# Evidence for Veratryl Alcohol as a Redox Mediator in Lignin Peroxidase-Catalyzed Oxidation†

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ABSTRACT: We have examined the hypothesis that veratryl alcohol (VA) may act as a redox mediator in lignin peroxidase (LiP)-catalyzed oxidations. The oxidation of chlorpromazine (CPZ) by this system was used to evaluate this hypothesis. Chlorpromazine can be oxidized by one electron to form a stable cation radical ($CPZ^{\bullet+}$). This cation radical can be oxidized by another electron to the sulfoxide (CPZSO). These oxidation steps are easily monitored, making CPZ a useful chemical to investigate redox mediation by VA. Lignin peroxidase oxidized CPZ to $CPZ^{\bullet+}$ whether or not VA was present. The inclusion of VA, however, stimulated CPZ oxidation to $CPZ^{\bullet+}$ and subsequent oxidation of $CPZ^{\bullet+}$ to CPZSO. In the absence of VA, the initial rates of CPZ oxidation by LiP were CPZ concentration-dependent. However, when saturating concentrations of VA were added, the oxidation of CPZ and $CPZ^{\bullet+}$ became independent of CPZ concentration. When the oxidation of VA to veratryl aldehyde was examined, increasing concentrations of CPZ produced a lag in veratryl aldehyde appearance proportional to the concentration of CPZ. Conversely, increasing concentrations of VA never inhibited CPZ oxidation. Transient-state kinetic studies indicated that both VA and CPZ reduced the compound I and compound II forms of LiP. However, when saturating concentrations of VA were utilized, LiP turnover was independent of CPZ concentration. We suggest these data demonstrate that VA may act as a redox mediator for the indirect oxidation of compounds by LiP.

Lignin peroxidase (LiP)[1] of *Phanerochaete chrysosporium* is thought to play an integral role in the degradation of lignin and environmental pollutants (Bumpus et al., 1985). Similar to other peroxidases, LiP catalyzes the oxidation of a number of organic compounds by $H_2O_2$ (Tien & Kirk, 1984). The catalytic cycle of LiP is similar to most peroxidases and is as follows (Chance, 1952; Tien, 1987). Hydrogen peroxide oxidizes the ferric heme of LiP by two electrons to a ferryl iron porphyrin $\pi$ cation radical species known as compound I. The porphyrin cation radical accepts one electron from an organic substrate molecule, yielding a substrate-free radical and a ferryl heme-peroxidase known as compound II. Compound II accepts one electron from another substrate molecule, yielding a second substrate-free radical, returning LiP to its ferric state. Compound II may also accept an electron from the substrate radical produced in the reaction with compound I, resulting in a net two-electron oxidation of the substrate.

It has been shown that in addition to secreting LiP, *P. chrysosporium* synthesizes and secretes a number of other enzymes and small molecules which are believed to participate in lignin and pollutant degradation (Barr & Aust, 1994). One such molecule is veratryl alcohol (VA) (Faison & Kirk, 1985), a substrate for LiP. The exact role of VA in lignin and pollutant degradation remains controversial. Indeed, the role of VA in LiP-catalyzed oxidation of other compounds is still disputed.

The observation that VA stimulated the oxidation of a number of compounds, including anisyl alcohol, led to the conclusion that VA may act as a redox mediator (Harvey et al., 1986). In this mechanism, VA oxidized by LiP to $VA^{\bullet+}$ acts as the oxidant of a secondary molecule (R), returning $VA^{\bullet+}$ to VA and yielding $R^{\bullet}$. These and other investigators hypothesized that this mediation phenomenon could explain the oxidation of compounds such as lignin which may be unable to access the active site of LiP. This idea has been challenged by some who contend that VA is not acting as a redox mediator, but instead protects LiP from irreversible inactivation by $H_2O_2$ (Valli et al., 1990).

More recently, it has been shown that neither mechanism is adequate to explain the stimulation of anisyl alcohol oxidation in the presence of VA (Koduri & Tien, 1994). These investigators explain that VA acts to complete the catalytic cycle of LiP by reducing compound II to ferric enzyme. As anisyl alcohol is unable to reduce LiP compound II, the inclusion of VA extends LiP turnover, stimulating anisyl alcohol oxidation.

