Articles

Oxidation of Monomethoxylated Aromatic Compounds by Lignin Peroxidase: Role of Veratryl Alcohol in Lignin Biodegradation†

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ABSTRACT: Lignin peroxidase (LiP), an extracellular heme enzyme from the lignin-degrading fungus *Phanerochaete chrysosporium*, catalyzes the H_2O_2-dependent oxidation of a variety of nonphenolic lignin model compounds. The oxidation of monomethoxylated lignin model compounds, such as anisyl alcohol (AA), and the role of veratryl alcohol (VA) in LiP reactions were studied. AA oxidation reached a maximum at relatively low H_2O_2 concentrations, beyond which the extent of the reactions decreased. The presence of VA did not affect AA oxidation at low molar ratios of H_2O_2 to enzyme; however, at ratios above 100, the presence of VA abolished the decrease in AA oxidation. Addition of stoichiometric amounts of AA to LiP compound II (LiPII) resulted in its reduction to the native enzyme at rates that were significantly faster than the spontaneous rate of reduction, indicating that AA and other monomethoxylated aromatics are directly oxidized by LiP, albeit slowly. Under steady-state conditions in the presence of excess H_2O_2 and VA, a visible spectrum for LiPII was obtained. In contrast, under steady-state conditions in the presence of AA a visible spectrum was obtained for LiPIII*, a noncovalent complex of LiPIII and H_2O_2. AA competitively inhibited the oxidation of VA by LiP; the K_i for AA inhibition was 32 μM. Addition of VA to LiPIII* resulted in its conversion to the native enzyme. In contrast, AA did not convert LiPIII* to the native enzyme; instead, LiPIII* was bleached in the presence of AA. Thus, AA does not protect LiP from inactivation by H_2O_2. These results support a mechanism whereby VA protects the enzyme from inactivation by H_2O_2, thus making more enzyme available for the oxidation of recalcitrant substrates, such as AA and probably polymeric lignin. The results do not support a mechanism whereby VA acts as a radical mediator in the LiP oxidation of AA and other monomethoxylated aromatics.

When cultured under ligninolytic conditions, the white rot basidiomycete *Phanerochaete chrysosoprium* secretes two extracellular heme peroxidases, lignin peroxidase (LiP)[1] and manganese peroxidase, which along with an H_2O_2 generating system are the major components of its lignin degradation system (Buswell & Odier, 1987; Kirk & Farrell, 1987; Tien, 1987; Gold et al., 1989). LiP is a glycoprotein with a molecular weight of ~41 000, contains one Fe protoporphyrin IX, and exists as a series of isozymes (p*I* = 3.2–4.0) (Kirk & Farrell, 1987; Leisola et al., 1987; Gold et al., 1989). Spectroscopic studies indicate that the heme iron in the native resting enzyme is in the high-spin, predominantly pentacoordinate, ferric state with a histidine coordinated as the fifth ligand (Andersson et al., 1985, 1987; Kuila et al., 1985; Gold et al., 1989). Spectral and kinetic characterization of the reactions of the oxidized intermediates LiPI, LiPII, and LiPIII indicates that the oxidation states and catalytic cycle of LiP are similar to HRP (Dunford & Stillman, 1976; Renganathan & Gold, 1986; Andrawis et al., 1988; Marquez et al., 1988; Harvey et al., 1989; Wariishi & Gold, 1990). However, the enzyme catalyzes the unique one-electron oxidation of nonphenolic lignin model compounds with high redox potentials via the initial formation of a substrate aryl cation radical (Higuchi, 1986; Buswell & Odier, 1987; Kirk & Farrell, 1987; Palmer et al., 1987; Tien, 1987; Gold et al., 1989).

Veratryl (3,4-dimethoxybenzyl) alcohol (VA), a secondary metabolite of *P. chrysosporium*, is a LiP substrate (Buswell & Odier, 1987; Kirk & Farrell, 1987; Tien, 1987; Gold et al., 1989). Harvey et al. (1986) showed that VA also stimulated the oxidation of monomethoxy-substituted aromatic substrates such as AA and postulated that LiP oxidizes VA to an aryl cation radical; and that the latter, acting as a radical mediator, in turn oxidizes AA and other recalcitrant substrates. In contrast, several other reports have suggested that the role of VA is to prevent the inactivation of LiP by H_2O_2 (Haemmerli et al., 1986a; Tonon & Odier, 1988) by preventing the formation of LiP compound III and by converting LiP compound III* back to the native enzyme (Wariishi & Gold, 1989, 1990). In an attempt to distinguish between these two proposed roles for VA in LiP reactions, we have reexamined the oxidation of AA and *p*-methoxymandelic acid by the enzyme under a variety of conditions. Our results strongly suggest that VA stimulates the oxidation of these monomethoxylated aromatics by preventing enzyme inactivation rather than by acting as a radical mediator.

