mechanism and role of bicarbonate, and, perhaps, the significance of the unusual quaternary structure of cyanase.

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Registry No. OCN^-, 661-20-1; HCO_3^-, 71-52-3; N_3^-, 14343-69-2; HOAc, 64-19-7; Cl^-, 16887-00-6; Br^-, 24959-67-9; HCO_2H, 64-18-6; NO_3^-, 14797-55-8; NO_2^-, 14797-65-0; $CH_2(CO_2H)_2$, 141-82-2; $(CO_2H)_2$, 144-62-7; $HO_2CCOCH_2CO_2H$, 328-42-7; cyanase, 37289-24-0.

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Spectral Characterization of the Oxidized States of Lignin Peroxidase, an Extracellular Heme Enzyme from the White Rot Basidiomycete *Phanerochaete chrysosporium*†

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ABSTRACT: Lignin peroxidase (diarylpropane oxygenase, ligninase, LiP), an H_2O_2-dependent lignin degrading heme enzyme from the basidiomycetous fungus *Phanerochaete chrysosporium*, catalyzes the oxidation of a variety of lignin model compounds. In this paper, we characterize the H_2O_2-oxidized forms of LiP compounds I, II, and III by electronic absorption spectroscopy. The native enzyme contains high-spin ferric iron and has its Soret maximum at 407.6 nm and α and β bands at 496 and 630 nm. Addition of 1 molar equiv of H_2O_2 to the native enzyme in 20 mM sodium phosphate, pH 6.0, yields compound I, characterized by a Soret maximum at 408 nm with reduced intensity and by additional maxima at 550, 608, and 650 nm. Addition of 2 molar equiv of H_2O_2 to the native enzyme yields compound II, identified by absorption maxima at 420, 525, and 556 nm. Addition of a molar excess of H_2O_2 to the native enzyme yields compound III, which is characterized by absorption maxima at 419, 540, and 578 nm. These spectral characteristics are very similar to those of horseradish peroxidases (HRPs) I, II, and III. In 20 mM sodium phosphate, pH 6.0, LiP-I spontaneously converts to LiP-II with a $t_{1/2}$ of $\sim$1.0 min, and this conversion is characterized by a single isosbestic point in the visible absorption spectrum at 565 nm. The organic peroxides *m*-(chloroperoxy)benzoic acid and *p*-(nitroperoxy)benzoic acid oxidize LiP to compounds I and II, respectively. Addition of 0.5 molar equiv of veratryl alcohol, a two-electron substrate, to LiP-I rapidly reduces it to LiP-II; further addition of 0.5 molar equiv of veratryl alcohol to LiP-II reduces it to the native ferric enzyme. This indicates that like HRP-I and HRP-II compounds I and II of lignin peroxidase contain 2 and 1 oxidizing equiv over the ferric resting state of the enzyme. It also suggests that the dehydrogenation of veratryl alcohol by this enzyme proceeds via two single-electron oxidation steps rather than through a single two-electron oxidation mechanism.

Lignin is a complex, optically inactive, heterogeneous, and random biopolymer (Sarkanen, 1971) that comprises 20–30% of woody plants. Since the biodegradation of cellulose is also retarded by the presence of lignin (Crawford, 1981), the catabolism and utilization of this intractable phenylpropanoid polymer are of enormous importance and interest. In nature, white not basidiomycetous fungi are primarily responsible for the decomposition of lignin (Crawford, 1981). Recent studies have shown that when cultured under aerobic conditions, the white rot basidiomycete *Phanerochaete chrysosporium* produces two extracellular H_2O_2-dependent enzymes (Kuwahara et al., 1984; Glenn & Gold, 1985), lignin peroxidase and an Mn(II) peroxidase.

Lignin peroxidase (ligninase, diarylpropane oxygenase, LiP)[1] has been purified to homogeneity from the extracellular me-

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[1] Abbreviations: EPR, electron paramagnetic resonance; LiP, lignin peroxidase; HRP, horseradish peroxidase; KTBA, 2-keto-4-(methylthio)butyric acid; CPO, chloroperoxidase; CAT, catalase; mCPBA, *m*-(chloroperoxy)benzoic acid; pNPBA, *p*-(nitroperoxy)benzoic acid.