It is clear that VA does not act as a redox mediator for anisyl alcohol oxidation. Indeed, the oxidation of anisyl alcohol by $VA^{\bullet+}$ is thermodynamically unfavorable (Zweig et al., 1964). We propose, however, that VA can act as a redox mediator for substrates with redox potentials lower than VA. One such compound is chlorpromazine (CPZ). The oxidation of CPZ is well characterized. It is first oxidized by one electron to a stable cation radical ($CPZ^{\bullet+}$),

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[1] Abbreviations: CPZ, chlorpromazine; $CPZ^{\bullet+}$, chlorpromazine cation radical; CPZSO, chlorpromazine sulfoxide; VA, veratryl alcohol (3,4-dimethoxybenzyl alcohol); $VA^{\bullet+}$, veratryl alcohol cation radical; LiP, lignin peroxidase.

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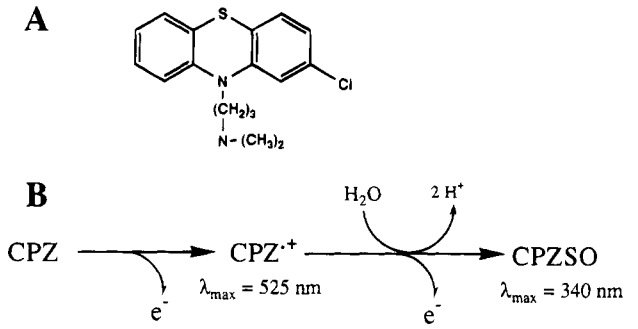



FIGURE 1: Diagram representing the structure (A) and oxidation (B) of chlorpromazine.

and subsequently by another electron to the sulfoxide (CPZSO) (Figure 1) (Borg & Cotzias, 1962). As CPZ oxidation to $CPZ^{\bullet+}$ and $CPZ^{\bullet+}$ oxidation to CPZSO are easily monitored, we have used CPZ to investigate redox mediation by VA.

## MATERIALS AND METHODS

*Chemicals.* Hydrogen peroxide, CPZ, and sodium acetate were purchased from Sigma (St. Louis, MO). Veratryl alcohol (3,4-dimethoxybenzyl alcohol) was purchased from Aldrich (Milwaukee, WI) and purified by redistillation. Sodium acetate buffer was prepared using purified water (Barnstead NANOpure II system; specific resistance 18.0 MΩ/cm). The buffer was then passed through a column of Chelex 100 (BioRad, Richmond, CA).

*Enzyme Production and Purification.* The culture conditions used to produce lignin peroxidases and their purification and activity assay were as described previously (Tuisel et al., 1990). The concentrated extracellular fluid was dialyzed overnight against 10 mM sodium acetate buffer, pH 6.0, and the proteins were purified on a Mono Q HR 5/5 column (Pharmacia LKB Biotechnology Inc.). Lignin peroxidase isozyme H2 (p*I* 4.4) was used throughout this study.

*Stopped-Flow Kinetics.* LiP compound I and compound II reductions were as described by Koduri and Tien (1994) using a three-syringe stopped-flow apparatus from KinTek Instruments (State College, PA) (Kuan et al., 1993). Compound I and compound II preparations were as described previously (Koduri & Tien, 1994). All reactions were performed at 25 °C in 50 mM acetate buffer, pH 4.5.

*Enzyme Reactions.* The effect of VA on $CPZ^{\bullet+}$ formation by LiP was monitored at 525 nm using the extinction coefficient 12 100 $M^{-1}$ $cm^{-1}$ (Sackett & McCreery, 1979). The effect of VA on CPZSO formation by LiP was monitored at 340 nm using an extinction coefficient of 5900 $M^{-1}$ $cm^{-1}$ (Kelder et al., 1994). The effect of CPZ on the oxidation of VA to veratryl aldehyde by LiP was monitored at 310 nm and quantified using an extinction coefficient of 9300 $M^{-1}$ $cm^{-1}$ (Tien & Kirk, 1984). All reactions were performed at room temperature in 50 mM acetate buffer, pH 4.5.

