Lignin Peroxidase Compound III
MECHANISM OF FORMATION AND DECOMPOSITION*

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Lignin peroxidase compound III (LiP111) was prepared via three procedures: (a) ferrous LiP + O2 (LiP111a), (b) ferric LiP + O2 (LiP111b), and (c) LiP compound II + excess H2O2 followed by treatment with catalase (LiP111c). LiP111a, b, and c each have a Soret maximum at ~414 nm and visible bands at 543 and 578 nm. LiP111a, b, and c each slowly reverted to native ferric LiP, releasing stoichiometric amounts of O2 in the process. Electronic absorption spectra of LiP111 reversion to the native enzyme displayed isosbestic points in the visible region at 470, 525, and 597 nm, suggesting a single-step reversion with no intermediates. The LiP111 reversion reactions obeyed first-order kinetics with rate constants of ~1.0 x 10^{-3} s^{-1}. In the presence of excess peroxide, at pH 3.0, native LiP, LiP111, and LiP111c, b, and c are all converted to a unique oxidized species (LiP111*) with a spectrum displaying visible bands at 543 and 578 nm, but with a Soret maximum at 419 nm, red-shifted 5 nm from that of LiP111. LiP111* is bleached and inactivated in the presence of excess H2O2 via a biphasic process. The fast first phase of this bleaching reaction obeys second-order kinetics, with a rate constant of 1.7 x 10^{-1} M^{-1} s^{-1}. Addition of veratryl alcohol to LiP111* results in its rapid reversion to the native enzyme, via an apparent one-step reaction that obeys second-order kinetics with a rate constant of 0.5 x 10^{-1} M^{-1} s^{-1}. Stoichiometric amounts of O2 are released during this reaction. When this reaction was run under conditions that prevented further reactions, HPLC analysis of the products demonstrated that veratryl alcohol was not oxidized. These results suggest that the binding of veratryl alcohol to LiP111* displaces O2, thus returning the enzyme to its native state. In contrast, the addition of veratryl alcohol to LiP111 did not affect the rate of spontaneous reversion of LiP111 to the native enzyme.

White rot basidiomycetous fungi are primarily responsible for the initiation of the decomposition of lignin in wood (1–3). When cultured under ligninolytic conditions, the white rot fungus Phanerochaete chrysosporium secretes two extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase, which, along with an H2O2-generating system, are the major components of its lignin degradative system (1, 2, 4, 5). LiP has been purified to homogeneity and partially characterized. The enzyme is a glycoprotein with a molecular weight of ~41,000, contains 1 mol of Fe protoporphyrin IX, and exists as a series of isozymes (pl 3.2–4.0) (1, 2, 4, 5).

The enzyme catalyzes the H2O2-dependent oxidation of a variety of nonphenolic lignin model compounds via the initial formation of a substrate aryl cation radical, with subsequent nonenzymatic reactions yielding the final products (1–4, 6, 7). Electronic absorption (1, 8), EPR, and resonance Raman (9–11) spectroscopic studies indicate that the heme iron in the native resting enzyme is in the high-spin, predominantly pentacoordinate, ferric state with histidine coordinated as the fifth ligand. The nucleotide sequences of several LiP cDNAs indicate that the proximal histidine and the distal histidine are conserved (12, 13).

Initial characterization of the formation and the reactions of the oxidized intermediates LiP1, LiP111, and LiP111 indicate that the oxidation states and catalytic cycle of LiP are similar to horseradish peroxidase (1, 14–18). LiP has a typical peroxidase catalytic cycle but with several unique features. Although LiP has an unusually low pH optimum, ~3.0 (18–20), the rate of formation of LiP1 is independent of pH from 3.0 to 8.0 (15, 16). In addition, the ready formation of a LiP111-like species occurs with considerably less H2O2 than is required with other peroxidases (14, 16, 17). Since this compound III-like species is irreversibly inactivated in the presence of excess H2O2 yet can be converted to the native enzyme by addition of the P. chrysosporium secondary metabolite veratral alcohol (17), we decided to study the mechanisms of the formation and decomposition of LiP111 in greater detail. We have prepared LiP111 via three different pathways (18, 21) and have studied its reversion to the native enzyme as well as its reaction with H2O2 and subsequent inactivation.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—P. chrysosporium strain OGC 101 (22) was grown in agitation, acetate-buffered cultures as previously described (8, 23), except that the medium contained 0.1% Tween 80 and 6-fold concentrated trace elements. Veratral alcohol (3 mM) was added on day 3 (24). Cultures were harvested when maximal LiP activity was observed (day 6 or 7). The enzyme was purified by DEAE-Sepharose chromatography (8, 23) and fast protein liquid chromatography (mono Q) (35). The purified protein was electrophoretically homogeneous and had an RZ (A280/A207) value of ~5.0. Enzyme concentrations were determined at 407.6 nm using an extinction coefficient of 130 mM^-1 cm^-1 (3). The enzyme was dialyzed exhaustively against deionized water before use.

