Kinetic Analysis of Lignin Peroxidase: Explanation for the Mediation Phenomenon by Veratryl Alcohol†

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ABSTRACT: We investigated the role of veratryl alcohol in lignin peroxidase-catalyzed oxidation of anisyl alcohol with pre-steady-state and steady-state kinetic methods. Veratryl alcohol has been proposed to act as a redox mediator for substrates that are not directly oxidized by the enzyme. Alternatively, its mediation activity has also been attributed to its ability to protect the enzyme from H_2O_2-dependent inactivation. As previously reported, veratryl alcohol was able to stimulate the oxidation of anisyl alcohol. However, this stimulation is not due to mediation or protection of the enzyme. The stimulation can be attributed to the relative reactivity of anisyl alcohol with compounds I and II of lignin peroxidase. We found that anisyl alcohol reacts with compound I, but not with compound II. Therefore, inclusion of veratryl alcohol or another substrate, which reacts with compound II, is essential for completion of the catalytic cycle.

Lignin peroxidases were first isolated from the white-rot fungus *Phanerochaete chrysosporium* (Tien & Kirk, 1983; Glenn et al., 1983). Like other peroxidases, lignin peroxidase is first oxidized by H_2O_2, also produced by the fungus (Forney et al., 1982), to form a two-electron-oxidized intermediate, compound I (Chance, 1952). Compound I then is reduced by one electron to form compound II and a radical product. Compound II, the one-electron-oxidized enzyme intermediate, then oxidizes a second substrate molecule by one electron to complete the catalytic cycle. Lignin peroxidases catalyze the oxidation of a large number of substrates. In addition to lignin, its natural substrate, it also catalyzes the oxidation of phenolic and nonphenolic substrates (Paszczynski et al., 1986; Kirk et al., 1986b). The mechanism by which lignin is depolymerized by lignin peroxidase has been the subject of intense study. Central to this study is the mechanism by which lignin peroxidase oxidizes veratryl alcohol. Veratryl alcohol is a secondary metabolite also produced by ligninolytic cultures of *P. chrysosporium* (Lundquist & Kirk, 1978). Harvey et al. (1986) were the first to propose that veratryl alcohol acts as a redox mediator between lignin peroxidase and lignin. In this scheme, veratryl alcohol is first oxidized by a single electron to form the cation radical. This radical then diffuses for an unknown distance and oxidizes other substrates, such as anisyl alcohol (Figure 1). This proposal was based on the observation that certain substrates that were not oxidized by lignin peroxidases were oxidized if veratryl alcohol was included in the incubation mixture (Harvey et al., 1986). These substrates include anisyl alcohol and 4-methoxymandelic acid. This mechanism was attractive for many reasons, one of which was the explanation of how a large bulky enzyme could interact with large bulky insoluble substrates.

Valli et al. (1990) proposed another role for veratryl alcohol. They proposed that veratryl alcohol prevented H_2O_2-dependent inactivation of the enzyme. These workers demonstrated that

FIGURE 1: Mediation scheme. The mechanism of Harvey et al. (1986) proposes that the veratryl alcohol cation radical, generated by lignin peroxidase, oxidizes anisyl alcohol to generate the anisyl alcohol radical. The anisyl alcohol then undergoes further nonenzymatic reactions to form anisaldehyde.

anisyl alcohol is an extremely poor substrate for lignin peroxidase. With poor substrates, the enzyme undergoes more rapid H_2O_2-dependent inactivation. We found that neither hypothesis explained all of the data. The present study addresses this mediation phenomenon using pre-steady-state and steady-state techniques. Our results provide an explanation for the enhancement of anisyl alcohol oxidation by veratryl alcohol. We found that protection of the enzyme by veratryl alcohol from inactivation is only a secondary consequence. The basis for the mediation phenomenon is explained by the inertness of anisyl alcohol to compound II but not compound I. Inclusion of veratryl alcohol allows the enzyme to return to the ferric state and complete the catalytic cycle.

