Veratryl Alcohol Oxidation by Lignin Peroxidase†

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ABSTRACT: Lignin peroxidase (LiP) from the white rot fungus *Phanerochaete chrysosporium* catalyzes the H_2O_2-dependent oxidation of veratryl alcohol (VA), a secondary metabolite of the fungus, to veratryl aldehyde (VAD). The oxidation of VA does not seem to be simply one-electron oxidation by LiP compound I (LiPI) to its cation radical ($VA^{\bullet+}$) and the second by LiP compound II (LiPII) to VAD. Moreover, the rate constant for LiPI reduction by VA (3×10^5 M^{-1} s^{-1}) is certainly sufficient, but the rate constant for LiPII reduction by VA (5.0 ± 0.2 s^{-1}) is insufficient to account for the turnover rate of LiP (8 ± 0.4 s^{-1}) at pH 4.5. Oxalate was found to decrease the turnover rate of LiP to 5 s^{-1}, but it had no effect on the rate constants for LiP with H_2O_2 or LiPI and LiPII, the latter formed by reduction of LiPI with ferrocyanide, with VA. However, when LiPII was formed by reduction of LiPI with VA , an oxalate-sensitive burst phase was observed during its reduction with VA. This was explained by the presence of LiPII, formed by reduction of LiPI with VA, in two different states, one that reacted faster with VA than the other. Activity during the burst was sensitive to preincubation of LiPI with VA, decaying with a half-life of 0.54 s, and was possibly due to an unstable intermediate complex of $VA^{\bullet+}$ and LiPII. This was supported by an anomalous, oxalate-sensitive, LiPII visible absorption spectrum observed during steady state oxidation of VA. The first order rate constant for the burst phase was 8.3 ± 0.2 s^{-1}, fast enough to account for the steady state turnover rate of LiP at pH 4.5. Thus, it was concluded that oxalate decreased the turnover of LiP by reacting with $VA^{\bullet+}$ bound to LiPII. The $VA^{\bullet+}$ concentration measured by electron spin resonance spectroscopy (ESR) was 2.2 μM at steady state (10 μM LiP, 250 μM H_2O_2, and 2 mM VA) and increased to 8.9 μM when measured after the reaction was acid quenched. Therefore, we assumed the presence of two states of $VA^{\bullet+}$ bound to LiPII, one ESR-active and one ESR-silent. The ESR-silent species, which could be detected after acid quenching, would be responsible for the burst phase. Both of the $VA^{\bullet+}$ species disappeared in the presence of 5 mM oxalate. The ESR-active species reached a maximum (3.5 μM) at 0.5 mM VA under steady state. From these studies, a mechanism for VA oxidation by LiP is proposed in which a complex of LiPII and $VA^{\bullet+}$ reacts with an additional molecule of VA, leading to veratryl aldehyde formation.

The wood-rotting fungus *Phanerochaete chrysosporium* is known for its ability to degrade lignin and environmental pollutants (Barr & Aust, 1994; Kirk & Farrell, 1987; Buswell & Odier, 1987; Hammel et al., 1994). The lignin-degrading system, secreted extracellularly during nutrient limitation (Tien & Kirk, 1983), is partly responsible for this ability of *P. chrysosporium*. Among other constituents, the lignin-degrading system consists of heme-containing peroxidase enzymes (Tien & Kirk, 1983), H_2O_2-generating enzymes (Forney et al., 1982; Faison & Kirk, 1983), veratryl alcohol (VA, 3,4-dimethoxy benzyl alcohol) (Lundquist & Kirk, 1978), and oxalate (Kuan & Tien, 1993; Barr et al., 1992). Lignin is degraded through one-electron oxidations by the peroxidase enzymes (Kersten et al., 1985; Hammel et al., 1986).

These fungal peroxidases are similar to other plant peroxidases in their catalytic cycle (Chance, 1952; Tien, 1987; Dunford, 1990). Ferric peroxidase is oxidized by two electrons of H_2O_2 to compound I. Compound I contains ferryl iron and a porphyrin π cation radical. It can oxidize substrates by one electron, with a concomitant reduction of the porphyrin π cation radical yielding compound II. Compound II can also oxidize substrates by one electron, returning the enzyme to the ferric state.

P. chrysosporium peroxidases are classified as lignin, and manganese-dependent peroxidases. Pertinent to this study is lignin peroxidase (LiP),[1] which catalyzes one-electron oxidation of aromatic hydrocarbons (Kersten et al., 1985, 1990; Hammel et al., 1986), inorganics (Shah & Aust, 1993a,b; Shah et al., 1991), and Mn(II) (Khindaria et al., 1995a). One of the more important substrates, VA, is a secondary metabolite of the fungus (Lundquist & Kirk, 1978). LiP catalyzes the oxidation of VA to veratryl aldehyde (VAD) through the one-electron-oxidized intermediate, the veratryl alcohol cation radical ($VA^{\bullet+}$). We (Khindaria et al., 1995b) recently reported the direct ESR detection of LiP-formed $VA^{\bullet+}$. The oxidation of $VA^{\bullet+}$ to VAD, though, is not well understood. It has been proposed that molecular oxygen oxidizes $VA^{\bullet+}$ to VAD (Bono et al., 1990). However, identical stoichiometry (1:1) has been

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[1] Abbreviations: LiP, lignin peroxidase; LiPI, lignin peroxidase compound I; LiPII, lignin peroxidase compound II; $LiPII_{VA}$, lignin peroxidase compound II prepared by reduction of LiPI with veratryl alcohol; VA, veratryl alcohol; $VA^{\bullet+}$, veratryl alcohol cation radical; $VA^{\bullet}$, veratryl alcohol neutral radical; LiPII−$VA^{\bullet+}$, complex of lignin peroxidase compound II and veratryl alcohol cation radical; ESR, electron spin resonance spectroscopy.

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reported for VAD formation under aerobic and anaerobic conditions, suggesting that the mechanism involving O_2 may not be significant.

The presence of an intermediate complex of LiP compound II (LiPII) and $VA^{\bullet+}$ (LiPII−$VA^{\bullet+}$) was originally suggested by Harvey et al. (1989) [but was subsequently questioned by Wariishi et al. (1991)]. Recently, Candeias and Harvey (1995) suggested that the proposed enzyme-bound $VA^{\bullet+}$ must have a longer lifetime than the free $VA^{\bullet+}$ in bulk solution. This conclusion was based upon a pulse radiolysis study on the formation and decay of $VA^{\bullet+}$. They observed that chemically generated $VA^{\bullet+}$ could not oxidize 4-methoxymandelic acid, while in the enzyme system, incubated with equimolar amounts of 4-methoxymandelic acid and VA, only the former was oxidized, even though it alone is not a substrate for LiP (Harvey et al., 1986). They assumed this to be due to indirect oxidation of 4-methoxymandelic acid by an enzyme-bound $VA^{\bullet+}$ that either is longer-lived or has a higher oxidation potential. But they did not provide any direct evidence for the existence of this complex or its half-life. We report in this study ESR evidence demonstrating a LiPII−$VA^{\bullet+}$ complex, its half-life, and the catalytic functions of this complex. Kinetic evidence, both steady state and under single turnover pseudo first order conditions, which supports the formation of this complex, is presented. Spectral evidence also suggests the presence of an LiPII−$VA^{\bullet+}$ complex.

EXPERIMENTAL PROCEDURES

Materials. Hydrogen peroxide, Tempol, and tetranitromethane were purchased from Sigma Chemical Co. Veratryl alcohol was obtained from Aldrich Chemical Co. Prior to use, VA was vacuum distilled to free it of a trace contaminant (Tien et al., 1986). Sodium succinate, succinic acid, and HNO_3 (69%) were purchased from Mallinckrodt. All chemicals were reagent grade and were used without further purification, unless otherwise stated. All buffers and solutions were prepared using purified water (Barnstead NANOpure II system; specific resistance, 18.0 Ω M cm^{-1}). Pure lignin peroxidase isozyme H2 (pI = 4.4), purified as described previously (Tuisel et al., 1990), was used throughout the study.