Materials and Methods

Enzyme Preparation. LiP was purified from acetate-buffered cultures of *P. chrysosoprium* by DEAE-Sepharose

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[1] Abbreviations: AA, anisyl (4-methoxybenzyl) alcohol; GC–MS, gas chromatography–mass spectrometry; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; KTBA, 2-keto-4-(methylthio)butyric acid; LiP, lignin peroxidase; LiPI, -II, -III, and -III*, lignin peroxidase compounds I, II, III, and III*; VA, veratryl (3,4-dimethoxybenzyl) alcohol.

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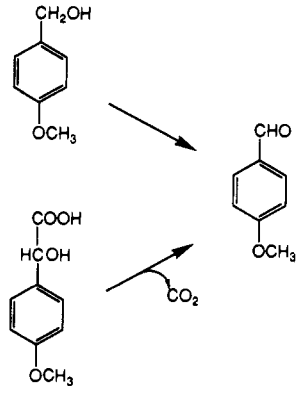

FIGURE 1: Oxidation of AA and *p*-methoxymandelic acid by homogeneous lignin peroxidase. Reactions were conducted and products were analyzed as described in the text.

Table I: Oxidation of Anisyl Alcohol and *p*-Methoxymandelic Acid by Lignin Peroxidase[a]

LiP (μM)	H_2O_2 (μM)	veratryl alcohol (20 μM)	anisaldehyde formed (μM) for substrate (500 μM)	
			anisyl alcohol	*p*-methoxymandelic acid
0.2	100	-	3.6	6.0
0.2	100	+	35.0	33.0
0.2	20	-	5.0	4.0
0.2	20	+	36.0	29.0
1.0	100	-	12.5	18.0
1.0	100	+	125.0	108.0
1.0	20	-	34.0	38.0
1.0	20	+	34.0	37.0

[a] Reactions (1 mL) were initiated by the addition of enzyme and carried out for 10 min at 37 °C. Products were analyzed as described in the text.

and fast protein liquid chromatography as previously described (Gold et al., 1984; Wariishi & Gold, 1990). The purified protein LiP isozyme 2 (H8) was electrophoretically homogeneous and had an RZ ($A_{407/280}$) value of ~5.0. Enzyme concentrations were determined at 407.6 nm by using an extinction coefficient of 133 mM^{-1} cm^{-1} (Gold et al., 1984).

Enzyme Reactions. Substrate oxidations were carried out at 37 °C for 30 min in 1 mL of 20 mM sodium succinate at pH 3.0, containing LiP and substrate as indicated. Reactions were initiated by adding enzyme (1 μM) or as indicated. After the reaction, mixtures were filtered by using a Centricon-10 microconcentrator (Amicon) to remove protein. Products were analyzed quantitatively by HPLC using an HP LiChrospher 100 RP-18 column and a linear gradient consisting of 5% methanol in water to 100% methanol. HPLC product peaks were identified by using GC–MS at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A GC and a 25-m fused silica column (DB-5, J & W Science). VA oxidations were measured at 310 nm as previously described (Buswell & Odier, 1987; Kirk & Farrell, 1987; Tien, 1987; Gold et al., 1989).

Spectral and Kinetic Studies. LiPII was prepared by the successive addition of stoichiometric amounts of potassium ferrocyanide and H_2O_2 to the native enzyme (5 μM) in 20 mM sodium succinate buffer (pH 6.0) (Wariishi & Gold, 1990). Reducing substrates were subsequently added at the concentrations indicated.

LiP compound III* was prepared by adding 40 equiv of H_2O_2 to LiPII (4.3 μM) in 20 mM sodium succinate, pH 3.0, as previously described (Wariishi & Gold, 1990). Substrates were added to LiPIII* at the concentrations indicated. Absorption spectra and kinetic data were recorded by using a Shimadzu UV260 at room temperature.