MATERIALS AND METHODS

Enzyme Purification. Lignin peroxidase isozyme H1 (p*I* = 4.7) was purified from the extracellular fluid on a day 5 stationary culture of *P. chrysosporium* strain PSBL-1 using the method of Kirk et al. (1986a). The H1 fraction obtained by FPLC was further purified by preparative isoelectric focusing. After isoelectric focusing, the enzyme was dialyzed and concentrated against 5 mM sodium phosphate buffer and stored at −20 °C. For stopped-flow experiments, enzyme was dialyzed against distilled, deionized water. The concentration of lignin peroxidase was determined at 408 nm using an extinction coefficient of 169 mM^{-1} cm^{-1} (Tien et al., 1986).

Chemicals. Hydrogen peroxide solution was prepared daily, and the concentration was determined at 240 nm by using an extinction coefficient of 39.4 M^{-1} cm^{-1} (Nelson & Kiesow, 1972). Veratryl alcohol, anisyl alcohol, and 3,4,5-trimethoxybenzyl alcohol were obtained from Aldrich.

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Enzyme Reactions. The effect of anisyl alcohol on veratryl alcohol oxidation was characterized by inhibition studies. These mixtures contained varying concentrations of the veratryl and anisyl alcohols, 0.4 mM H_2O_2, and 0.1 μM lignin peroxidase in 25 mM sodium tartrate buffer (pH 3.5). Reactions were run at 28 °C and initiated by addition of H_2O_2. Veratryl alcohol oxidation was quantitated by veratraldehyde formation using an extinction coefficient of 9300 M^{-1} cm^{-1} at 310 nm.

In studies characterizing the effect of veratryl and trimethoxybenzyl alcohols on anisyl alcohol oxidation, the incubation mixtures contained 4 mM anisyl alcohol, 0.3 mM H_2O_2, 0.5 μM lignin peroxidase, and varying concentrations of either veratryl or trimethoxybenzyl alcohol in 25 mM sodium tartrate buffer (pH 3.5). Anisaldehyde, the oxidation product of anisyl alcohol, was quantitated by HPLC. The detector was set at 280 nm, and standard curves were generated from known standards of anisaldehyde. Aliquots of 100 μL from a 500-μL reaction mixture were removed at specified times, added to 400 μL of methanol, and injected onto a Vydac RP-C_{18} column. A 5–100% linear methanol gradient in water at 1 mL/min was used.

Stopped-Flow Apparatus. The reduction of compound I to compound II was monitored at 417 nm, the isosbestic wavelength between resting ferric enzyme and compound II. The reduction of compound II was monitored at 426 nm, the isosbestic wavelength between native enzyme and compound I and/or at 397 nm, the isosbestic wavelength between compounds I and II. All reactions were run at 28 °C. The stopped-flow apparatus was obtained from KinTek Instruments (State College, PA) and contained a 2.6-cm light path. Each kinetic trace is composed of the average of three shots. Reactions of lignin peroxidase compounds I and II were studied under single-turnover pseudo-first-order conditions with a three-syringe stopped-flow apparatus (Kuan et al., 1993). The stopped flow is driven by a stepper motor without a stopping syringe to allow for double mixing experiments, as previously described (Kuan et al., 1993). The double mixing allows for studies on unstable intermediates such as compounds I and II. For compound I studies, the first syringe contained enzyme in distilled, deionized water, and the second syringe contained 1 equiv of H_2O_2. These two solutions were mixed in the first shot and allowed to age for 1.5 s in the delay line. The second push from the stepper motor mixed compound I with the contents of the third syringe, which contained the alcohol substrate of choice in 75 mM sodium tartrate buffer (pH 3.5) (this yielded a final buffer concentration of 25 mM).

Due to the difference in rates between compounds I and II, rate constants for compound II could be determined from experiments where compound II was formed from the reaction of compound I with the reductant of choice. Alternatively, compound II could be generated from one-electron reduction of compound I by ferrocyanide. These experiments were identical to those where compound I was generated, except that the syringe containing the enzyme also contained 1 equiv of ferrocyanide.

Simulation of Steady-State Kinetics. Computer simulation of steady-state data using rate constants obtained from pre-steady-state studies was performed with the kinetics simulation program KINSIM (Barshop et al., 1983), provided by Carl Frieden and Bruce Barshop (Washington University, St. Louis, MO).