Steady State Kinetic Analysis. The initial velocity of VAD formation was measured by the increase in 310 nm absorbance [VAD $E_{310\ nm}$ = 9300 M^{-1} cm^{-1} (Tien et al., 1986)].

Single Turnover Pseudo First Order Kinetic Analysis. Rate constants for the reaction of LiP with H_2O_2 and reactions of LiPI and LiPII with VA were determined using a three-syringe stopped-flow spectrophotometer at pH 4.5. All rate constants were determined under single turnover pseudo first order conditions, with the substrate concentration (H_2O_2 or VA) being at least 10 times the enzyme concentration. Oxidation of LiP by H_2O_2 was followed at 417 nm, the isosbestic wavelength between ferric LiP and LiPII. The reduction of LiPI by VA was also followed at 417 nm. The reduction of LiPII by VA was followed at 397 nm. All reactions were performed at 25 °C.

To determine rate constants for the reaction of LiPI and VA, ferric LiP was first mixed with 1 equiv of H_2O_2 for 1.5 s to allow for complete formation of LiPI. It was then reacted with excess VA in the presence and absence of 5 mM oxalate. Rate constants for the reaction of LiPII were determined using LiPII generated by one-electron reduction of LiPI by either ferrocyanide or VA. In this case, LiP and 1 equiv of reductant were mixed with 1 equiv of H_2O_2 to form LiPII. The delay time for reaction of the enzyme with ferrocyanide as a reductant was 2.0 s, while with VA, it was 0.75 s to allow for complete formation of LiPII. The three-syringe stopped-flow apparatus used for determination of the rate constants was purchased from KinTek Instruments (State College, PA). Operation and data collection were as described previously (Kuan et al., 1993).

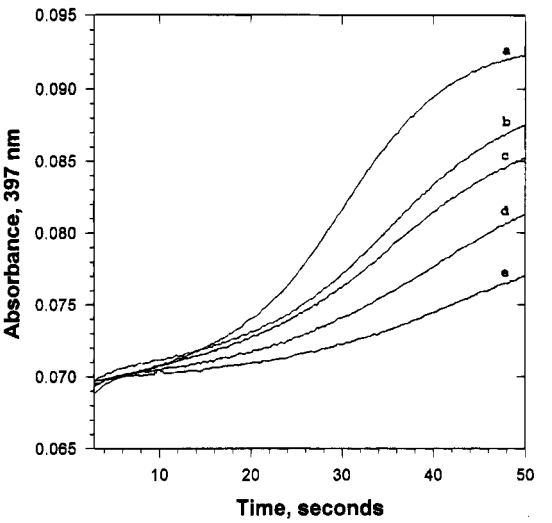


FIGURE 1: Effect of oxalate on the turnover rate of LiP. Turnover of LiP was monitored by the change in absorbance at 397 nm in a stopped-flow spectrophotometer. One syringe contained LiP (1 μM after mixing), and the other syringe contained VA (2 mM after mixing), H_2O_2 (50 μM after mixing), and various concentrations of oxalate in pH 4.5 succinate buffer. Trace a was obtained in the absence of oxalate, while traces b−e were obtained with 3.3, 5, 10, and 13.3 mM oxalate, respectively. Each trace is an average of three acquisitions.

ESR Detection and Quantitation of $VA^{\bullet+}$. The $VA^{\bullet+}$ formed by LiP was detected using a two-syringe fast-flow system as described previously (Khindaria et al., 1995b). For ESR detection under acid-quenched conditions, a three-syringe flow system was used. The contents of the first two syringes, one containing LiP and the other containing VA and H_2O_2, were first mixed together for 0.5 s, after which 10% HNO_3 was added using the third syringe to acid quench the reaction and denature the enzyme.

Radical concentrations were determined by double integration of the first derivative spectra using Tempol as a standard. A standard solution of Tempol was prepared using an extinction coefficient of 1440 M^{-1} cm^{-1} at 240 nm (Morrisett, 1976). Typical spectrometer settings were as follows: modulation amplitude, 1 G; time constant, 320 ms; scan time, 80 s; microwave power, 20 mW; and receiver gain, 2 × 10^4.

RESULTS

Effect of Oxalate on the Turnover Rate of LiP. Enzyme-monitored reactions of incubations containing LiP (1 μM), VA (2 mM), H_2O_2 (50 μM), and varying concentrations of oxalate at pH 4.5 are shown in Figure 1. In the presence of excess VA , LiP undergoes multiple turnovers until the H_2O_2 is depleted, whereupon the enzyme returns to the ferric state and the rate of return is reflective of the rate of catalysis. During steady state, the predominant form of LiP is LiPII

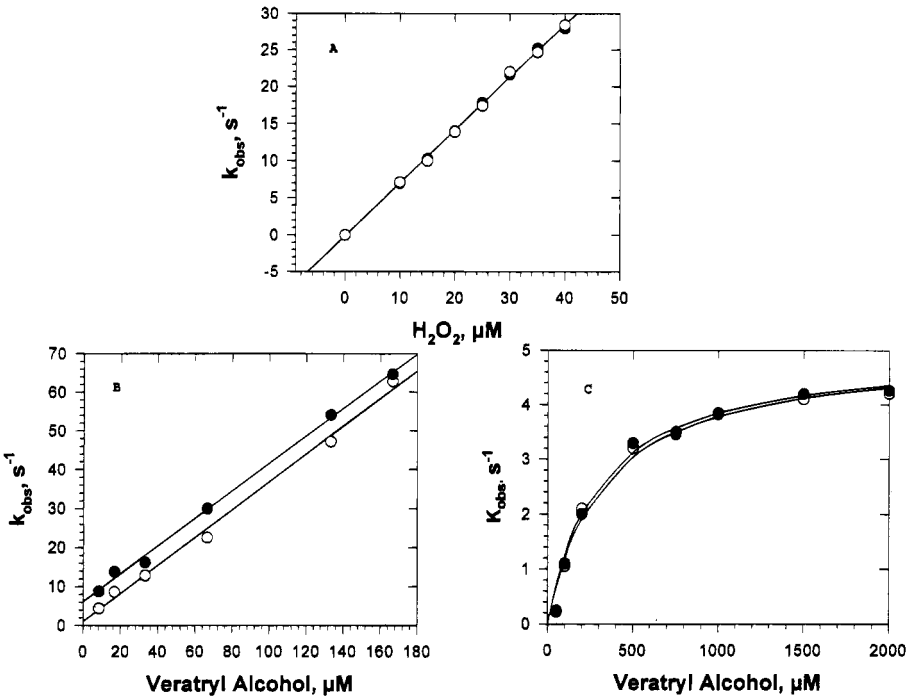


FIGURE 2: Oxidation of ferric LiP by H_2O_2 and reductions of LiPI and LiPII by VA in the absence or presence of oxalate. All reactions were carried out under pseudo first order conditions in a stopped-flow spectrophotometer. Incubations contained 1 μM LiP as ferric LiP (A), LiPI (B), and LiPII (C) in pH 4.5 succinate buffer and indicated concentrations of H_2O_2 (A) or VA (B and C) with 5 mM oxalate (●) or without (○). The k_{obs} values were determined by fitting the stopped-flow traces to an exponential equation. Each data point is an average of three traces.

because the reaction of LiPII with VA is rate-limiting. Due to the spectral changes associated with LiP compound I (LiPI) and LiPII formation, enzyme turnover can be monitored by measuring absorbance changes at 397 nm, the isosbestic wavelength for LiPI and LiPII (ϵ_{397} = 57 mM^{-1} cm^{-1}) at which ferric LiP exhibits an increased extinction coefficient (ϵ_{397} = 90 mM^{-1} cm^{-1}) (Tien et al., 1986). The initial decrease at 397 nm associated with LiPI formation was too rapid to detect under the experimental conditions used. Increasing oxalate concentrations (Figure 1b–e) decreased the rate of LiP turnover, as shown by the increased time required for LiP to return to the ferric state. That the steady state intermediate during turnover was LiPII was confirmed by the visible absorption spectrum of LiP (data not shown). Tetranitromethane (50 μM) had no effect on LiP turnover with or without oxalate.

