Haloperoxidase Reactions Catalyzed by Lignin Peroxidase, an Extracellular Enzyme from the Basidiomycete *Phanerochaete chrysosporium*†

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ABSTRACT: Lignin peroxidase (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 examine the haloperoxidase reactions of LiP. In the presence of H_2O_2, homogeneous LiP oxidized bromide and iodide but not chloride. Halide oxidation was measured by the halogenation of monochlorodimedone (MCD) and a variety of other aromatic compounds. Bromination of MCD produced monochloromonobromodimedone. The pH optimum for the bromination of MCD was 3.5. Both chloride and fluoride inhibited the bromination reaction. LiP binds halides to produce characteristic optical difference spectra. From these spectra apparent dissociation constants for fluoride and chloride were determined to be 0.3 and 20 mM, respectively. Incubation of LiP with bromide and H_2O_2 in the absence of organic substrate led to the bleaching of the heme as measured by a decrease in Soret maximum. LiP brominated a variety of aromatic substrates including 3,4-dimethoxybenzyl alcohol (veratryl alcohol) to produce 6-bromoveratryl alcohol (VII). LiP hydrobrominated cinnamic acid (IV) to produce 2-bromo-3-hydroxy-3-phenylpropionic acid (XII). In an analogous reaction LiP hydrobrominated 1-(4-ethoxy-3-methoxyphenyl)propene (II) to produce 2-bromo-1-(4-ethoxy-3-methoxyphenyl)-1-hydroxypropane (XIII). Finally, with 3,4-dimethoxycinnamic acid as the substrate, three bromination products were identified: *trans*-2-bromo-1-(3,4-dimethoxyphenyl)ethylene (IX), 2,2-dibromo-1-(3,4-dimethoxyphenyl)-1-hydroxyethane (X), and 2-bromo-3-(3,4-dimethoxyphenyl)-3-hydroxypropionic acid (XI).

The lignin-degrading basidiomycete *Phanerochaete chrysosporium* secretes at least two extracellular heme peroxidases during its secondary metabolic phase of growth. These enzymes, the manganese peroxidase (MnP)[1] (Kuwahara et al., 1984; Glenn & Gold, 1985; Paszcyznski et al., 1986) and lignin peroxidase (LiP) (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985), have been purified to homogeneity. The main isozymic form of lignin peroxidase is a glycoprotein with M_r 41 000, and all forms of the enzyme have a single iron protoporphyrin IX prosthetic group. Electronic absorption (Gold et al., 1984), EPR, and resonance Raman (Andersson et al., 1985; Kuila et al., 1985) data suggest that the native form is in the high-spin ferric state with histidine coordinated as the fifth ligand. We have characterized the H_2O_2 reaction intermediates, compounds I–III of LiP, using elecronic absorption spectroscopy. Like the analogous intermediates of horseradish peroxidase, LiP compounds I and II appear to have formal oxidation states of V and IV, respectively (Renganathan & Gold, 1986; Dunford, 1982). Recent resonance Raman studies have confirmed that the heme iron in LiPII is in the ferryl form (Andersson et al., 1987).

Homogeneous LiP is H_2O_2-dependent and catalyzes the oxidation of a variety of lignin model compounds such as diarylpropanes, β-aryl ethers, and phenylpropanes (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). These oxidative reactions include α,β bond cleavage, β-aryl ether bond cleavage (Gold et al., 1984; Tien & Kirk, 1984), intramolecular displacements and rearrangements (Miki et al., 1986a,b), and aromatic ring opening (Umezawa et al., 1986; Miki et al., 1987). A proposed mechanism that accounts for all of these reactions involves an initial one-electron oxidation of the aromatic substrate by lignin peroxidase to form an aryl π-cation radical which undergoes subsequent nonenzymatic reactions to yield the final products (Kersten et al., 1985; Schoemaker et al., 1985; Renganathan et al., 1986; Renganathan & Gold, 1986). Like CPO (Geigert et al., 1983a), LiP can dehydrogenate some benzyl alcohols. For example, veratryl alcohol (3,4-dimethoxybenzyl alcohol) is oxidized to the corresponding benzaldehyde, and this reaction appears to proceed through two single-electron oxidation steps, probably through an aryl cation radical intermediate (Renganathan & Gold, 1986; Tien et al., 1986), as has been observed for the oxidation of dimethoxybenzene (Kersten et al., 1985). During the course of our investigations on the aryl alcohol dehydrogenations catalyzed by LiP we observed inhibition with bromide and chloride anions, suggesting that halides may also function as substrates for LiP. In this report, we demonstrate that, like some other peroxidases, LiP can function as a bromo/iodoperoxidase.