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The abbreviations used are: LiP, lignin peroxidase; HPLC, high-pressure liquid chromatography; LiP1, LiP111, LiP111, lignin peroxidase compounds I, II, III; TMN, tetranitromethane.
Preparation of LiPIIIa (Ferrous Oxy Complex)—The addition of a large excess of dithionite during the reduction of horseradish peroxidase adversely affects the subsequent yield of the ferrous oxy complex (16). For this reason, ferrous LiP was prepared by adding only 50 eq of sodium dithionite to the native enzyme under anaerobic conditions. Dithionite was dissolved in N2-saturated H2O to prepare a 1 mM stock solution immediately prior to use. LiPIIIa was generated by purging the ferrous enzyme with O2 gas.

Preparation of LiPIIIb (Ferric Superoxide Complex)—LiPIII was also prepared from native LiP by the addition of superoxide anion generated with xanthine/xanthine oxidase at pH 6.0 (18, 21, 27). Superoxide anion was monitored by measuring its capacity to reduce ascorbate to dehydroascorbate at 249.6 nm (27).

Preparation of LiPIIIc from LiPIIIb—LiP compound II was prepared by the successive addition of stoichiometric amounts of ferrocyanide and H2O2 to the native enzyme, followed by dilution with buffer to obtain the desired pH and ionic strength. LiPIII was prepared by adding 40 eq of H2O2 (buffered at pH 3.0) to LiP (6.25 μM). Addition of xanthine/xanthine oxidase (0.01 eq) to LiPIII at pH 3.0 to remove excess peroxide resulted in its conversion to LiPIIIc. LiPIIIc was also prepared by separating the LiPIII* from H2O2 on a Sephadex G-25 column equilibrated with 50 mM sodium succinate, pH 4.5.

Electronic absorption spectra and rate measurements were recorded on a Shimadzu UV-260 spectrophotometer with a spectral bandwidth of 1.0 nm and cuvettes of 1-cm light path. Reactions were carried out at room temperature in 20 mM sodium succinate, pH 3.0, as indicated in the text. Some kinetic data were obtained with the spectrophotometer equipped with a SEA-11 stopped-flow apparatus (Hi-Tech Scientific). H2O2 (30%), xanthine oxidase, cytochrome c, and catalase were obtained from Sigma. The concentration of H2O2 was determined by the horseradish peroxidase assay (28) or spectrophotometrically (29). Veratryl alcohol and tetraniromethane (TNM) were purchased from Aldrich. TNM was dissolved in ethanol, then diluted with H2O to prepare a 10 mM stock solution immediately prior to use (17, 30). Possible veratryl alcohol oxidation products were separated by HPLC as described (17).

RESULTS

Formation and Decomposition of LiPIIIa—Addition of molecular oxygen to ferrous LiP resulted in the immediate formation of LiPII (ferrous oxy complex) (14), with absorption maxima at 414, 543, and 578 nm (Fig. 1, Table I). At pH 3.0, LiPIIIa spontaneously reverted to the native enzyme with a half-life of ~20 min. The spectrum displayed isosbestic points at 413, 470, 525, and 597 nm (Fig. 1), suggesting a direct conversion without intermediates. To study the kinetics of the spontaneous reversion of LiPIIIa, its disappearance was followed at 543 and 578 nm and the appearance of native LiP was followed at 407.6 and 500 nm (Fig. 2A). LiPIIIa was generated by purging the ferrous enzyme with O2 gas. For this reason, ferrous LiP was prepared by adding only 50 eq of sodium dithionite to the native enzyme under anaerobic conditions. Dithionite was dissolved in N2-saturated H2O to prepare a 1 mM stock solution immediately prior to use. LiPIIIa was generated by purging the ferrous enzyme with O2 gas.