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dium of large agitated (Gold et al., 1984) and small stationary cultures (Tien & Kirk, 1984) of *P. chrysosporium*. Three different molecular forms of lignin peroxidase have recently been identified (Renganathan et al., 1985). The main isozymic form of lignin peroxidase has a M_r 41 000, and all forms have a single iron protoporphyrin IX prosthetic group (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). Electronic absorption (Gold et al., 1984), EPR, and resonance Raman (Andersson et al., 1985; Kuila et al., 1985) data suggest that the heme iron in the native protein is in the high-spin ferric state, with histidine coordinated as the fifth ligand and a possible weakly bound water as a sixth ligand. Homogeneous lignin peroxidase is H_2O_2-dependent and catalyzes the oxidation of a variety of lignin model compounds such as 1-(3′,4′-diethoxyphenyl)-1,3-dihydroxy-2-(4″-methoxyphenyl)propane (diarylpropane), 1-(4′-ethoxy-3′-methoxyphenyl)glycerol 2-guaiacyl ether (β-aryl ether), 1-(4′-ethoxy-3′-methoxyphenyl)propane (phenylpropane), 1-(4′-ethoxy-3′-methoxyphenyl)propene (phenylpropene), and 3,4-dimethoxybenzyl alcohol (veratryl alcohol) (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). Initially this enzyme was characterized as an oxygenase since under aerobic conditions one atom of dioxygen was incorporated into various products (Gold et al., 1983; Kuwahara et al., 1984; Tien & Kirk, 1984). However, our most recent studies indicate that oxygenation proceeds even under rigorously anaerobic conditions, with incorporation of ^{18}O from $H_2{}^{18}O$ into the products (Renganathan et al., 1986). This suggests that this enzyme functions as a peroxidase rather than as an oxygenase and O_2 serves only as a scavenger of radicals produced during the peroxidase reaction. In order to more fully understand the mechanism of lignin peroxidase, we have prepared all of the oxidation states and peroxide reaction intermediates of this enzyme using H_2O_2, *m*-(chloroperoxy)benzoic acid (mCPBA), and *p*-(nitroperoxy)benzoic acid (pNPBA) as oxidants. In addition, using various substrates we have reduced the oxidized forms of the enzyme back to the native ferric state. These experiments indicate that the lignin peroxidase catalytic cycle is essentially similar to that of HRP (Dunford & Stillman, 1976; Dunford, 1982).

Materials and Methods

Chemicals. Cumene hydroperoxide and H_2O_2 were obtained from Sigma Chemical Co. *tert*-Butyl hydroperoxide, *m*-(chloroperoxy)benzoic acid, and *p*-(nitroperoxy)benzoic acid were obtained from Aldrich Chemical Co. All other chemicals were reagent-grade. Buffers were prepared in water that had been purified initially by deionization and then by triple glass distillation, once with potassium permanganate and twice by normal methods (Schultz et al., 1984).

Lignin peroxidase was purified from the extracellular medium of acetate buffered agitated cultures of *P. chrysosporium* as previously described (Gold et al., 1984; Renganathan et al., 1985). The major molecular form (II) was separated from two minor forms by DEAE-Sepharose chromatography and further purified by gel filtration on a Sephadex G-100 column (Renganathan et al., 1985). The purified protein was electrophoretically homogeneous and had an R_z value (A_{407}/A_{280}) of ~5.0. The enzyme preparation used for spectral studies was dialyzed exhaustively against 20 mM sodium phosphate, pH 6.0.

Electronic absorption spectra were recorded on a Shimadzu UV-260 spectrophotometer at room temperature with a spectral band width of 1.0 nm and cuvettes of 1-cm light path. Compounds I, II, and III of lignin peroxidase were prepared by adding 1, 2, and 25 equiv of H_2O_2 to the enzyme in 20 mM sodium phosphate, pH 6.0. mCPBA and pNPBA were dissolved in 2-methyl-2-propanol and diluted 100-fold in triply distilled water to a final stock concentration of 5 mM. One or two molar equivalents of these oxidants was then added to the enzyme as described. *tert*-Butyl hydroperoxide and cumene hydroperoxide were added to the enzyme as concentrated DMF solutions. Veratryl alcohol (3,4-dimethoxybenzyl alcohol), 2-keto-4-(methylthio)butyric acid (KTBA), phenol, ascorbic acid, syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), and vanillic acid (4-hydroxy-3-methoxybenzoic acid) were each added as water solutions to the oxidized enzymes in the concentrations described.