*Kinetic Simulation.* Simulation of kinetic data was performed using two methods, a steady-state method and a transient-state method. For the steady-state method, Michaelis–Menten constants ($K_m$'s) were obtained for LiP with each substrate (VA or CPZ). Expected initial rates of CPZ oxidation to $CPZ^{\bullet+}$ under various conditions were then plotted using the rate equation for $CPZ^{\bullet+}$ formation:

$$\frac{d[CPZ^{\bullet+}]}{dt} = \frac{V_{max}[VA]}{K_{mVA}(1 + [CPZ]/K_{mCPZ}) + [VA]} + \frac{V_{max}[CPZ]}{K_{mCPZ}(1 + [VA]/K_{mVA}) + [CPZ]} \quad (1)$$

Simulation was also done using a transient-state method. Rate constants utilized for this simulation were obtained from LiP compound I and compound II reduction by VA and CPZ as monitored by stopped-flow spectrophotometry (see above). For the transient-state method, KINSIM software (Barshop et al., 1983) was used for kinetic simulation.

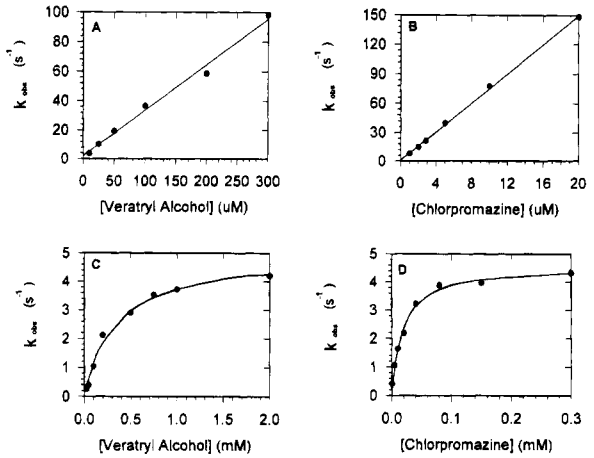



FIGURE 2: Reduction of LiP compound I to compound II (A and B), and reduction of LiP compound II to ferric enzyme (C and D) by VA and CPZ. Compound I was mixed with varying concentrations of VA (A) or CPZ (B). Compound II was also mixed with varying concentrations of VA (C) or CPZ (D).

## RESULTS

Both VA and CPZ reduced LiP compound I to compound II (Figure 2A,B). The plots of $k_{obs}$ versus the concentration of both substrates were linear, indicating a bimolecular reaction with second-order rate constants for each substrate. A rate constant of $3.1 \times 10^5$ $M^{-1}$ $s^{-1}$ was calculated for VA (Figure 2A). The rate constant for CPZ reduction of compound I was $7.4 \times 10^6$ $M^{-1}$ $s^{-1}$ (Figure 2B).

Veratryl alcohol and CPZ also reduced LiP compound II to ferric enzyme (Figure 2C,D). The plots of $k_{obs}$ versus the concentration of both substrates were hyperbolic, indicating a binding step. Consequently, dissociation constants ($K_d$) and first-order rate constants ($k$) were determined using the equation:

$$k_{obs} = \frac{k}{1 + K_d/[substrate]} \quad (2)$$

The first-order rate constants for VA (Figure 2C) and CPZ (Figure 2D) were both 5 $s^{-1}$. The $K_d$, however, for VA (330 μM) was much higher than that determined for CPZ (18 μM).

Chlorpromazine oxidation to $CPZ^{\bullet+}$ in the presence and absence of VA is shown in Figure 3. In the absence of VA (Figure 3A), CPZ was rapidly oxidized to $CPZ^{\bullet+}$. The $K_m$ of LiP for CPZ was approximately 8 μM, whereas the $K_m$ of LiP for VA was 135 μM (data not shown). Following the rapid accumulation of $CPZ^{\bullet+}$, $CPZ^{\bullet+}$ decayed at a rate dependent on its concentration. When VA was included in the reaction (Figure 3B), the rates of both appearance and