RESULTS

Steady-State Oxidation of Anisyl Alcohol. In accord with published results (Harvey et al., 1986; Valli et al., 1990), anisyl alcohol does not readily support steady-state turnover of lignin peroxidase. Incubation of the enzyme with 4 mM anisyl alcohol and 0.3 mM H_2O_2 resulted in low levels of anisaldehyde formation (Table 1). The stoichiometry indicates that the enzyme went through 3.3 turnovers. Lowering of the H_2O_2 concentration resulted in a slight increase in the amount of anisaldehyde formed. At a H_2O_2 concentration equal to 20 equiv of enzyme and an anisyl alcohol concentration of 4 mM, only 12 equiv of anisaldehyde was formed. An increase in the anisyl alcohol concentration resulted in an increase in the amount of anisaldehyde formed. At the highest concentration of 80 mM, we were able to obtain approximately 140 turnovers (Table 1). All of these yields are much lower than the amount of anisaldehyde formed in the presence of 0.1 mM veratryl alcohol. The yield here was greater than 260 equiv.

Table 1: Oxidation of Anisyl Alcohol by Lignin Peroxidase[a]

incubation conditions (mM)			product yield[b] (μM)
[H_2O_2]	[AA]	[VA]	
0.3	1	0	3.3
0.3	4	0	5.3
0.3	8	0	7.6
0.3	80	0	140
0.020[c]	4	0	12
0.001[d]	4	0	0.48
0.001	0	2	1.0
0.3	4	0.1	260

[a] Incubations contained 1 μM lignin peroxidase in 25 mM tartrate buffer (pH 3.5). Reactions were initiated by the addition of H_2O_2 and terminated at 30 min by the addition of 1 vol of methanol. The terminated reaction mixtures were analyzed by HPLC for product formation as described in Materials and Methods. [b] Yield is given in micromolar, this is also equal to equivalents of enzyme. [c] This H_2O_2 concentration is equal to 20 equiv of enzyme. [d] This H_2O_2 concentration is equal to 1 equiv of enzyme.

Oxidation of Anisyl Alcohol by Compound I. The H_2O_2 concentration was further lowered to equal 1 equiv of enzyme. This would allow for the formation of compound I without any excess H_2O_2 remaining. Reaction of anisyl alcohol with this compound I preparation resulted in slightly less than one-half of the expected amount of anisaldehyde (Table 1). This suggests that only compound I and not compound II reacts with anisyl alcohol. In contrast, oxidation of veratryl alcohol by compound I resulted in near-stoichiometric formation of veratraldehyde (Table 1).

These findings were further confirmed by spectral characterization of the enzyme. Incubations containing 1 (or 5) equiv of H_2O_2 and 50 μM anisyl alcohol yielded the compound II spectrum (data not shown). In contrast, similar incubations containing 50 μM veratryl alcohol yielded the spectrum of resting ferric enzyme, thus indicating immediate turnover of the enzyme.

Enzyme-Monitored Turnover of Anisyl Alcohol Oxidation. To further investigate the reactivity of compound II with anisyl alcohol, enzyme-monitored turnover studies were performed. In these experiments only two syringes were used. Syringe A contained ferric enzyme in water, and syringe B contained anisyl alcohol, H_2O_2, and buffer. All incubations contained H_2O_2 and excess anisyl alcohol. If a substrate can support steady-state turnover, then the enzyme undergoes multiple turnovers until the H_2O_2 is depleted. Upon H_2O_2 depletion, the enzyme returns to the ferric state. During steady state, the predominant form of the enzyme is compound II because the reaction of compound II with anisyl alcohol is rate-limiting. Due to the spectral changes associated with compound I and compound II formation, enzyme turnover can be monitored by measuring absorbance changes in the Soret region; 408 nm