Steady State and Single Turnover Kinetic Analyses of LiP. To further investigate the effect of oxalate on LiP during turnover, we determined the rate constants for reaction of ferric LiP with H_2O_2 and for reaction of LiPI and LiPII with VA, in both the presence and absence of oxalate. These reactions were studied under single turnover pseudo first order conditions. The plots of k_{obs} versus the concentration of H_2O_2 for ferric LiP oxidation to LiPI were linear with or without oxalate (Figure 2A). The two second order rate constants (5.9 × 10^5 M^{-1} s^{-1} and 5.6 × 10^5 M^{-1} s^{-1} with and without oxalate, respectively) were within experimental error and were similar to the value reported earlier for LiP oxidation by H_2O_2 (Tien et al., 1986).

For the reaction of LiPI with VA, LiPI was prepared by reaction of 1 μM H_2O_2 with 1 μM ferric LiP for 1.0 s and then reacted with varying concentrations of VA without or with 5 mM oxalate. The plots of k_{obs} versus the concentration of VA for LiPI reduction to LiPII by VA were also linear, in both the absence and presence of oxalate (Figure 2B). The plots were parallel with identical slopes (rate constant for the reaction of LiPI and VA, 3.3 × 10^5 M^{-1} s^{-1}), though with oxalate in the reaction mixture, the k_{obs} versus VA concentration plot exhibited a positive y-intercept. The magnitude of the y-intercept was proportional to oxalate concentration (data not shown) and was explained by the slow reduction of LiPI by oxalate (8 × 10^2 M^{-1} s^{-1}). The rate constant for reaction of LiPI with VA obtained in this study agreed closely with that reported earlier (Wariishi et al., 1991) and was not affected by oxalate.

The reaction of LiPII with VA was studied by forming LiPII two different ways, first by reduction of LiPI with ferrocyanide and second by reduction of LiPI with VA. The plots of k_{obs} versus VA concentration (with or without 5 mM oxalate) were hyperbolic when LiPII was formed by reduction of LiPI with ferrocyanide (Figure 2C). This hyperbolic concentration dependence of k_{obs} can be explained by a two-step reaction, involving a binding step between LiPII and VA, for the oxidation of VA by LiPII.

If rapid equilibrium is assumed for the binding of VA to LiPII, then the rate of VA oxidation can be given by

$$k_{obs} = \frac{k}{1 + \frac{K_d}{[VA]}} \quad (1)$$

where k is the first order rate constant and K_d is the binding constant. The plots in Figure 2C are best fit according to eq 1 with k = 5.1 ± 0.2 s^{-1} and K_d = 326 ± 7 μM without oxalate and k = 5.0 ± 0.3 s^{-1} and K_d = 300 ± 3 μM with oxalate for VA oxidation by LiPII. These values are consistent with the rate constants reported by Wariishi et al. (1991). No reaction of LiPII with oxalate was observed, and oxalate did not affect the reaction of VA with LiPII.

Table 1: Comparison of Single Turnover and Steady State Kinetic Constants for the Oxidation of VA by LiPII

	steady state kinetic constants[a]		single turnover kinetic constants[b]	
pH	k_{cat} (s^{-1})	K_m (μM)	k (s^{-1})	K_d (μM)
3.5	17.0	260	17.0	280
4.5	8.0	135	5.0	300
5.5	1.9	80	0.8	360

[a] This study and Tuisel et al. (1990). The k_{cat} and K_m for LiP oxidation of VA were determined as described elsewhere (Tien et al., 1986). [b] This study and Wariishi et al. (1991). k is the first order rate constant for the reaction of VA with LiPII formed by reduction of LiPI by ferrocyanide or ascorbate, and K_d is the dissociation constant of VA and LiPII. Additional experimental details are given in Experimental Procedures.

These results suggested that none of the catalytic steps involved in LiP-catalyzed oxidation of VA were affected by oxalate. However, each step was studied separately, which may not be the case under steady state conditions. The first order rate constant of 5.1 ± 0.2 s^{-1} is inconsistent with the k_{cat} = 8.0 s^{-1} [Table 1 of this study and Tuisel et al. (1990)] obtained from steady state turnover of the enzyme. Comparisons of the k_{cat} and K_m values, obtained through steady state analysis, and the first order rate constant and K_d, obtained under single turnover conditions, for the reaction of LiPII and VA at three different pH were tabulated (Table 1). It was observed that, at pH 3.5, the first order rate constant could account for the steady state turnover of the enzyme but, at pH 5.5, the k_{cat} was twice the slowest step involved in catalysis.

To further investigate this discrepancy, the reaction of LiPII, prepared by the second method, i.e., by reduction of LiPI with VA, with VA was studied. In this case, the stopped-flow syringe that contained the enzyme also contained an equimolar amount of VA. Compound II formed in this manner ($LiPII_{VA}$) was then reacted with at least a 10-fold excess of VA to ensure pseudo first order kinetics. The kinetics, monitored at 397 nm, of the reduction of $LiPII_{VA}$ are shown in part a of Figure 3. Biphasic kinetics were observed, with approximately 40% of the enzyme reacting in the faster phase. This phase, characterized by a burst, is more easily identified in a semilogarithmic plot of the stopped-flow trace by its deviation from linearity (part a of Figure 3B). The magnitude of the burst phase decreased with increasing concentrations of oxalate, and the phase completely disappeared at 5 mM oxalate (part b of Figure 3A). At this concentration of oxalate, all of the $LiPII_{VA}$ reacted with VA at the slower rate, as evident from the semilogarithmic plot (Figure 3B).

To confirm that the observed burst phase was due to the reduction of $LiPII_{VA}$ by VA, a difference spectrum of LiP was obtained during the reaction (Figure 4). The difference spectrum exhibited an isosbestic point at 417 nm, which corresponds to the isosbestic wavelength for LiPII and ferric LiP. The changes in absorbance were of the expected order, as predicted from the known extinction coefficients of the two LiP intermediates (Tien et al., 1986).

The stability of the species responsible for the burst phase was investigated by preincubation of the enzyme with H_2O_2 and VA in the delay line for varying time periods. The reaction mixture was then reacted with a fixed concentration of VA (500 μM). Preincubation did not affect the rate during the initial burst phase but had a marked effect on its extent.

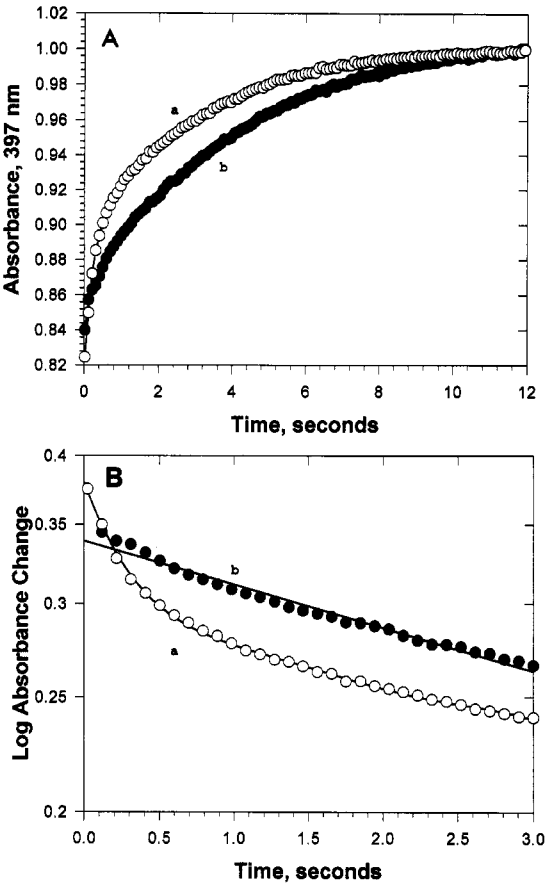


FIGURE 3: Stopped-flow kinetic trace obtained during reduction of $LiPII_{VA}$ by VA (part a in panel A) and the effect of 5 mM oxalate (part b in panel A). LiPI was prepared by mixing 1 equiv of H_2O_2 with 1 μM ferric LiP which was then reacted with 1 equiv of VA (1 μM) to form $LiPII_{VA}$. $LiPII_{VA}$ was then reacted with 500 μM VA. Panel B shows the semilogarithmic plot for the kinetic traces shown in panel A. The filled circles correspond to the trace obtained with 5 mM oxalate, and the open circles correspond to the trace obtained without oxalate.