Fig. 1. Spontaneous decomposition of LiPIIIa (ferrous LiP + O2). LiPIIIa was prepared by the addition of O2 to ferrous LiP (5.0 μM). In the Soret region, spectra were taken at intervals of 1 min (0–6 min), 2 min (6–20 min), and 5 min (20–120 min). In the visible region, spectra were taken at intervals of 2 min (0–18 min) and 5 min (25–60 min).

\[ \ln [A] - A_0 = -kt + \ln [A] - A_0 \] (1)

where \( A_0, A_t, \) and \( A_w \) are the absorbances at times 0, \( t, \) and 120 min. The replotted lines (Fig. 2B) for each wavelength were linear and parallel, indicating that the conversion of LiPIIIa to ferric LiP followed first-order kinetics. The rate constant \( (k_t) \) determined from the slope of the line was \((1.1 \pm 0.1) \times 10^{-3} \text{s}^{-1}\) (Table II).

Formation and Decomposition of LiPIIIb—The oxidation of native horseradish peroxidase to horseradish peroxidase III in the presence of superoxide has been reported (18, 21). Superoxide anion was generated in the presence of LiP with xanthine/xanthine oxidase at pH 6.0. Catalase was added to eliminate H2O2 formed via the dismutation of O2. Approximately 70 μM O2 was generated in 20 min under the conditions described in the legend to Fig. 3. When the reaction was initiated by the addition of xanthine oxidase, ferric LiP was converted to LiPIIIb (Fe3+-O2 complex). Under these conditions, LiPIIIb started to revert to the native enzyme after ~18 min. The spectral changes for the formation and reversion of LiPIIIb showed the same isosbestic points in the visible region at 525 and 578 nm (Fig. 3), suggesting that both the forward and reverse reactions occurred in a single reversible step. The kinetics of the reversion of LiPIIIb at pH 3.08 were measured by following the absorbance at 407.6, 500, 543, and 578 (as described in Fig. 2) using the pH jump method in which LiPIIIb was prepared at pH 6.0 (5 mM succinate); then buffer (25 mM sodium succinate, pH 3.0) was added to obtain the final pH (data not shown). A plot of In A versus time according to Equation 1 yielded a first-order rate constant \((k_t)\) for the reversion of LiPIIIb of \((1.0 \pm 0.2) \times 10^{-3} \text{s}^{-1}\) (Table II).

Reaction of LiPII with H2O2—Previously, we prepared a LiPIII species by adding excess H2O2 to the native enzyme (14, 17). In the present study, this LiPIII species was prepared by the reaction of LiP compound II with H2O2. At pH 6.0, LiPIIIa, prepared as described above, was stable for several minutes. In contrast, as described previously, at pH 3.0 LiPII
was unstable with a $t_{1/2}$ of less than 1 s (14, 16). Addition of 5 eq of ferrocyanide to LiP11 resulted in its rapid reduction to ferric LiP, with isosbestic points at 417.5, 462, and 518 nm. In Fig. 4. The spectra displayed isosbestic points at 497, 529, and 578 nm (disappearance of LiPIIIA). LiPIIIA (5.0 $\mu$M) was prepared as described in the text.