Materials and Methods

Enzyme. The major isozymic form (LiPII) was purified from acetate-buffered agitated cultures of *P. chrysosporium*

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[1] Abbreviations: MnP, manganese peroxidase; LiP, lignin peroxidase; CPO, chloroperoxidase; HRP, horseradish peroxidase; MPO, myeloperoxidase; LPO, lactoperoxidase; CAT, catalase; MS, mass spectrum; GC/MS, gas chromatography–mass spectrometry; MCD, monochlorodimedone; TLC, thin-layer chromatography; FT-NMR, Fourier transform nuclear magnetic resonance spectrometer; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

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as previously described (Gold et al., 1984; Renganathan et al., 1985). The purified protein was electrophoretically homogeneous and had an R_z value of ~5.0.

Chemicals. Monochlorodimedone (MCD) (I) was prepared as previously described (Hager et al., 1966). 1-(4-Ethoxy-3-methoxyphenyl)propene (II) was prepared by the ethylation of isoeugenol as previously described (Enoki et al., 1981). Veratryl alcohol (III), cinnamic acid (IV), and 3,4-dimethoxycinnamic acid (V) were obtained from Aldrich Chemical Co. Sodium halides were all analytical grade.

MCD Haloperoxidase Assay. Reaction mixtures (1 mL) consisted of sodium halide (20 mM), MCD (0.1 mM) (added as a solution in dimethyl formamide), and H_2O_2 (0.1 mM) in 20 mM sodium succinate (pH 3.5). Reactions were initiated by the addition of enzyme (0.5 μg) and incubated at room temperature. Sodium chlorite ($NaClO_2$) dependent halogenation reaction mixtures (1 mL) consisted of $NaClO_2$ (20 mM), MCD (0.1 mM), and enzyme (0.5 μg) in 20 mM sodium succinate, pH 3.5. Halogenation of MCD was measured by following the decrease in absorbance at 280 nm as previously described (Hager et al., 1966); an extinction coefficient for MCD of 12.2 mM^{-1} cm^{-1} was used.

Halogenation of Aromatic Substrates. The aromatic substrates veratryl alcohol (III), 3,4-dimethoxycinnamic acid (V), or 1-(4-ethoxy-3-methoxyphenyl)propene (II) (0.1 mM) were incubated in 1 L of 20 mM sodium succinate, pH 3.5, containing NaBr (20 mM), H_2O_2 (0.1 mM), and LiP (0.5 mg). MCD (I) or cinnamic acid (IV) (0.1 mM) were incubated in 20 mL of sodium succinate buffer, pH 3.5, containing NaBr (20 mM), H_2O_2 (0.1 mM), and enzyme (10 μg). Reaction mixtures were stirred for 0.5 h at room temperature. The mixtures were then extracted with ethyl acetate, dried over anhydrous Na_2SO_4, evaporated under vacuum, and redissolved in chloroform. Products were analyzed by TLC using one of the following solvent systems: (A) hexane/ethyl acetate (7:3); (B) hexane/ethyl acetate (1:1); (C) hexane/ethyl acetate/acetic acid (15:50:1). ^1H NMR spectra were determined with a JEOL FX-90Q FT-NMR with chemical shifts expressed as parts per million (δ) downfield from an internal standard of tetramethylsilane. In addition, aromatic products were silylated (BSTFA/pyridine, 2:1 v/v) and identified by capillary GC and GC/MS. GC/MS was performed at 70 eV with a VG Analytical 7070E mass spectrometer fitted with an HP 5790A GC and a 15-m fused silica column (DB-5, J & W Science).

Halide Binding. Both the reference and sample cuvettes contained lignin peroxidase (1 μM) in 20 mM sodium succinate, pH 3.0. Halides were added only to the sample cuvettes, and difference spectra were determined from 500 to 300 nm. The apparent dissociation constants K_D were calculated from plots of $[halide]^{-1}$ vs. ΔA^{-1} (the difference between maximum and minimum absorptions).

