Oxidation of Guaiacol by Lignin Peroxidase

ROLE OF VERATRYL ALCOHOL*

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Rao S. Koduri and Ming Tien‡

From the Department of Biochemistry and Molecular Biology and the Center for Biomolecular Structure and Function, Pennsylvania State University, University Park, Pennsylvania 16802

We have investigated the lignin peroxidase-catalyzed oxidation of guaiacol and the role of veratryl alcohol in this reaction by steady-state and pre-steady-state methods. Pre-steady-state kinetic analyses demonstrated that guaiacol is a good substrate for both compounds I and II, the two- and one-electron oxidized enzyme intermediates, respectively, of lignin peroxidase. The rate constant for the reaction with compound I is 1.2 × 10^6 M^-1 s^-1. The reaction of guaiacol with compound II exhibits a K_m of 64 μM and a first-order rate constant of 17 s^-1. Oxidation of guaiacol leads to tetraguaiacol formation. This reaction exhibits classical Michaelis-Menten kinetics with a K_m of 160 μM and a k_cat of 7.7 s^-1. Veratryl alcohol, a secondary metabolite of ligninolytic fungi, is capable of mediating the oxidation of guaiacol. This was shown by steady-state inhibition studies. Guaiacol completely inhibited the oxidation of veratryl alcohol, whereas veratryl alcohol had no corresponding inhibitory effect on guaiacol oxidation. In fact, at low guaiacol concentrations, veratryl alcohol stimulated the rate of guaiacol oxidation. These results collectively demonstrate that veratryl alcohol can serve as a mediator for phenolic substrates in the lignin peroxidase reaction.

This study investigates the ability of 3,4-dimethoxybenzyl (veratryl) alcohol to mediate the lignin peroxidase-catalyzed oxidation of guaiacol. Lignin peroxidases are hemeproteins secreted by the white rot fungus Phanerochaete chrysosporium during secondary metabolism (1, 2). These enzymes catalyze the oxidation of lignin and a large number of phenolic and non-phenolic substrates (3, 4). The catalytic cycle of lignin peroxidase is similar to that of other peroxidases (5, 6) where ferric enzyme is first oxidized by H_2O_2 to generate the two-electron oxidized intermediate, compound I (7). Compound I is then reduced by one electron donated by a substrate molecule, yielding the 1-electron oxidized enzyme intermediate, compound II, and a free radical product. The catalytic cycle is completed by the one-electron reduction of compound II by a second substrate molecule.

In the absence of a reducing substrate, the enzyme can undergo a series of reactions with H_2O_2 to form compound III, oxyperoxidase (6, 8). It is also well documented that prolonged incubation of enzyme with H_2O_2 in the absence of a reducing substrate such as veratryl alcohol can cause irreversible inactivation of the enzyme (9). In the presence of veratryl alcohol, however, lignin peroxidase undergoes multiple turnovers without any detectable inactivation. Because veratryl alcohol is normally produced by ligninolytic cultures of P. chrysosporium (10), workers have proposed that its physiological function is to protect the enzyme from H_2O_2-dependent inactivation (11).

An alternate role for veratryl alcohol in lignin biodegradation has been proposed by Harvey et al. (12). These workers observed that substrates that are not oxidized by lignin peroxidase such as anisyl alcohol and 4-methoxymandelic acid are oxidized in the presence of veratryl alcohol (12). They proposed that the one-electron oxidized product of veratryl alcohol, the aryldation radical, is able to mediate the oxidation of substrates typically not oxidized by the enzyme. They further proposed that the arylation radical is a diffusible species, capable of acting at a distance. In contrast to Harvey et al. (12), Valli et al. (13) proposed that the stimulation of 4-methoxymandelic acid and anisyl alcohol oxidation is due solely to the ability of veratryl alcohol to prevent inactivation of lignin peroxidase. They claimed that enzyme in the presence of anisyl alcohol and excess H_2O_2 leads to the formation of inactive compound III* (13). Veratryl alcohol is then capable of converting inactive compound III* back to the native state (14). The existence of compound III* has been subsequently questioned (15).