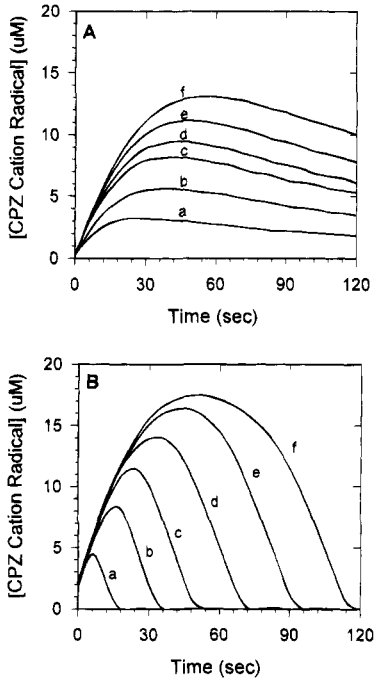



FIGURE 3: Effect of VA on CPZ oxidation by LiP. (A) Reaction mixtures contained 100 nM LiP, 100 $\mu$M $H_2O_2$, and 5 $\mu$M CPZ (a), 10 $\mu$M CPZ (b), 15 $\mu$M CPZ (c), 20 $\mu$M CPZ (d), 25 $\mu$M CPZ (e), or 30 $\mu$M CPZ (f). (B) All reactants were the same except that 1 mM VA was included in each reaction.

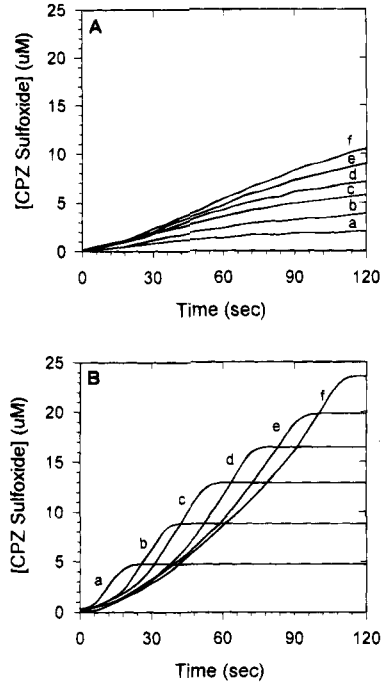



FIGURE 4: Effect of VA on CPZSO formation by LiP. All reactant concentrations were identical to those in Figure 3. All letters used in Figure 3 (A and B) correspond to letters used in Figure 4 (A and B). Change in absorbance at 340 nm due to veratryl aldehyde formation was subtracted to obtain changes due to CPZSO formation.

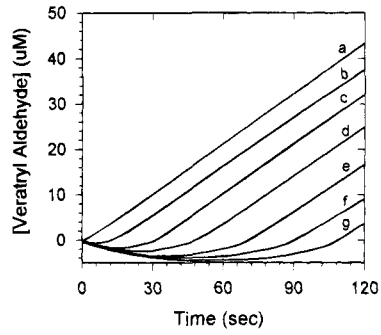



FIGURE 5: Effect of increasing CPZ concentrations on oxidation of VA to veratryl aldehyde by LiP. Reactions contained 100 nM LiP, 100 $\mu$M $H_2O_2$, 1 mM VA, and no CPZ (a), 5 $\mu$M CPZ (b), 10 $\mu$M CPZ (c), 15 $\mu$M CPZ (d), 20 $\mu$M CPZ (e), 25 $\mu$M CPZ (f), or 30 $\mu$M CPZ (g).

disappearance of $CPZ^{\bullet+}$ were stimulated above those observed in the absence of VA. In the absence of VA, the initial rate and extent of $CPZ^{\bullet+}$ appearance and the rate of $CPZ^{\bullet+}$ disappearance were all dependent on the initial concentration of CPZ. With the inclusion of VA, however, the rates of $CPZ^{\bullet+}$ appearance and disappearance were equal to each other and independent of CPZ concentration.

Chlorpromazine sulfoxide production by LiP in the absence and presence of VA is shown in Figure 4. Without VA (Figure 4A), the rates and extent of CPZSO appearance were proportional to initial CPZ concentrations. When VA was present (Figure 4B), initial rates of CPZSO production were similar to those obtained in the absence of VA. With time, however, CPZSO production accelerated such that the maximal rates were independent of initial CPZ concentration. Only the duration of the initial slow phase and the extent of CPZSO accumulation were dependent on the initial CPZ concentration. In the presence of VA, CPZSO production ended abruptly at the same time that all $CPZ^{\bullet+}$ had disappeared in corresponding reactions (Figure 3B).

The effect of increasing CPZ concentration on the oxidation of VA to its aldehyde by LiP is shown in Figure 5. The presence of CPZ resulted in a lag in VA oxidation, and the length of the lag time was directly proportional to the initial CPZ concentration. Furthermore, the end of the lag period in veratryl aldehyde formation, the end of $CPZ^{\bullet+}$ disappearance (Figure 3B), and the end of CPZSO production (Figure 4B) all occurred at the same time point in corresponding reactions.