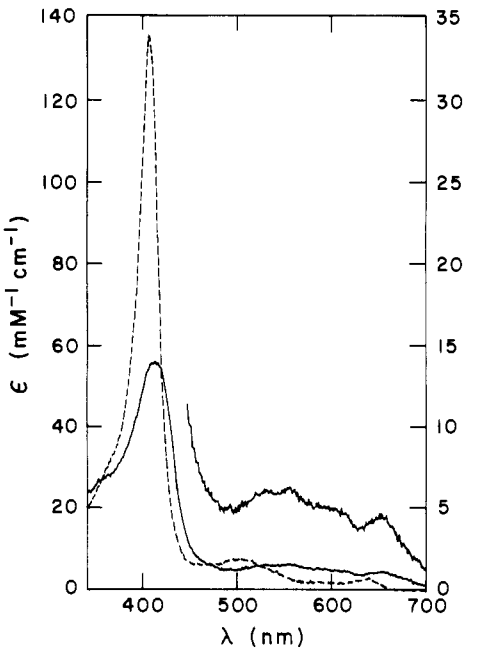

FIGURE 1: Electronic absorption spectrum of the native enzyme (--) and of compound I (—). To produce LiP-I, 1 molar equiv of H_2O_2 was added to the native enzyme (1.4 μM) in 1 mL of 20 mM sodium phosphate, pH 6.0. The spectra were recorded at room temperature in a cell with a 1-cm light path vs. a blank containing only 20 mM sodium phosphate, pH 6.0.

Table I: Oxidation States of Lignin Peroxidase

enzyme species	absorption maxima (nm) [ε (mM^{-1} cm^{-1})]				
Fe^{3+}[a,b]	407.6 (133)	496 (8.1)			630 (2.7)
Fe^{2+}[a,b]	435.6 (92.8)		552 (11.0)		
compound I	408.0 (55.0)		550	608	650 (4.5)
compound II	420.0 (70.6)	525 (6.8)	556 (7.5)		
compound III	419.0 (74.0)	543 (7.7)	578 (6.7)		
$Fe^{2+}O_2$[b]	414.2 (104.0)	543 (13.3)	578 (10.2)		
pNPBA oxidized	412.0 (63.0)	528 (6.5)	552 (6.5)		
mCPBA oxidized	408.0 (56.7)	525	550	608	650 (4.2)

[a] Gold et al., 1984. [b] Renganathan et al., 1985.

Results

The absorption spectrum of the native enzyme (Gold et al., 1984; Renganathan et al., 1985) shown in Figure 1, Table I, had a Soret maximum at 407.6 nm with α and β bands at 496 and 630 nm. Upon the addition of 1 equiv of H_2O_2, the Soret absorption band broadened, and the intensity was reduced to ~40% of that of the native enzyme. The visible region showed a broad absorption with peaks at 550, 608, and 650 nm (Figure 1, Table I). These characteristics are similar to those of HRP-I (Dunford, 1982). The addition of 2 equiv of H_2O_2 produced

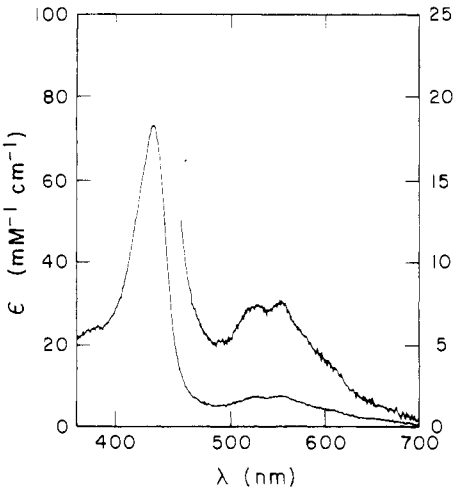

FIGURE 2: Electronic absorption spectrum of lignin peroxidase compound II. Two molar equivalents of H_2O_2 was added to the enzyme (1.4 μM) in 1 mL of 20 mM sodium phosphate, pH 6.0. Other procedures are as described in the text.