Spectra of the enzyme intermediates in the steady state were recorded on an HP8542A diode array spectrophotometer equipped with a SEA-11 stopped-flow apparatus (Hi-Tech Scientific). One drive syringe contained native LiP (5.0 μM after mixing) and reducing substrate (60 μM) in water, while the other syringe contained H_2O_2 (200 μM) in 40 mM succinate buffer, pH 3.0. H_2O_2, VA, AA, anisaldehyde, and *p*-methoxymandelic acid were all obtained from the Aldrich Chemical Co. H_2O_2 concentrations were determined according to the HRP method (Cotton & Dunford, 1973).

RESULTS

As shown in Figure 1, LiP oxidized both AA and *p*-methoxymandelic acid to anisaldehyde. The effects of enzyme, substrate, and peroxide concentrations on this reaction are shown in Table I. In reaction mixtures with 0.2 μM enzyme and 100 μM H_2O_2, the presence of 20 μM VA stimulated the formation of anisaldehyde from either AA or *p*-methoxymandelic acid approximately 5- to 10-fold, confirming previouis observations (Harvey et al., 1986). A similar stimulatory effect was observed with 1.0 μM enzyme and 100 μM H_2O_2. However, when reaction mixtures contained 1.0 μM enzyme and 20 μM H_2O_2, no stimulation of anisaldehyde formation by VA was observed.

The effect of H_2O_2 concentration on AA oxidation is shown in Figure 2A. For each concentration of AA, anisaldehyde formation increased with increasing H_2O_2 concentration up to a maximum beyond which the extent of the reaction decreased. Maximum anisaldehyde formation and the concentration of H_2O_2 required to reach the maximum increased with increasing AA concentration. The presence of VA (20 μM) in the reaction mixture did not affect anisaldehyde formation at low concentrations of H_2O_2, nor did it affect the maximum level of anisaldehyde formation. However, beyond the optimal concentration of H_2O_2 (~20–25 μM with 500 μM AA), AA oxidation in the presence of VA leveled off, while the rate in the absence of VA decreased significantly.

By comparison, Figure 2B shows the effect of H_2O_2 concentration on the oxidation of VA. In this case, veratraldehyde formation increased as a hyperbolic function of H_2O_2 concentration, and no large decrease was observed above the optimal H_2O_2 concentration.

The reduction of LiPII to the native enzyme at pH 6.0 is shown in Figure 3. This nonoptimal pH was utilized so that the rates could be followed without rapid kinetic analysis. The addition of 1 equiv of AA to LiPII resulted in a time-dependent change in the visible spectrum (Figure 3A). The spectra display isosbestic points at 462, 518, 621, and 656 nm, suggesting that the conversion occurred via a single reduction step. The kinetics of LiPII reduction at pH 6.0 were followed at 407.6 nm. Although even at pH 6.0 LiPII spontaneously reverts to the native enzyme, the rate of reduction is significantly increased in the presence of either VA or AA, demonstrating that both compounds are substrates for the enzyme.

Figure 4 shows the reaction of LiP with VA or AA under pseudo-first-order conditions with excess H_2O_2. If the rate constant for the reduction of LiPI is larger than for the reduction of LiPII and for the conversion of LiPII to LiPIII by H_2O_2, and if sufficient H_2O_2 is present so that LiPI formation is not limiting, then accumulation of LiPII would be expected. Rapid scan spectra of LiP were recorded in the presence of either VA or AA at pH 3.0. When the concentrations of H_2O_2, VA, and LiP were 200, 60, and 5 μM, respectively, a spectrum for the visible region of LiPII (maxima at 525 and 555 nm)