Chlorpromazine oxidation to $CPZ^{\bullet+}$ and subsequent $CPZ^{\bullet+}$ oxidation to CPZSO by LiP in the presence of increasing VA concentrations are shown in Figure 6. Increasing VA concentrations stimulated the rate of CPZ and $CPZ^{\bullet+}$ oxidation up to a concentration of 1 mM. At higher concentrations of VA, the rate of CPZ and $CPZ^{\bullet+}$ oxidation remained constant at the maximal rate. Within the VA concentration range utilized (0−4 mM), VA never inhibited the rate of CPZ oxidation to $CPZ^{\bullet+}$, or $CPZ^{\bullet+}$ oxidation to CPZSO. Moreover, at saturating concentrations of VA, these rates were identical.

The proposed mechanism of redox mediation by electron transfer is represented in Figure 7. The catalytic cycle of LiP was simplified to a simple Michaelis−Menten model (Figure 7A). This was done by recognizing that the reaction of compound II (LiP II) with substrate (S) is the rate-limiting step of the catalytic cycle (Dunford, 1990). According to Koduri and Tien (1994), this reaction is of the form:

$$\text{LiPII} + \text{S} \overset{K_d}{\rightleftharpoons} \text{LiPII}-\text{S} \overset{k}{\rightarrow} \text{ferric-LiP} + \text{P} \qquad (3)$$

The same saturating concentration of $H_2O_2$ was used in all of our steady-state experiments. Under such conditions, the proposed "ping-pong bi-bi" mechanism of a peroxidase (Dunford, 1990) simplifies to a simple Michaelis−Menten

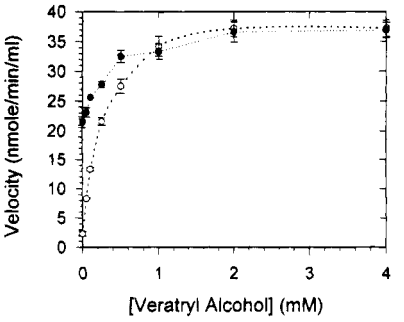
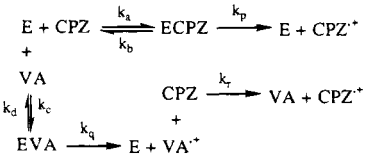



FIGURE 6: Effect of VA concentration on the rates of CPZ oxidation to $CPZ^{\bullet+}$ and $CPZ^{\bullet+}$ oxidation to CPZSO by LiP. Reaction mixtures contained 100 nM LiP, 100 $\mu$M $H_2O_2$, 10 $\mu$M CPZ, and varying concentrations of VA. Chlorpromazine oxidation to $CPZ^{\bullet+}$ was measured by $CPZ^{\bullet+}$ appearance (closed circles), and $CPZ^{\bullet+}$ oxidation to CPZSO was measured by $CPZ^{\bullet+}$ disappearance (open circles).

**A**




**B**

$$\text{If } \frac{d[EVA]}{dt} = \frac{d[ECPZ]}{dt} = \frac{d[VA^{\bullet+}]}{dt} = 0, \quad \text{or} \quad k_q[EVA] \ll k_r[CPZ][VA^{\bullet+}],$$

$$\text{then } \frac{d[CPZ^{\bullet+}]}{dt} = k_p[ECPZ] + k_q[EVA]$$

$$\frac{d[CPZ^{\bullet+}]}{dt} = \frac{V_{max}[VA]}{K_{mVA}(1 + [CPZ]/K_{mCPZ}) + [VA]} + \frac{V_{max}[CPZ]}{K_{mCPZ}(1 + [VA]/K_{mVA}) + [CPZ]}$$

