (1957), who showed a pH dependency of the oxidation–reduction potential of HRP (pK_a = 7.38). Studies by Yamada and Yamazaki (1974) confirmed the presence of such an ionization by demonstrating a pH-dependent spectral change in the ferrous form of HRP. Similar ionizations in the ferrous enzyme were observed by Ricard et al. (1972) for two isozymes of turnip peroxidase, P_1 and P_7. pK_a values of 6.3 and 5 were calculated for isozymes P_1 and P_7, respectively. Residues in the distal pocket were proposed to be responsible for the ionizations, a histidine for isozyme P_1 and a carboxyl group for isozyme P_7. The ionizations observed for both the lignin and Mn-dependent peroxidases are within the range for a histidine residue. Nucleotide sequencing of cDNA clones encoding the lignin (Tien & Tu, 1987a,b; de Boer et al., 1987) and Mn-dependent peroxidases (Pribnow et al., 1989; Pease et al., 1989) shows that a histidine is found in the distal pocket. Residues flanking this histidine are also conserved with the

lignin and Mn-dependent peroxidases.

The presence of a distal histidine in lignin and Mn-dependent peroxidases and similarities in its effect on the spectral and electrochemical properties would suggest similarities in environment for this histidine with HRP and turnip peroxidase P_1. This same histidine has been proposed to be the ionization controlling compound I formation where the acid form of the enzyme does not react with H_2O_2 (Poulos & Kraut, 1980). In contrast, the reaction of the lignin (Andrawis et al., 1988; Harvey et al., 1989) and Mn-dependent peroxidases (Wariishi et al., 1989) with H_2O_2 to form compound I exhibits no pH dependency from pH 2 to 7.5. A shift of nearly 5 for the pK_a value of the distal histidine from the ferrous to the ferric form is unlikely because this would require approximately 5 kcal/mol. An alternate residue, aspartic acid [Asp43 in HRP (Welinder, 1979), Asp48 in lignin peroxidase (Tien & Tu, 1987a,b), and Asp47 in the Mn-dependent peroxidase (Pribnow et al., 1989; Pease et al., 1989)], has been proposed by Dunford and Araiso (1979) to control compound I formation. The carboxylic acid group of an aspartic acid, if located near a positively charged residue, can have its pK_a value shifted to below 2. The participation of such a group in compound I formation is more consistent with the results obtained with the lignin and Mn-dependent peroxidases.

The E_{m7} values for the lignin and Mn-dependent peroxidases are not affected by their respective substrates, veratryl alcohol and Mn(II). These results suggest that substrates probably do not directly interact with the ferric heme iron as axial ligands. This is consistent with the present model for peroxidase catalysis. Suicide inhibitor (Ator & Ortiz de Montellano, 1987; Ator et al., 1987) and NMR studies (La Mar & de Ropp, 1979; Sakurada et al., 1986) indicate that the heme iron of HRP is not fully accessible to bulky substrates and suggest that the oxidation of substrates occurs at the periphery of the heme. Our results using the mechanism-based inhibitor phenylhydrazine (C. Millis and M. Tien, unpublished results) are consistent with such a model for lignin peroxidase. They indicate that only the heme periphery is accessible to the organic substrate.

A factor which can affect the oxidation–reduction potential of heme proteins is the basicity of the axial ligand (Falk, 1964; Falk & Perrin, 1961). The axial ligand of metmyoglobin, CCP, and HRP (as well as the enzymes studied here) is the imidazole of the proximal histidine. Variation in H bonding strength is observed for the N_δ of this imidazole with neighboring amino acid residues. The N_δ proton is weakly H bonded to the carbonyl of Leu89 in metmyoglobin (Takano, 1977). The H bonding is somewhat stronger in CCP; the N_δ proton is bonded to Gln239 which in turn is H bonded to Glu187 (Poulos & Kraut, 1980). Although no crystal structure is available for HRP, resonance Raman (Teraoka & Kitagawa, 1981) and ^1H NMR (de Ropp et al., 1985; La Mar & de Ropp, 1982) studies have revealed the H bonding of the N_δ proton of the proximal histidine to be even stronger. Strong H bonding of the axial imidazole would impart more basicity to the axial ligand which in turn has numerous structural consequences which have been experimentally substantiated: (i) Increased basicity would also tend to repel a bonding pair of electrons from a sixth ligand such as water. Thus, metmyoglobin, which is weakly H bonded, is hexacoordinated with water as the axial ligand, and HRP, which is strongly H bonded (La Mar & de Ropp, 1982), is pentacoordinated. The H-bonding strength of CCP appears to be intermediate between metmyoglobin and HRP (Stein et al., 1980; Poulos & Finzel, 1984). CCP shows a temperature-dependent equilibrium in coordination state. At room temperature, a high percentage of CCP is hexacoordinated (Evangelista-Kirkup et al., 1985). (ii) Increased basicity would rather render the imidazole more capable of stabilizing both the ferric state of the resting enzyme (decreased E_{m7}) and the Fe(IV) states of compounds I and II (and thus decreasing their E_{m7} values). The E_{m7} values for the ferric/ferrous couple of metmyoglobin, CCP, and HRP shown in Table I are consistent with this observation.

The degree of H bonding of the axial imidazole for lignin peroxidase and Mn-dependent peroxidase is not as well characterized. However, resonance Raman spectroscopy suggests that for both enzymes, the axial imidazole is more strongly hydrogen bonded than metmyoglobin and less strongly hydrogen bonded than that of HRP (Kuila et al., 1985; Andersson et al., 1987). The Fe(II)–histidine stretching frequency is approximately 240 cm^{-1} for both enzymes, similar to that of HRP but higher than those of globins at ~220 cm^{-1} (Kuila et al., 1985; Mino et al., 1988). This has been attributed to stronger H bonding of the imidazole (Mino et al., 1988). For the Mn-dependent peroxidase, this stretch exhibits a lack of deuterium sensitivity (Mino et al., 1988) consistent with a high degree of H bonding.

The temperature-dependent change in coordination number of CCP has been observed for lignin peroxidase. Raman spectroscopy studies show that at 25 °C lignin peroxidase is largely pentacoordinated whereas at 2 °C the equilibrium shifts to a large proportion being hexacoordinated with water as the sixth ligand (Kuila et al., 1985; Andersson et al., 1987). This temperature-dependent shift in equilibrium has also been observed for Mn-dependent peroxidase (Mino et al., 1988). This, again, would place the lignin and Mn-dependent peroxidases between metmyoglobin and HRP in degree of H bonding of the axial imidazole. The results presented here reveal E_{m7} values for the lignin and Mn-dependent peroxidases higher than that for HRP but lower than that for metmyoglobin. This is in keeping with the heme active site of these two classes of enzymes being more electron deficient than HRP but less than that of metmyoglobin.

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