# Oxidation of Ferrocytochrome *c* by Lignin Peroxidase†

Hiroyuki Wariishi,‡ Dawei Sheng, and Michael H. Gold*

*Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science and Technology, Portland, Oregon 97291-1000*

*Received August 3, 1993; Revised Manuscript Received March 9, 1994*®

ABSTRACT: We demonstrate direct oxidation of ferrocytochrome *c* by lignin peroxidase (LiP) from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Steady-state kinetic data fit a peroxidase ping-pong mechanism rather than an ordered bi-bi ping-pong mechanism, suggesting that the reductions of LiP compounds I and II by ferrocytochrome *c* are irreversible. The pH dependence of the overall reaction apparently is controlled by two factors, the pH dependence of the electron-transfer rate and the pH dependence of enzyme inactivation in the presence of $H_2O_2$. In the presence of 100 $\mu$M $H_2O_2$, veratryl alcohol (VA) significantly enhanced cytochrome *c* oxidation at pH 3.0 but had little effect above pH 4.5. In the presence of <10 $\mu$M $H_2O_2$, the stimulating effect of VA on the reaction is greatly diminished. As with cytochrome *c* peroxidase reactions, LiP oxidation of ferrocytochrome *c* decreased as the ionic strength increased, implying the involvement of electrostatic interactions between the polymeric substrate and enzyme. The reaction product ferricytochrome *c* inhibited VA oxidation by LiP in a noncompetitive manner, suggesting that cytochrome *c* binds to LiP at a site different from the small aromatic substrate binding site. Recent crystallographic studies show that the heme is buried in the LiP protein and unavailable for direct interaction with polymeric substrates, suggesting that electron transfer from ferrocytochrome *c* to LiP occurs over a relatively long range. The role of VA in this electron-transfer reaction is discussed. This observation of ferrocytochrome *c* oxidation by LiP, which is stimulated at low pH and high [$H_2O_2$] by VA, suggests that this system may serve as a useful model for probing the mechanism of electron transfer between the buried heme of the enzyme and other polymeric substrates, such as lignin.

Lignin is a heterogeneous, random, phenylpropanoid polymer that constitutes 20–30% of woody plant cell walls (Sarkanen & Ludwig, 1971). White rot basidiomycetous fungi are primarily responsible for initiating the depolymerization of lignin, which is a key step in the earth's carbon cycle (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987). The best-studied lignin-degrading fungus, *Phanerochaete chrysosporium*, secretes two extracellular heme peroxidases, manganese peroxidase and lignin peroxidase (LiP),[1] which, along with an $H_2O_2$-generating system, are apparently major extracellular components of its lignin degradative system (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987; Hammel et al., 1993; Wariishi et al., 1991b; Higuchi, 1991; Schoemaker, 1990). Nucleotide sequences of several LiP cDNAs and genes (Tien & Tu, 1987; Gold & Alic, 1992; Ritch et al., 1991) as well as the recently published LiP X-ray crystal structures (Edwards et al., 1993; Poulos et al., 1993; Piontek et al., 1993) demonstrate that important catalytic residues including the proximal and distal His, the distal Arg, and an H-bonded Asp are all conserved within the heme pocket.

LiP also shares mechanistic features with other plant and fungal peroxidases. Characterization of the formation and reactions of the oxidized intermediates LiPI, LiPII, and LiPIII indicates that the oxidized states and catalytic cycle of LiP are similar to those of horseradish peroxidase (Gold et al., 1989; Renganathan & Gold, 1986; Marquez et al., 1988; Dunford & Stillman, 1976; Tien et al., 1986). Yet LiP has several unique features, including an apparently higher redox potential (Gold et al., 1989; Kirk & Farrell, 1987; Schoemaker, 1990), a very low pH optimum of ~3.0 (Tien et al., 1986; Renganathan et al., 1987; Wariishi et al., 1991a), and an unusually high reactivity of compound II with $H_2O_2$ (Wariishi & Gold, 1991; Wariishi et al., 1990). LiP slowly depolymerizes synthetic polymeric lignin in the presence of VA (Hammel et al., 1993) and effectively oxidizes nonphenolic lignin models and aromatic pollutants with redox potentials beyond the reach of other peroxidases (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987; Schoemaker, 1990; Valli et al., 1992). Since, as in CCP, the heme group in LiP is buried (Edwards et al., 1993; Poulos et al., 1993; Piontek et al., 1993), it is not available to interact directly with polymeric substrates. In this report, we utilize ferrocytochrome *c* as a model polymeric substrate for LiP and discuss the implications of our findings for lignin oxidation by the enzyme.

## MATERIALS AND METHODS

*Protein Preparation.* LiP isozyme 2 (H8) was purified from cultures of *P. chrysosporium* strain OGC101 as previously described (Wariishi & Gold, 1990; Gold et al., 1984). The purified enzyme was electrophoretically homogeneous and had an RZ value ($A_{408}/A_{280}$) of ~5.0. Enzyme concentrations were determined at 408 nm using an extinction coefficient of 133 $mM^{-1}$ $cm^{-1}$ (Gold et al., 1984). Horse heart ferricytochrome *c* was obtained from Sigma. Stock solutions of ferrocytochrome *c* were prepared by dissolving ferricytochrome

† This work was supported by Grants MCB 9207997 from the National Science Foundation and FG06292ER from the U.S. Department of Energy, Office of Basic Energy Sciences.

* To whom correspondence should be addressed at the Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science and Technology, P.O. Box 91000, Portland, OR 97291-1000. Telephone: 503-690-1076; Fax: 503-690-1464.

‡ Present address: Department of Forest Products, Kyushu University, Fukuoka 812, Japan.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; LiP, lignin peroxidase; LiPI, LiPII, and LiPIII, LiP compounds I, II, and III; VA, 3,4-dimethoxybenzyl (veratryl) alcohol; $VA^{\bullet+}$, VA cation radical.

0006-2960/94/0433-5545$04.50/0 

*c* in water, and then adding excess potassium dithionite as described (Kang & Erman, 1982). Excess dithionite was removed by gel filtration on a Sephadex G-10 column equilibrated with an argon-saturated 10 mM $KNO_3$ solution. After gel filtration, the ferrocytochrome *c* solution (>97% in the reduced state) was stored under argon. The stock solution concentration was determined spectroscopically after each series of experiments, and data were used for kinetic analysis only with solutions which were >95% ferrocytochrome *c*. Concentrations of ferri- and ferrocytochrome *c* were determined using an $\epsilon_{410}$ of 106 $mM^{-1}$ $cm^{-1}$ and an $\epsilon_{550}$ of 27.7 $mM^{-1}$ $cm^{-1}$, respectively (Margoliash & Frohwirt, 1959). HRP (type VI) was purchased from Sigma, and yeast CCP was kindly supplied by Dr. A. G. Mauk (University of British Columbia, Canada). Concentrations of HRP and CCP were determined using an $\epsilon_{402}$ of 102 $mM^{-1}$ $cm^{-1}$ (Dunford & Stillman, 1976) and an $\epsilon_{408}$ of 98 $mM^{-1}$ $cm^{-1}$ (Vitello et al., 1990), respectively.