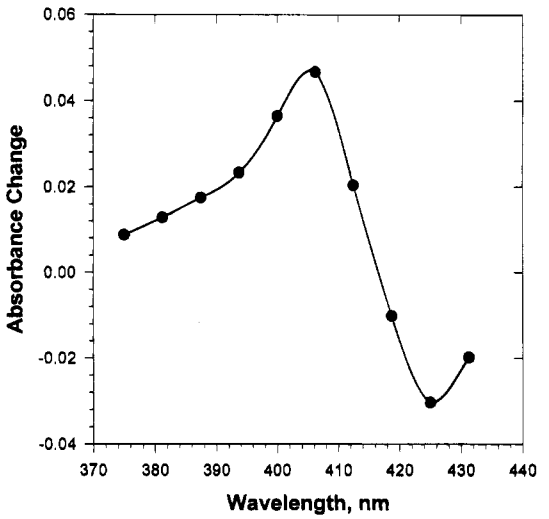


FIGURE 4: Difference spectrum of LiP obtained during the reduction of $LiPII_{VA}$ (formed as described in the legend to Figure 3) by VA. The difference spectrum exhibits an isosbestic point at 417 nm, the isosbestic wavelength between LiPII and ferric LiP.

The fraction of enzyme reacting in the faster phase was determined from the absorbance change at 397 nm. The percent $LiPII_{VA}$ reacting in the faster phase as a function of preincubation time is plotted in Figure 5. The plot best fits

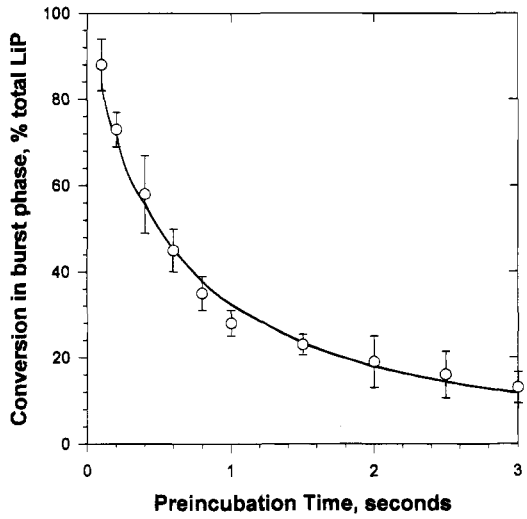


FIGURE 5: Effect of preincubation time on the extent of the burst observed during the reduction of $LiPII_{VA}$ by VA. The experimental conditions used were the same as described in the legend to Figure 3 except that equimolar amounts of LiPI and VA were mixed and preincubated for varying time periods as indicated. The percent of $LiPII_{VA}$ reacting in the faster phase, characterized by a burst, was quantitated by monitoring the absorbance changes observed in the two phases.

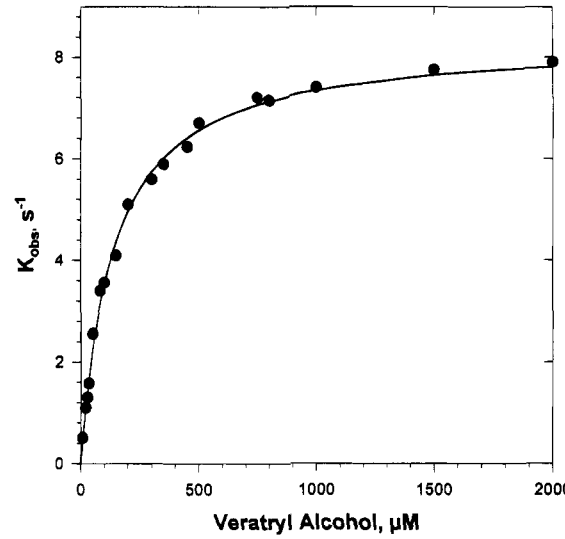


FIGURE 6: Reduction of $LiPII_{VA}$ (formed as described in the legend to Figure 3) by VA under pseudo first order conditions. The experimental conditions were the same as described in the legend to Figure 3 except that varying concentrations of VA were used as indicated. The k_{obs} values were obtained by fitting the kinetic traces to a burst first exponential equation ($r^2 > 0.95$). Each datum point is an average of three traces.

a first order decay equation with $t_{1/2} = 0.54$ s. This suggested the presence of a transient enzyme intermediate. At this stage, it was assumed that the unstable intermediate was a complex of LiPII and $VA^{\bullet+}$ (hereafter referred to as LiPII−$VA^{\bullet+}$), resulting from the one-electron reduction of LiPI by VA. LiPII−$VA^{\bullet+}$ reacted faster with VA than did free LiPII. The rate constant for the reaction of VA with LiPII−$VA^{\bullet+}$ was determined by fitting the faster phase to burst first exponential kinetics to obtain the k_{obs}. The plot of k_{obs} versus the concentration of VA is shown in Figure 6. The plot is hyperbolic, suggesting a binding interaction between the reactants, resulting in a two-step reaction mechanism (eq 2).

$$\text{LiPII}-\text{VA}^{\bullet+} + \text{VA} \rightleftharpoons \text{VA}-\text{LiPII}-\text{VA}^{\bullet+} \rightarrow \text{LiP} + \text{VA} + \text{VAD} \quad (2)$$

If a rapid equilibrium is assumed for the binding of VA to LiPII−$VA^{\bullet+}$, then the reaction rate can be given by eq 3, analogous to eq 1.

$$k_{obs} = \frac{k'}{1 + \frac{K'_d}{[\text{VA}]}} \quad (3)$$

The data shown in Figure 6 best fit according to eq 3 with $k' = 8.3 \pm 0.2$ s^{-1} and $K'_d = 133$ μM. The first order rate constant is fast enough to account for the steady state turnover rate of LiP at pH 4.5, and the binding constant was in close agreement with the K_m obtained from the steady state kinetic data.