The addition of excess $H_2O_2$, buffered at pH 3.0, to LiPII prepared in aqueous solution resulted in the formation of a LiPII-like species with absorption maxima at 419, 543, and 578 nm (Fig. 4, Table I). This spectrum was identical to those reported earlier (14, 17). The dependence of the conversion of LiPII to this LiPIIII species on $H_2O_2$ concentration is shown in Fig. 4. The spectra displayed isosbestic points at 497, 529, 556, 565, and 588 nm. The LiPIIIII species derived from LiPII and excess $H_2O_2$ had properties which distinguished it from LiPIIIA and LiPIIIB. Although the visible spectra of all LiPIIIII species were identical, the Soret of LiPII derived from LiPII and $H_2O_2$ was red-shifted from 412-414 nm, and -15-20% decrease in Soret intensity. From 2-10 min after addition of catalase, the Soret shifted from 414 nm. After the first Soret shift was complete, the Soret increased slowly and shifted to 407.6 nm, the wavelength characteristic of native LiP, with isosbestic points at 407.6 nm (data not shown). The time course for spectral changes at 543 and 578 nm (disappearance of LiPIII* or LiPIIIc) and at 407.6 and 500 nm (appearance of ferric LiP) was measured. Replotting in A versus time yielded linear lines in the time frame 7-20 min after initiation of the reaction. These lines fit Equation 1 with the first-order rate constant ($k_1$) of (1.0 $\pm$ 0.1) x 10^{-5} s^{-1} which is essentially identical to $k_1$ and $k_2$ (Table II). These results suggest that the addition of catalase to LiPIII* converts it to LiPIIIc by reacting with bound $H_2O_2$ and that the resultant LiPIIIc reverts to native LiP through a first-order process.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme intermediates</th>
<th>Absorption maxima ($\alpha$)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native ferric LiP</td>
<td>407 (133) 500 (8.1)</td>
<td>1, 8</td>
</tr>
<tr>
<td>LiP</td>
<td>408 (55) 550 (7.5)</td>
<td>14, 16</td>
</tr>
<tr>
<td>LiPII</td>
<td>420 (105) 525 (7.5)</td>
<td>14, this work</td>
</tr>
<tr>
<td>LiPIII</td>
<td>414 (106) 543 (8.8)</td>
<td>23, this work</td>
</tr>
<tr>
<td>LiPIIIa (ferrous LiP + $O_2$)</td>
<td>412 (115) 543 (7.1)</td>
<td>This work</td>
</tr>
<tr>
<td>LiPIIIc (LiPII + $H_2O_2 +$ catalase)</td>
<td>414 (105) 453 (8.9)</td>
<td>This work</td>
</tr>
<tr>
<td>LiPIII*</td>
<td>419 (91) 433 (8.0)</td>
<td>14, 17, this work</td>
</tr>
<tr>
<td>LiP-CN*</td>
<td>360 (35) 425 (76)</td>
<td>8</td>
</tr>
<tr>
<td>LiPIII* + KCN + veratryl alcohol</td>
<td>360 (31) 425 (65)</td>
<td>This work</td>
</tr>
</tbody>
</table>

Values in parentheses are extraction coefficients expressed in mM$^{-1}$ cm$^{-1}$.

The table is a summary of the spectral characteristics of oxidized intermediates of lignin peroxidase. The reaction of LiP with $H_2O_2$ results in the formation of LiPPIII, which can be further oxidized to LiPPIII*, LiPPIIIa, LiPPIIIb, and LiPPIIIc. The conversion of LiPPIII to LiPPIII* is a first-order process, and the resulting LiPPIII* reverts to LiP through a first-order process.

![Fig. 2. Time course for the spontaneous decomposition of LiPPIIIa (ferrous LiP + $O_2$)]. A: appearance of native LiP was monitored at 407.6 (C) and 500 (A) nm. Disappearance of LiPPIIIa was followed at 543 (B) and 578 (A) nm. B: first-order plot for the decomposition of LiPPIIIa. LiPPIIIa (5.0 $\mu$M) was prepared as described in the text.](http://www.jbc.org/)

![Fig. 5A. Catalase to LiPPIII*](http://www.jbc.org/)

![Fig. 5B. Catalase to LiPPIII*](http://www.jbc.org/)
Lignin Peroxidase Compound III

The abbreviation used is: VA, veratryl alcohol.

### TABLE II

<table>
<thead>
<tr>
<th>Compound III species</th>
<th>Conditions</th>
<th>First-order rate constant ( \text{s}^{-1} )</th>
<th>Second-order rate constant ( \text{M}^{-1} \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conversion to ferric LIP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LiP**IIIa (ferrous LiP + O(_2))</td>
<td>Spontaneous</td>
<td>1.1 \times 10^{-3} (k(_a))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ VA(^b)</td>
<td>1.3 \times 10^{-3} (k(_{3VA}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ TNM(^b)</td>
<td>2.1 \times 10^{-3} (k(_{TNM}))</td>
<td></td>
</tr>
<tr>
<td>LiP**IIb (ferric LiP + O(_2))</td>
<td>Spontaneous</td>
<td>1.0 \times 10^{-3} (k(_a))</td>
<td></td>
</tr>
<tr>
<td>LiP<strong>IIc (LiP</strong>II + H(_2)O(_2) + catalase)</td>
<td>Spontaneous</td>
<td>1.0 \times 10^{-3} (k(_a))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ VA(^b)</td>
<td>1.3 \times 10^{-3} (k(_{3VA}))</td>
<td></td>
</tr>
<tr>
<td>LiP<strong>II* (LiP</strong>II + H(_2)O(_2))</td>
<td>+ VA (excess)</td>
<td>3.5 \times 10^3 (k(_{app}))</td>
<td></td>
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</table>

Inactivation

| LiP**II* (LiP**II + H\(_2\)O\(_2\)) | + H\(_2\)O\(_2\) (excess) | (5.0-6.8) \times 10^{-4} (k\(_{s}\)) | 1.7 \times 10^4 (k\(_{app}\)) |

\(^a\) The rate was measured under pseudo-first-order conditions with VA in excess (100 eq).