Results

Haloperoxidase Activity. Chloride and bromide inhibition of the LiP-catalyzed dehydrogenation of veratryl alcohol to veratrylaldehyde is shown in Figure 1. Bromide (3 mM) and chloride (32 mM) each effected 50% inhibition of the dehydrogenation. In the presence of H_2O_2 and bromide, lignin peroxidase brominated MCD (Table I). Halogenation was measured by following the decrease in absorbance at 280 nm as previously described (Hager et al., 1966). The LiP- and CPO-catalyzed MCD bromination products migrated identically on TLC (solvent system C, R_f = 0.87), demonstrating that LiP produces monobromomonochlorodimedone (VI). Although chloride-dependent halogenation of MCD was insignificant, chloride (5 mM) and fluoride (1 mM) inhibited bromination of MCD 15% and 50%, respectively (Table I). Unlike HRP and CPO (Hollenberg et al., 1974), lignin peroxidase does not appear to catalyze the sodium chlorite dependent chlorination of MCD (Table I), nor is the enzyme inactivated by sodium chlorite (Renganathan & Gold, 1986).

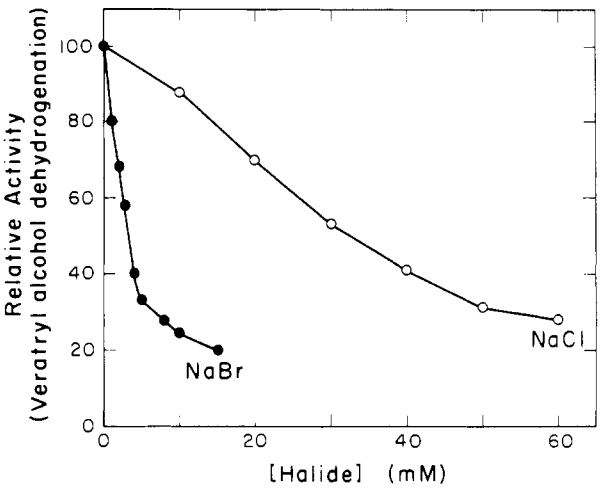

FIGURE 1: Chloride (○) and bromide (●) inhibition of veratryl alcohol dehydrogenation by lignin peroxidase. The reaction was followed by measuring the production of veratryl aldehyde as an increase in absorbance at 310 nm. Reaction mixtures were as described in the text.

Table I: Halogenation of Monochlorodimedone by Peroxidases[a]

enzyme system	rate of MCD halogenation or oxidation (μmol min^{-1} mg^{-1})
LiP + H_2O_2 + Br^-	47.0
LiP + H_2O_2	14.6
LiP + H_2O_2 + Br^- + F^- (1 mM)	24.0
LiP + H_2O_2 + Br^- + Cl^- (5 mM)	40.0
HRP + $NaClO_2$[b]	≫200.0
LiP + $NaClO_2$	<1.5

[a] Reaction mixtures (1 mL) consisted of 20 mM sodium succinate, pH 3.5, containing MCD (600 μM), NaBr (20 mM), H_2O_2 (100 μM), and enzyme (0.5 μg). When $NaClO_2$ (20 mM) replaced NaBr as the substrate, H_2O_2 was excluded. MCD halogenation or oxidation was monitored as a decrease in the absorbance at 280 nm as previously described (Hager et al., 1966). [b] Rate is very fast.

The pH dependence of the LiP-catalyzed bromination of MCD is shown in Figure 2. At the optimum pH of 3.5, the specific activity for the bromination of MCD was 50 μmol min^{-1} mg^{-1}. In comparison, the peroxidative activity as assayed by veratryl alcohol dehydrogenation increased continuously as the pH was lowered to 3.0 (Figure 2).

The LiP-catalyzed oxidation of bromide to bromine was measured by the formation of the tribromide complex (Libby et al., 1982) as shown in Figure 3. LiP also catalyzed the oxidation of iodide as measured by the increase in absorbance at 350 nm owing to the formation of the triiodide complex (data not shown) Brown & Hager, 1967).

Identification of Aromatic Bromination Products. TLC analysis (system A) demonstrated the formation of one major (VII) and one minor (VIII) bromination product when veratryl alcohol (III) was used as a substrate. VII was identified as 6-bromoveratryl alcohol. ^1H NMR ($CDCl_3$) δ 3.87 (6 H, s, 2-OCH_3), 4.68 (2 H, s, benzylic H), 7.0 (2 H, s, ArH); MS *m/z* [trimethylsilyl (TMS) ether] 320, 318 (M^+), 305, 303 ($M^+ - CH_3$), 231, 229 (M^+ - OTMS). VII was identified as 6-bromoveratrylaldehyde. ^1H NMR ($CDCl_3$) δ 3.92, 3.96 (6 H, 2 s, 2-OCH_3), 7.05 (1 H, s, ArH), 7.4 (1 H, s, ArH), 10.87