If such a complex of LiPII and $VA^{\bullet+}$ does form, which reacts faster with VA than does free LiPII, it would be expected that the absorption spectrum of the enzyme complexed with $VA^{\bullet+}$ would be altered from the one of the free LiPII. Recently, we have reported (Khindaria et al., 1995b) that the $VA^{\bullet+}$ absorbs strongly in the visible region, with a maximum at 529 nm ($\epsilon = 11\,000$ M^{-1} cm^{-1}). Thus, it would be expected to also alter the absorption spectrum of the enzyme in the visible region. The visible absorption

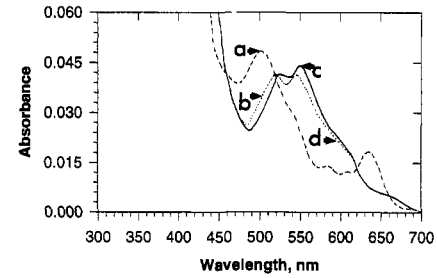


FIGURE 7: Visible absorption spectra of ferric LiP and LiPII during steady state oxidation of VA and the effect of oxalate on it. Trace a is the spectrum of 6.0 μM LiP in pH 4.5 succinate buffer. Trace b is the spectrum obtained when LiPII was formed by addition of 2 equiv of H_2O_2 to ferric LiP. Trace c is the spectrum obtained during the steady state oxidation of VA by LiP. Trace d, overlapping with b, is the spectrum obtained under the same conditions as in trace c except that the mixture also contained 10 mM oxalate.

spectrum of LiPII, formed by reaction of ferric LiP either with 2 equiv of H_2O_2 or with equimolar concentrations of H_2O_2 and ferrocyanide, is shown in trace b of Figure 7. The LiPII spectrum obtained under steady state turnover (during oxidation of VA) is shown in trace c of Figure 7. The α and β bands of the steady state intermediate of the enzyme, LiPII−$VA^{\bullet+}$, were found to be shifted as compared to the spectrum of free LiPII. When 10 mM oxalate was added during turnover of LiP with VA, a spectrum identical to the one obtained in trace b was observed (trace d). This suggested that oxalate was scavenging the species responsible for altering the LiPII spectrum, possibly the $VA^{\bullet+}$, as oxalate reacts with $VA^{\bullet+}$ with appreciable rates (Akamatsu et al., 1990). The effect of oxalate on the spectrum and the burst phase was assumed to be due to its reaction with $VA^{\bullet+}$.

Electron Spin Resonance Studies on $VA^{\bullet+}$. The involvement of $VA^{\bullet+}$ and the effect of oxalate were further investigated by ESR. A two-syringe fast-flow system was used for direct ESR detection of $VA^{\bullet+}$ (Khindaria et al., 1995a,b). The ESR signal obtained in a fast-flow incubation (24 mL/min) with 10 μM LiP, 2 mM VA, and 250 μM H_2O_2 in 20 mM pH 4.5 succinate buffer is shown in Figure 8A.

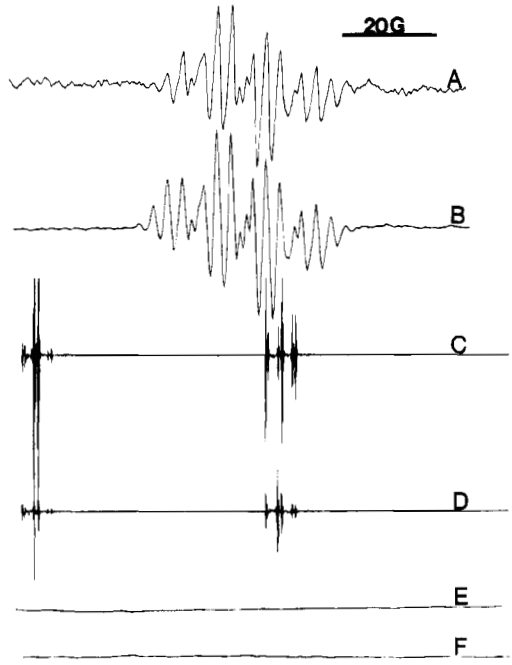


FIGURE 8: The ESR spectra of LiP-formed $VA^{\bullet+}$ at pH 4.5. Trace A is the spectrum obtained in a fast-flow incubation containing 10 μM LiP, 2 mM VA, and 250 μM H_2O_2 in 20 mM succinate buffer (pH 4.5). Trace B is the spectrum obtained when the reaction in trace A was acid quenched with 10% HNO_3, also in a fast-flow incubation. Traces C and D are the Fourier transforms of the signals in A and B, respectively. Traces E and F were recorded under the same conditions as for trace B except that H_2O_2 and LiP were left out of the reaction mixture, respectively.

The ESR hyperfine-splitting constants were identical to those reported earlier for $VA^{\bullet+}$ (Khindaria et al., 1995b). The radical concentration was 2.2 $\pm$ 0.2 μM (n = 3), as determined by double integrating the spectrum and using Tempol as a standard. The low radical concentration, as compared to the LiP concentration (10 μM), could be explained if the majority of $VA^{\bullet+}$ generated by LiP was ESR-silent. This was investigated by acid quenching the reaction to denature the protein and release the $VA^{\bullet+}$ in acid solution. For this experiment, a three-syringe fast-flow system was used. One syringe contained LiP in pH 4.5 buffer, the second contained VA and H_2O_2 in the same buffer, and the third contained 10% HNO_3. The contents of the first and second syringes were reacted for 0.5 s in a delay line, after which 10% HNO_3 was added. The combined flow rate through the ESR cell was 36 mL/min. The ESR signal detected from this incubation is shown in Figure 8B. The ESR hyperfine-splitting constants of the radical signal were identical to those for the signal in Figure 8A. This was supported by identical Fourier transforms of the two signals (Figure 8C,D). However, the radical concentration increased to 8.9 $\pm$ 0.3 μM. Since the two signals were identical, it was assumed that the increase in $VA^{\bullet+}$ ESR spectral signal intensity was due to the presence of two forms of $VA^{\bullet+}$, one ESR-silent and one ESR-active. The ESR-silent form could only be detected when the reaction was acid quenched. The increase in signal intensity was not an artifact of acid quenching, as no signal was detected in the absence of H_2O_2 or LiP (Figure 8E,F).

To validate the earlier premise that the effect of oxalate was due to its reaction with $VA^{\bullet+}$, the effect of oxalate on $VA^{\bullet+}$ was investigated by ESR. The ESR signal of $VA^{\bullet+}$ obtained in the presence of oxalate is shown in Figure 9A.

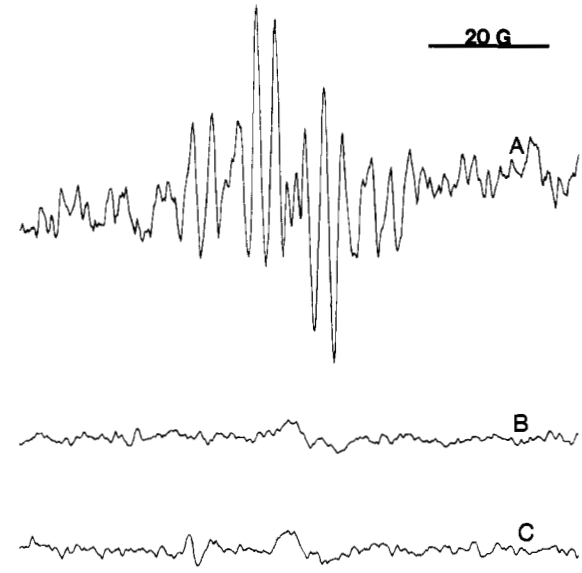


FIGURE 9: Effect of oxalate on the LiP-formed $VA^{\bullet+}$ ESR radical signal. Trace A is the spectrum obtained under the same conditions as described in the legend to Figure 8A except that 0.5 mM oxalate was also included in the incubation. Trace B was obtained when the oxalate concentration was increased to 5 mM. Trace C was obtained when the reaction in B was acid quenched with 10% HNO_3.

The reaction conditions used were the same as described in the legend to Figure 8A except that the syringe that contained VA and H_2O_2 also contained 0.5 mM oxalate. The radical concentration was ~1.0 μM. No $VA^{\bullet+}$ ESR signal could be detected when the oxalate concentration was increased to 5 mM (Figure 9B). When the reaction in B was acid quenched using the three-syringe fast-flow system, no ESR radical signal was detected (Figure 9C).