**C**

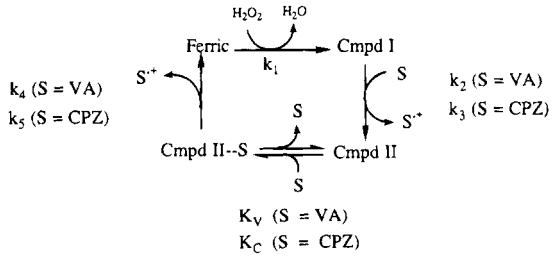



FIGURE 7: Kinetic models used for simulations of LiP-catalyzed CPZ oxidation to $CPZ^{\bullet+}$ through mediation by VA. Simulations were performed using a steady-state method and a transient-state method. The model used for the steady-state method (A) yielded the rate equation given in (B). The $K_m$ for LiP with VA ($K_{mVA}$) was 135 $\mu$M, and the $K_m$ for LiP with CPZ ($K_{mCPZ}$) was 8 $\mu$M. Because enzyme turnover with saturating concentrations of each substrate was identical, $V_{max}$ was taken to be the same for VA and CPZ. The model used for the transient-state method is given in (C). All simulations by this method were performed using KINSIM software. The rate constants used were as follows: $k_1 = 5.8 \times 10^5\ M^{-1}\ s^{-1}$, $k_2 = 3.1 \times 10^5\ M^{-1}\ s^{-1}$, $k_3 = 7.4 \times 10^6\ M^{-1}\ s^{-1}$, $K_V = 330\ \mu M$, $K_C = 18\ \mu M$, and $k_4 = k_5 = 5\ s^{-1}$. A rate constant of $10^7\ M^{-1}\ s^{-1}$ was estimated for $VA^{\bullet+}$ reaction with CPZ.

model. Both VA and CPZ are substrates for LiP. So it is assumed that at any one time LiP can react with either substrate to form the corresponding product. Mediation by electron transfer presumes that the product ($VA^{\bullet+}$) of one reaction with LiP can react with the second substrate (CPZ) to form the second product ($CPZ^{\bullet+}$). Provided the rate of the reaction between $VA^{\bullet+}$ and CPZ is faster than LiP turnover, then the observed kinetics should be determined

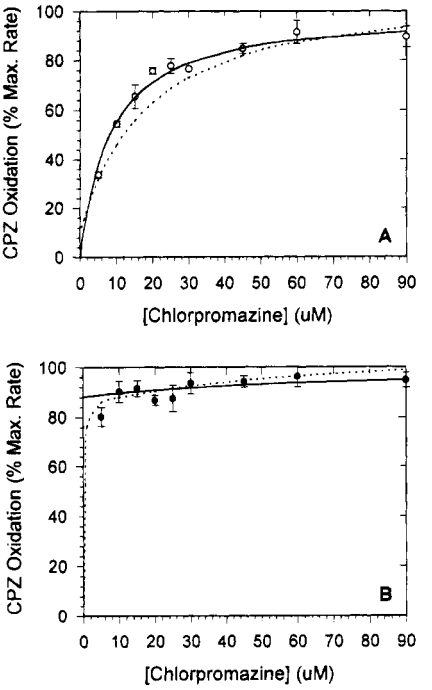



FIGURE 8: Effect of 1 mM VA on the rate of oxidation of CPZ to $CPZ^{\bullet+}$ by LiP. Reactions contained 100 nM LiP, 100 $\mu$M $H_2O_2$, variable CPZ concentrations, and either no VA (A) or 1 mM VA (B). Data points represent the mean of triplicate measurements, and the error bars indicate standard deviations. The lines represent kinetic simulations using a steady-state method (solid lines), or a transient-state method (dotted lines). The reactant conditions used for both simulations are described above. The kinetic models and rate constants used for these simulations are shown in Figure 7.

by the turnover of LiP. This can be obtained mathematically by one of two methods (Figure 7B): (1) The steady-state approximation is applied to each enzyme–substrate complex and to $VA^{\bullet+}$, or (2) the rate-determining-step approximation is applied to the enzyme reaction with VA (i.e., $k_r[VA^{\bullet+}][CPZ] > k_q[EVA]$).

We also constructed a kinetic model which utilized the rate constants obtained by transient-state methods (Figure 7C). For this model, all of the steps of the LiP catalytic cycle were considered. We were unable to obtain a rate constant for the reaction of $VA^{\bullet+}$ with CPZ. However, based upon the reactivity of methoxybenzene cation radicals with similar structure to $VA^{\bullet+}$ (O'Neill et al., 1975), we estimated a rate constant of at least $1 \times 10^7\ M^{-1}\ s^{-1}$.