\(^b\) The rate was measured under pseudo-first-order conditions with TNM in excess (100 eq).

\(^c\) The reaction is biphasic (Fig. 7).

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**Fig. 3.** Formation and spontaneous decomposition of LiP**IIb** (ferric LiP + O\(_2\)). Reaction mixtures (1 ml) contained xanthine (0.2 mM), xanthine oxidase (50 \( \mu \)g), EDTA (70 \( \mu \)M), catalase (22 \( \mu \)g), and LiP (2.1 \( \mu \)M) in 20 mM succinate, pH 6.0. The reference cuvette contained the same mixture, minus LiP. Spectra were taken at 0, 4, 10, and 25 min. The reaction was initiated by the addition of xanthine oxidase.

The first-order rate constant \( (k_0) \) for the slow reaction was determined to be (5.0-6.8) \( \times 10^{-4} \) \( \text{s}^{-1} \) (Table II).

**Release of Superoxide from LiP**III**—LiP**IIIa, b, and c each slowly reverted to the native state via first-order reactions (Table II) with no apparent intermediate involved. Thus, the mechanism of reversion of LiP**III to native LiP is likely to involve the dissociation of a ferric-superoxide complex to ferric LiP + O\(_2\). In order to examine the reaction, we used the TNM assay method (17, 30) to detect released O\(_2\). Fig. 8A shows the time course for the reduction of TNM to trinitromethane (\( \epsilon_{350} = 14,600 \text{ M}^{-1} \text{cm}^{-1} \)) during the conversion of LiP**IIc** to ferric LiP, in the presence of catalase. Reduced cytochrome c reduction assay. Reduced cytochrome c has intense maxima at 520 and 550 nm. Spectra obtained during the conversion of LiP**IIc** (55 \( \mu \)M) to ferric LiP, in the presence of cytochrome c, displayed two new maxima at 520 and 550 nm (data not shown). Fig. 8B shows the time course of TNM reduction during the conversion of LiP**IIc**. The kinetics of TNM reduction corresponded to the disappearance of LiP**IIc** and the appearance of ferric LiP. During the conversion of 2.9 \( \mu \)M of LiP**IIc** to ferric LiP (5 \( \mu \)M), the indicated amounts of O\(_2\) were added and the spectra were recorded when the spectral change was maximal (0.5 min).

**Reactions of LiP**III** and LiP**III** with Veratryl Alcohol—** Because the oxidized species formed in the presence of excess H\(_2\)O\(_2\) is actually LiP**II* which may be a LiP**II + H\(_2\)O\(_2\) complex, we reinvestigated the reactions of LiP**II* and LiP**III** with veratryl alcohol (17). Fig. 9 (inset) shows the conversion of LiP**II* to ferric LiP (\( A_{607.6} \)) under pseudo-first-order conditions with veratryl alcohol in excess. A linear dependence of \( k_{obs} \) versus veratryl alcohol concentration was observed between 0-500 \( \mu \)M veratryl alcohol (Fig. 9). The second-order rate constant \( (k_{obs}) \) calculated from the slope of the line was (3.5 \( \pm 0.1 \)) \( \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) (Table II). Since this suggested that veratryl alcohol is involved in the reaction, we reinvestigated the possible oxidation of veratryl alcohol during this reaction (17). The veratryl alcohol-stimulated conversion of LiP**II* to
FIG. 5. Effect of catalase on LiP**. A, LiP** was prepared by adding 40 eq of \( \text{H}_2\text{O}_2 \) buffered at pH 3.0 to LiP (6.25 \( \times \) 10\(^{-5} \)) \( \mu \text{M} \) in H.O. Catalase (6.25 \( \times \) 10\(^{-6} \) \( \mu \text{M} \) final concentration) was then added and spectra were recorded at 2-min intervals. B, a time course of the conversion was followed at 407.6 (O), 500 (\( \Delta \)), 543 (O), and 578 (\( \Delta \)) nm, and the absorption (\( \ln [A] = A_0 \) was plotted against time according to Equation 1 in the text.