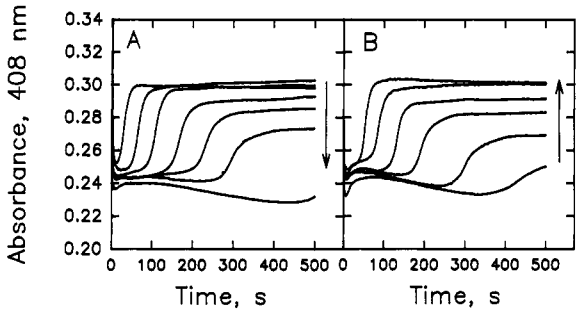

FIGURE 2: Enzyme-monitored turnover studies on anisyl alcohol oxidation. Turnover of lignin peroxidase was monitored by changes in absorbance at 408 nm. One syringe contained lignin peroxidase (1.0 μM after mixing) in water. In panel A, the other syringe contained sodium tartrate buffer (pH 3.5) (25 mM after mixing), anisyl alcohol (4 mM after mixing), and increasing concentration of H_2O_2 (the direction of the arrow indicates increasing concentrations of 5, 10, 15, 20, 25, 30, and 40 μM after mixing). In panel B, the other syringe contained sodium tartrate buffer (pH 3.5) (25 mM after mixing), H_2O_2 (60 μM after mixing), and increasing concentrations of anisyl alcohol (the direction of the arrow indicates increasing concentrations of 8, 10, 12, 20, 28, and 40 mM after mixing).

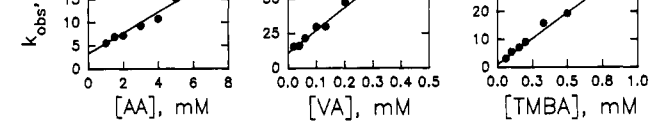

FIGURE 3: Reduction of lignin peroxidase compound I to compound II by benzyl alcohol congeners. Compound I, generated as described in Materials and Methods, was mixed with varying concentrations of anisyl alcohol (AA) (A), veratryl alcohol (VA), (B), and 3,4,5-trimethoxybenzyl alcohol (TMBA) (C).

is the Soret maximum of ferric lignin peroxidase. Compounds I and II exhibit decreased extinction coefficients at 408 nm. Figure 2A shows the actual 408-nm traces from incubations containing varying concentrations of H_2O_2 and excess anisyl alcohol (4 mM). At low H_2O_2 concentrations of 5, 10, and 15 μM, the enzyme does indeed return to the ferric state, as demonstrated by the plateau in the 408-nm absorbance. The initial decrease at 408 nm associated with compound I formation is too rapid to detect under the present experimental conditions. At H_2O_2 concentrations above 15 μM, the enzyme does not return to the ferric state. Figure 2B shows similar experiments, except that the H_2O_2 concentration was held constant at 60 μM and the anisyl alcohol concentration was varied. Only at concentrations above 20 mM was the enzyme able to return to the ferric state. Calculations indicate that only when the H_2O_2 concentration is below 1% of the anisyl alcohol concentration is the enzyme able to return to the ferric state. These results are consistent with the presence of a 1% contaminant in anisyl alcohol, which can convert compound II to ferric enzyme.

Reaction of Compound I with Methoxylated Benzyl Alcohol Congeners. The reactivity of anisyl, veratryl, and 3,4,5-trimethoxybenzyl alcohols with compound I was studied by pre-steady-state techniques. Compound I was generated in the stopped flow with 1 equiv of H_2O_2 and reacted with the alcohols within 1.5 s. The plot of k_{obs} *versus* anisyl alcohol concentration is linear (Figure 3). A rate constant of 2.3×10^3 M^{-1} s^{-1} was calculated from the slope. Also plotted in Figure 3 are the rate constants for veratryl alcohol and 3,4,5-trimethoxybenzyl alcohol. Rate constants of 1.5×10^5 M^{-1} s^{-1} for veratryl alcohol and 2.4×10^4 M^{-1} s^{-1} for 3,4,5-trimethoxybenzyl alcohol were calculated. All three plots

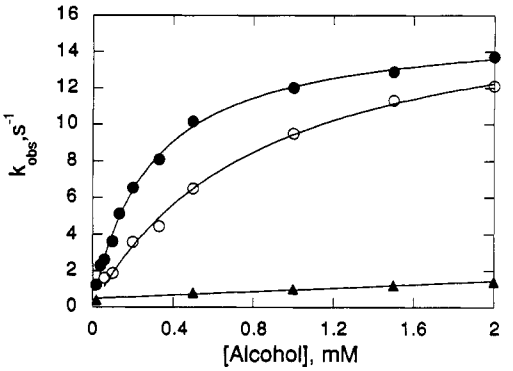

FIGURE 4: Reduction of lignin peroxidase compound II to the ferric enzyme by benzyl alcohol congeners. Compound II, generated as described in Materials and Methods, was mixed with varying concentrations of anisyl (▲), veratryl (●), and 3,4,5-trimethoxybenzyl alcohol (○).