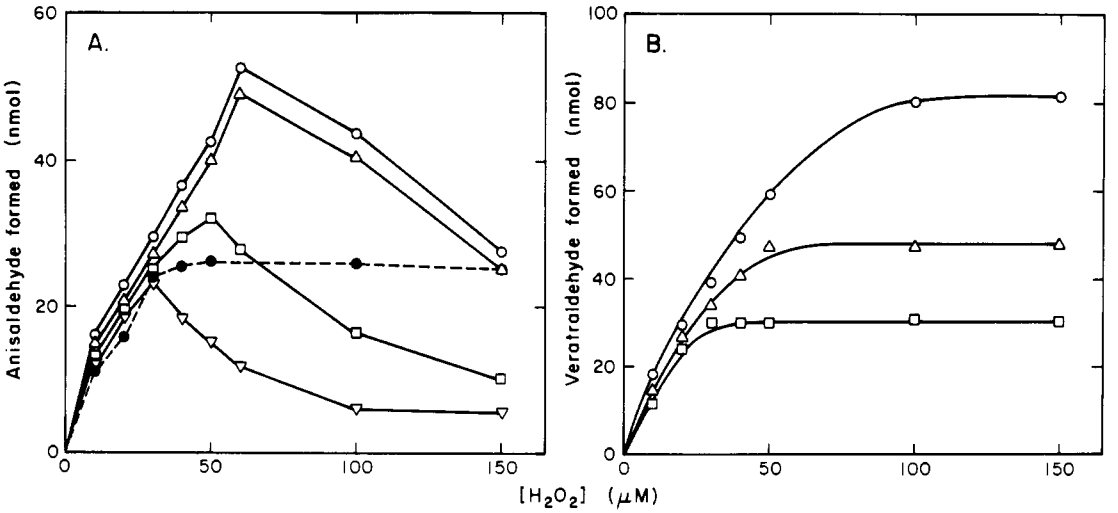

FIGURE 2: (A) Dependence of AA oxidation on H_2O_2 concentration at various concentrations of substrate. AA concentrations were (▽) 0.5 mM, (□) 1.0 mM, (△) 2 mM, and (○) 4 mM. In one experiment (---), reaction mixtures contained 0.5 mM AA and 20 μM VA. Reactions (1 mL) were initiated by the addition of enzyme (1 μM) incubated for 30 min and analyzed by HPLC as described in the text. The total product yield (nanomoles) is indicated. (B) Dependence of VA oxidation on H_2O_2 at various concentrations of substrate. VA concentrations were (□) 30 μM (△) 60 μM, and (○) 100 μM. Reactions (1 mL) were initiated by the addition of 0.2 nmol of enzyme, incubated for 10 min, and analyzed as described in the text.

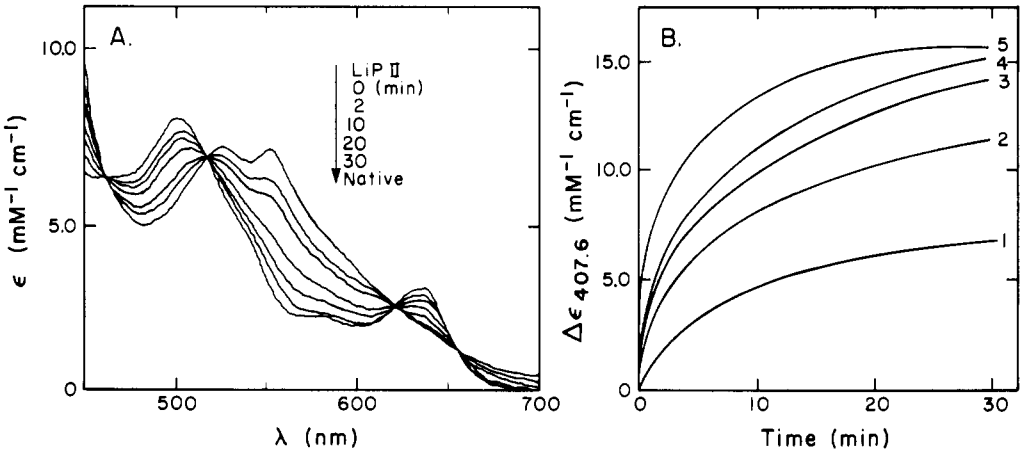

FIGURE 3: Reduction of LiP compound II. (A) LiPII (5.0 μM) was prepared by adding 1 equiv of ferrocyanide followed by 1 equiv of H_2O_2 to the native enzyme in 20 mM sodium succinate, pH 6.0. Subsequently, 1 equiv of AA was added, and the absorption spectrum was scanned at the indicated intervals. (B) Kinetics of the reduction of LiPII. LiPII (5.0 μM) was prepared as above, after which no reducing substrate (1), 5μM AA (2), 50 μM AA (3), 5 μM VA (4), or 50 μM VA (5) was added, and the reduction of LiPII was followed at 407.6 nm.