*Chemicals.* $H_2O_2$ (30% solution) was obtained from Sigma, and the stock solution concentration was determined as described (Cotton & Dunford, 1973). VA was purchased from Aldrich. All other chemicals were reagent grade. All solutions were prepared using deionized water from a Milli Q 50 system (Millipore).

*Determination of Oxidation Rates.* Ferrocytochrome *c* oxidation was monitored at room temperature at 550 nm using a Shimadzu UV-260 spectrophotometer with a 1-cm light path. The initial oxidation rate was calculated using a $\Delta\epsilon_{550}$ of 19.5 $mM^{-1}$ $cm^{-1}$ between ferro- and ferricytochrome *c* (Kang & Erman, 1982). The initial velocities were corrected for the uncatalyzed $H_2O_2$ oxidation of ferrocytochrome *c*. The oxidation of VA to veratraldehyde was monitored at 310 nm ($\epsilon_{310}$ = 9.3 $mM^{-1}$ $cm^{-1}$) (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987).

$1/v$ versus 1/[ferrocytochrome *c*] was plotted at several fixed concentrations of $H_2O_2$. Reaction mixtures contained LiP (1 μg/mL), ferrocytochrome *c* (8–100 μM), and $H_2O_2$ (20–100 μM). The reaction was initiated by the addition of $H_2O_2$. Buffers used were 10 mM potassium succinate for the LiP and HRP reactions and 10 mM potassium phosphate for the CCP reaction at the indicated pHs. The ionic strength of the reaction mixture was adjusted with $KNO_3$. Owing to the low ionic strength, the pH was remeasured after the reactions were completed. The pH change over the course of the reaction was less than 0.03 in all cases. Reactions were carried out under both air and argon.

*Protection of Lignin Peroxidase from Inactivation by $H_2O_2$.* The ability of ferrocytochrome *c* and VA to protect LiP from $H_2O_2$ was investigated. Reaction mixtures consisted of LiP (50 μg/mL, ~1.2 μM) and $H_2O_2$ (120 μM) in 10 mM sodium succinate, pH 3.0. Reactions were run at 25 °C in the presence of ferrocytochrome *c* (250 μM), VA (250 μM), or no additives. Periodically during the reaction a 20-μL aliquot was removed and LiP activity was measured in an assay mixture consisting of enzyme, 5 mM VA, and 0.1 mM $H_2O_2$ in 20 mM sodium succinate, pH 3.0. The VA oxidation rate was corrected for the inhibition caused by ferricytochrome *c* in the aliquot.

*Inactivation of Lignin Peroxidase during the Reaction.* LiP inactivation during the course of the reaction was monitored via a previously reported procedure (Selwyn, 1965). In these Selwyn tests the rate of ferricytochrome *c* produced at pH 3.0 was plotted against (LiP concentration × time) at three LiP concentrations. LiP concentrations of 1, 2, and 4 μg/mL were used for reactions conducted in the absence of VA. LiP concentrations of 0.1, 0.2, and 0.4 μg/mL were used for reactions conducted in the presence of VA. To compensate for the differences in enzyme concentration, the recorder chart speed was varied accordingly.

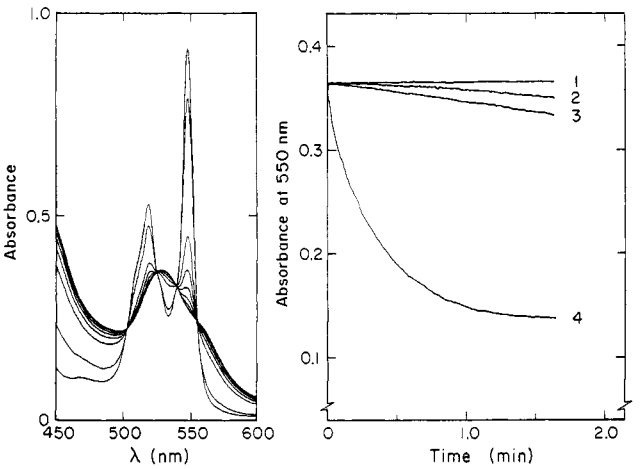


FIGURE 1: Oxidation of ferrocytochrome *c* by LiP. (A) Spectra were recorded at 2-min intervals. Reaction mixtures consisted of LiP (0.5 μg/mL), ferrocytochrome *c* (30 μM), $H_2O_2$ (100 μM) in potassium succinate, pH 6.2 (μ 0.01). (B) Ferrocytochrome *c* oxidations were monitored at 550 nm under aerobic conditions. Trace 1 (spontaneous autoxidation): ferrocytochrome *c* (13 μM) in potassium succinate, pH 4.5 (μ 0.01). Trace 2: Same as trace 1 with addition of 100 μM $H_2O_2$. Trace 3: Same as trace 2 with addition of HRP (1 μg/mL). Trace 4: Same as trace 2 with addition of LiP (1 μg/mL).

*Effect of Ferricytochrome c on the Lignin Peroxidase Oxidation of Veratryl Alcohol.* Reaction mixtures contained LiP (1 μg/mL), VA (50–500 μM), and $H_2O_2$ (100 μM) in the presence of ferricytochrome *c* (0–10 μM). The generation of superoxide during aerobic VA oxidation by LiP has been reported (Schmidt et al., 1989), and ferricytochrome *c* is readily reduced by superoxide to form ferrocytochrome *c*. To avoid interference from the possible reduction of ferricytochrome *c* by superoxide, the isosbestic point between ferri- and ferrocytochrome *c* (337 nm) was used to monitor VA oxidation, using an $\epsilon_{337}$ of 2.06 $mM^{-1}$ $cm^{-1}$. The reaction was initiated by the addition of $H_2O_2$ and carried out under aerobic conditions.

## RESULTS

*Ferrocytochrome c Oxidation by LiP.* The visible spectral change observed when ferrocytochrome *c* was treated with LiP/$H_2O_2$ is shown in Figure 1A. During the course of the reaction, the 550- and 520-nm bands indicative of ferrocytochrome *c* decreased and a new peak appeared at 528 nm. This conversion has several isosbestic points (Figure 1A) which are identical to those observed during the CCP oxidation of ferrocytochrome *c* and during autoxidation (data not shown), strongly suggesting that LiP oxidizes ferrocytochrome *c* to ferricytochrome *c* via a single-electron step. The LiP-catalyzed oxidation of cytochrome *c* is much faster than autoxidation or oxidation with $H_2O_2$ alone (Figure 1B). As shown in Figure 1B, HRP has minimal activity with ferrocytochrome *c*.

*Effect of pH and Veratryl Alcohol.* The effect of pH on the LiP-catalyzed oxidation of ferrocytochrome *c* in the presence and the absence of VA is shown in Figure 2. In the presence of VA, ferrocytochrome *c* oxidation increased continuously as the pH was lowered to 3.0. This is similar to the pH dependence for VA oxidation by LiP (Tien et al., 1986; Renganathan et al., 1987). In the absence of VA, the activity increased with decreasing pH to a maximum at pH 4.0, beyond which the activity decreased considerably. The addition of VA had little effect above pH 4.5, but it enhanced the rate significantly below pH 3.5.