exhibit a non-zero intercept, suggesting reversibility. The rates vary from 1 s^{-1} for trimethoxybenzyl alcohol to 12 s^{-1} for veratryl alcohol. Although the reverse reaction, the oxidation of compound II by the corresponding cation radical, is conceivable, this possibility is not easily investigated.

Reaction of Compound II with Methoxylated Benzyl Alcohol Congeners. Very low reactivity is observed for anisyl alcohol with compound II. The plot of the k_{obs} *versus* anisyl alcohol concentration is shown in Figure 4. For comparative purposes, the plot also contains the rate constants for veratryl and 3,4,5-trimethoxybenzyl alcohols. The rates observed with these two alcohols show a hyperbolic concentration dependence, suggesting at least a two-step pathway leading to the oxidation of veratryl and 3,4,5-trimethoxybenzyl alcohols by compound II:

$$\text{LP II} + \text{VA} \overset{K_d}{\rightleftharpoons} \text{LP II}\cdots\text{VA} \overset{k}{\rightarrow} \text{LP} + \text{VA}^{\bullet+}$$

If rapid equilibrium is assumed for the first step, the rate of alcohol oxidation can be given by

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

The lines drawn in Figure 4 are best fits according to eq 1, with K_d = 0.28 mM and k = 16 s^{-1} for veratryl alcohol and K_d = 0.84 mM and k = 17 s^{-1} for 3,4,5-trimethoxybenzyl alcohol.

An increase in the anisyl alcohol concentration up to 80 mM did result in an increase in k_{obs} (data not shown). A fit of these data revealed a K_d of 27 mM for anisyl alcohol. However, these results are most likely due to the 1% contaminant, which was detected in the enzyme-monitored turnover experiments. If so, then the K_d for this contaminant would be 0.27 mM. This K_d is in line with the K_d values obtained for other substrates of lignin peroxidase. The nature of the contaminant is not known. It may be phenolic; such a contaminant has been found in commercial veratryl alcohol preparations (Tien et al., 1986). It may also be a benzyl alcohol that the enzyme is capable of oxidizing.

Effect of Anisyl Alcohol on Veratryl Alcohol Oxidation. Anisyl alcohol is a poor inhibitor of veratryl alcohol oxidation. The mechanism of inhibition was investigated using steady-state techniques where the concentrations of substrate and inhibitor were varied in a systematic manner. The results were analyzed by the method of Cleland (1979). The data were fit to both competitive inhibition and noncompetitive inhibition. The competitive inhibition yielded a K_i of 3.8 mM;

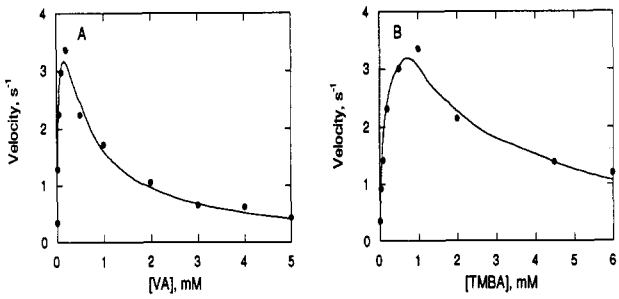

FIGURE 5: Effect of veratryl alcohol (VA) (A) and 3,4,5-trimethoxybenzyl alcohol (TMBA) (B) on the rate of anisaldehyde formation. Reaction mixtures, as described in Materials and Methods, contained varying concentrations of veratryl alcohol or 3,4,5-trimethoxybenzyl alcohol and 4 mM anisyl alcohol. Velocities were determined by sampling the incubations at 15, 30, and 45 s and quantitating anisaldehyde by HPLC. The points represent the data and the line is the kinetic simulation obtained from KINSIM. The mechanism and rate constants used for the simulation are provided in Figure 6.

the noncompetitive inhibition yielded a K_{is} of 4 mM and K_{ii} of 39 mM. The error analysis indicates that both mechanisms fit equally well. The σ values (for error) are 1.7×10^{-4} for competitive and 1.6×10^{-4} for noncompetitive inhibition.