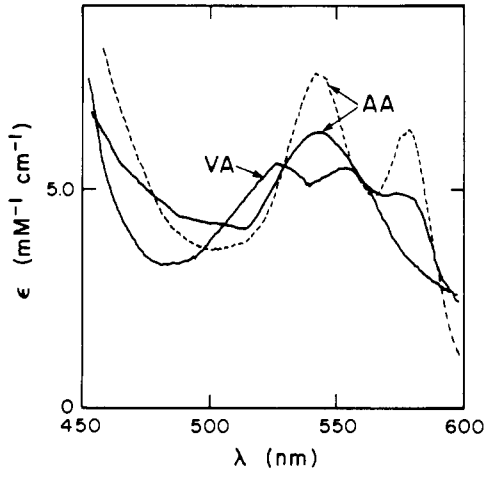

FIGURE 4: Formation of compound II and compound III* in the steady state. Reaction mixtures contained LiP (5.0 μM), reducing substrate (60 μM), and H_2O_2 (200 μM) and were buffered at pH 3.0. The visible spectrum of LiPII prepared in the presence of VA was recorded 5.2 s after mixing. The visible spectrum of LiPIII* prepared in the presence of AA was recorded 5.2 s (—) and 10.2 s (---) after mixing.

was obtained. In contrast, when AA replaced VA in the reaction mixture, the LiPIII* visible spectrum (maxima at 543 and 578 nm) was obtained. The latter suggests that the rate constant for LiPII conversion to LiPIII is greater than for the AA reduction of LiPI or LiPII.

The family of plots $1/v$ versus $1/[VA]$ at various fixed concentrations of AA (0, 25, 50, 100 μM) intercepts on the ordinate, indicating that AA is a competitive inhibitor. The apparent K_m for VA calculated from this plot is 71 μM, confirming our earlier results (Renganathan et al. 1985). The replot of the slopes from this family of plots vs [AA] is linear. From this slope replot, a K_i for AA of 32 μM at 100 μM H_2O_2 was determined.

The reactions of VA and AA with LiPIII* are shown in Figure 5. Addition of 50 equiv of VA to ~4.3 μM LiPIII* (maxima at 419, 543, and 578 nm) prepared by the reaction of LiPII with excess peroxide resulted in the rapid conversion of LiPIII* to the native enzyme (maxima at 408, 500, and 632 nm), confirming our previous results (Wariishi & Gold, 1989, 1990). In contrast, addition of AA to LiPIII* in the presence of excess H_2O_2 did not result in its conversion to the native enzyme and did not prevent the bleaching of LiPIII*. Spectral

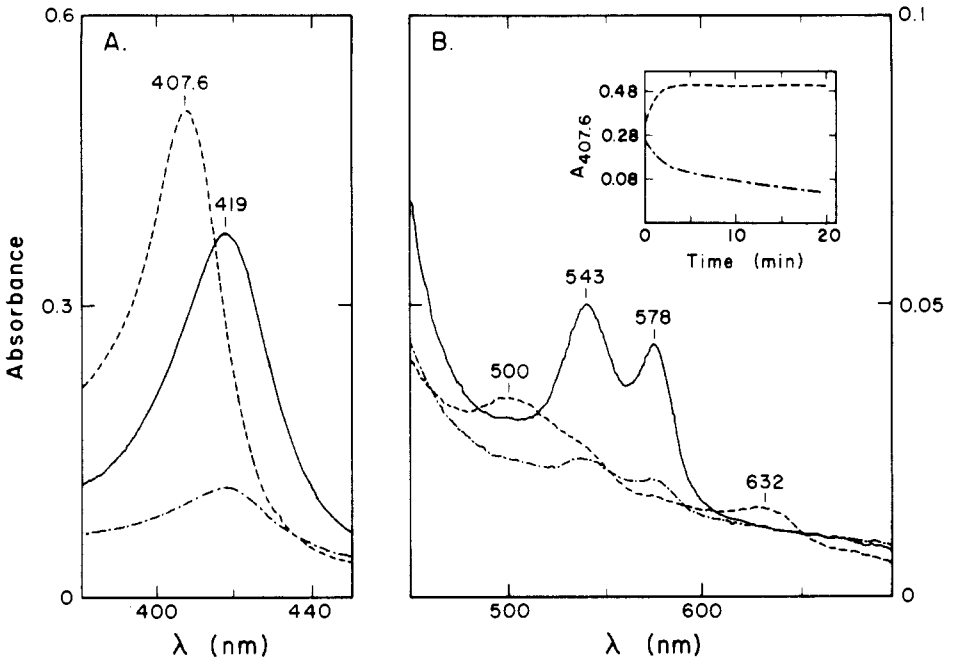

FIGURE 5: Reactions of LiPIII* in the presence of VA and AA. LiPIII* (—) was prepared from LiPII (4.3 μM) by adding 40 equiv of H_2O_2 at pH 3.0. Subsequently, 50 equiv of either VA (---) or AA (-·-) was added, and the Soret spectrum (a) and visible spectrum (B) were recorded after an additional 6 min. The kinetics of LiPIII* conversion to the native enzyme in the presence of VA and of LiPIII* bleaching in the presence of AA are shown in the inset to B.