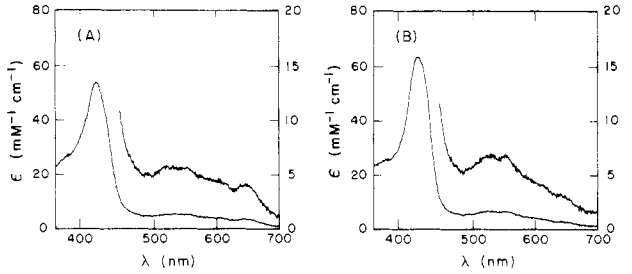

FIGURE 3: Absorption spectra of mCPBA- and pNPBA-oxidized lignin peroxidase: (A) 1 molar equiv of mCPBA was added to the enzyme (1.4 μM); (B) 2 molar equiv of pNPBA was added to the enzyme (1.4 μM). Other procedures were as described in the text. The oxidants were added to the enzyme as solutions in 2-methyl-2-propanol/water (1:100).

a spectrum similar to that of HRP-II (Dunford & Stillman, 1976; Dunford, 1982) with absorption maxima at 420, 525 and 556 nm (Figure 2, Table I). However, the extinction of the Soret maximum (420 nm) was only ~60% of that of the native enzyme. The oxidation of lignin peroxidase by several organic peroxides was also investigated. The addition of 1 molar equiv of mCPBA oxidized lignin peroxidase to a compound I intermediate (Figure 3A). However, the small band at 525 nm (Figure 3A, Table I) suggests that compound I prepared in this manner may be contaminated with a small amount of compound II. In contrast, the addition of 1 molar equiv of pNPBA had no observable effect on the enzyme. However, 2 molar equiv of this oxidant converted lignin peroxidase to a compound II like intermediate (Figure 3B). Finally, excess *tert*-butyl hydroperoxide, cumene hydroperoxide, or sodium chlorite did not result in the oxidation of lignin peroxidase.

The addition of excess H_2O_2 (25 molar equiv) to the native enzyme led to the formation of lignin peroxidase III. The absorption maxima of this intermediate were at 419, 543, and 578 nm (Figure 4, Table I).

Lignin peroxidase I was unstable at room temperature, spontaneously converting to lignin peroxidase II with a $t_{1/2}$ of ~1.0 min. The time dependence of the change in the visible spectrum from 500 to 700 nm is shown in Figure 5. There was a decrease in the maximum at 650 nm and an increase in the maxima at 525 and 550 nm. The spectra displayed a single isosbestic point at 565 nm. At pH 4.5, 1 molar equiv of the one-electron substrates *p*-cresol, syringic acid, and

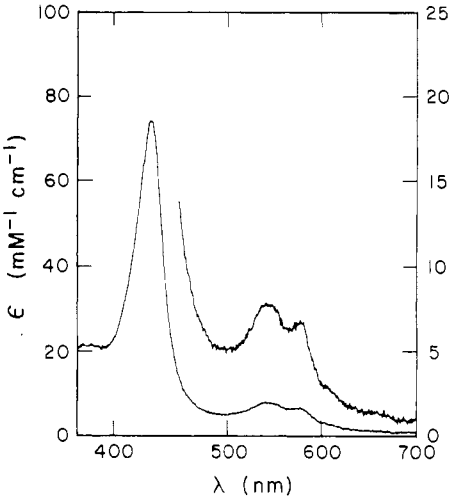

FIGURE 4: Electronic absorption spectrum of lignin peroxidase compound III. A total of 25 molar equiv of H_2O_2 was added to the enzyme (1.5 μM) in 1 mL of 20 mM sodium phosphate, pH 6.0. Other procedures are as described in the text.