Simulated rates of CPZ oxidation to $CPZ^{\bullet+}$ were obtained based on the models and rate constants presented in Figure 7. Simulations of the effect of 1 mM VA on CPZ oxidation to $CPZ^{\bullet+}$ by LiP are shown in Figure 8 along with the real data. The close fit of the experimental points to the theoretically expected values using either steady-state or transient-state rate constants supports the proposed model for stimulation of CPZ oxidation by VA.

## DISCUSSION

The role of VA in LiP catalysis has been widely disputed since the discovery that the presence of VA enhanced the oxidation of anisyl alcohol and 4-methoxymandelic acid (Harvey et al., 1986). Central to this controversy has been the question of whether or not VA can act as a redox mediator in LiP-catalyzed oxidations. The ability of VA to act as a redox mediator has been extensively tested for anisyl

alcohol (Valli et al., 1990; Koduri & Tien, 1994). It has been shown that in this case, anisyl alcohol is a poor substrate for LiP compound II. Therefore, VA is needed to complete the catalytic cycle of LiP and does not act as a redox mediator (Koduri & Tien, 1994). These authors leave open the possibility, however, that VA may act as a redox mediator for compounds other than anisyl alcohol. We have used CPZ to demonstrate that VA may act as a redox mediator for the oxidation of some compounds.

Lignin peroxidase was able to catalyze the oxidation of CPZ to $CPZ^{\bullet+}$ in the presence or absence of VA. In the absence of VA, the rate of $CPZ^{\bullet+}$ formation was dependent on CPZ concentration. When saturating concentrations of VA were present, however, the rate of CPZ oxidation was independent of CPZ concentration. This indicates that in the presence of VA, CPZ was no longer solely oxidized by LiP directly, but that $VA^{\bullet+}$ also oxidized CPZ to $CPZ^{\bullet+}$.

If two compounds (e.g., CPZ and VA) can only act as substrates for LiP, then the presence of each should inhibit the oxidation of the other. On the other hand, if $VA^{\bullet+}$, formed by LiP, can also oxidize CPZ to $CPZ^{\bullet+}$ (Figure 7), then $CPZ^{\bullet+}$ will be formed regardless of which substrate is initially oxidized by LiP. If the rate of the reaction between $VA^{\bullet+}$ and CPZ is faster than the turnover of LiP, then the observed $CPZ^{\bullet+}$ formation should be determined solely by the turnover of LiP. Therefore, in the presence of saturating VA, the oxidation of CPZ to $CPZ^{\bullet+}$ by LiP would appear to be independent of CPZ concentration. This is precisely what we observed.

It is clear from this and previous work that both VA (Tien & Kirk, 1984; Wariishi et al., 1991) and CPZ are substrates for LiP. Furthermore, CPZ is oxidized by both compound I and compound II of LiP, rather than by only compound I as is the case with anisyl alcohol (Koduri & Tien, 1994). As both CPZ and VA are substrates for LiP, each should competitively inhibit the oxidation of the other, and the $K_i$ should equal the $K_m$ for each substrate (Segel, 1975). We have demonstrated, however, that the presence of VA never inhibited CPZ oxidation by LiP, even at VA concentrations (1−4 mM) which far exceeded the $K_m$ of this substrate for LiP (135 $\mu$M). Indeed, increasing concentrations of VA stimulated LiP-catalyzed CPZ oxidation to $CPZ^{\bullet+}$. These data can be explained if $VA^{\bullet+}$, produced by LiP, results in $CPZ^{\bullet+}$ formation. As stated previously, the observed rate of $CPZ^{\bullet+}$ formation should be determined by the rate of LiP turnover. It follows that in the presence of saturating concentrations of either substrate, maximal rates of CPZ oxidation to $CPZ^{\bullet+}$ will be observed.

If oxidation of CPZ to $CPZ^{\bullet+}$ by LiP occurs through mediation, then the presence of CPZ should completely inhibit veratryl aldehyde formation. Conversely, if CPZ and VA are only acting as substrates for LiP, then CPZ should competitively inhibit VA oxidation. Furthermore, the $K_i$ for CPZ should be 8 $\mu$M, the approximate $K_m$ for this substrate. However, all concentrations of CPZ completely inhibited veratryl aldehyde formation, indicating that $VA^{\bullet+}$ formed by LiP was consumed through reaction with CPZ.