maxima remained at 419, 543, and 578 nm, but their intensity diminished rapidly. The kinetics of these processes are shown in the inset to Figure 5B.

Discussion

LiP oxidizes a variety of nonphenolic lignin model compounds via the formation of a substrate aryl cation radical intermediate (Buswell & Odier, 1987; Kirk & Farrell, 1987; Palmer et al., 1987; Tien, 1987; Higuchi, 1986; Gold et al., 1989). Electron-donating groups appear to be necessary for the formation or stabilization of the aryl cation radical (Walling et al., 1984), and our work with β-aryl ether dimers (Miki et al., 1988) led us to conclude that the number of electron-donating alkoxy groups on each aromatic ring has a profound influence on whether the ring will be oxidized. Aromatic rings with two alkoxy groups are apparently oxidized preferentially over rings with just one alkoxy group (Miki et al., 1988).

VA is a secondary metabolite of *P. chrysosporium* and a LiP substrate (Buswell & Odier, 1987; Kirk & Farrell, 1987; Tien, 1987; Gold et al., 1989). Several roles for VA in lignin degradation have been proposed. Our laboratory first demonstrated that the LiP oxidation of KTBA was dependent on the presence of VA (Kuwahara et al., 1984; Renganathan et al., 1985). Subsequently, the oxidation of a variety of compounds was shown to be stimulated by VA (Haemmerli et al., 1986b; Harvey et al., 1986; Umezawa & Higuchi, 1989). Specifically, on the basis of their finding that LiP oxidations of the monomethoxylated compounds AA and *p*-methoxymandelic acid are stimulated in the presence of VA, Harvey et al. (1986) proposed that LiP oxidizes VA to an aryl cation radical, and the VA cation radical in turn oxidizes the monomethoxylated substrates. Thus, VA was proposed to act as a radical mediator (Harvey et al., 1986). Recently, Harvey et al. (1989) suggested a modified mechanism whereby the cation radical of VA in association with LiPII somehow mediated the oxidation of monomethoxylated compounds.

In contrast, the results of several studies have suggested that the role of VA is to prevent the inactivation of LiP by H_2O_2 (Haemmerli et al., 1986a; Tonon & Odier, 1988). We have shown previously that at its pH optimum, in the absence of a reducing substrate, LiP is oxidized readily to a LiPIII-like species, LiPIII*, with considerably less H_2O_2 than is required for other peroxidases (Dunford & Stillman, 1976; Renganthan & Gold, 1986; Marquez et al., 1988; Wariishi & Gold, 1989, 1990) and that LiPIII is an intermediate in the conversion of LiPII to LiPIII* (Wariishi & Gold, 1990; Wariishi et al., 1990). In addition, we demonstrated that, in the absence of VA and in the presence of excess H_2O_2, LiPIII* is inactivated (Wariishi & Gold, 1989, 1990). Finally, we demonstrated that VA protects the enzyme from inactivation by reducing LiPII to the native enzyme, thereby diverting the cycle from LiPIII formation and by converting LiPIII* to the native enzyme (Wariishi & Gold, 1989, 1990). In an attempt to distinguish between the radical mediation and enzyme protection roles for VA, we have reexamined the role of VA in the oxidation of AA and *p*-methoxymandelic acid by LiP under a variety of conditions.