![Graph](image)

Fig. 6. Effect of excess \( \text{H}_2\text{O}_2 \) on LiP. A, \( \text{H}_2\text{O}_2 \) (30 eq) was added to LiP** (ferrous LiP + \( \text{O}_2 \)) (2.0 \( \mu \text{M} \)). B, \( \text{H}_2\text{O}_2 \) (30 eq) was added to LiP** (ferric LiP + \( \text{O}_2 \)) (2.0 \( \mu \text{M} \)), prepared via the pH jump method described in the text. C, \( \text{H}_2\text{O}_2 \) (40 eq) was added to LiP** (LiP** (2.0 \( \mu \text{M} \)) + \( \text{H}_2\text{O}_2 \) + catalase (0.02 \( \mu \text{M} \)). In each case the spectrum of LiP** was recorded (solid line); \( \text{H}_2\text{O}_2 \) was then added, and the resultant LiP** spectrum was recorded (dashed line).

![Graph](image)

Fig. 7. Reaction of LiP** with \( \text{H}_2\text{O}_2 \). LiP** (3.0 \( \mu \text{M} \)) was prepared as described in the legend to Fig. 4 at pH 3.0 with excess \( \text{H}_2\text{O}_2 \) (100–400 \( \mu \text{M} \)). Kinetic traces were obtained at 419 nm and plotted according to Equation 1. The concentrations of \( \text{H}_2\text{O}_2 \) used were 100 (O), 200 (\( \circ \)), 300 (\( \Delta \)), and 400 (\( \Delta \)) \( \mu \text{M} \). Inset, the slopes of the first phase of the reaction were replotted against [\( \text{H}_2\text{O}_2 \)]. Each determination of \( k_{\text{obs}} \) is the mean of five traces.

![Graph](image)

**Discussion**

Although LiP has a typical peroxidase catalytic cycle (1, 14, 16, 18), several features of this enzyme distinguish it from other plant peroxidases. LiP oxidizes methoxy benzenes to aryl cation radicals (1–4, 6, 7). It has an unusually low pH optimum, ~3.0 (19, 20), and at its pH optimum, LiP is easily oxidized to a LiP**-like species (LiP**) with \( \text{H}_2\text{O}_2 \) concentrations that are considerably less than those required for other peroxidases (16, 17). LiP**, which was previously referred to as LiP** (17), is irreversibly inactivated in the presence of excess \( \text{H}_2\text{O}_2 \) (17) and can be converted to the native enzyme by the addition of veratryl alcohol (17). These unusual properties of LiP encouraged us to study the formation and reactions of LiP** and LiP** in greater detail. Peroxidase compound III-like species can be prepared via three different pathways (18, 21) as described below for LiP.
Lignin Peroxidase Compound III

**FIG. 8.** Release of superoxide during the decomposition of LiPIII. A, LiPIIIc (6.0 μM) was prepared from feryl LiP, H₂O₂ (40 eq), followed by catalase (0.06 μM). The time course of the spontaneous release of O²⁻ from LiPIIIc was followed at 350 nm using TNM (0.1 mM). B, LiPIIIa (2.9 μM) was prepared from ferrous LiP + O₂. TNM (0.1 mM) was added and the release of O²⁻ was followed at 350 nm (solid line). Formation of native LiP (407.6 nm) and disappearance of LiPIIIc (543 nm) were also monitored. Dashed lines, spontaneous conversion of LiPIIIc to ferric LiP in the absence of TNM.

**FIG. 9.** Plot of kobs versus veratryl alcohol concentration for the reaction of LiPIII* with veratryl alcohol. Inset, typical exponential trace (veratryl alcohol, 190 μM and LiPIII*, 9.5 μM) from which kobs was calculated. The linear plot of kobs versus veratryl alcohol concentration indicates that any complex between LiPIII*, and veratryl alcohol is too short-lived to detect under these conditions.

These compound III species have been characterized as Fe³⁺-O₂ or as Fe³¹⁺⁻O₇ complexes (18, 21). Since compound III is not involved in the peroxidatic cycle, the reversibility of its formation has been investigated (18, 21, 26, 33-35).