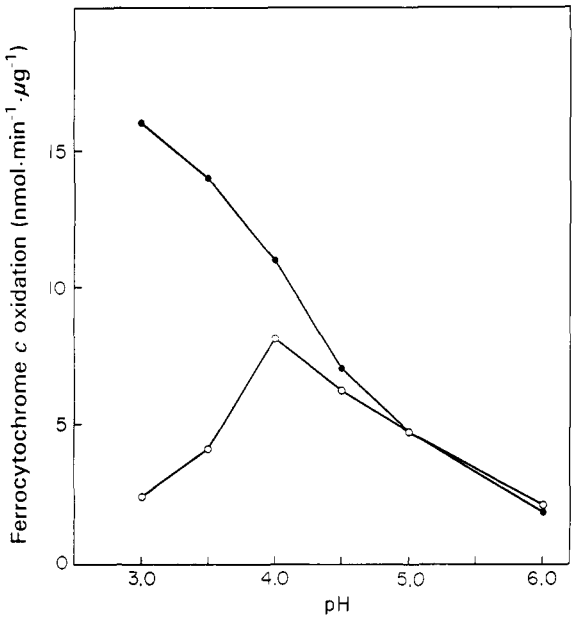


FIGURE 2: pH profile for LiP oxidation of ferrocytochrome c in the presence or absence of veratryl alcohol. Reaction mixtures consisted of LiP (1 μg/mL), ferrocytochrome c (20 μM), and $H_2O_2$ (100 μM) in potassium succinate (μ 0.01) without VA (○) and with VA (0.5 mM) (●).

Table 1: Veratryl Alcohol and Ferrocytochrome c Oxidation by LiP

| | | nmol·min⁻¹·μg⁻¹ | |
|---|---|---|---|
| pH | assay method[a] | VA oxidation (%)[b] | ferrocytochrome c oxidation (%)[b] |
| 3.04 | A | 21.34 | 2.90 |
| | B | 18.90 (89) | 1.56 (54) |
| 3.97 | A | 11.03 | 8.32 |
| | B | 10.81 (98) | 6.41 (73) |
| 5.04 | A | 2.87 | 4.83 |
| | B | 2.84 (99) | 4.62 (96) |
| 6.12 | A | 1.61 | 2.80 |
| | B | 1.58 (98) | 2.73 (98) |

[a] Method A: 20-s preincubation of the reducing substrate and LiP in buffer, prior to addition of $H_2O_2$ to initiate the reaction. Method B: 20-s preincubation of LiP and $H_2O_2$ in buffer, prior to addition of reducing substrate to initiate the reaction. Reaction mixtures consisted of LiP (1 μg/mL), $H_2O_2$ (0.1 mM), and VA (0.5 mM) or ferrocytochrome c (20 μM) in potassium succinate (μ 0.01). [b] Percentage of the rate for method B over that for method A.

To explore the role of VA in this reaction, two assay methods were used to measure the initial velocities. In the first assay (method A), the reducing substrate and LiP were preincubated in the buffer solution for 20 s, after which $H_2O_2$ was added to initiate the reaction. In the second assay (method B), $H_2O_2$ and LiP were preincubated for 20 s in the buffer, after which the reducing substrate was added to initiate the reaction. Table 1 shows the initial rates at several pHs with either VA or ferrocytochrome c as substrate.

The direct effect of cytochrome c or VA on $H_2O_2$ inactivation of LiP also was investigated (Figure 3). When LiP was incubated with 120 μM $H_2O_2$ at pH 3.0, almost all activity was lost within 2 min at 25 °C. However, in the presence of 250 μM VA, essentially all LiP activity was retained. Under the same conditions in the presence of ferrocytochrome c, 70% of the LiP activity was lost within the first 2 min.

The effect of LiP inactivation on the rate of cytochrome c oxidation at pH 3.0 is shown in Figure 4. In these experiments, the rate of ferricytochrome c formed was plotted against (LiP concentration × time) at three different LiP concentrations (Selwyn, 1965). At pH 3.0, in the presence of 65 μM $H_2O_2$ and in the absence of VA, the Selwyn test demonstrated that the enzyme was inactivated during the course of the reaction (Figure 4A). When the reaction was conducted under the same conditions in the presence of 500 μM VA, the Selwyn test indicated that the enzyme was not inactivated significantly (Figure 4B). Finally, at pH 3.0 with 6.5 μM $H_2O_2$, the enzyme was not inactivated significantly even in the absence of VA (Figure 4C).

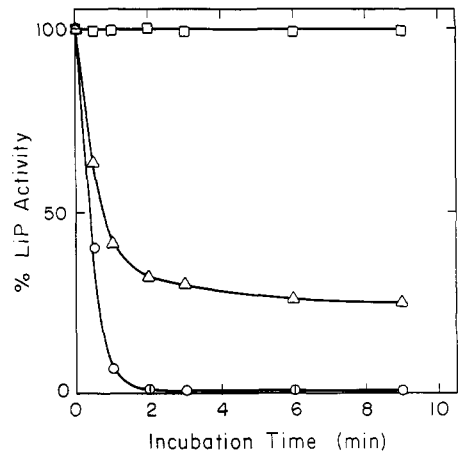


FIGURE 3: Effect of veratryl alcohol and ferrocytochrome c on lignin peroxidase stability and activity. LiP (50 μg/mL) was incubated with $H_2O_2$ (120 μM) in 10 mM potassium succinate, pH 3.0, in the absence (○) or in the presence of either VA (250 μM) (□), or ferrocytochrome c (250 μM) (△). Periodically, aliquots (20 μL) were removed and assayed for LiP activity as described in the text.

The results in Figure 5 show the stimulatory effect of VA on the oxidation of ferrocytochrome c at various $H_2O_2$ concentrations. At pH 3.0, in the presence of 100 μM $H_2O_2$, VA stimulated the reaction approximately 7-fold with maximal effect at ~250 μM VA. In contrast, at pH 3.0 in the presence of 3 or 6 μM $H_2O_2$, the effect of VA was minimal. These results show that the VA stimulatory effect increased with increasing concentrations of $H_2O_2$ in the reaction mixture.

*Steady-State Kinetics.* The family of plots, $1/v$ versus $1/[\text{ferrocytochrome } c]$ at various fixed concentrations of $H_2O_2$, yielded a set of parallel lines, indicating a ping-pong mechanism for the LiP oxidation of ferrocytochrome c. Reactions were carried out at pH 4.0 under aerobic conditions. The secondary plot of primary y-intercepts versus $1/[H_2O_2]$ showed a linear relationship but passed through the origin within experimental error, suggesting that the reaction obeys peroxidase ping-pong kinetics rather than classical ordered bi-bi ping-pong kinetics (data not shown). For this reason, the $K_m$ for $H_2O_2$ could not be determined. However, at a fixed $[H_2O_2]$, the apparent $K_m$ and $k_{cat}$ for ferrocytochrome c could be determined. CCP reactions were carried out at pH 7.3 in potassium phosphate buffer. Table 2 summarizes these kinetic parameters at an $[H_2O_2]$ of 100 μM. CCP did not oxidize VA under the conditions used.

*Effect of Ionic Strength.* A plot of the logarithm of the initial velocity versus the square root of the ionic strength exhibited a linear relationship at pH 4.0 (Figure 6). As the ionic strength increased, the rate of ferrocytochrome c oxidation by LiP decreased.

