Spectroscopic and Kinetic Properties of the Oxidized Intermediates of Lignin Peroxidase from *Phanerochaete chrysosporium*

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Stopped-flow rapid scan techniques were used to obtain a spectrum of nearly homogeneous lignin peroxidase compound I (LiP) under pseudo-first order conditions at the unusually low pH optimum (3.0) for the enzyme. The LiPI spectrum had a Soret band at 407 nm with ~60% reduced intensity and a visible maximum at 650 nm. Under steady-state conditions a Soret spectrum for lignin peroxidase compound II (LiPII) was also obtained. The Soret maximum of LiPII at 420 nm was only ~15% reduced in intensity compared to native LiP. Transient state kinetic results confirmed the pH independence of LiPII formation over the pH range 3.06–7.39. The rate constant was (6.5 ± 0.2) × 10^6 M⁻¹ s⁻¹. Addition of excess veratryl alcohol to LiPII resulted in its reduction to LiPII with subsequent reduction of LiPII to the native enzyme. Reactions of LiPI and LiPII with veratryl alcohol exhibited marked pH dependencies. For the LiPI reaction the rate constants ranged from 2.5 × 10^6 M⁻¹ s⁻¹ at pH 3.06 to 4.1 × 10^5 M⁻¹ s⁻¹ at pH 7.39; for the LiPII reaction, 1.6 × 10^6 M⁻¹ s⁻¹ (pH 3.06) to 2.3 × 10^5 M⁻¹ s⁻¹ (pH 5.16). These single turnover experiments demonstrate directly that the pH dependence of these reactions dictates the overall pH dependence of this novel enzyme. These results are consistent with the one-electron oxidation of veratryl alcohol to an aryl cation radical by LiPI and by LiPII.

Under secondary metabolic conditions the wood-rotting fungus *Phanerochaete chrysosporium* secretes two extracellular heme peroxidases which are involved in the degradation of lignin. These enzymes, manganese peroxidase and lignin peroxidase (LiP), have been purified and characterized (1–5). LiP, a glycoprotein with a molecular weight of 41,000 and a single iron protoporphyrin IX prosthetic group (3–5), catalyzes the H₂O₂-dependent oxidation of lignin model compounds via the initial formation of a substrate aryl cation radical with subsequent nonenzymatic reactions to yield the final products (6–9). Our initial characterization of the oxidized intermediates LiPI, LiPII, and LiPIII has been reported (10). Herein we have utilized rapid-scan spectrophotometry to determine the spectra of the oxidized intermediates of LiP generated both at the pH optimum (3.0) and at pH 6.0 under pseudo-first order conditions with H₂O₂ in excess. We have also investigated the pH dependence of LiPII formation and the rate of reduction of LiPI and LiPII using VA as the reducing substrate.

**EXPERIMENTAL PROCEDURES**

The major isozyme of LiP was purified from cultures of *P. chrysosporium* as previously described (3, 5). The purified protein was homogeneous and had an Rf value of ~5.0. Enzyme concentrations were determined at 407.6 nm using a molar absorptivity of 133 M⁻¹ cm⁻¹ (3, 5). The enzyme was dialyzed exhaustively against triply distilled water before use (10). The concentration of H₂O₂ (BDH Chemicals) was determined as reported (11).

Rapid scan spectra were recorded with a Union Giken RA601 Rapid Reaction Analyzer equipped with a 1-cm cell. The absorption spectra were measured by means of a multichannel photodiode array and memorized in a digital computer system (Sord M200 Mark III). The analogue replicon was plotted on an X-Y recorder. Spectral regions of 96 nm were scanned from 380 to 700 nm.

Kinetic measurements were conducted using the Union Giken RA601 in the stopped-flow mode. One of the drive syringes contained native LiP or LiPII in deionized water while the other syringe contained the substrate (H₂O₂ or VA) and buffer. All experiments were performed at (25.0 ± 0.5) °C in 0.1 M sodium citrate buffer (pH 4.5) and in 0.1 M sodium phosphate >pH 4.5.

LiPI formation was followed at 407.6 nm using a final enzyme concentration of 1.5 μM and various concentrations of excess H₂O₂ (20–200 μM) to maintain pseudo-first order conditions. The reaction was observed from pH 3.06 to 7.39. LiPIII formation and decay were followed at 426 nm. LiPI (1.5 μM) was freshly prepared for each experiment. Various concentrations of VA or p-cresol were buffered at the pH indicated.