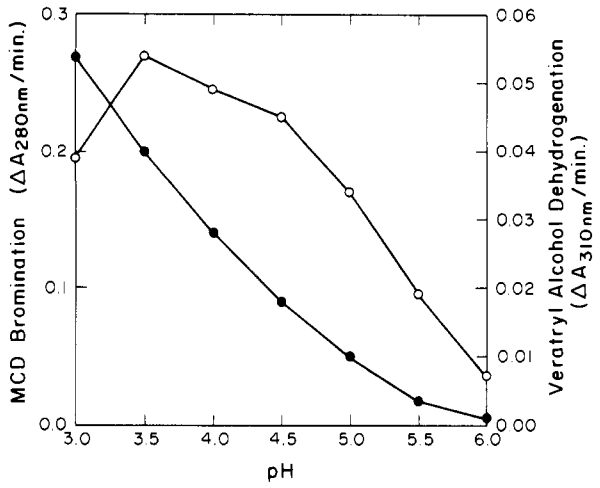

FIGURE 2: pH dependence of lignin peroxidase catalyzed dehydrogenation and haloperoxidation reactions. Dehydrogenation of veratryl alcohol (●) was followed by measuring the increase in absorbance at 310 nm. Bromination of monochlorodimedone (○) was followed by measuring the decrease in absorbance at 280 nm. Reaction mixtures were as described in the text.

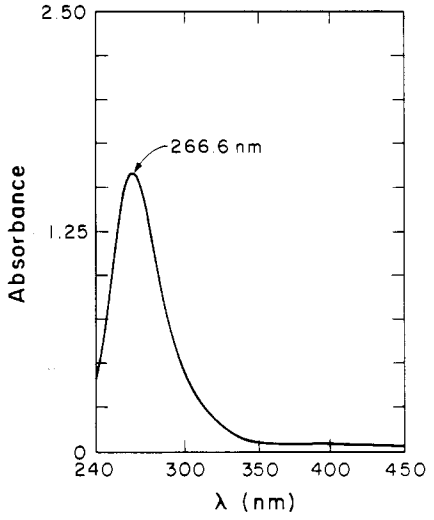

FIGURE 3: Lignin peroxidase catalyzed formation of bromine. Reaction mixtures (1 mL) consisted of LiP (0.5 μM), bromide (20 mM), and H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.5. Bromine was detected as its tribromide complex (Libby et al., 1982).

(1 H, s, –CHO); MS *m/z* 246, 244 (M^+), 231, 229 ($M^+ - CH_3$).

GC analysis of 3,4-dimethoxycinnamic acid (V) bromination products demonstrated three major products and several minor products. Two of the major products were separated by TLC (system B; R_f for IX = 0.72, R_f for X = 0.53) and were identified as *trans*-2-bromo-1-(3,4-dimethoxyphenyl)ethylene (IX) and 2,2-dibromo-1-(3,4-dimethoxyphenyl)-1-hydroxyethane (X).

IX: ^{1}H NMR ($CDCl_3$) δ 3.87 (6 H, s, 2-OCH_3), 6.6 (1 H, d, J = 13.6 Hz), 6.82 (3 H, s, ArH), 7.04 (1 H, d, J = 14.0 Hz); MS *m/z* 244, 242 (M^+), 229, 227 ($M^+ - CH_3$), 201, 199 ($M^+ - CH_3HCHO$).

X: ^{1}H NMR ($CDCl_3$) δ 2.96 (1 H, br s, –OH disappears on the addition of D_2O), 3.88, 3.9 (6 H, 2 s, 2-OCH_3), 5.01 (1 H, d), 5.76 (1 H, d), 6.8–7.0 (3 H, m, ArH); MS *m/z* (trimethylsilyl ether) 410, 412, 414 (M^+), 318, 316 ($M^+ - CH_3$, – Br), 239.

3,4-Dimethoxybenzaldehyde (veratrylaldehyde) was identified by comparing its retention time on capillary GC and its mass spectrum with a standard: MS *m/z* 166 (M^+), 151