*Inhibition of Veratryl Alcohol Oxidation by Ferricytochrome c.* The family of plots, 1/v versus 1/[VA] at various fixed concentrations of ferricytochrome c, intercepted on the x-axis (Figure 7). A linear relationship of the secondary plot, y-intercept versus [ferricytochrome c], demonstrated non-

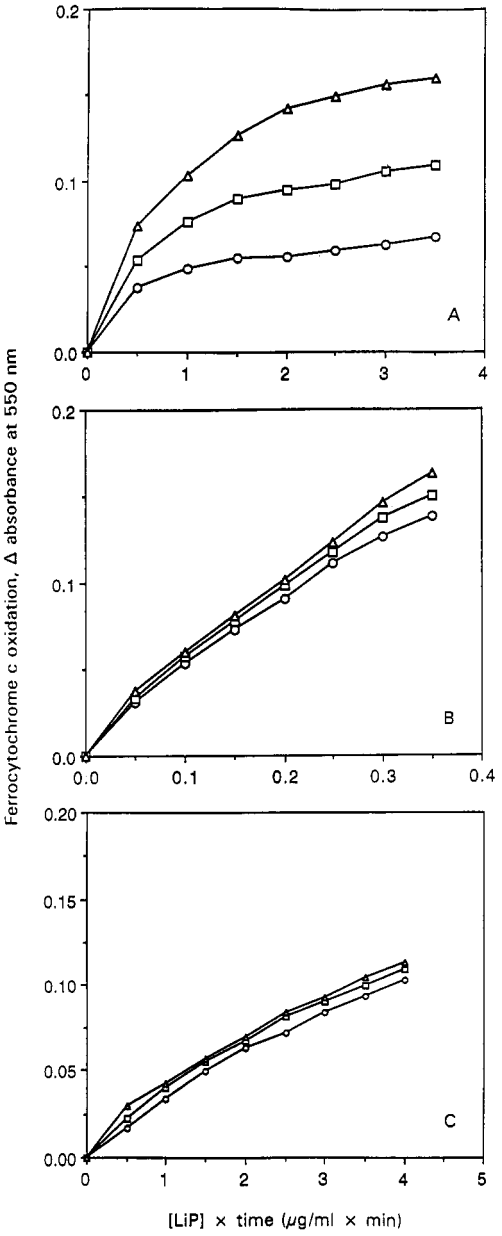


FIGURE 4: Inactivation of LiP by $H_2O_2$ during the oxidation of ferrocytochrome *c*. The amount of ferricytochrome *c* produced is plotted against (enzyme concentration × time) as described (Selwyn, 1965). All reaction mixtures contained 20 μM ferrocytochrome *c* in 10 mM potassium succinate, pH 3.0. (A) Reactions contained 65 μM $H_2O_2$, and either 1.0 (○), 2.0 (□), or 4.0 (△) μg of LiP but no VA. (B) Reactions contained 65 μM $H_2O_2$, 500 μM VA, and either 0.1 (○), 0.2 (□), or 0.4 (△) μg of LiP. (C) Reactions contained 6.5 μM $H_2O_2$ and either 1.0 (○), 2.0 (□), or 4.0 (△) μg of LiP but no VA. The recorder chart speed was run at 1, 2, or 4 cm/min to compensate for the enzyme concentrations used.

competitive inhibition (Figure 7, inset). The $K_i$ for ferricytochrome *c* was determined to be 2.5 μM.

## DISCUSSION

Although considerable work has been reported on the mechanism of LiP, the precise role of this enzyme in polymeric lignin degradation remains unclear. The role that VA plays in the lignin degradative system also is still under discussion. VA stimulates the LiP-catalyzed depolymerization of synthetic lignin (Hammel et al., 1993) and the LiP-catalyzed oxidation of a variety of poor reducing substrates (Valli et al., 1992; Harvey et al., 1986; Renganathan et al., 1985; Haemmerli et al., 1986). *P. chrysosporium* produces VA under ligninolytic

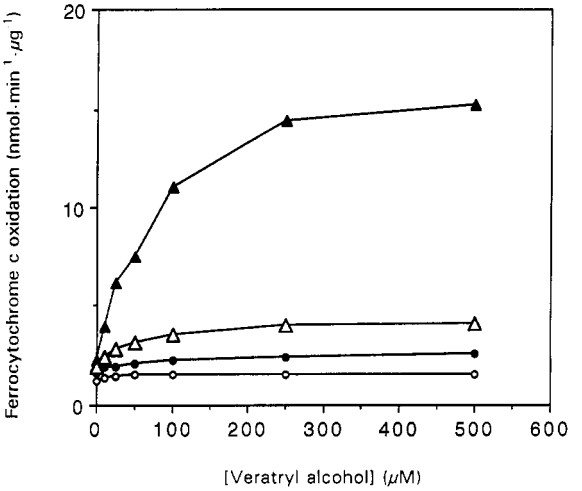


FIGURE 5: Effect of $H_2O_2$ concentration on the stimulation of cytochrome *c* oxidation by VA. Reaction mixtures contained LiP (1 μg/mL), VA (0.0–0.5 mM), and ferrocytochrome *c* (20 μM) in 10 mM potassium succinate, pH 3.0. Reactions were initiated by addition of $H_2O_2$ to a final concentration of 3 (○), 6 (●), 9 (△), or 100 (▲) μM, and ferrocytochrome *c* oxidation was monitored as described in the text.

Table 2: Kinetic Parameters of LiP and CCP Reactions[a]

| enzyme | substrate | $K_{m(app)}$ (μM) | $k_{cat(app)}$ ($s^{-1}$) |
|---|---|---|---|
| LiP | ferrocytochrome *c* | 68.2 | 25.7 |
| | veratryl alcohol | 113 | 19 |
| CCP | ferrocytochrome *c* | 18.3 | 175 |

[a] Values were determined at an $H_2O_2$ concentration of 100 μM. The LiP reaction with VA was carried out in potassium succinate, pH 3.04 (μ 0.01). The LiP reaction with ferrocytochrome *c* was carried out at pH 4.0. CCP reactions were carried out in potassium phosphate, pH 7.3 (μ 0.01). Reaction mixtures consisted of enzymes (LiP, 1 μg/mL; CCP, 0.5 μg/mL), reducing substrates (ferrocytochrome *c*, 10–100 μM; VA, 50–500 μM), and $H_2O_2$.