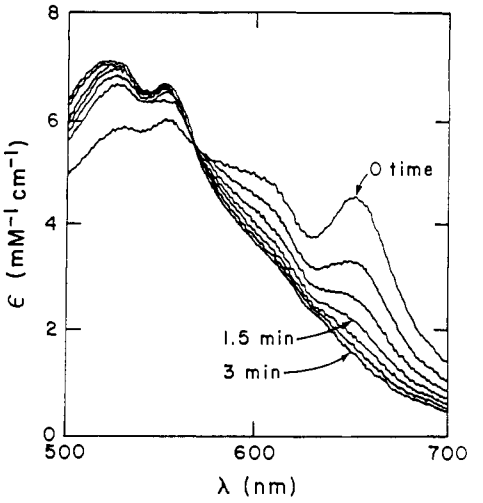

FIGURE 5: Spontaneous conversion of lignin peroxidase compound I to compound II. LiP-I (9 μM) was prepared as described in the text. The absorption spectrum between 500 and 700 nm was scanned at 30-s intervals.

vanillic acid or 0.5 molar equiv of the two-electron substrates veratryl alcohol or ascorbic acid rapidly reduced lignin peroxidase I to lignin peroxidase II. The addition of 2 molar equiv of *p*-cresol or 1 molar equiv of veratryl alcohol reduced lignin peroxidase I to the native enzyme. Finally, at pH 4.5, 1 molar equiv of *p*-cresol or 0.5 molar equiv of veratryl alcohol reduced lignin peroxidase II to the native enzyme. The addition of KTBA, a noncompetitive inhibitor of veratryl alcohol oxidation (Renganathan et al., 1985), did not result in the reduction of lignin peroxidase compound I or II.

Discussion

Lignin peroxidase is a heme-containing enzyme isolated from the extracellular medium of ligninolytic cultures of the white rot basidiomycete *P. chrysosporium* (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). The purified enzyme required H_2O_2 as a cosubstrate and oxidizes a variety of lignin model compounds (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985) including diarylpropanes, β-aryl ethers, phenylpropenes, and phenylpropanes. Like monooxygenases, under aerobic conditions it incorporates one atom of dioxygen into some products. The prosthetic group

Table II: Spectral Characteristics (nm) of H_2O_2 Reaction Intermediates of Several Peroxidases

peroxidase[a]	compound I	compound II	compound III	ref
LiP	408, 550, 608, 650	420, 525, 556	419, 543, 578	this work
HRP	400, 577, 622, 650	420, 527, 554	418, 546, 583	Dunford, 1976
CPO	367, 545, 610, 688	438, 542, 571	432, 555, 586	Palcic et al., 1980; Nakajima et al., 1985
LPO	410, 562, 600, 662	433, 537, 568	428, 551, 590	Kimura & Yamazaki, 1979

[a] Abbreviations: LiP, lignin peroxidase; HRP, horseradish peroxidase; CPO, chloroperoxidase; LPO, bovine lactoperoxidase.

of lignin peroxidase is iron protoporphyrin IX (Gold et al., 1984; Tien & Kirk, 1984). In this respect, lignin peroxidase resembles the globins, the P-450 cytochromes, and the peroxidases. Previous studies using electronic absorption (Gold et al., 1984), EPR, and resonance Raman spectroscopy (Andersson et al., 1985) indicate that the iron in the native protein is in the high-spin ferric oxidation state. These studies also suggest that a histidine is coordinated to the iron as a fifth ligand in lignin peroxidase. Myoglobin (Spiro, 1983; Desbois et al., 1984), yeast cytochrome *c* peroxidase (Poulos et al., 1980), and intestinal peroxidase (Kimura et al., 1981) all appear to be hexacoordinate with a histidine coordinated to the iron as a fifth ligand. Native HRP is pentacoordinate with a histidinate (Teraoka & Kitagawa, 1981) or a strongly hydrogen-bonded histidine (La Mar & De Ropp, 1982) as the fifth ligand. Lignin peroxidase is either hexacoordinate with a loosely bound water as the sixth ligand (Andersson et al., 1985) or pentacoordinate (Kuila et al., 1985).