Oxalate reacts with $VA^{\bullet+}$ (Shimada et al., 1990); however, to investigate the possible role of a negatively charged anion, the effect of malonate on $VA^{\bullet+}$ ESR signal intensity was studied. The ESR signal observed in a reaction mixture containing LiP, VA, and H_2O_2 at pH 4.5 in the presence of 5 mM malonate is shown in Figure 10A. The $VA^{\bullet+}$ concentration was 1.4 $\pm$ 0.1 μM. Increasing the malonate concentration to 10 mM further decreased the steady state concentration of $VA^{\bullet+}$ to 0.8 $\pm$ 0.1 μM (Figure 10B). When the above reaction was acid quenched with 10% HNO_3, a strong $VA^{\bullet+}$ cation radical signal was observed (Figure 10C). The $VA^{\bullet+}$ concentration was 8.0 $\pm$ 0.2 μM. The control experiments in the absence of either LiP, H_2O_2, or VA did not yield any radical signal.

If VA reacted with the LiPII$-VA^{\bullet+}$ complex, then increasing VA concentration would result in a decreased ESR signal due to $VA^{\bullet+}$. The plot of $VA^{\bullet+}$ versus concentrations of VA at pH 4.5 is shown in Figure 11. The concentration of $VA^{\bullet+}$ was determined during steady state turnover of the enzyme in continuous flow incubation with varying concentrations of VA. The steady state concentration of $VA^{\bullet+}$ first increased with increasing VA concentration up to 500 μM VA and then decreased with further increase in VA concentration. The maximum $VA^{\bullet+}$ concentration at 500 μM VA was 3.5 μM.

DISCUSSION

The reactions of $VA^{\bullet+}$ and its oxidation to VAD have not been characterized previously, and it has been assumed,

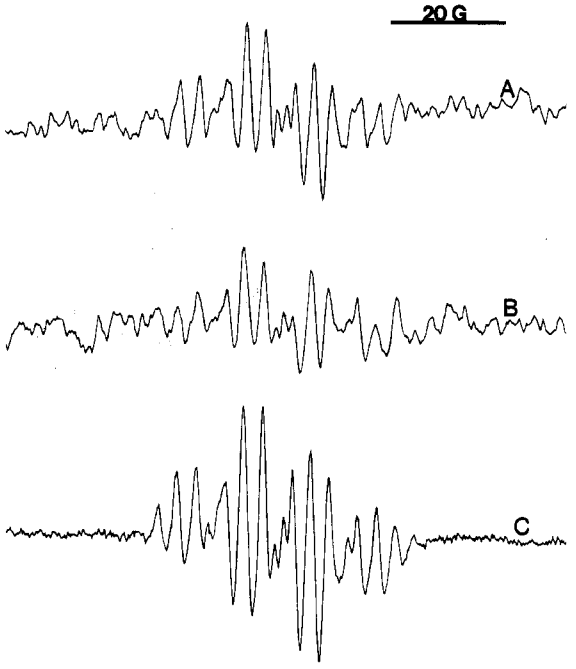


FIGURE 10: Effect of malonate on the LiP-formed $VA^{\bullet+}$ ESR radical signal. Trace A is the spectrum obtained under the same conditions as described in the legend to Figure 8A except that 5 mM malonate was also included in the incubation. Trace B was obtained when the malonate concentration was increased to 10 mM. Trace C was obtained when the reaction in B was acid quenched with 10% HNO_3.

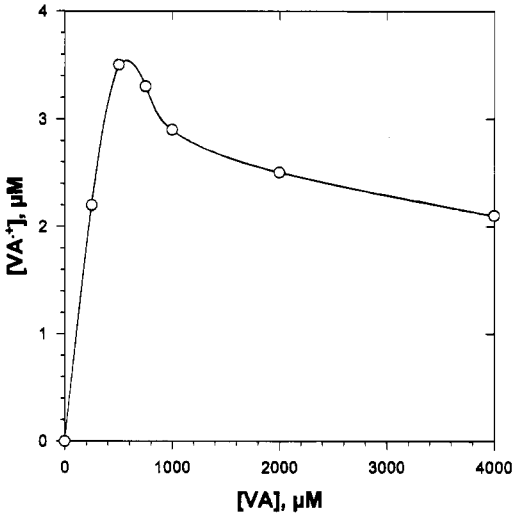
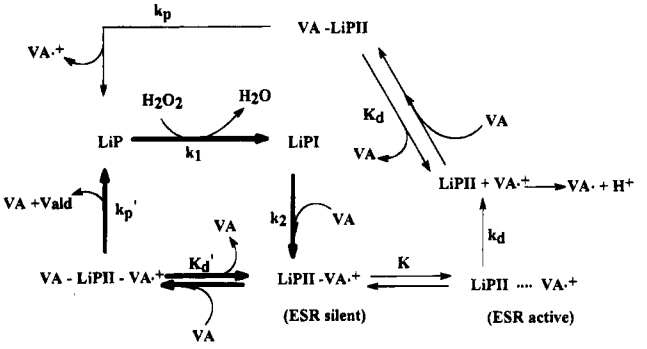


FIGURE 11: Effect of VA concentration on the LiP-catalyzed formation of $VA^{\bullet+}$. The experimental conditions were the same as described in the legend to Figure 8A except that the concentration of VA was varied as indicated.

without evidence, that $VA^{\bullet+}$ is oxidized to VAD by LiP catalysis (Haemmerli et al., 1987; Bono et al., 1990). Our data allow us to propose a series of reactions that may entail the fate of $VA^{\bullet+}$ leading to the formation of VAD. The formation of VAD is suggested to involve the formation of a unique ternary complex of LiPII, VA, and $VA^{\bullet+}$. We also present here direct ESR evidence for the role of $VA^{\bullet+}$ as a redox mediator in LiP catalysis.

The results of this study allow us to propose an overall scheme for the oxidation of VA to VAD by LiP (Scheme 1). According to this scheme, LiP is oxidized by H_2O_2 to LiPI, which oxidizes VA by one electron to $VA^{\bullet+}$ with the concominant formation of LiPII. These first two steps of

Scheme 1: Proposed Scheme for the Oxidation of VA by LiP[a]



[a] The values of the kinetic constants obtained in this study at pH 4.5 are as follows: $k_1 = 5.6 \times 10^5\ M^{-1}\ s^{-1}$, $k_2 = 3.3 \times 10^5\ M^{-1}\ s^{-1}$, $K'_d = 133\ \mu M$, $k'_p = 8.3\ s^{-1}$, $K = 0.14$, $k_d = 1.85\ s^{-1}$, $K_d = 326\ \mu M$, and $k_p = 5.0\ s^{-1}$.

the reaction mechanism are generally accepted. At this stage, our scheme digresses from the earlier published mechanisms in that we assumed the formation of a transient intermediate complex of LiPII and $VA^{\bullet+}$. Several lines of evidence support the formation of this LiPII−$VA^{\bullet+}$ complex.

Biphasic kinetics were observed during the reduction of LiPII−$VA^{\bullet+}$ by VA, consisting of burst and slow phases. It was confirmed by the difference spectrum of the enzyme that the burst was not an artifact and resulted from the reduction of LiPII−$VA^{\bullet+}$ to ferric LiP by VA. Since the reaction was monitored by following the conversion of LiPII−$VA^{\bullet+}$ to ferric LiP, the most likely explanation for the burst phenomenon was the existence of LiPII in two states. One state reacted faster with VA than the other which resulted in the rapid formation of ferric LiP for the part of the enzyme that was complexed with $VA^{\bullet+}$. It may be argued that, for other ping−pong bi−bi enzymes, e.g., phosphatases, that exhibit the pre-steady state burst phenomenon, the entire enzyme preparation reacts in the burst phase till steady state is attained. However, with LiP, which also exhibits a ping−pong bi−bi mechanism, only a fraction reacts in the burst phase due to the transient nature of the intermediate species responsible for the burst. This was demonstrated by the decrease in the extent of the burst phase with an increase in the preincubation time for the reaction mixture with equimolar LiPI and VA. The fact that the rate of the reaction in the burst phase was unaffected by preincubation and only the extent was decreased revealed the presence of a short-lived species ($t_{1/2} = 0.54$ s). This would be expected if the species involved is a free radical that is short-lived, as is $VA^{\bullet+}$. Moreover, this burst was not observed when any other reductant (ferrocyanide or ascorbate) was used to form LiPII. Additionally, the faster burst phase was sensitive to oxalate which did not affect the reaction of LiPII with VA when LiPII was formed from LiPI by using ferrocyanide. Hence, the effect of oxalate was on the species different from LiPII and could only be on $VA^{\bullet+}$.