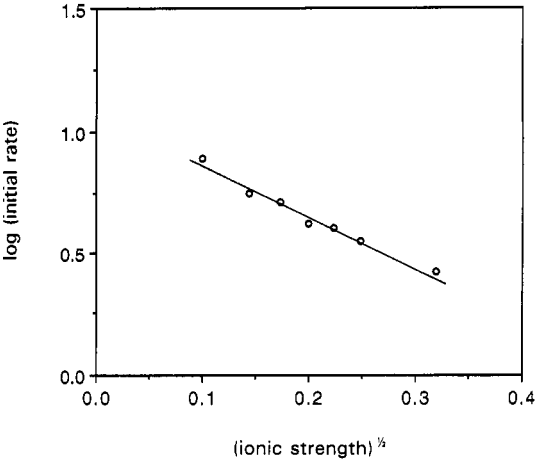


FIGURE 6: Effect of ionic strength on the initial velocity of ferrocytochrome *c* oxidation by LiP. The logarithm of initial velocities was plotted against the square root of the ionic strength at pH 4.0. Reaction mixtures consisted of LiP (1 μg/mL), ferrocytochrome *c* (17 μM), and $H_2O_2$ (100 μM).

conditions; VA is a good reducing substrate for LiP (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987). A variety of mechanisms have been postulated to account for the stimulation of LiP reactions by VA. One role of VA appears to be protection of the enzyme from $H_2O_2$-derived irreversible inactivation (Wariishi & Gold, 1989, 1990), although the exact protection mechanism(s) has not been clarified. A second proposed role for VA is that of a diffusible

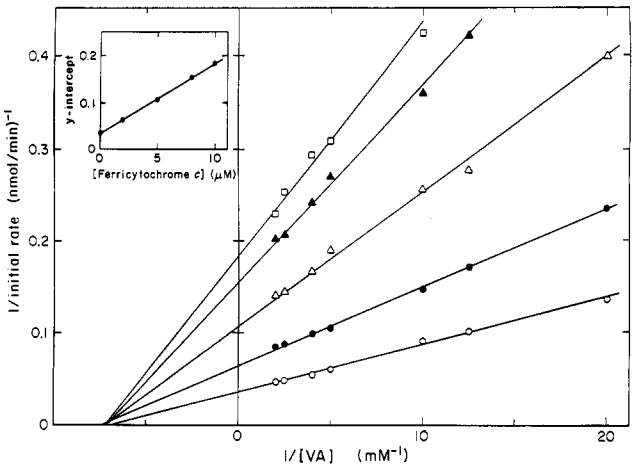


FIGURE 7: Inhibitory effect of ferricytochrome c on veratryl alcohol oxidation by LiP. Reaction mixtures contained LiP (1 μg/mL), VA (0.05–0.5 mM)), $H_2O_2$ (100 μM), and fixed [ferricytochrome c] of 0 (○), 1.97 (●), 4.95 (△), 7.92 (▲), and 9.89 (□) μM in potassium succinate, pH 3.04. Inset: Replot of the y-intercept versus [ferricytochrome c] (μM).

radical mediator, wherein the $VA^{\bullet+}$ produced via the one-electron oxidation of VA diffuses and, in turn, oxidizes the terminal substrate (lignin) some distance from the surface of the enzyme (Harvey et al., 1986, 1987; Akamatsu et al., 1990; Popp et al., 1990). However, both the long-range mediation by a diffusible $VA^{\bullet+}$ and the formation of a catalytic LiPII–$VA^{\bullet+}$ complex (Harvey et al., 1987) have been challenged (Wariishi et al., 1991a, 1992a; Valli et al., 1990; Cui & Dolphin, 1990). Recent crystal structures of LiP demonstrate that the heme group is buried in the protein (Edwards et al., 1993; Poulos et al., 1993; Piontek et al., 1993) and that a narrow access channel may be available to $H_2O_2$ and small aromatic compounds such as VA. Indeed, a protein-binding site for VA has been postulated via computer modeling (Poulos et al., 1993). This raises the question of whether LiP can directly attack polymeric lignin or whether VA plays some role in electron transfer between the buried heme and the polymeric substrate. To investigate the interaction between the enzyme and a polymeric substrate, we utilized ferrocytochrome c as a model polymer. Ferrocytochrome c was selected as a polymeric substrate since (i) its heme iron is low spin and hexacoordinated (Ferguson-Miller et al., 1979), assuring that it does not react with $H_2O_2$ to form higher redox states such as compounds I and II of peroxidases; (ii) ferro-ferricytochrome c is a well-characterized one-electron couple, limiting complexities in the reactions; (iii) the molecular mass of horse heart cytochrome c (~12 300 Da) is considerably larger than the average molecular mass of chemically synthesized lignin (3000–5000 Da); and finally, (iv) the heme in cytochrome c is buried (Pelletier & Kraut, 1992; Ferguson-Miller et al., 1979).

*Effect of pH and Veratryl Alcohol.* Below pH 3.5, ferrocytochrome c oxidation by LiP is enhanced significantly by VA. The lower the pH, the greater the effect of VA. In contrast, above pH 4.5 the effect of VA on the reaction is negligible (Figure 2). To examine the role of VA in the reaction, several experiments were conducted. Initially, two assay methods were used to measure the initial velocities for LiP oxidation of ferrocytochrome c and VA (Table 1).

Preincubation of LiP with $H_2O_2$ at pH 3 for 20 s before initiating the reaction results in ~50% loss of the ferrocytochrome c oxidation activity. In contrast, only ~10% loss of LiP activity is observed with VA as the substrate (Table 1). This may be explained by our previous finding that LiPII has an unusually high reactivity with $H_2O_2$, generating LiPIII (Wariishi & Gold, 1990; Wariishi et al., 1990). The latter reacts further with $H_2O_2$, causing irreversible heme bleaching via the intermediate formation of LiPIII*. We have shown previously that VA both reduces LiPII efficiently and reacts with the intermediate LiPIII* to regenerate native LiP (Wariishi & Gold, 1990; Wariishi et al., 1990). The 10% activity loss with respect to VA oxidation probably is caused by the irreversible inactivation of the enzyme during the 20-s incubation period. In contrast, ferrocytochrome c does not react with the intermediate LiPIII* to regenerate native LiP (see below), and at pH 3, ferrocytochrome is a relatively poor substrate as compared to VA. Thus, in the presence of both ferrocytochrome c and $H_2O_2$ but in the absence of VA, the enzyme does not complete its catalytic cycle. Instead, it is rapidly converted to LiPIII*, followed by slow irreversible enzyme inactivation. Since LiPIII* is not catalytically active, 50% loss of activity is seen for ferrocytochrome c oxidation; most likely 40% of the loss is caused by LiPIII* formation and 10% by enzyme inactivation.

We have reported that the reaction of LiPII with $H_2O_2$ is pH dependent, with a $pK_a$ of 4.2, and that the reaction rate dramatically decreases above pH 4 (Wariishi et al., 1990). Thus, the rate for irreversible inactivation also decreases above pH 4 (Wariishi et al., 1990). This previous finding may explain why above pH 4 no loss is observed for VA oxidation activity by preincubation of LiP with $H_2O_2$ (Table 1). As the pH increases, the loss of ferrocytochrome c oxidation activity also decreases, correlating well with our previous findings on the pH dependence of LiP inactivation (Wariishi & Gold, 1990).

The electron-transfer reaction increases as the pH decreases. The protonated forms of LiPI and LiPII are most active for one-electron transfer from VA (Wariishi et al., 1991a). However, the enzyme inactivation rate also rapidly increases as pH decreases, possibly explaining why LiP oxidation of ferrocytochrome c has an apparent pH optimum at 4.0 in the absence of VA.