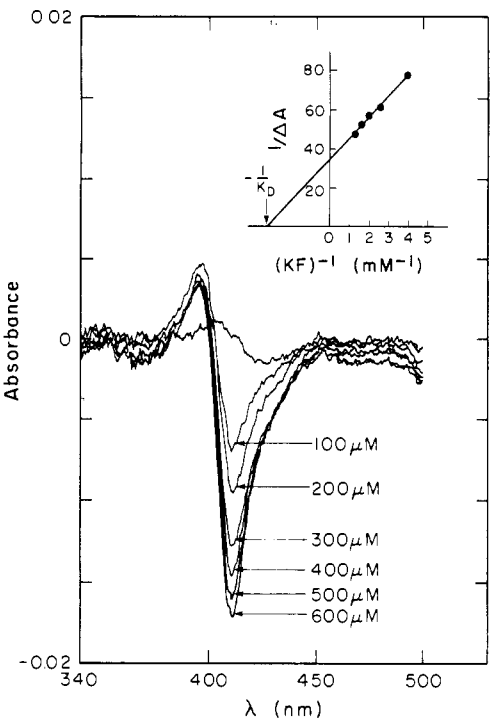

FIGURE 4: Fluoride binding to lignin peroxidase. Both the reference and sample cuvettes contained lignin peroxidase (1 μM) in 20 mM sodium succinate (pH 3.0). Spectra were recorded after each addition of fluoride to the sample cuvette. Inset: Reciprocal plot of fluoride concentration vs. absorbance change (ΔA). ΔA is the difference between the maximum and minimum absorbance at a given halide ion concentration.

(–CH_3), 137 (–CHO). Finally, 2-bromo-3-(3,4-dimethoxyphenyl)-3-hydroxypropionic acid (XI) was identified from its mass spectrum: MS *m/z* (di TMS ether) 450, 448 (M^+), 369 (M^+ – Br), 239 (M^+ – CHBr, CO_2, trimethylsilyl). With cinnamic acid (IV) as the substrate, one product, 2-bromo-3-hydroxy-3-phenylpropionic acid (XII) was identified from its mass spectrum: MS *m/z* (di TMS ether) 375, 373 ($M^+ - CH_3$), 309 (M^+ – Br), 179 (M^+ – CHBr, CO_2, trimethylsilyl). The mass spectral data correspond to those recently reported (Yamada et al., 1985).

In an analogous reaction the phenylpropene (II) was converted to 2-bromo-1-(4-ethoxy-3-methoxyphenyl)-1-hydroxypropane (XIII). ^{1}H NMR ($CDCl_3$) δ 1.45 (3 H, t, –OCH_2CH_3), 1.55 (3 H, d, –CH_3), 3.87 (3 H, s, –OCH_3), 4.09 (2 H, q, –OCH_2CH_3), 4.24–4.5 (1 H, m, –CHBr), 4.94 (1 H, d, –CHOH), 6.84 (2 H, s, ArH), 6.92 (1 H, s, ArH); MS *m/z* (trimethylsilyl ether) 362, 360 (M^+), 347, 345 ($M^+ - CH_3$), 254 ($M^+ - C_2H_4Br$).

Halide Binding Spectra. Lignin peroxidase binds halides to produce characteristic difference spectra (Figures 4 and 5). The fluoride–LiP difference spectrum (Figure 4) exhibited a maximum of 397 nm and a minimum at 410 nm. The apparent dissociation constant of 0.3 mM was determined from these data. The chloride difference spectrum (Figure 5) exhibited a maximum at 418 nm and a minimum at 405 nm. An apparent chloride dissociation constant K_D of 20 mM was determined. Binding of bromide and iodide resulted in a decrease in the Soret absorption. However, owing to the relative weakness of binding, an apparent K_D could not be determined. Binding of halides decreased with increasing pH as observed with CPO (Thomas et al., 1970).

Oxidation of the Heme. In the absence of substrate, addition of H_2O_2 to a mixture of LiP and bromide results in a rapid loss of the Soret maximum (Figure 6), attributable to

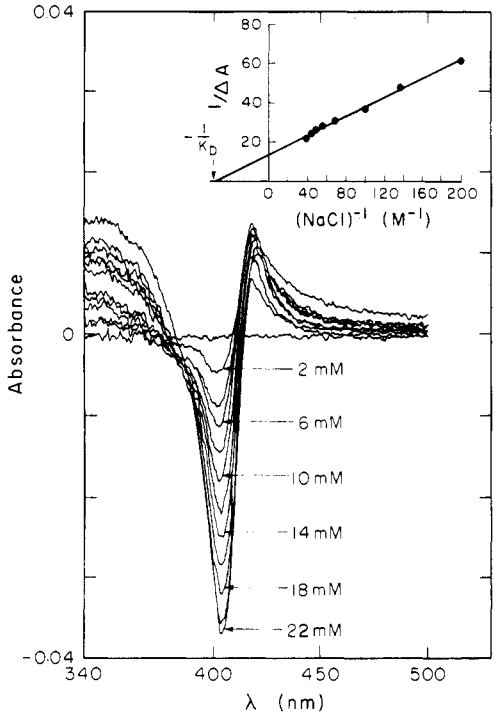

FIGURE 5: Chloride binding to lignin peroxidase. Both the reference and sample cuvettes contained lignin peroxidase (1 μM) in 20 mM sodium succinate (pH 3.0). Spectra were recorded after each addition of chloride to the sample cuvette. Inset: Reciprocal plot of chloride concentraiton vs. absorbance change (ΔA). ΔA is the difference between the maximum and minimum absorbance at a given halide ion concentration.