Effect of Veratryl Alcohol on Anisyl Alcohol Oxidation. Inclusion of veratryl alcohol into anisyl alcohol reaction mixtures stimulated the rate of anisaldehyde formation. This is in agreement with the results of other researchers (Harvey et al., 1986; Valli et al., 1990). At an anisyl alcohol concentration 40 times greater than that of veratryl alcohol, a much larger percentage of the veratryl alcohol is oxidized than anisyl alcohol. This would indicate that if mediation is occurring, the efficiency of mediation is very poor (i.e., only a small percentage of the veratryl alcohol cation radical reacts with anisyl alcohol to yield the anisyl alcohol cation radical). This is also consistent with a relatively high K_{ii} value obtained for noncompetitive inhibition. The enhancement of anisaldehyde formation as a function of veratryl alcohol concentration is shown in Figure 5A. The enhancement phenomenon increases up to 200 μM veratryl alcohol followed by a decrease in rate.

Effect of 3,4,5-Trimethoxybenzyl Alcohol on Anisyl Alcohol Oxidation. Similar to veratryl alcohol, 3,4,5-trimethoxybenzyl alcohol is also able to stimulate anisyl alcohol oxidation. The concentration dependence of this stimulation is shown in Figure 5B. Similar to stimulation by veratryl alcohol, an initial increase is observed followed by a decrease in the rate of anisaldehyde formation. The maximal rate, however, was observed at a higher concentration of 3,4,5-trimethoxybenzyl alcohol of near 1 mM. This is in contrast to a concentration of 200 μM for veratryl alcohol.

Simulation of Kinetic Results. In light of the finding that anisyl alcohol does not react with compound II, an alternate explanation is possible for the "mediation" phenomenon. The stimulation can be explained by the necessity of a second substrate to convert compound II to resting enzyme. This would complete the catalytic cycle and allow for continued turnover of the enzyme. This mechanism is illustrated in Figure 6. The rate of anisaldehyde formation was simulated with the mechanism shown in Figure 6 and with rate constants given in the figure legend. The change in this rate as a function of veratryl alcohol or 3,4,5-trimethoxybenzyl alcohol was simulated with KINSIM. The simulations are shown in Figure 5A,B for veratryl alcohol and 3,4,5-trimethoxybenzyl alcohol along with the real data. This clearly indicates that the mechanism and the rate constants obtained in the present

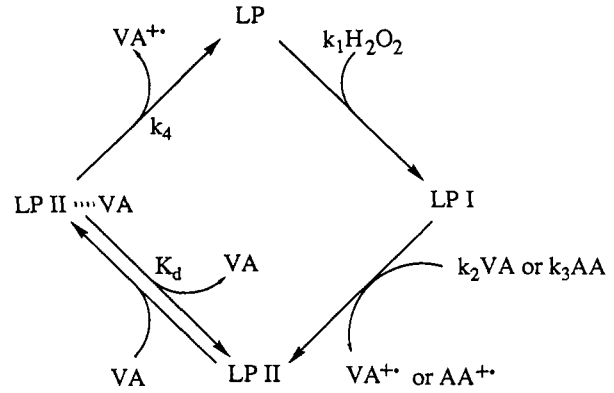

FIGURE 6: Mechanism used in the kinetic simulation. Rate constants are as follows: $k_1 = 5.8 \times 10^5$ M^{-1} s^{-1}; $k_2 = 1.5 \times 10^5$ M^{-1} s^{-1}; $k_3 = 2.3 \times 10^3$ M^{-1} s^{-1}; $K_d = 0.28$ mM; $k_4 = 16$ s^{-1}. The figure shows only the mechanism for veratryl alcohol. This mechanism was also used for simulation with trimethoxybenzyl alcohol. Those rates constants (replacing veratryl alcohol (VA) with trimethoxybenzyl alcohol (TMBA) in the figure) are as follows: $k_1 = 5.8 \times 10^5$ M^{-1} s^{-1}; $k_2 = 2.4 \times 10^4$ M^{-1} s^{-1}; $k_3 = 2.3 \times 10^3$ M^{-1} s^{-1}; $K_d = 0.84$ mM; $k_4 = 17$ s^{-1}.

study can readily account for the observed stimulation of anisyl alcohol oxidation.