Proposing the formation of such a complex, on the basis of the kinetic studies, is not without precedent in the peroxidase field. Dunford and Cotton (1975) proposed, from kinetic analyses, that, during oxidation of *p*-aminobenzoic acid by horseradish peroxidase, the free radical product of one-electron oxidation of *p*-aminobenzoic acid by HRPI forms a complex with HRPII. The HRPII−free radical complex reacts slower with an additional molecule of

p-aminobenzoic acid than the pure HRPII. In contrast, we have found that VA reacts faster with the LiPII−$VA^{\bullet+}$ complex than with free LiPII. The contrast observed between LiP and HRP may be attributed both to the fact that the active sites of LiP and HRP are markedly different (Poulos et al., 1993; Banci et al., 1993, 1994) and to the fact that the nature of the free radical that forms the complex with compound II of the enzyme. The neutral radical of *p*-aminobenzoic acid that formed a complex with HRPII is a weak oxidant, while the $VA^{\bullet+}$, which is proposed to complex with LiPII, is a strong oxidant (Harvey et al., 1989; Goodwin et al., 1995; Chung & Aust, 1995). This might explain why, in one case (i.e., HRP), the rate of a subsequent reaction is slower while, in the other (i.e., LiP), it is accelerated.

Perhaps the most convincing evidence for the formation of the LiPII−$VA^{\bullet+}$ complex came from ESR studies. Direct ESR was used to detect LiP-formed $VA^{\bullet+}$ at pH 4.5. The proton hyperfine-splitting constants obtained in this study were identical to those reported earlier (Khindaria et al., 1995b). This was further confirmed by the computer simulation of the ESR spectrum using the earlier published magnetic parameters. The interaction of $VA^{\bullet+}$ with LiPII became evident by ESR under acid-quenched conditions. While under normal turnover conditions only 2.2 μM $VA^{\bullet+}$ could be detected (using 10 μM LiP), the concentration reached a maximum of 8.9 μM when an identical reaction was acid quenched. This suggested that a higher concentration of the radical was formed but only a fraction of it was ESR-active during LiP turnover at pH 4.5. The rest, or the ESR-silent $VA^{\bullet+}$, was detected only after the reaction was acid quenched. This could be due to two types of restrictions imposed on the radical in the enzyme active site. The $VA^{\bullet+}$ could interact with LiP, resulting in constraint of the physical tumbling or spin coupling between the $VA^{\bullet+}$ and the ferryl iron, leading to broadening of the ESR signal beyond detection. Upon acid quenching, the enzyme would be denatured, releasing the $VA^{\bullet+}$ into the solution which could then be detected by ESR.

The formation of this putative complex is also supported by the anomalous LiPII spectrum observed during steady state turnover of LiP with excess VA and H_2O_2, as was reported by Harvey et al. (1989). These workers also suggested the formation of such a complex that accelerated the rate of VA reaction with LiPII. In contrast, Wariishi et al. (1991) discounted the possible formation of such a complex on the basis of an unambiguous LiPII visible absorption spectrum observed by them during VA oxidation (Valli et al., 1990). However, under their (Valli et al., 1990) experimental conditions, the reaction would be expected to be over in approximately 1 s (at pH 3.0; $k_{cat} = 38\ s^{-1}$, [LiP] = 5.0 μM, [VA] = 60 μM, and [H_2O_2] = 200 μM; 5 × 38 = 190 μM H_2O_2 consumed per second), while the spectral scan was recorded after 5.2 s, possibly accounting for their inability to observe the anomalous LiPII spectrum resulting from the LiPII−$VA^{\bullet+}$ complex. The LiPII spectrum observed under steady state is different from the free LiPII spectrum and was assigned to the LiPII−$VA^{\bullet+}$ complex. This spectrum was sensitive to oxalate, as was the burst phase, due to the scavenging of the $VA^{\bullet+}$ associated with LiPII. The visible absorption spectrum of the $VA^{\bullet+}$ in 98% H_2SO_4 has recently been published (Khindaria et al., 1995a,b); however, it is very difficult at this stage to quantitate the contribution of the $VA^{\bullet+}$ to the LiPII spectrum, on the basis of the previous study, because of the difference in conditions. It is evident though that a visible absorption spectrum of an enzyme species different from LiPII is observed during steady state oxidation of VA, and supporting evidence obtained in this study allows us to propose that this spectrum was due to the LiPII−$VA^{\bullet+}$ complex.

The effect of oxalate on the free radical species complexed with the enzyme was confirmed by ESR studies. In confirmation of the ealier published data (Akamatsu et al., 1990), we observed that 0.5 mM oxalate was able to inhibit the formation of $VA^{\bullet+}$ by 50% during the steady state. We reported the physiological concentration of oxalate in the shaking cultures of *P. chrysosporium* to be about 3 mM (Barr et al., 1992). Concentrations much above this would inhibit the oxidation of other chemicals by $VA^{\bullet+}$. In accord, with 5 mM oxalate, no ESR signal could be detected. This also substantiated the claim that the effect of oxalate on the burst phase and the anomalous LiPII spectrum was due to its reaction with $VA^{\bullet+}$. The effect of oxalate on the radical was due to its reducing ability and the negative charges; oxalate is completely deprotonated at pH 4.5. Malonate would also be expected to be deprotonated, but it did not scavenge the $VA^{\bullet+}$ to the same extent as did oxalate. Malonate has a more positive reduction potential than oxalate and does not reduce $VA^{\bullet+}$ as efficiently. Furthermore, it may only be able to interact with the ESR-active $VA^{\bullet+}$. This was supported by the fact that malonate had minimal effect on the $VA^{\bullet+}$ concentration under acid-quenched conditions.

Direct ESR evidence was also obtained for the reaction of LiPII−$VA^{\bullet+}$ with VA. It was found that the concentration of the ESR-active $VA^{\bullet+}$ species was dependent on the VA concentration. The $VA^{\bullet+}$ concentration first increased with increasing VA concentration up to 500 μM VA. This was due to the increase in the rate of VA oxidation by LiP as the K_m of LiP for VA is 135 μM. A further increase in VA concentration led to a decrease in $VA^{\bullet+}$ concentration. We propose that this is due to the increase in reaction rate between LiPII−$VA^{\bullet+}$ and VA. As this rate increased, the equilibrium was pulled toward the formation of the ternary complex of LiPII−$VA^{\bullet+}$ and VA, which resulted in the decrease of the ESR-active $VA^{\bullet+}$. These studies support the presence of two forms of the LiPII−$VA^{\bullet+}$ complex, one ESR-active and one ESR-silent. Both ESR-silent and ESR-active $VA^{\bullet+}$ can mediate electron transfer to molecules that may not react with LiP. However, the ESR-silent $VA^{\bullet+}$ was assumed to be responsible for the burst phase observed during the reduction of LiPII−$VA^{\bullet+}$ by VA.