Redox mediation by VA also appeared to occur in the subsequent oxidation of $CPZ^{\bullet+}$ to CPZSO. Without VA, $CPZ^{\bullet+}$ disappeared at a rate proportional to $CPZ^{\bullet+}$ concentration. The presence of 1 mM VA increased the rate of $CPZ^{\bullet+}$ disappearance by 20-fold. The rate of $CPZ^{\bullet+}$ oxidation to CPZSO became independent of $CPZ^{\bullet+}$ concentration in reactions containing 1 mM VA. Moreover, the rates of CPZ and $CPZ^{\bullet+}$ oxidation were identical in the presence of saturating VA.

If subsequent oxidation of $CPZ^{\bullet+}$ to CPZSO is also mediated by VA, then $CPZ^{\bullet+}$ should also inhibit veratryl aldehyde formation. We were unable to fully characterize $CPZ^{\bullet+}$ inhibition of VA oxidation monitored at 310 nm because of interfering absorbance changes due to CPZ disappearance and CPZSO formation. It is clear, however, that $CPZ^{\bullet+}$ was able to inhibit veratryl aldehyde formation. Maximal rates of veratryl aldehyde formation by LiP in the presence of CPZ were not observed until the end of $CPZ^{\bullet+}$ disappearance. This suggests that, in the presence of VA, CPZ oxidation to CPZSO by LiP occurs via two single electron transfer steps both of which can be mediated by VA.

Based on these and previous results (Koduri & Tien, 1994; Harvey et al., 1986), it seems that compounds may be divided into three categories with regard to reactivity with LiP. The first are those which have redox potentials above that of LiP. These compounds, of course, will not be oxidized by LiP even if a mediating compound like VA is present. The second group contains substances like anisyl alcohol which have redox potentials that are less than that of compound I but presumably greater than that of compound II. Substrates which are in this second group will not be significantly oxidized unless another molecule like VA is added which has a lower potential than compound II, and thus can complete the catalytic cycle of LiP. This group of substrates would be expected to display similar kinetics to those observed for anisyl alcohol by Koduri and Tien (1994).

The third group includes substrates which have redox potentials lower than those of both compound I and compound II of LiP. We have shown that CPZ is one such substrate, and it is well established that VA also belongs in this group (Wariishi et al., 1991; Koduri & Tien, 1994). When two compounds from this group are present with LiP and $H_2O_2$, it is likely that one will behave as a redox mediator for oxidation of the other. It follows that the mediator will have the higher reduction potential of the two molecules. When the rate of mediated oxidation exceeds the rate of direct oxidation by LiP, kinetics indictaive of mediation will be observed. This is what we have demonstrated for VA and CPZ.

Redox mediation is then most important for the oxidation of chemicals which have potentials lower than compound I or compound II but are, for other reasons, unreactive with LiP. Two examples of such chemicals are EDTA and oxalate (Bagotzky & Vasilyev, 1964; Moratille & Trémillon, 1961). These compounds are not oxidized by LiP unless VA is present in the reaction system (Shah et al., 1992; Akamatsu et al., 1990). Therefore, the oxidation of these compounds and their subsequent reactions (Shah et al., 1992; Barr et al., 1992; Akamatsu et al., 1990) are likely to occur through redox mediation by VA as proposed.

It has been suggested that VA may mediate the oxidation of large bulky compounds (e.g., lignin) that may be unable to reach the active site of LiP (Harvey et al., 1986). We are unable to make any conclusions regarding the stability of $VA^{\bullet+}$ as a diffusible oxidant. On the other hand, we suggest that small molecules which are free to approach the active site of LiP (e.g., phenols, oxalate, etc.) can be oxidized by $VA^{\bullet+}$. Therefore, VA may mediate the oxidation of many

compounds with lower reduction potentials, such as organopollutants, their metabolites, and the phenolic products of initial lignin breakdown. This would suggest that redox mediation may have a role in lignin and pollutant degradation by white rot fungi.

Chlorpromazine is a good substrate for LiP. Therefore, there is little practical significance for its oxidation through redox mediation. However, the kinetics of CPZ oxidation in the presence of VA demonstrate that VA can act as a redox mediator for LiP-catalyzed oxidation. We propose that if CPZ oxidation can be mediated by VA, then the oxidation of other compounds (where redox potential allows it) may also occur by this mechanism.

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