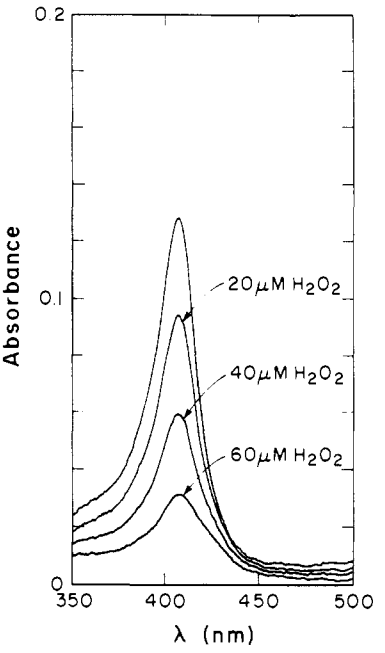

FIGURE 6: Bleaching of the heme prosthetic group of LiP. In the absence of an organic substrate, aliquots of H_2O_2 (20 μM) were added to LiP ($\sim$1 μM) and bromide (40 μM) in 20 mM sodium succinate, pH 3.5. The spectrum of the enzyme was subsequently recorded as described in the text.

the loss of intact heme. This bromide-dependent reaction occurred between pH 3.0 and 4.5 with bromide concentrations as low as 40 μM. Above pH 4.5 the same reaction mixture yielded the spectrum of compound II, indicating that the oxidation of bromide is slow above this pH. This is consistent with the decreased bromination of MCD above pH 4.5 as shown in Figure 2.

Discussion

Haloperoxidase-mediated halogenation reactions are the major source of natural organohalogen compounds (Neidleman & Geigert, 1986). The haloperoxidase reaction pathway includes the initial oxidation of the native ferric peroxidase by H_2O_2 to produce the oxidized species compound I. This intermediate accepts both oxidizing equivalents of H_2O_2 and is thus in the formal oxidation state V (Dunford, 1982; Rutter et al., 1983). Lignin peroxidase appears to be oxidized in an analogous manner (Renganathan & Gold, 1986). In the case of chloroperoxidase, compound I in turn oxidizes the halide by two electrons to produce an Fe(III) hypohalite intermediate (Libby et al., 1982). The latter rapidly transfers the halonium ion to the substrate and regenerates the ferric enzyme (Libby et al., 1982; Morrison & Schonbaum, 1976). CPO and MPO catalyze the oxidation of chloride, bromide, and iodide (Neidleman & Geigert, 1986), and LPO catalyzes the oxidation of bromide and iodide (Morrison & Schonbaum, 1976).

Homogeneous LiP oxidizes a variety of lignin model compounds, and these various oxidations have been discussed in terms of an aryl cation radical intermediate produced via a single-electron oxidation of the substrate by an oxidized form of the enzyme (Kersten et al., 1985; Renganathan et al., 1985; Renganathan & Gold, 1986; Shoemaker et al., 1985). LiP appears to dehydrogenate veratryl alcohol via two single-electron oxidation steps rather than via a single two-electron step as observed in ethyl alcohol dehydrogenations with CAT and CPO (Schonbaum & Chance, 1976; Thomas et al., 1970). During our study of benzyl alcohol dehydrogenation by LiP we observed inhibition of the reaction by halides. In the current study, we demonstrate that, like several other peroxidases, LiP can catalyze the oxidation of halides.

LiP oxidizes iodide and bromide but not chloride. The enzyme brominates MCD at pH 3.5 with a specific activity of $\sim$50 μmol min^{-1} mg^{-1}. As observed with chloroperoxidase (Thomas et al., 1970), halogenation activity of LiP occurs at a low pH with optimum activity at pH 3.5. The chloride-dependent halogenation of MCD by LiP is insignificant, suggesting that LiP, like LPO (Morrison & Schonbaum, 1976), HRP (Griffin & Ashley, 1984), and the bromoperoxidases from *Penicillus capitatus* (Manthey & Hager, 1981) and *Pseudomonas aureofaciens* (van Pee & Lingens, 1985), oxidizes only bromide and iodide.