The discrepancy in the rate constant for VA reduction of LiPII, formed from LiPI with ferrocyanide as a reductant, and the steady state kinetic constants is also explained by the existence of LiPII−$VA^{\bullet+}$, formed by the reduction of LiP by VA. The former reacts with VA with the first order rate equal to 5 s^{-1}, which is slower than what would be expected from the steady state kinetic constants. If a step is involved in catalysis, then the rate of that step cannot be lower than the k_{cat}. In LiP catalysis, the steady state and single turnover kinetic parameters are in accord only when the reduction of LiPII−$VA^{\bullet+}$ by VA was measured. The faster rate of reaction of VA with LiPII−$VA^{\bullet+}$ could result from two mechanisms. The $VA^{\bullet+}$ may increase the oxidation potential of LiPII, as was proposed by Harvey et al. (1989). Second, $VA^{\bullet+}$ may serve as a proton donor in the reduction

of LiPII to ferric LiP. If a strong proton donor ($VA^{\bullet+}$) is associated with LiPII in addition to the electron donor (VA), then the rate of reduction of LiPII would increase. This may be the case due to the formation of the ternary complex of LiPII, $VA^{\bullet+}$, and VA (Scheme 1). The mechanism for VA oxidation may then be

$$\text{LiPI} + \text{VA} \rightarrow \text{LiPII}-\text{VA}^{\bullet+}$$

$$\text{LiPII}-\text{VA}^{\bullet+} + \text{VA} \rightarrow \text{VA}-\text{LiPII}-\text{VA}^{\bullet+} \rightarrow \text{LiP} + \text{VA}^{\bullet+} + \text{VA}^{\bullet}$$

$$\text{VA}^{\bullet+} + \text{VA}^{\bullet} \rightarrow \text{VA} + \text{VAD} + \text{H}^{+}$$

where, following the formation of the ternary complex, $VA^{\bullet+}$ donates the proton while VA is the electron donor to LiPII. The proton donor is converted to a neutral radical and the electron donor to the cation radical. The cation radical then oxidizes the neutral radical to the aldehyde while it is reduced back to VA. This most likely occurs, or the enzyme for the neutral radical would otherwise react very rapidly with oxygen. This would also result in the formation of 1 mol of VAD per turnover of the enzyme.

The two mechanisms, one where $VA^{\bullet+}$ increases the reduction potential of LiPII and the other where $VA^{\bullet+}$ acts as a proton donor, assume the formation of the LiPII$-VA^{\bullet+}$ complex. In the former case, the aldehyde would probably form by dismutation of two $VA^{\bullet+}$ and in the latter from the oxidation of $VA^{\bullet}$ by $VA^{\bullet+}$. Our kinetic data fit both the mechanisms equally well. Although from a kinetic standpoint it is not possible to distinguish between the two mechanisms, the ESR studies on the concentration dependence of $VA^{\bullet+}$ on VA concentration favor the latter mechanism. The latter mechanism is also more plausible, considering the structure and nature of $VA^{\bullet+}$ and analyzing the spin and charge densities on the radical (Khindaria et al., 1995b). Cation radicals are more prone to dimerization than dismutation (Hammerich & Parker, 1982), especially those with asymmetric charge distribution, as is the case with $VA^{\bullet+}$; hence, a dication would be the most likely product of two $VA^{\bullet+}$ molecules reacting, not VAD.

The $VA^{\bullet+}$ can dissociate from the enzyme to yield free LiPII. The dissociation, leading to the decay of the complex, is probably not reversible. The decay of the LiPII$-VA^{\bullet+}$ complex occurs with a half-life of 0.54 s, as determined from the affect of preincubation time on the burst phase. In contrast to this relative stability of the complex, the $VA^{\bullet+}$ is very unstable in solution and decays with a half-life of nano- to milliseconds (Candeias & Harvey, 1995). They reported that $VA^{\bullet+}$ in bulk solution has a decay constant of 17 s^{-1} and a half-life of 0.5 ms. This implies that the $VA^{\bullet+}$ is stabilized by the enzyme system as the LiPII$-VA^{\bullet+}$ complex decays with a half-life of 0.54 s. This suggests that the enzyme stabilizes the radical and shields it from the nucleophilic attack by the solvent water. Additionally, the microenvironment of the enzyme may have a low pH that might prevent the deprotonation of the $VA^{\bullet+}$ (Khindaria et al., 1995b). We have investigated the decay of free $VA^{\bullet+}$ and the LiP-bound $VA^{\bullet+}$ by ESR and have found that the LiP-bound $VA^{\bullet+}$ decays with a first order mechanism with a rate constant of 1.85 s^{-1} while free $VA^{\bullet+}$ decays much faster ($k_d = 1.2 \times 10^3$ s^{-1}) (manuscript in preparation). However, once dissociated, the $VA^{\bullet+}$ quickly deprotonates to yield the neutral radical, free in solution. The neutral radical in solution can react with oxygen, probably with diffusion-limited rates (Schmidt et al., 1989). The LiPII resulting from the dissociation of the $VA^{\bullet+}$ can then bind a molecule of VA, forming a complex and oxidizing it to the cation radical and concomitantly being reduced to ferric LiP. As such, our results and the mechanism agree with the earlier published work (Wariishi, 1990).

Since a ternary complex of VA, LiPII, and $VA^{\bullet+}$ is assumed to form in the proposed mechanism for VAD formation, it is speculated that the VA and $VA^{\bullet+}$ bind at different sites in the enzyme. This is supported by the fact that VA never inhibited the burst phase and hence probably does not compete with the $VA^{\bullet+}$ for binding. The formation of VAD, though, is hard to characterize as a one- or two-electron oxidation by LiP. A general introduction to one-electron and two-electron transfers in enzyme substrate reactions has been given elsewhere (Yamazaki, 1971). According to his terminology, the LiP$-$VA reaction occurs via a two-electron mechanism if only VAD is released from the enzyme (bold line in Scheme 1). However, since $VA^{\bullet+}$ is released with a half-life of 0.54 s (13% of the product), the LiP$-$VA reaction would be classified as a mixed type as are the HRP$-$sulfite (Araiso et al., 1976) and the xanthine oxidase$-O_2$ reactions (Nakamura & Yamazaki, 1969).

On the basis of crystallographic studies, Poulos et al. (1993) suggested that the δ-meso edge of the heme is accessible to the solvent and is the site for VA binding. We have investigated a binding site for $VA^{\bullet+}$ using energy minimization calculations, ESR, kinetic inhibition, and dynamic simulation studies (to be published). Interestingly, we found a site, near the heme propionates, similar to the Mn^{2+} oxidation site in manganese peroxidase reported by Sundaramoorthy et al. (1994), in which the $VA^{\bullet+}$ fit in a more energy favorable manner. Electrostatic potential calculations showed that this site could stabilize the radical and that the potential field was symmetric such that it would only slightly constrain the tumbling of the radical and may not broaden the ESR signal of the $VA^{\bullet+}$ bound to this site. Therefore, this may be the site for the ESR-active $VA^{\bullet+}$. The ESR-silent $VA^{\bullet+}$ may exist closer to the heme and would not be detected due to spin interaction with the iron.

Dynamic simulation studies showed that, even if the VA bound in the site proposed by Poulos et al. (1993) near the δ-meso edge, once oxidized to $VA^{\bullet+}$ it can actively diffuse to the site by the propionates in the catalytic time frame. Furthermore, we have previously shown that Mn^{2+} inhibits VA oxidation by LiP (Khindaria et al., 1995a), and since Mn^{2+} binds in the site by the propionates, it is likely that $VA^{\bullet+}$ also binds in this site, which may be specific for cations. This could then explain the proposed mechanism where LiPI oxidizes one VA to $VA^{\bullet+}$ which then moves to the site by the heme propionates, while another VA binds near the δ-meso edge. The first $VA^{\bullet+}$ then donates the proton at the same time as the second VA donates an electron to the δ-meso edge. The site near the heme propionates may even be more important for mediation and lignin degradation. It is more open and more accessible to larger substrates like lignin. Even though lignin may not be able to approach the active site or the heme, the stabilized $VA^{\bullet+}$ can act as an electron shuttle by orbital overlap.

In conclusion, we have shown that $VA^{\bullet+}$ complexes with LiPII and accelerates its reaction with VA. We have also

provided strong evidence for a mechanism for formation of VAD by LiP. We have also provided direct evidence for the role of $VA^{\bullet+}$ as a redox mediator by monitoring its reaction with oxalate by ESR. In the future, we would like to probe the two proposed active sites of LiP and how the $VA^{\bullet+}$ is stabilized in the site by the propionates.

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