The ability of VA and ferrocytochrome c to protect LiP directly from $H_2O_2$-derived inactivation also was examined (Figure 3). Both VA and ferrocytochrome c protect the enzyme, but the extent of protection is much greater with VA. We have reported that VA protects the enzyme via two mechanisms: (i) by stimulating the reduction of LiPII to the native enzyme, thus preventing the reaction of LiPII with $H_2O_2$; and (ii) by converting LiPIII* back to the native enzyme (Wariishi & Gold, 1990). Ferrocytochrome c is a good reducing substrate for LiP above pH 4.0 (Figure 2). The reactivity of LiPIII* with cytochrome c was examined at pH 3.0. Owing to the strong absorptivity of ferrocytochrome c, the spectral shifts due to LiPIII* conversion are difficult to observe. However, a shoulder at 578 nm attributable to LiPIII* remains for several minutes after addition of ferrocytochrome c. This shoulder is observed in LiPIII* spectra but is not observed with native LiP or with ferro- or ferricytochrome c. Furthermore, this shoulder disappears upon addition of VA to LiPIII* (Wariishi & Gold, 1990).

These results suggest that at pH 3.0 and in the presence of 100 μm $H_2O_2$, ferrocytochrome c reduction of LiPII is unable to compete with the rapid conversion of LiPII to LiPIII*. In addition, ferrocytochrome c is unable to convert LiPIII* to the native enzyme. Thus, at pH 3.0 in the presence of 100 μM $H_2O_2$ and in the absence of VA, LiP would be inactivated during the oxidation of ferrocytochrome c. This inactivation also accounts, at least in part, for the slow rate of ferrocytochrome c oxidation observed at pH 3.0. Under the same

conditions but in the presence of VA, LiP should not be inactivated.

To examine the possible inactivation of LiP during the course of ferrocytochrome *c* oxidation, we conducted a simple test. Selwyn (1965) showed that when the product formed during the reaction is plotted against (enzyme concentration × time), enzyme inactivation during the course of the reaction will become apparent. The results shown in Figure 4A demonstrate that LiP is inactivated during the course of the reaction at pH 3.0 in the presence of 65 $\mu$M $H_2O_2$. Figure 4B shows that VA protects against this inactivation. Finally, Figure 4C shows that significant inactivation does not occur when the reaction is conducted in the presence of only 6.5 $\mu$M $H_2O_2$. The exact mechanism of this protection has not been elucidated. However, it is likely to involve the comparative facile reduction of LiPII in the presence of VA and conversion of LiPIII* to the native enzyme. At pH 3.0, LiPII is readily converted to LiPIII in the presence of $H_2O_2$. Apparently, the oxidation of VA by LiPII effectively competes with the conversion of LiPII to LiPIII. At pH 3.0 and in the presence of 100 $\mu$M $H_2O_2$, the rate of ferrocytochrome *c* oxidation apparently does not compete with the rate of LiPII conversion to LiPIII. At pH 3.0, the presence of VA significantly stimulates ferrocytochrome *c* oxidation, at least in part by protecting LiP from inactivation, but the mechanism(s) for this remains unclear. At least three possibilities are feasible: (i) VA may act as an enzyme-binding activator such that the binding of VA stimulates the direct transfer of an electron from the alternate substrate (ferrocytochrome *c*) to the heme in LiP; (ii) by reacting rapidly with LiPII, VA stimulates completion of the peroxidatic cycle and prevents the formation of LiPIII* (Wariishi & Gold, 1990; Paszczyński & Crawford, 1991); alternatively, (iii) VA bound in the heme pocket may mediate the electron transfer directly. In this case, VA would act as a cofactor cycling between the fully reduced and the radical cation oxidation states. The radical cation would accept an electron from ferrocytochrome *c* and in turn be reoxidized by LiPI and LiPII. This alternative does not require VA to diffuse into the aqueous medium as proposed by Harvey et al. (1986) with subsequent rapid loss of a proton. As shown in Figure 4, when the reaction is conducted at pH 3.0 in the presence of 6.5 $\mu$M $H_2O_2$, LiP is not inactivated, suggesting that VA stimulates the oxidation of ferrocytochrome *c* only at relatively high concentrations of $H_2O_2$. The results shown in Figure 5 confirm that VA stimulates the oxidation of ferrocytochrome *c* in the presence of high concentrations of $H_2O_2$; but in the presence of low concentrations of $H_2O_2$ (3–6 $\mu$M), there is minimal stimulation by VA. Furthermore, there is minimal stimulation by VA above pH 4.0, even in the presence of high concentrations of $H_2O_2$. These observations suggest that the VA stimulation of cytochrome *c* oxidation is due to its capacity to foster efficient enzyme turnover at pH 3.0 and 100 $\mu$M $H_2O_2$, conditions which are optimal for activity. In the absence of VA, the enzyme is rapidly inactivated under these conditions.

*Peroxidase Ping-Pong Mechanism.* A linear relationship is obtained for the plot of $1/v$ against $1/$[ferrocytochrome *c*], suggesting that only one ferrocytochrome *c* equivalent is involved in each reduction step of the catalytic cycle. The series of plots of $1/v$ against $1/$[ferrocytochrome *c*] at various fixed [$H_2O_2$] yield parallel lines. The secondary plot of primary *y*-intercepts ($1/V_{max}$) against $1/[H_2O_2]$ passes through the origin. In classical ordered bi-bi ping-pong kinetics, $K_m$ and $V_{max}$ are assigned from the secondary plot. This difference between classical ping-pong and peroxidase ping-pong mechanisms is well documented, resulting from the irreversible reactions of the peroxidase catalytic cycle (Dunford, 1991). Both the irreversible formation of LiPI by $H_2O_2$ and irreversible reductions of LiPI and LiPII by VA have been reported (Marquez et al., 1988; Wariishi et al., 1991a,b). The LiP oxidation of ferrocytochrome *c* obeys peroxidase ping-pong kinetics rather than classical ordered bi-bi ping-pong kinetics. This strongly suggests that the reductions of LiPI and LiPII by ferrocytochrome *c* also are irreversible. Peroxidase ping-pong kinetics also have been observed for $Mn^{II}$ oxidation by manganese peroxidase (Wariishi et al., 1992b).

To compare the rates of ferrocytochrome *c* oxidation by LiP and CCP, steady-state kinetic parameters were determined. The kinetics of CCP reactions depend upon the ferrocytochrome *c* concentration (Kang & Erman, 1982; Margoliash et al., 1976; Kang et al., 1977). With the concentrations used in this study, the kinetics display no such complexity. For CCP, a double-reciprocal plot of the initial velocity versus ferrocytochrome *c* concentration shows a linear relationship at 100 $\mu$M $H_2O_2$ (data not shown). At pH 7.3 and 100 $\mu$M $H_2O_2$, a $K_{m(app)}$ for ferrocytochrome *c* of 18.2 $\mu$M and a $k_{cat(app)}$ of 175 $s^{-1}$ are obtained for CCP (Table 2). A $K_{m(app)}$ of 15.2 $\mu$M and a $k_{cat(app)}$ of 209 $s^{-1}$ at 170 $\mu$M [$H_2O_2$] were recently reported for CCP (DePillis et al., 1991). In contrast, Yonetani and Ray (1965) reported a $k_{cat(app)}$ of 1800 $s^{-1}$ at 176 $\mu$M [$H_2O_2$] at pH 6.0 with a different CCP preparation. At pH 4.0 and an $H_2O_2$ concentration of 100 $\mu$M, LiP shows a $K_{m(app)}$ of 68 $\mu$M and a $k_{cat(app)}$ of 25.7 $s^{-1}$ for ferrocytochrome *c*. Although CCP/$H_2O_2$ is a better catalyst of ferrocytochrome *c* oxidation than LiP/$H_2O_2$, ferrocytochrome *c* oxidation by LiP is significant. Indeed, LiP has a $K_{m(app)}$ of 113 $\mu$M and a $k_{cat(app)}$ of 19 $s^{-1}$ for VA at 100 $\mu$M $H_2O_2$ (Table 1). The faster rate for ferrocytochrome *c* oxidation may be explained in part by the lower redox potential of ferrocytochrome *c* (0.26 V) vs ~1.5 V for VA. However, VA oxidation to veratraldehyde requires 2 one-electron steps, which also accounts for the lower turnover number. The $K_{m(app)}$ for these two substrates suggests that the polymeric substrate may bind more strongly to the enzyme. We carried out additional experiments to characterize the binding of ferrocytochrome *c* to LiP.