HRP and CPO catalyze the sodium chlorite dependent chlorination of MCD in the absence of chloride and H_2O_2 (Hollenberg et al., 1974). Furthermore, in the absence of a halogen acceptor, HRP is oxidized by $NaClO_2$ to compound X. The absorption spectrum of this intermediate is similar to that of compound II. It has been postulated that compound X is converted to a compound I type intermediate with a hypochlorite ligand and then to the native enzyme via reduction (Hewson & Hager, 1979a,b; Shahangian & Hager, 1982). Although MPO does not catalyze this reaction, chlorite has been reported too inactivate this peroxidase (Harrison & Schultz, 1976). In contrast, (a) LiP did not catalyze the chlorite-dependent halogenation of MCD; and (b) our previous results (Renganathan & Gold, 1986) indicate that LiP is not oxidized by a 50-fold excess of sodium chlorite.

Bromination of Aromatic Substrates. Each aromatic ring in the lignin polymer contains from one to three alkoxy substitutions, thus increasing its electron density. In addition, on the periphery of the polymer, double bonds from the precursor *p*-hydroxycinnamyl alcohols are conjugated to the aromatic rings (Sarkanen, 1971). Since these functions are very susceptible to halogenation, we chose to study the halogenation

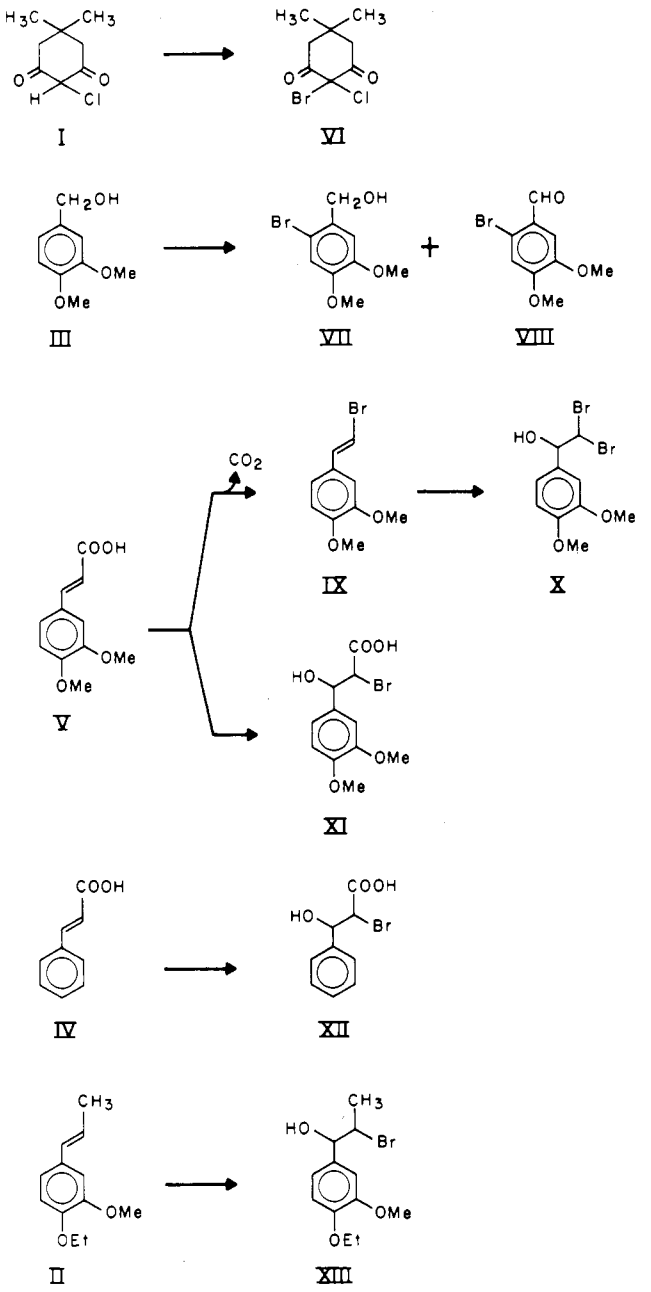

FIGURE 7: Products obtained from the bromination of various substrates by lignin peroxidase.

of several C_6–C_3 lignin-related aromatic compounds, as well as veratryl alcohol, a secondary metabolite of *P. chrysosporium*. CPO catalyzes the halogenation of a wide variety of substrates including β-keto acids (Shaw & Hager, 1961), β-diketones (Hager et al., 1966), steroids (Levine et al., 1968), alkenes (Giegert et al., 1983b), and cinnamic acids (Yamada et al., 1985). LiP quantitatively brominates veratryl alcohol in the 6-position of the aromatic ring (Figure 7). This reaction proceeds at bromide concentrations as low as 100 μM (8 ppm).