The primary reaction product of peroxidases and catalase with H_2O_2 is the oxidized intermediate compound I. This intermediate accepts both oxidizing equivalents of H_2O_2 and is thus in the formal Fe(V) oxidation state (Dunford, 1982; Rutter et al., 1983); i.e., it contains 2 additional oxidizing equiv over the native ferric form of the enzyme (Theorell, 1947). Mössbauer studies (Schultz et al., 1984) indicate a ferryl [Fe^{IV}=O] state of the heme iron in compound I; thus, only 1 oxidizing equiv is present in the iron. The electronic absorption maxima of compounds I of various proteins display varied features, and this has been attributed to the localization site of the second oxidizing equivalent. For HRP, the second oxidizing equivalent is on the porphyrin ring (Dolphin et al., 1971; Rutter et al., 1983), and for yeast cytochrome *c* peroxidase, it is on an amino acid side chain of the protein (Yonetani, 1976). The reduced absorption of the Soret band is a distinctive feature of compound I of HRP, CPO, and CAT (Dunford, 1982; Palcic et al., 1980). Spectral studies show that in these intermediates the second oxidation equivalent is stored as a porphyrin π-cation radical (Dolphin et al., 1971; Schultz et al., 1979, 1984; Rutter & Hager, 1982). The reduced Soret absorption of compound I has been attributed to the decreased conjugation of the porphyrin ring owing to its π-cation radical nature (Dolphin et al., 1971; Palcic et al., 1980). Furthermore, CAT-I and CPO-I show a strong optical absorption band at 680 nm while HRP-I does not absorb in this region. There is still discussion as to whether this difference is due to different electronic ground states of the π-cation radical produced by HRP, CAT, and CPO (DiNello & Dolphin, 1981; Rutter et al., 1983). In contrast, the electronic absorption spectrum of the primary oxidized species of yeast cytochrome *c* peroxidase compound ES (equivalent to compound I) is similar to that of HRP-II with maxima at 420, 530, and 561 nm (Yonetani, 1976). Magnetic resonance studies of compound ES have established the presence of the second oxidizing equivalent on an amino acid residue, which has been hypothesized to be either a tryptophan or a methionine (Lang et al., 1976; Hoffman et al., 1979).

Lignin Peroxidase Compound I. The electronic absorption spectrum produced on the addition of 1 molar equiv of H_2O_2 to lignin peroxidase at room temperature has a reduced Soret maximum at 408 nm and additional maxima at 550, 608, and 650 nm (Figure 1, Table II), and thus, this appears to be a compound I species. The absorption maxima for several peroxidase compound I species are compared in Table II. The spectral characteristics of lignin peroxidase I are most similar to those of HRP-I. The reduced Soret intensity suggests the π-cation radical nature of the lignin peroxidase I porphyrin ring (Dolphin et al., 1971). Furthermore, absence of absorbance in the 680-nm region suggests that this Fe^{IV}=O porphyrin π-cation radical has an A_{2u}-type or HRP-type electronic ground state (Dolphin et al., 1971; DiNello & Dolphin, 1981). Previously, we obtained compound II by the addition of 1 equiv of H_2O_2 to lignin peroxidase (Renganathan et al., 1985). In that study, the reaction was conducted in 20 mM sodium succinate, pH 4.5, prepared in singly distilled water. Previous work (Hewson & Dunford, 1976; Schultz et al., 1984) has shown that rigorous precautions should be taken to remove oxidizable impurities from the water, which may foster the reduction of compound I.

Lignin Peroxidase Compound II. One-electron reduction of HRP compound I by a peroxidase substrate results in the formation of compound II (Dunford & Stillman, 1976; Dunford, 1982). This intermediate has a formal oxidation state of IV, containing one additional oxidizing equivalent over the native enzyme (Dunford, 1982; Schultz et al., 1984). The electronic absorption spectrum of HRP compound II is characterized by an intense red-shifted Soret maximum at 420 nm and by visible maxima at 527 and 554 nm (Table II). The addition of 2 molar equiv of H_2O_2 to lignin peroxidase at pH 6.0 results in the formation of an intermediate with the absorption spectrum shown in Figure 2 and Table II. As with HRP-II, the Soret maximum is red-shifted to 420 nm and the visible maxima are at 525 and 556 nm. However, the intensity of the Soret maximum of lignin peroxidase II is considerably reduced compared to that of the HRP-II (Figure 2).