LiPIII was prepared via three reaction paths: (a) ferrous LiP + O₂ (Fig. 1, Table I) (LiPIIIa), (b) ferric LiP + O₂ (Fig. 3, Table I) (LiPIIIb), and (c) LiPII + H₂O₂ + catalase (Fig. 5A, Table I) (LiPIIIc). In the latter case, excess H₂O₂ was removed by catalase or by gel filtration to form pure LiPIIIc from LiPIII*. The spectral features of LiPIIIa, b, and c are almost identical (Table I) with spectral maxima at ~414, 543, and 578 nm. LiPIIIb had a Soret maximum at 412 nm, but the visible spectrum indicated that it was only ~80% pure. Thus, this 2-nm shift from 414 to 412 nm is probably due to contamination with native ferric LiP (Soret maximum at 407.6 nm). The addition of H₂O₂ to LiPIIIa, b, or c or to LiPIII results in the formation of LiPIII* (Fig. 6, Table I), which has visible maxima identical to those of LiPIII at 543 and 578 nm, but which has a red-shifted Soret maximum at 419 nm (Table I) (14, 16, 17). Removal of excess H₂O₂ from LiPIII* by catalase (Fig. 5A) or by gel filtration (data not shown) converts this intermediate to LiPIII (Soret maximum at 414 nm). This suggests that LiPIII* may be a complex of LiPIII and one or more molecules of bound H₂O₂.

**Fig. 10.** Reaction of LiPIII* with veratryl alcohol in the presence of KCN. LiPIII* (12 μM) was prepared as described for Fig. 4. KCN (80 mM) was added and the spectrum (solid line) was recorded. Finally, veratryl alcohol (400 μM) was added, and the resultant spectrum (dashed line) was recorded after 3 min.

Decomposition of LiPIII—In the absence of H₂O₂, LiPIIIa, b, and c all slowly revert to native LiP via first-order processes. The similarity of the first-order rate constants for these reversions (~1.0 × 10⁻³ s⁻¹) (Table II) and the identical isosbestic points at 413, 470, and 525 nm (Figs. 1, 3, and 5A) suggest that these reversions all occur via the same single step mechanism. Furthermore, during the spontaneous decomposition of LiPIIIa and c, stoichiometric amounts of O²⁻ are released (Fig. 8) and the conversion of LiPIII to native LiP is accelerated in the presence of an O²⁻ scavenger, in a manner similar to that reported for the conversion of HRPIII (34). All of these results strongly suggest that LiPIIIa, b, and c have the identical Fe⁴⁺⁻O₇⁻ structure. Resonance Raman spectroscopic evidence for LiPIIIa and c is consistent with a ferric oxidation state.²

**Mechanism of LiPIII Formation—**It has been proposed that upon the binding of O₂ to the ferrous heme, electron density migrates from the iron to the oxygen (35). Thus, the predomin-

² M. Mylrajran, K. Valli, H. Wariishi, T. M. Loehr, and M. H. Gold, unpublished results.
Lignin Peroxidase Compound III

The addition of H$_2$O$_2$ to Lip$_{111}$ results in the formation of LiPIII$^*$ (Fig. 6) which is followed by enzyme inactivation and bleaching of the heme (data not shown). Identical isosbestic points for the forward and reverse reactions indicate that the reaction is reversible (Fig. 3).

In the absence of a reducing substrate, excess H$_2$O$_2$ reacts with compound II to form LiPIII$^*$ (Fig. 4) and LiPIII is probably an intermediate in this process (Fig. 6). The formation of LiPIII$^*$ from LiPIII and H$_2$O$_2$ is not inhibited by TNM (data not shown), a strong O$_2^-$ scavenger (31-33), suggesting that the mechanism probably does not involve the reduction of LiPIII to the native enzyme and the reaction of the latter with O$_2^-$ (33). Instead, the mechanism of LiPIII conversion to LiPIII may involve a ligand-exchange reaction as described in Ref. 33, and as shown:

$$\text{Fe}^{III} + O_2^- + H^+ \rightarrow \text{Fe}^{III} - O_2^- \rightarrow H^+ \rightarrow \text{Fe}^{IV} - O_2^- \rightarrow \text{Fe}^{III} - O_2^- \rightarrow H^+ \rightarrow \text{Fe}^{IV}$$

Unlike horseradish peroxidase, LiP is oxidized to LiPIII in the presence of a relatively low concentration of H$_2$O$_2$ (14, 17, 18, 21, 33). This may reflect the relative strengths of the iron-oxygen bond in the two Fe$^{III}$=O enzyme species.