The olefin II is also a substrate for LiP. In the absence of halide the enzyme hydroxylates II to form a diol (Gold et al., 1984), but the olefin can also function as a substrate for haloperoxidase reactions. Hydrobromination by LiP yields 2-bromo-1-(4-ethoxy-3-methoxyphenyl)-1-hydroxypropane (XIII) (Figure 7). In an analogous manner, LiP-catalyzed addition of HOBr across the double bond of cinnamic acid yields 2-bromo-3-hydroxy-3-phenylpropionic acid (XII) (Figure 7). The major products of the LiP-catalyzed bromination of 3,4-dimethoxycinnamic acid (V) are the decarboxylated product 2-bromo-1-(3,4-dimethoxyphenyl)ethylene (IX) and its hydrobrominated derivative 2,2-dibromo-1-(3,4-dimethoxyphenyl)-1-hydroxyethane (X) in addition to the hydrobromination product 2-bromo-3-(3,4-dimethoxyphenyl)-3-hydroxypropionic acid (XI) (Figure 7). CPO has been reported to catalyze the bromination of the 5-position of the aromatic ring of 3,4-dimethoxycinnamic acid (Yamada et al., 1985). This product is not detectable in LiP-catalyzed reactions under the conditions described. Taken together, the various bromoperoxidase reactions of LiP suggest that, under appropriate conditions, the enzyme might brominate lignin itself.

There is still discussion as to whether chloroperoxidase-catalyzed halogenation involves an enzyme-bound halogenating intermediate, EOX, or a diffusible agent such as HOX/X_2 (Neidleman & Geigert, 1986; Libby et al., 1982). LiP forms molecular bromine under the assay conditions (Figure 3), and many of its bromoperoxidase reactions can be explained qualitatively by the reaction of $HOBr/Br_2$ with the substrates examined. At room temperature HOBr is considerably less stable in aqueous solution than HOCl. Furthermore, at low pH the equilibrium

$$H^+ + HOBr + Br^- \rightleftharpoons H_2O + Br_2$$

would favor the formation of Br_2, which is stable (Cotton & Wilkinson, 1972). Increased halogenating activity of the enzyme as the pH is lowered to 3.5 may reflect an increased concentration of Br_2. Further studies will be needed to determine whether the brominating agent is enzyme bound or free in solution.

The peroxidases bind halide ions irrespective of their ability to oxidize them (Morrison & Schonbaum, 1976). It has been suggested that an anionic site near the heme is normally occupied by a dissociable carboxylate ion. At low pH values, the carboxyl group is protonated, releasing the site for halide binding (Thomas et al., 1970). Binding of halide ions to LiP increases with decreasing pH. Bromide and iodide form only weak complexes. Chloride and fluoride form relatively strong complexes with apparent K_D values of 20 mM and 3 mM, respectively (Figure 4). The inability of LiP to oxidize chloride suggests, therefore, that the redox potential of LiP compound I is lower than the equivalent forms of CPO and MPO. In the absence of an organic substrate, incubation of LiP with H_2O_2 and bromide results in the bleaching of the heme prosthetic group. The bleaching process is rapid and occurs at concentrations of bromide as low as 40 μM. Similar oxidative inactivation of MPO by chloride and H_2O_2 has been reported (Matheson et al., 1981). In the absence of a suitable halogen acceptor, an enzyme-produced HOBr may react directly with the heme instead. Free heme is known to be bleached by hypohalous acids (Albrich et al., 1981).

Inhibition of veratryl alcohol oxidation by bromide (Figure 1) may be due either to competition between bromination and dehydrogenation reactions or to heme inactivation. Since chloride is not oxidized by LiP, inhibition of dehydrogenation by chloride is probably a consequence of chloride binding to the enzyme (Kimura & Yamazaki, 1978).

In summary, we have demonstrated that LiP oxidizes bromide and iodide and have characterized various LiP-catalyzed bromoperoxidase reactions. Like CPO, LiP catalyzes the bromination of aromatic rings, 1,3-diketones, and α,β olefins conjugated to aromatic rings. Recently, it has been reported that white rot fungi are an important natural source of halomethanes (Harper, 1985). To our knowledge, this is the first report on the identification of a halogenation reaction by an enzyme from a white rot fungus and of the character-

ization of the haloperoxidase involved. The possible physiological significance of this reaction will require the identification of halogenated compounds produced by *P. chrysosporium*.

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