Lignin peroxidase I spontaneously converts to lignin peroxidase II in sodium phosphate, pH 6.0, with a $t_{1/2}$ of 1.0 min. Monitoring the change in the visible absorption spectrum during the course of this conversion yields a single isosbestic point at ~565 nm (Figure 5), suggesting that this is a direct conversion without the involvement of another intermediate (Schonbaum & Lo, 1972). Formation of lignin peroxidase I from the native enzyme occurs only in the pH region 4–6; at acidic pH (3.0) or alkaline pH (8.0), only the compound II spectrum is obtained upon addition of 1 molar equiv of H_2O_2.

Lignin Peroxidase Compound III. The addition of excess H_2O_2 converts HRP to compound III, which is considered to be either a ferric superoxide complex ($Fe^{III}O_2^{-}\cdot$) or a ferrous oxy complex ($Fe^{II}O_2$) (Dunford & Stillman, 1976; Dunford, 1982). This intermediate is not involved in the normal peroxidase reaction, although peroxidase III has been implicated in the oxidation of IAA (Yamazaki, 1974; Ricard & Job, 1974) where O_2 is the cosubstrate. The absorption spectrum of the ferrous oxy species of HRP is very similar to that of compound III (Table I) (Wittenberg et al., 1967). The addition of a 25-fold molar excess of H_2O_2 to lignin peroxidase

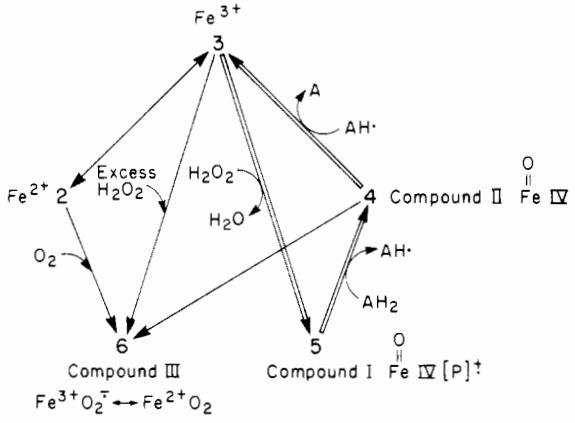

FIGURE 6: Interrelationship between the five redox states of lignin peroxidase. Reaction paths 3 → 5 → 4 → 3 indicate one catalytic cycle of the enzyme. AH_2 = substrate.

yields an intermediate with absorption maxima at 419, 540, and 578 nm (Figure 4), which is very similar to those observed with HRP-III. Since lignin peroxidases II and III have very similar Soret maxima at ~420 nm, the spectra of these intermediates can be differentiated only by their visible maxima. As stated, lignin peroxidase II has visible absorption bands at 525 and 556 nm (Figure 2) while lignin peroxidase III has visible bands red-shifted to 540 and 578 nm (Figure 4). The ferrous form of the enzyme (Gold et al., 1984) readily formed a ferrous oxy complex (Renganathan et al., 1985) with absorption bands similar to those of lignin peroxidase III (Table I). In preliminary experiments, previous workers (Tien & Kirk, 1984; Harvey et al., 1985) have suggested that treatment of lignin peroxidase with 3 and >400 molar equiv, respectively, of H_2O_2 at room temperature leads to the formation of a compound I species. However, the spectra produced by those authors had visible maxima at ~420, 540, and 578 nm. These characteristics indicate that the intermediate formed in both previous studies was compound III, which is not part of the peroxidase catalytic cycle. Thus, this is the first study in which lignin peroxidase compounds I and II have been demonstrated.

Effect of Other Oxidants. HRP is oxidized to compound I by such organic peroxides as methyl and ethyl hydroperoxide and pNPBA (Schonbaum & Lo, 1972). Both mCPBA and pNPBA oxidize lignin peroxidase (Figure 3), although 2 molar equiv of pNPBA are required and then only the spectrum of compound II is obtained. mCPBA can be used as an oxidant in the oxidation of veratryl alcohol by this enzyme (Renganathan et al., 1986). Neither *tert*-butyl nor cumene hydroperoxide has any effect on lignin peroxidase.

Chloroperoxidase (CPO) and HRP are oxidized by sodium chlorite to compound X, which is subsequently converted through compound II to compound I (Hewson & Hagar, 1979a,b). The addition of a 50-fold excess of sodium chlorite to lignin peroxidase did not oxidize this enzyme.