In this study, we reinvestigated the ability of veratryl alcohol to mediate the oxidation of guaiacol. We find that unlike anisyl alcohol (15), guaiacol is a substrate for compounds I and II of lignin peroxidase. Nevertheless, our results support the findings of Harvey et al. (12) and show that veratryl alcohol can mediate the oxidation of guaiacol. We also show that guaiacol, like veratryl alcohol, is capable of converting the enzyme from the compound III state back to the resting ferric state in the presence of H_2O_2.

MATERIALS AND METHODS

Enzyme Purification—Lignin peroxidase isozyme H1 (pI 4.7) was purified by Mono Q chromatography from P. chrysosporium strain PSBL-1 as described previously (16). The H1 fraction from the Mono Q column was further purified by preparative isoelectric focusing. For stopped-flow experiments, the enzyme was dialyzed against distilled deionized water. The concentration of lignin peroxidase was determined at 409 nm using an extinction coefficient of 169 mM^-1 cm^-1 (5).

Chemicals—Hydrogen peroxide solution was prepared daily, and the concentration was determined at 240 nm using an extinction coefficient of 9300 (310 nm) and 26,600 (470 nm) M^-1 cm^-1 were used for veratraldehyde and
tetraguaiacol, respectively (19, 20).

Stopped-flow Apparatus—Stopped-flow experiments were performed at 28 °C using a Model SF-2001 stopped-flow apparatus (KinTek Instruments, State College, PA). This instrument utilizes three driving syringes and is driven by a stepper motor without a stop syringe (21). The three-syringe system permits kinetic studies on the unstable intermediates compounds I and II as described previously (21). For compound I formation, ferric enzyme was mixed with 1 eq of H₂O₂. For generation of compound II, ferric enzyme was mixed with 1 eq of H₂O₂ and 1 eq of potassium ferrocyanide. The resultant reaction mixtures were allowed to age in the delay line for 4 s. The contents of the delay line (compound I or II) were then mixed with the contents of the third syringe, which contained the reducing substrate. Conversion of compound I to compound II was monitored at 417 nm, and the conversion of compound II to resting enzyme was monitored at 426 or 397 nm.

RESULTS

Steady-state Kinetics of Guaiacol Oxidation—High pressure liquid chromatography analysis (data not shown) demonstrated that the oxidation of guaiacol by lignin peroxidase produces tetraguaiacol, as does for horseradish peroxidase (22). The rate of tetraguaiacol formation was linear relative to the enzyme concentration (data not shown). Results from steady-state experiments shown in Fig. 1 indicate that lignin peroxidase-catalyzed guaiacol oxidation follows classical Michaelis-Menten kinetics. The $K_m$ and $k_{cat}$ for guaiacol were calculated to be 160 μM and 7.7 s⁻¹, respectively, at pH 3.5.

Steady-state analyses with H₂O₂ as the variable substrate indicated that H₂O₂ inhibits the enzyme at high concentration (Fig. 2A). This has also been observed for veratryl alcohol oxidation (5). The rate of tetraguaiacol formation increased as the H₂O₂ concentration increased to 125 μM. A further increase in the H₂O₂ concentration resulted in a decrease in the rate of tetraguaiacol formation.

The observed decrease in the rate of tetraguaiacol formation at high H₂O₂ concentrations was reflected by the decreased yield of tetraguaiacol (Fig. 2B). In these experiments, the tetraguaiacol concentration was monitored by absorbance at 470 nm, and the maximal absorbance value was recorded. This maximal value was reached anywhere from 10 to 50 min. In incubations containing 2 mM guaiacol and low concentrations of H₂O₂, the yield of tetraguaiacol was slightly less than the 4:1 ratio of H₂O₂ to tetraguaiacol, similar to that reported for horseradish peroxidase (22). At H₂O₂ concentrations above 300 μM, the amount of tetraguaiacol decreased.

Harvey and Palmer (23) proposed that compound III was formed during steady-state oxidation of guaiacol. Formation of compound III would account for the decreased rate and yield of tetraguaiacol. To further investigate this possibility, we added tetranitromethane to the incubations. Tetranitromethane scavenges superoxide and also reacts directly with compound III, yielding ferric enzyme (8). This addition dramatically increased the yield of tetraguaiacol formation (Fig. 2B). This observation is consistent with the proposal that compound III formation accounts for the decreased yield at high H₂O₂ concentrations. The decrease in the yield of tetraguaiacol at higher H₂O₂ concentrations could be partially reversed by increasing the guaiacol concentration (Fig. 2B).