DISCUSSION

The ability of veratryl alcohol to mediate the oxidation of other substrates was first proposed by Harvey et al. (1986). These workers observed the stimulation in lignin peroxidase-catalyzed oxidation of recalcitrant substrates such as anisyl alcohol by veratryl alcohol. They argued that because anisyl alcohol was poorly oxidized by the enzyme itself, its oxidation was brought about by veratryl alcohol cation radicals generated by lignin peroxidase catalysis. Harvey et al. (1986) further proposed that the physiological role of veratryl alcohol, produced by ligninolytic cultures of *P. chrysosporium*, was to mediate the oxidation of lignin.

Indeed, the oxidation of many substrates including lignin (Haemmerli et al., 1986a,b; Harvey et al., 1986; Hattori & Higushi, 1991) is stimulated by the inclusion of veratryl alcohol in the incubation mixture. However, some have argued against the ability of veratryl alcohol to mediate the oxidation of these substrates. The oxidation of monomethoxylated aromatics by a dimethoxylated (or trimethoxylated) aromatic cation radical is thermodynamically unfavorable. The redox potential of methoxybenzenes is roughly inversely related to the number of methoxyl groups (Zweig et al., 1964). Others have questioned the stability of the veratryl alcohol cation radical as a diffusible oxidant.

Valli et al. (1990) suggested an alternate mechanism for the phenomenon of veratryl alcohol mediation. These workers proposed that the stimulation is due to the ability of veratryl alcohol to protect the enzyme from H_2O_2-dependent inactivation. These workers first demonstrated that anisyl alcohol is oxidized by compound I of lignin peroxidase. They also noted that veratryl alcohol is a much better reductant. This is in accord with our present results. They found that anisyl alcohol is oxidized by compound II very slowly. In light of this poor reactivity, the reaction between compound II and H_2O_2 would be more favorable. This would eventually lead to enzyme inactivation. Valli et al. (1990) reported that anisyl alcohol is a competitive inhibitor with respect to veratryl alcohol, with a K_i of 32 μM. This finding is not consistent with their own data indicating that anisyl alcohol is a poor substrate for compound II. If an inhibitor, such as anisyl

alcohol, is an alternate substrate as suggested by Valli et al. (1990), then its inhibition constant K_i should be equal to its apparent K_m (Segel, 1975). If such were the case for anisyl alcohol, then its apparent K_m should be 32 μM. This would indicate that it is an excellent substrate, even better than veratryl alcohol, whose K_m is 72 μM (Tien et al., 1986). This would predict that anisyl alcohol should be able to support steady-state turnover at higher concentrations ($10K_m$). Our data here indicate that anisyl alcohol cannot support steady-state turnover of the enzyme, even at a concentration greater than 2000 times (80 mM) the K_i (or putative K_m) value. The K_i reported in this article for anisyl alcohol is 3.8 mM.

We also used steady-state kinetic techniques in the present study. Anisyl alcohol was used as an inhibitor for veratryl alcohol oxidation. Pure competitive inhibition would rule out mediation by veratryl alcohol and indicate that anisyl alcohol is only interacting at the active site of lignin peroxidase. Noncompetitive inhibition would be consistent with mediation. Here, anisyl alcohol is not only oxidized at the active site but is also oxidized by the veratryl alcohol radical. The two inhibition constants obtained from noncompetitive inhibition would reflect the relative contribution of each mode of oxidation. The K_{is} value would be reflective of inhibition at the active site. The K_{ii} value would reflect inhibition from anisyl alcohol reacting with the veratryl alcohol radical (mediation). A high K_{ii} value would also be consistent with the inefficiency of mediation. This would be reflected by minor amounts of anisaldehyde formed relative to the amount of veratraldehyde formed. The analyses indicated that the data could be fit to either mechanism equally. Therefore, we concluded that steady-state kinetics is not a useful method for resolving the mechanism of anisyl alcohol oxidation.