**RESULTS**

**Spectroscopic Characterization of Oxidized Intermediates**

**Formation of Compound I**—The pH optimum for LiP is ~3.0 (5, 12, 13); thus, where possible, spectral measurements were made at this pH. Measurements were also made at pH 6.0 where LiPI and LiPII are more stable (10). The spectrum of native LiP has maxima at 407, 496, and 632 nm (3, 5, 10) (Fig. 1). Within 0.2 s after addition of excess H₂O₂ (30 eq) to the native enzyme at pH 3.06, the Soret peak height was reduced by ~60%, and a new peak appeared at 650 nm, suggesting the formation of LiPI (10). Isosbestic points between native LiP and LiPI occur at 426 and 540 nm.

**Formation of Compounds II and III**—Upon addition of 30 eq of H₂O₂ to the native enzyme at pH 3.06, a sequence of spectral changes was recorded over a 10-s time span. During the first 0.2 s, LiPI formation was observed (Fig. 1); subse-

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The abbreviations used are: LiP, lignin peroxidase; LiPI, LiPII, LiPIII, compounds I, II, and III of lignin peroxidase; VA, veratryl alcohol.
obtained 2 and 10 pseudo-first order conditions with excess \( \text{H}_2\text{O}_2 \), steady-state situations. In contrast, within peaks appeared in the visible region at of native Lip.

In order to prepare LipII under experimental conditions as in Fig. 1, except pH is 6.21. Spectra were recorded 2 sec after mixing. The reference spectrum is that of native Lip.

Within the transient formation of LiPII and LiPIII.

The Soret maximum red-shifted to 426 nm, the isosbestic point between native Lip and LiPIII. At this wavelength the absorptivity of the native enzyme. A steady-state spectrum for LiPIII could also be obtained at pH 3.0 with VA as the reducing substrate (data not shown).

**Kinetic Experiments**

**Compound I Formation**—All kinetic traces were of a single exponential character. The observed rate constants were linearly proportional to the \( \text{H}_2\text{O}_2 \) concentration in the range used. The formation of LiPI exhibited no pH dependence from pH 3.06 to 7.39 (data not shown). The mean rate constant was \((6.5 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\).

**Compound II Formation and Conversion to the Native Enzyme**—The formation of LiPIII and its subsequent conversion to the native enzyme in the presence of VA was followed at 426 nm, the isosbestic point between native Lip and LiPI. At this wavelength the absorptivity of LiPIII is \(>2\) times that of native Lip and LiPI. Fig. 4 shows a typical time course trace of the changes in absorbance. The biphasic curve shows an initial increase in absorbance (formation of LiPIII) which is completed in less than 20 ms (shown on the expanded scale) followed by a decline (reduction of LiPII). The traces were exponential in character when followed for sufficiently short intervals. The rate of formation of LiPIII was followed at different pH values ranging from 3.06 to 7.39. Fig. 5A shows the pH dependence of the reaction of LiPI with VA. The rate decreased dramatically with increasing pH, from \(2.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) at pH 3.06 to \(4.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}\) at pH 7.39. This result is consistent with published findings (12, 13) that the activity of Lip increases to a maximum near pH 2.5 and approaches zero at pH values above 6.

The second portion of the biphasic curve (Fig. 4) corresponds to the slower one-electron reduction of LiPII to the native enzyme. Fig. 5B shows the pH dependence of this reduction. Again, a striking decrease in rate constant is ob-
The spectrum of LiPI obtained at pH 3.06 with 30 eq of H₂O₂ (Fig. 1) is very similar to that obtained previously at pH 6.0 (10) and to those obtained for horseradish peroxidase (14) and indicates that this LiPI preparation is >95% pure. This is the first spectrum of a reasonably pure LiPI at the pH optimum. A recently published spectrum of LiPI, limited to the Soret region, shows contamination by native LiP (Soret intensity ~82% of native LiP) rather than, as the authors claim, an unusually high Soret intensity (15). The reduced absorption of the Soret region of LiPI compared to native LiP and the characteristic absorbance of 650 nm suggests that, like horseradish peroxidase I (14, 16, 17), LiPI contains 2 oxidizing eq over the native enzyme (10). The first oxidizing equivalent apparently is a FeII state in the ferryl state of the iron (14) as recently confirmed by our resonance Raman experiments (18); the second equivalent is contained as a porphyrin π-cation radical (16, 17).