Reactions of Lignin Peroxidase Compounds I and II. Compounds I and II of HRP are reduced back to the native resting state by such substrates as ascorbic acid, phenol, thiourea, *p*-aminobenzoic acid, and tyrosine (Dunford & Stillman, 1976; Dunford, 1982). Similar reductions are also observed with compounds I and II of lignin peroxidase. One molar equivalent of the one-electron substrates *p*-cresol, syringic acid, and vanillic acid or 0.5 molar equiv of the two-electron substrates veratryl alcohol or ascorbic acid rapidly reduced lignin peroxidase I to lignin peroxidase II. Two molar equivalents of *p*-cresol or 1 molar equiv of veratryl alcohol reduced compound I to the native enzyme. Finally, 1 molar equiv of *p*-cresol or 0.5 molar equiv of veratryl alcohol reduced lignin peroxidase II to the native ferric state of the enzyme. These results indicate that lignin peroxidase compounds I and II are in the formal oxidation states V and IV, respectively, and that they respectively contain 2 and 1 oxidizing equiv over the native ferric resting state of the enzyme.

In the presence of H_2O_2, lignin peroxidase oxidizes veratryl alcohol to veratryl aldehyde (Kuwahara et al., 1984; Gold et al., 1984; Tien & Kirk, 1984). In this respect, it shares the ability of CAT and CPO (Schonbaum & Chance, 1976; Thomas et al., 1970; Giegert et al., 1983) to dehydrogenate some alcohols. Alcohol dehydrogenation is not observed with HRP, lactoperoxidase, or myeloperoxidase (Geigert et al., 1983). As described above, through the measured addition of single-electron reducing equivalents of veratryl alcohol and other substrates, it is possible to reduce lignin peroxidase compound I to compound II and then to the native enzyme, thus completing a peroxidase catalytic cycle (Figure 6). This indicates that the enzyme dehydrogenates veratryl alcohol via two single-electron oxidation steps rather than via a single two-electron step mechanism as observed with CAT and CPO (Schonbaum & Chance, 1976; Thomas et al., 1970). An early assay for lignin peroxidase was the oxidation of KTBA to produce ethylene (Glenn et al., 1983; Gold et al., 1983). Later, it was observed that the purified enzyme oxidizes KTBA only in the presence of veratryl alcohol or other aromatic substrates (Kuwahara et al., 1984; Gold et al., 1984). Recently, we have observed that KTBA noncompetitively inhibits veratryl alcohol oxidation by this peroxidase (Renganathan et al., 1985). KTBA does not reduce either lignin peroxidase I or II, indicating that the inhibition of veratryl alcohol oxidation is not via the reduction of the enzyme. We propose that the one-electron oxidized veratryl alcohol intermediate, a radical, in turn oxidizes KTBA to yield ethylene. The removal of the veratryl alcohol radical intermediate by this subsequent reaction with KTBA would result in the observed noncompetitive inhibition of veratryl alcohol oxidation. Recently, EPR evidence demonstrated that lignin peroxidase oxidizes *p*-dimethoxybenzene to form a stable benzene cation radical (Kersten et al., 1985). By analogy, the veratryl alcohol radical intermediate may also be a benzene cation radical, and EPR studies designed to identify the nature of the veratryl alcohol radical are planned.

In this paper, we have spectrally characterized compounds I, II, and III of lignin peroxidase and found them to be analogous to the oxidized intermediates of HRP. Formally, peroxidases can exist in five redox states from 2+ to 6+ (Yamazaki, 1974). Earlier, we observed the native ferric and the reduced ferrous forms of the enzyme (Gold et al., 1984; Renganathan et al., 1985; Andersson et al., 1985) as well as the ferrous oxy complex of lignin peroxidase (Renganathan et al., 1985). With this spectral characterization of compounds I, II, and III of the enzyme, we have demonstrated all five oxidation states of the enzyme. Dehydrogenation of veratryl alcohol has been suggested to proceed through two single electron oxidation steps with the formation of a cation radical intermediate. Further characterization of the lignin peroxidase oxidation states by EPR and resonance Raman spectroscopy as well as by kinetic procedures is planned.

Registry No. Lignin peroxidase, 42613-30-9; veratryl alcohol, 93-03-8.

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