Valli et al. (1990) further claimed that enzyme in the presence of anisyl alcohol and excess H_2O_2 leads to the formation of compound III*, a noncovalent complex of compound III and H_2O_2. Compound III is then further inactivated upon longer incubation with H_2O_2. They proposed that the unique ability of veratryl alcohol to prevent this inactivation lies in its ability to react with compound III* (Wariishi & Gold, 1990). However, the existence of such a putative complex has subsequently been questioned and discounted (Cai & Tien, 1992).

Our results also clarify the question of whether compound II reacts with anisyl alcohol. Although it is difficult to distinguish between no reactivity and exceedingly low reactivity, our enzyme-monitored turnover experiment clearly shows that, at H_2O_2 concentrations above 1% of the anisyl alcohol concentration, the enzyme cannot undergo turnover. This is consistent with a 1% contaminant reducing compound II. Furthermore, when 1 equiv of H_2O_2 was added to resting enzyme to form compound I, this preparation yielded only one-half the expected amount of anisaldehyde from anisyl alcohol. This suggests that compound II does not oxidize anisyl alcohol. The presence of a 1% contaminant in anisyl alcohol would also predict that, at low H_2O_2 concentrations (less than 1% of anisyl alcohol), mediation by veratryl alcohol is not as significant. This is precisely the observation made by Valli et al. (1990).

Our findings indicate that veratryl alcohol (or 3,4,5-trimethoxybenzyl alcohol) serves as a necessary substrate for compound II. The protection from inactivation is a secondary consequence of the inefficiency of anisyl alcohol to convert compound II to resting enzyme and complete the catalytic cycle. Our results provide an alternate mechanism for the mediation phenomenon. These studies reveal that the ideal "mediator"-like molecule would react poorly with compound I and be an excellent substrate for compound II. Substrates that would be susceptible to the mediation would be ones that are oxidized by compound I but not by compound II. Such is the case for veratryl alcohol and anisyl alcohol. In the absence of veratryl alcohol, enzyme turnover is not supported by anisyl alcohol. Turnover stops at compound II (which leads to compound III formation). The addition of low levels of veratryl alcohol would convert compound II to ferric enzyme. This would stimulate the rate of anisaldehyde formation by allowing for continued turnover. As the veratryl alcohol concentration is increased, however, veratryl alcohol would more effectively compete with anisyl alcohol for oxidation by compound I. This would result in the observed decrease in the rate of anisaldehyde formation. This observed decrease would not be observed if veratryl alcohol acted as a mediator because increasing its concentration should not result in decreased efficiency in mediation.

The requirement of veratryl alcohol for completion of the catalytic cycle has also been reported by Paszczynski and Crawford (1991) on the oxidation of azo dyes by lignin peroxidase. These workers found that the azo dye was only oxidized by compound I and not by compound II of the enzyme. Here again, veratryl alcohol would appear to act as a mediator while not serving such a function.

Finally, there is still the question of the role of veratryl alcohol in lignin biodegradation. It is difficult to extrapolate these *in vitro* results with fungal lignin depolymerization. With substrates like anisyl alcohol, we find no evidence for mediation. However, with phenolic substrates such as guaiacol, we observed that veratryl alcohol can act as a mediator (unpublished data). Thus, we can conclude that phenolic subunits, but not the more recalcitrant subunits, in lignin may indeed be oxidized by veratryl alcohol radicals. This is not a likely physiological role due to the presence of Mn peroxidases, which readily oxidize phenolic substrates (Paszczynski et al., 1986). We cannot exclude the proposal of Valli et al. (1990) that veratryl alcohol serves to protect the enzyme. This has long been shown by other groups (Haemmerli et al., 1986a; Tonon & Odier, 1988), and undoubtedly, veratryl alcohol certainly does extend the half-life of lignin peroxidase in fungal cultures (Tonon & Odier, 1988). We can, however, exclude the proposal that stimulation of anisyl alcohol oxidation is solely due to the protective effect of veratryl alcohol; this is a secondary consequence. We have clearly demonstrated that veratryl alcohol stimulates the oxidation of highly recalcitrant substrates by supplying a reducing equivalent for compound II.

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