H_2O_2 Dependence of Anisyl Alcohol Oxidation. The results in Table I suggest that the ratio of H_2O_2 to enzyme determines whether the oxidation of AA or *p*-methoxymandelic acid is stimulated by the presence of VA. When the molar ratio of H_2O_2 to enzyme is ~20, no stimulation is observed. In contrast, when the molar ratio is greater than 100, significant stimulation occurs. We calculate that the ratio used by Harvey et al. was >1000 (Harvey et al., 1986). The lack of stimulation at low H_2O_2:enzyme ratios suggests that VA is not acting as a radical mediator. AA oxidation increases with increasing H_2O_2 at various concentrations of substrate until it reaches a maximum, beyond which it decreases. The maximal oxidation increases with increasing substrate (Figure 2A). The addition of VA to the reaction mixture does not affect AA oxidation at low H_2O_2 concentrations. However, addition of VA does inhibit the decrease in AA oxidation observed at high H_2O_2 concentrations. As shown in Figure 2B, no decrease in VA oxidation is observed at high H_2O_2 concentrations. These results are consistent with a mechanism involving protection

of the enzyme from inactivation at high H_2O_2 concentrations by VA but not by AA. The results are not consistent with a mechanism requiring VA to act as a radical mediator (Harvey et al., 1986), since that mechanism would predict VA stimulation of the AA oxidation at any H_2O_2 concentration.

Reduction of LiP Compound II by Anisyl Alcohol. The results in Figure 3 confirm that AA is a reducing substrate for LiP. Addition of AA to LiPII results in the reduction of LiPII back to the native enzyme. While the rate of reduction of LiPII with AA is slower than with an equivalent amount of VA, it is significantly faster than the spontaneous rate, indicating that AA is oxidized directly by the enzyme and does not require mediation by VA.

Figure 4 shows the results of a steady-state experiment in which LiPII was prepared under pseudo-first-order conditions with H_2O_2 in excess and in the presence of VA. The usual visible maxima at 525 and 555 nm are observed, suggesting that LiPII prepared under these conditions contains a normal Fe^{IV}=O structure (Dunford & Stillman, 1976; Renganathan & Gold, 1986; Marquez et al., 1988). The results do not support the proposal (Harvey et al., 1989) that a complex of LiPII and a VA cation radical, akin to LiPI, is formed and that this hypothetical complex is required for oxidation of AA.

Competitive Inhibition of Veratryl Alcohol Oxidation by Anisyl Alcohol. The VA cation radical mediation hypothesis implies that AA and other indirect substrates do not bind to LiP (Harvey et al., 1986). To determine whether AA binding occurs, we examined the effect of AA on the oxidation of VA. The results indicate that AA is a competitive inhibitor of VA oxidation and that the K_i for AA is approximately 32 μM. These results strongly suggest that AA binds to the same site on the enzyme as VA, implying that VA is not acting as a mediator.

Effect of Anisyl Alcohol and Veratryl Alcohol on LiP Compound III.* In order to understand why AA oxidation decreases at high H_2O_2 concentrations in the absence of VA, we examined the effect of VA and AA on LiPIII*. We have shown previously that the formation of LiPIII*, a complex of LiP and H_2O_2, results in the inactivation of the enzyme (Wariishi & Gold, 1989, 1990; Wariishi et al., 1990). As shown in Figure 5, addition of 50 equiv of VA to LiPIII* results in its conversion to the native enzyme, confirming our previous observations (Wariishi & Gold, 1989, 1990). In contrast, addition of 50 equiv of AA to LiPIII* does not prevent the bleaching of LiPIII* and subsequent inactivation of the enzyme. Thus, at ratios of H_2O_2 to enzyme which result in the generation of LiPIII*, VA is apparently required to prevent bleaching of the enzyme. In contrast, AA, a poorer substrate, cannot prevent the formation of LiPIII* and cannot convert LiPIII* to the native enzyme. Therefore, AA does not protect the enzyme from inactivation by excess H_2O_2.

In conclusion, these results establish that LiP is capable of directly oxidizing monomethoxylated lignin model compounds such as AA and that a VA cation radical probably does not mediate this reaction. The presence of VA does stimulate the oxidation of AA in the presence of high concentrations of H_2O_2. The results indicate that, in this case, VA acts to protect the enzyme both by efficiently reducing LiPII and by converting LiPIII* to the native enzyme. In both of these roles, VA protects the enzyme from inactivation (Wariishi & Gold, 1989, 1990). In contrast, AA does not reduce LiPII as efficiently as does VA. Furthermore, AA is not capable of converting LiPIII* to the native enzyme, and in the absence of VA and the presence of high concentrations of H_2O_2, AA is not capable of protecting the enzyme from inactivation. Thus, VA apparently stimulates the oxidation of AA in the presence of high concentrations of H_2O_2 by allowing more catalytically active enzyme to react with the substrate. Experiments designed to elucidate the mechanism of conversion of LiPIII* to the native enzyme by VA are in progress.

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