The addition of H$_2$O$_2$ to LiPIII results in the formation of LiPIII$^*$ (Fig. 6) which is followed by enzyme inactivation and bleaching of the heme (data not shown) (17). Removal of excess H$_2$O$_2$ from LiPIII$^*$ by the addition of catalase or by gel filtration converts it back to LiPIII (Fig. 5), indicating that the formation of LiPIII$^*$ is reversible. Previous work has demonstrated the formation of a horseradish peroxidase oxidized intermediate compound IV upon exposure of that enzyme to H$_2$O$_2$. The formation of horseradish peroxidase compound IV (18, 36) is also followed by its inactivation (36, 37). Two types of bromoperoxidase compound III species with identical visible maxima but a small difference in the Soret band IV (18, 36) is also followed by its inactivation (36, 37).

Lip$_{111}$ recently we reported that the addition of veratryl alcohol to Lip$_{111}$, prepared by the addition of excess H$_2$O$_2$ to the native enzyme, leads to the rapid single-step conversion of this LiPIII species back to the native enzyme (17). Herein we have recharacterized the LiPIII species prepared from native Lip or from Lip with excess H$_2$O$_2$ as LiPIII$^*$ and with LiPIII. Addition of excess veratryl alcohol to LiPIII$^*$ results in its rapid conversion to the native enzyme via a second-order process (Fig. 9). The apparent second-order rate constant for this process is $3.5 \times 10^2$ M$^{-1}$ s$^{-1}$. The ferric produced by this reaction can be trapped with KCN to form a stable ferric-CN complex (Fig. 10) (1, 8), thus preventing subsequent catalytic cycles. HPLC analysis of the reaction products formed under these conditions indicates that veratryl alcohol is not oxidized during this reaction. In the absence of KCN, veratryl alcohol is oxidized to veratraldehyde since in the presence of excess H$_2$O$_2$, the recovered native enzyme begins a new catalytic cycle. We have previously shown that the reversion of LiPIII$^*$ to native Lip probably occurs in a single step (isosbestic point in the Soret region at 413 nm) and that O$_2^-$ is released during this reaction (17). Taken together, these results suggest that the binding of veratryl alcohol to LiPIII$^*$ leads to the displacement of O$_2^-$, resulting in the conversion of the Fe$^{III}$O$_2^-$ enzyme to the ferric enzyme. The effect of veratryl alcohol on the spontaneous reversion of LiPIIIa and LiPIIIc has also been examined. In each case the first-order rate constant is not accelerated (Table II). This contrasts with conclusions implied in our previous paper (17). It also suggests that conversion of LiPIII$^*$ to ferric Lip occurs via a single step and that LiPIII is probably not an intermediate in this process.

All of these results indicate that Lip undergoes the reactions shown in Fig. 11. Three different pathways for LiPIII formation have been established and these are similar to pathways previously described for HRP (18, 21). LiPIIIa, b, and c spontaneously revert to the native enzyme, releasing a stoichiometric amount of O$_2^-$ in the process, suggesting an Fe$^{III}$O$_2^-$ structure for LiPIII. Veratryl alcohol does not accelerate the rates of reversion of LiPIIIa, b, or c. In the presence of excess H$_2$O$_2$, LiPIII is converted to LiPIII$^*$ which is subsequently bleached and inactivated via a second-order process. In contrast to the negligible effect of veratryl alcohol on...
Lignin Peroxidase Compound III

LiPIII, addition of veratryl alcohol to LiPIII★ results in its rapid conversion to native LiP with the release of O2 (17). Veratryl alcohol is not oxidized in this reaction, suggesting a mechanism whereby the binding of veratryl alcohol displaces O2. Thus, an important role for veratryl alcohol, a secondary metabolite of the fungus, appears to be in the protection of LiP from inactivation by H2O2 (17,41).

Recent work has demonstrated that superoxide scavengers such as MnⅢ suppress the occurrence of ring-opening and quinone-forming reactions of LiP (42). Since LiPIII, like horseradish peroxidase (36), spontaneously releases O2, and LiPIII★ releases O2 upon binding veratryl alcohol, these reactions may have an indirect role in the oxidation of lignin. Thus, the ready formation of LiPIII and LiPIII★ may be a factor in the biodegradation of this polymer by P. chrysosporium.

Further characterization of the structure and function of LiPIII and LiPIII★ is in progress.

REFERENCES
Lignin peroxidase compound III. Mechanism of formation and decomposition.
H Wariishi and M H Gold


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