*Effect of Ionic Strength.* Ferrocytochrome *c* oxidation by CCP is inhibited at high ionic strength (Kim et al., 1990). Detailed kinetic studies have shown that ionic strength has a strong effect on the binding interaction of ferrocytochrome *c* with CCP compounds I and II, but not on compound I formation or the rate of electron transfer (Kim et al., 1990; Loo & Erman, 1975). The equilibrium dissociation constant for ferricytochrome *c* and native CCP is also dependent upon ionic strength (Erman & Vitello, 1980). These studies demonstrated a 1:1 binding between CCP and cytochrome *c* and suggest that an electrostatic interaction between CCP and cytochrome *c* is the rate-determining step (Kim et al., 1990; Loo & Erman, 1975; Erman & Vitello, 1980).

The initial velocity of ferrocytochrome *c* oxidation by LiP/$H_2O_2$ also decreases with increasing ionic strength. A plot of the logarithm of the initial rate versus the Debye–Hückel ionic strength function at pH 4.0 (Figure 6) indicates that the rate-determining step(s) of this reaction depends upon ionic strength. These results suggest that electrostatic binding of LiP and cytochrome *c* is the rate-determining step. However, a direct interaction between the LiP heme and the polymeric substrate is ruled out by recent X-ray crystallographic studies of LiP (Edwards et al., 1993; Poulos et al., 1993; Piontek et al., 1993) which show that the heme is buried and is not

available to interact directly with the substrate. This is supported by our previous results showing that the heme edge is not available for modification with arylhydrazine (DePillis et al., 1990). All of these results suggest that protein surface electrostatic interaction between LiP and cytochrome *c* is required for the reaction. Involvement of an electrostatic surface interaction is consistent with the binding of these two proteins which would be oppositely charged in the pH range of 4.5–6.0. LiP isozymes have p*I*s of 3.2–3.9 (Leisola et al., 1987), and cytochrome *c* has a p*I* of 10.7 (Ferguson-Miller et al., 1979). Like LiP, CCP also is an acidic protein (Yonetani, 1967).

*Cytochrome c Binding Site.* Recent crystallographic and modeling studies suggest that LiP may have a VA binding site in an access channel near the buried heme (Poulos et al., 1993). If electrostatic surface interactions are involved in the binding of LiP to cytochrome *c*, then the sites for the two substrates would be distinct and VA oxidation by LiP should be inhibited by cytochrome *c* in a noncompetitive manner. In this study ferricytochrome *c* was utilized as an inhibitor. Although it is not a substrate for LiP, the surface amino acids and charge which control the electrostatic binding are identical for ferri- and ferrocytochrome *c*.

The family of plots, $1/v$ versus $1/[VA]$ at various fixed concentrations of ferricytochrome *c*, demonstrate a noncompetitive inhibition pattern (Figure 7), suggesting that VA is oxidized at a site separate from the cytochrome *c* binding site. From the secondary plot of *y*-intercept versus [ferricytochrome *c*], the $K_i$ for ferricytochrome *c* was determined to be 2.5 $\mu$M (Figure 7, inset), demonstrating that it is an effective noncompetitive inhibitor of VA oxidation by LiP. Others have suggested that noncompetitive inhibition of VA oxidation by a terminal LiP substrate implies that VA is acting as a radical mediator in the reaction (Akamatsu et al., 1990; Popp et al., 1990). However, in this experiment, we have used ferricytochrome *c* which is not a substrate for LiP; therefore, the noncompetitive inhibition kinetics strongly suggest the existence of different binding sites rather than a radical mediation mechanism, at least in this case. These kinetics are consistent with the assumption that cytochrome *c* is oxidized through surface interaction with LiP and that VA may be oxidized in a binding pocket relatively closer to the heme edge.

Ferrocytochrome *c* also is oxidized by LiP/$H_2O_2$ in the absence of VA above pH 4.0. This electron-transfer reaction most likely occurs via surface interaction of the two proteins. In this case, an electron relay system through amino acid residues is possibly involved as proposed for the CCP reaction (Pelletier & Kraut, 1992). X-ray crystallographic studies on CCP–cytochrome *c* complexes have suggested that the interaction between these proteins is highly specific (Pelletier & Kraut, 1992) and LiP may have a similar surface docking site for cytochrome *c*. Recently, it has been demonstrated that CCP oxidizes monomeric phenols at the buried heme edge whereas it interacts with ferrocytochrome *c* on the surface of the protein (DePillis et al., 1991). A similar model could be proposed for LiP. However, the direct access of small aromatic substrates (such as VA) with the heme edge has not yet been demonstrated conclusively. Indeed, (i) arylhydrazine modifies the heme edge of CCP but not of LiP (DePillis et al., 1990, 1991), and (ii) LiP has a narrower heme access channel than CCP (Poulos et al., 1993).

This is the first report of ferrocytochrome *c* oxidation by LiP. Although ferrocytochrome *c* has a lower redox potential than the phenolic groups and aromatic rings in lignin, this system may serve as a useful model for probing the mechanism of electron transfer between the buried heme of the enzyme and polymeric substrates. In particular, study of ferrocytochrome *c* oxidation by LiP should aid our attempts to elucidate the stimulating role of VA in some LiP-catalyzed reactions. Manganese peroxidase is also capable of oxidizing ferrocytochrome *c*, but this reaction is strictly dependent upon the presence of $Mn^{II}$ (unpublished). Manganese peroxidase does not oxidize cytochrome *c* directly; rather, the substrate is oxidized by $Mn^{III}$ chelator complexes generated by the enzyme (Glenn et al., 1986). Thus, LiP oxidizes cytochrome *c* through a direct interaction, and this reaction is stimulated by VA at low pH and high [$H_2O_2$]. In contrast, manganese peroxidase oxidizes cytochrome *c* through the redox mediation of a diffusible $Mn^{III}$ complex. These observations may reflect the mechanisms used by these two peroxidases in the oxidation of polymeric lignin.

Further spectral, kinetic, and structural studies aimed at probing the role of VA in this reaction are planned.

## ACKNOWLEDGMENT

We thank Professor A. Grant Mauk of the University of British Columbia for his generous gift of yeast CCP and Dr. Margaret Alic for useful discussions.

## REFERENCES

Akamatsu, Y., Ma, D. B., Higuchi, T., & Shimada, M. (1990) *FEBS Lett. 269*, 261–263.