Effect of Veratryl Alcohol on Guaiacol Oxidation—The rate of guaiacol oxidation was studied with a saturating concentration of guaiacol (3 mM) in the presence of varying concentrations of veratryl alcohol. Fig. 3A shows that veratryl alcohol was not able to inhibit guaiacol oxidation at concentrations up to 2 mM. A concentration of 10 times its $K_m$ does not inhibit guaiacol oxidation (Fig. 3B). The effect of veratryl alcohol on the rate of guaiacol oxidation was also studied when the concentration of veratryl alcohol was held constant (2 mM) and the guaiacol concentration was varied. In contrast to the results shown in Fig. 1, a hyperbolic relationship was not observed. Surprisingly, at low guaiacol concentrations, the rate of its oxidation was even higher than the calculated $k_{cat}$ of 7.7 s⁻¹. At these low concentrations of guaiacol (with 2 mM veratryl alcohol), the rate approached that of the $k_{cat}$ for veratryl alcohol (Fig. 3B). These data suggest that 2 mM veratryl alcohol saturates the enzyme (thus, the velocity should approach the $k_{cat}$ for veratryl alcohol), resulting in the formation of the veratryl alcohol cation radical intermediate. This radical, in turn, mediates the oxidation of guaiacol. At higher guaiacol concentrations, competition by veratryl alcohol at the active site is minimized; thus, the rate approaches the $k_{cat}$ for guaiacol. At these low guaiacol concentrations, no detectable veratraldehyde is detected until the guaiacol is depleted (see below).

Steady-state Kinetic Analyses of Veratryl Alcohol Oxidation—Veratryl alcohol oxidation by lignin peroxidase was investigated in the presence and absence of guaiacol. In the absence of guaiacol, veratryl alcohol oxidation follows Michae-
lis-Menten kinetics, as reported earlier (5). The $K_m$ and $k_{cat}$ were calculated to be $168 \mu M$ and $15 \text{ s}^{-1}$, respectively, at pH 3.5 (Fig. 4). Guaiacol at 0.1 mM was capable of completely inhibiting veratryl alcohol oxidation at all concentrations of veratryl alcohol tested (Fig. 4). Typical traces from similar experiments are shown in Fig. 5, where the rate of veratryl alcohol oxidation was monitored in the presence of 0–50 mM guaiacol. Even at guaiacol concentrations below its $K_m$, complete inhibition of veratryl alcohol oxidation was observed. Even at the lowest guaiacol concentration of 10 mM, we noted a lag period in veratryl alcohol oxidation. The length of the lag period correlated with the amount of guaiacol added (Fig. 5). During the lag period, tetraguaiacol was detected in the reaction mixture (data not shown). Only when formation of tetraguaiacol ceased was veratraldehyde detected.

Pre-steady-state Kinetic Studies—The reaction of guaiacol with compound I (forming compound II) was studied under pseudo first-order conditions at 417 nm, the isosbestic point between compound II and resting enzyme (24). The rate is linearly proportional to the guaiacol concentration (Fig. 6A). A rate constant of $1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ was calculated. This compares with $1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for the reaction of compound I with veratryl alcohol (15).

Conversion of Compound II to Resting Enzyme—The reaction of guaiacol with compound II (forming resting enzyme) was followed at 426 nm, the isosbestic point between resting enzyme and compound I (Fig. 6B). The rate observed with guaiacol shows a hyperbolic concentration dependence, suggesting at least a two-step pathway leading to its oxidation by compound II:

$\text{LP II + Gu} \overset{k_{II\rightarrow\text{LP}}} {\longrightarrow} \text{LP II⋅Gu} \overset{k_{\text{LP⋅Gu}}} {\longrightarrow} \text{LP + Gu}$

REACTION I

where LP II is lignin peroxidase compound II and Gu is guaiacol. If rapid equilibrium is assumed for the first step, the rate of guaiacol oxidation can be given by Equation 1.