Our kinetic results (data not shown) for LiPI formation demonstrate no pH dependence over a pH range of 3.06–7.39, confirming previous results (15). Formation of compound I for other peroxidases is pH-dependent, and the distal ionizable group (for horseradish peroxidase and chloroperoxidase, pK₅ ≈ 3.0 (19, 20) and for yeast cytochrome c peroxidase, pK₅ ≈ 4.5 (21). Presumably, this ionizable group plays a role in the heterogeneous cleavage of H₂O₂. The unusually lack of pH dependence for LiPI formation suggests that such an ionization may not occur in this system. This may account for the decreased rate of LiPII formation compared to that found for other peroxidases (19–21).

The spectrum of LiPIII obtained at pH 3.06 with 30 eq of H₂O₂ and the absence of a reducing substrate, LiP is readily oxidized by LiPIII. Within 2 s after addition of 30 eq of H₂O₂ to native LiP at pH 3.0, a spectrum with characteristic peaks at 545 and 579 nm is observed (Fig. 2). Presumably (as mentioned above), this pH dependence of LiPIII at pH 3.0 is in contrast to a recent report (15) which claims the detection of native horseradish peroxidase at pH 3.0 whereas 250 eq of H₂O₂ are required to convert native horseradish peroxidase to horseradish peroxidase III at that pH. Our observation of the rapid formation of LiPIII at pH 3.0 is in contrast to a recent report (15) which claims the detection of LiPIII 6 s after addition of 20 eq of H₂O₂ to native LiP at pH 3.5. However, in that report only the Soret region was scanned and in that region LiPIII and LiPIII cannot be readily differentiated.

Evidence for the transient formation of LiPI in the presence of excess H₂O₂ at pH 6.0 is shown in Fig. 2B. Possibly, at low pH in the absence of another reducing substrate, H₂O₂ is readily oxidized by LiPII to form HO₂⁺ and native LiP: HO₂⁺ may then complex with the FeIII in native LiP to form the FeO⁻O₂⁻ structure of LiPIII (10, 14, 22). The formation of horseradish peroxidase III from horseradish peroxidase II and H₂O₂ has been reported (14, 22).

**Compound II Formation in the Steady State**—In order to prepare a relatively pure preparation of LiPIII under pseudo-first order conditions with H₂O₂ in excess, steady-state conditions were applied. Under these steady-state conditions a Soret spectrum for LiPIII was obtained (Fig. 3). The molar absorbivity of the Soret of LiPIII was 20% higher than the value we obtained previously (10). These results suggest that, as for horseradish peroxidase (19, 20), LiPIII has a higher reactivity than LiPII for the reducing substrate.

**Reduction of LiPI and LiPI**—Our earlier work suggested that the oxidation of VA by LiPI proceeds via two single-electron steps (10) with the intermediate formation of a VA.
cation radical (6–8) rather than by a single two-electron step (12). The results in Fig. 4 show this directly. The initial conversion of LiPI to LiPII takes place in 20 ms. This is followed by a slower conversion of LiPII to the native enzyme over 500 ms. Thus, at pH 3.0 in the presence of excess reducing substrate (VA), the normal catalytic cycle is completed via two single-electron steps as shown in Reactions 1–3. This result also demonstrates that LiPII is capable of efficiently oxidizing VA.

The pH dependence of the conversion of LiPI to LiPII ($k_2$) and LiPII to native LiP ($k_3$) is shown in Fig. 5. These results demonstrate directly for the first time that the rate of these conversions is strongly dependent on pH with optimum activity at a low pH. Apparently, it is the pH dependence of the reduction of LiPI and LiPII rather than the formation of LiPI which dictates the unusually low pH optimum for this enzyme (12, 13). These results suggest that an ionizable group(s) with a $pK_a < 5.0$ controls either the binding and/or oxidation of the reducing substrate by these oxidized enzyme intermediates. Preliminary results (data not shown) indicate that the binding of VA to the native enzyme has a similar dependence. The low pH optimum of the enzyme may facilitate the formation and stabilization of the aryl cation radical (6–8). Experiments designed to determine what ionizable group(s) on the enzyme dictate this pH dependence are planned.

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