Bull, H. B., & Breese, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 74–78.

Buswell, J. A., & Odier, E. (1987) *CRC Crit. Rev. Biotechnol. 6*, 1–60.

Cotton, H. L., & Dunford, H. B. (1973) *Can. J. Chem. 51*, 582–587.

Cui, F., & Dolphin, D. (1990) *Holzforschung 44*, 279–283.

DePillis, G. D., Wariishi, H., Gold, M. H., & Ortiz de Montellano, P. R. (1990) *Arch. Biochem. Biophys. 280*, 217–223.

DePillis, G. D., Sishta, B. P., Mauk, A. G., & Ortiz de Montellano, P. R. (1991) *J. Biol. Chem. 266*, 19334–19341.

Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 2, pp 1–24, CRC Press, Boca Raton, FL.

Dunford, H. B., & Stillman, J. S. (1976) *Coord. Chem. Rev. 19*, 187–251.

Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H., & Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. USA 90*, 750–754.

Erman, J. E., & Vitello, L. B. (1980) *J. Biol. Chem. 255*, 6224–6227.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1979) in *The Porphyrins* (Dolphin, D., Ed.) Vol. 7, pp 149–240, Academic Press, New York.

Glenn, J. K., Akileswaran, L., & Gold, M. H. (1986) *Arch. Biochem. Biophys. 251*, 688–696.

Gold, M. H., & Alic, M. (1993) *Microbiol. Rev. 57*, 605–622.

Gold, M. H., Kuwahara, M., Chiu, A. A., & Glenn, J. K. (1984) *Arch. Biochem. Biophys. 234*, 353–362.

Gold, M. H., Wariishi, H., & K. Valli (1989) in *Biocatalysis in Agricultural Biotechnology* (Whitaker, J. R., & Sonnet, P. E., Eds.) Series 389, pp 127–140, American Chemical Society, Washington, DC.

Haemmerli, S. D., Leisola, M. S. A., Sanglerd, D., & Fiechter, A. (1986) *J. Biol. Chem. 261*, 6900–6903.

Hammel, K. E., Jensen, K. A., Jr., Mozuch, M. D., Landucci, L. L., Tien, M., & Pease, E. A. (1993) *J. Biol. Chem. 268*, 12274–12281.

Harvey, P. J., Schoemaker, H. E., & Palmer, J. M. (1986) *FEBS Lett. 195*, 242–246.

Harvey, P. J., Schoemaker, H. E., & Palmer, J. M. (1987) in *Lignin Enzymic and Microbial Degradation* (Odier, E., Ed.) pp 145–150, Institut National de la Recherche Agronomique, Paris.
Higuchi, T. (1990) *Wood Sci. Technol. 24*, 23–63.
Kang, C. H., Ferguson-Miller, S., & Margoliash, E. (1977) *J. Biol. Chem. 252*, 919–926.
Kang, D. S., & Erman, J. E. (1982) *J. Biol. Chem. 257*, 12775-12779.
Kim, K. L., Kang, D. S., Vitello, L. B., & Erman, J. E. (1990) *Biochemistry 29*, 9150–9159.
Kirk, T. K., & Farrell, R. L. (1987) *Annu. Rev. Microbiol. 41*, 465–505.
Leisola, M. S. A., Kozulic, B., Meussdoerffer, F., & Fiechter, A. (1987) *J. Biol. Chem. 262*, 419–424.
Loo, S., & Erman, J. E. (1975) *Biochim. Biophys. Acta 481*, 279–282.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.
Margoliash, E., Ferguson-Miller, S., Kang, C. H., & Brautigan, D. L. (1976) *Fed. Proc. 35*, 2124–2130.
Marquez, L., Wariishi, H., Dunford, H. B., & Gold, M. H. (1988) *J. Biol. Chem. 263*, 10549–10552.
Paszczyński, A., & Crawford, R. L. (1991) *Biochem. Biophys. Res. Commun. 178*, 1056–1063.
Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748–1755.
Piontek, K., Glumoff, T., & Winterhalter, K. (1993) *FEBS Lett. 315*, 119–124.
Popp, J. L., Kalyanaraman, B., & Kirk, T. K. (1990) *Biochemistry 29*, 10475–10480.
Poulos, T. L., Edwards, S., Wariishi, H., & Gold, M. H. (1993) *J. Biol. Chem. 268*, 4429–4440.
Renganathan, V., & Gold, M. H. (1986) *Biochemistry 25*, 1626–1631.
Renganathan, V., Miki, K., & Gold, M. H. (1985) *Arch. Biochem. Biophys. 241*, 304–314.
Renganathan, V., Miki, K., & Gold, M. H. (1987) *Biochemistry 26*, 5127–5132.
Ritch, T. G., Jr., Nipper, V. J., Akileswaran, L., Smith, A., Pribnow, D., & Gold, M. H. (1991) *Gene 107*, 119–126.
Sarkanen, K. V., & Ludwig, C. H. (1971) *Lignins. Occurrence, Formation, Structure and Reactions*, Wiley-Interscience, New York.
Schmidt, H. W. H., Haemmerli, S. D., Schoemaker, H. E., & Leisola, M. S. A. (1989) *Biochemistry 28*, 1776–1783.
Schoemaker, H. E. (1990) *Recl. Trav. Chim. Pays-Bas 109*, 255–272.
Selwyn, M. J. (1965) *Biochim. Biophys. Acta 105*, 193–195.
Tien, M., & Tu, C.-P. D. (1987) *Nature 326*, 520–523.
Tien, M., Kirk, T. K., Bull, C., & Fee, J. A. (1986) *J. Biol. Chem. 261*, 1687–1693.
Valli, K., Wariishi, H., & Gold, M. H. (1990) *Biochemistry 29*, 8535–8539.
Valli, K., Wariishi, H., & Gold, M. H. (1992) *J. Bacteriol. 174*, 2131–2137.
Vitello, L. B., Huang, M., & Erman, J. E. (1990) *Biochemistry 29*, 4283–4288.
Wariishi, H., & Gold, M. H. (1989) *FEBS Lett. 243*, 165–168.
Wariishi, H., & Gold, M. H. (1990) *J. Biol. Chem. 265*, 2070–2077.
Wariishi, H., Marquez, L., Dunford, H. B., & Gold, M. H. (1990) *J. Biol. Chem. 265*, 11137–11142.
Wariishi, H., Huang, J., Dunford, H. B., & Gold, M. H. (1991a) *J. Biol. Chem. 266*, 20694–20699.
Wariishi, H., Valli, K., & Gold, M. H. (1991b) *Biochem. Biophys. Res. Commun. 176*, 269–275.
Wariishi, H., Huang, J., Dunford, H. B., & Gold, M. H. (1992a) in *Biotechnology in the Pulp and Paper Industry* (Kuwahara, M., & Shimada, M., Eds.) Uni Publishing Co., Ltd., Tokyo.
Wariishi, H., Valli, K., & Gold, M. H. (1992b) *J. Biol. Chem. 267*, 23688–23695.
Yonetani, T. (1967) *J. Biol. Chem. 242*, 5008–5013.
Yonetani, T., & Ray, G. (1966) *J. Biol. Chem. 241*, 700–706.