$$k_{\text{obs}} = \frac{k(\text{guaiacol})}{[\text{guaiacol}]+K_d}$$

(Eq. 1)

The line drawn in Fig. 6B is a best fit according to Equation 1 with $K_d = 64 \mu M$ and $k = 17 \text{ s}^{-1}$. This is in contrast to 280 $\mu M$ and 16 $\text{ s}^{-1}$ for $K_d$ and $k$, respectively, for veratryl alcohol (15).
The addition of 40 eq of H₂O₂ to native enzyme (maximum at 409 nm) resulted in the formation of compound III (maxima at 419, 543, and 575 nm). Guaiacol (50 eq) was then added to this compound III preparation (Fig. 7). The resultant spectrum shows the conversion of compound III to the native state.

**DISCUSSION**

Veratryl alcohol has been shown to stimulate the lignin peroxidase-catalyzed oxidation of monomethoxy substrates such as anisyl alcohol and 4-methoxymandelic acid (12). Harvey et al. (12) suggested that these substrates, which are not oxidized directly by the enzyme, could be oxidized by veratryl alcohol cation radicals generated by lignin peroxidase catalysis. This mediation phenomenon by veratryl alcohol, a secondary metabolite also produced by the fungus (10), led to the proposal by Harvey et al. (12) that the physiological role of veratryl alcohol is to mediate the oxidation of lignin. Our previous studies have provided a different interpretation for the stimulation of anisyl alcohol and 4-methoxymandelic acid oxidation by veratryl alcohol (15). Transient state kinetic studies showed that anisyl alcohol can be oxidized by compound I (but not by compound II) of lignin peroxidase. Therefore, inclusion of veratryl alcohol or another substrate that reacts with compound II is essential for completion of the catalytic cycle. This completion of the catalytic cycle explains the observed stimulation of anisyl alcohol oxidation by veratryl alcohol.

In contrast to anisyl alcohol, guaiacol is a very good substrate for lignin peroxidase. As summarized in the scheme shown in Fig. 8A, guaiacol is a good substrate for both compounds I and II. With guaiacol, a second substrate like veratryl alcohol would not be needed to complete the catalytic cycle. The rate constant for compound I reacting with guaiacol is actually greater than that with veratryl alcohol. The reactivity of compound II with these two substrates is comparable with the exception that guaiacol has a much lower K₅₇₆. These results indicate that the stimulation of guaiacol oxidation by veratryl alcohol cannot be attributed to the need of veratryl alcohol to complete the catalytic cycle.

Although our previous studies discounted a mediation role for veratryl alcohol in the oxidation of anisyl alcohol (15), this study clearly shows that veratryl alcohol can mediate the oxidation of a phenolic substrate such as guaiacol. This evidence comes from steady-state kinetic studies. Fig. 8B shows the proposed mechanism of guaiacol oxidation in the presence of veratryl alcohol. Both guaiacol and veratryl alcohol are substrates for lignin peroxidase. As shown in Fig. 8B, they are both oxidized by compounds I and II. If the interaction of guaiacol and veratryl alcohol was only at compounds I and II, the inhibition of the oxidation of one substrate by the other would be expected to be competitive (25). However, veratryl alcohol has no inhibitory effect on guaiacol oxidation. In fact, at low guaiacol concentrations (below K₅₇₆), saturating concentrations of veratryl alcohol (2 mM) stimulated guaiacol oxidation to rates higher than its k₅₇₆ of 7.7 s⁻¹ (Fig. 3B). This can be explained by veratryl alcohol saturating the enzyme (K₅₇₆ = 168 μM) at 2 mM (thus resulting in its maximal velocity) and the subsequent reaction of the veratryl alcohol cation radical with guaiacol. In contrast, guaiacol was able to completely inhibit veratryl alcohol oxidation at concentrations much lower than its K₅₇₆. Veratraldehyde production was not observed until all of the guaiacol was depleted from the reaction mixture. This indicates that the mode of inhibition is more than just competing at the active site and can only be explained by guaiacol very effectively reducing the veratryl alcohol cation radical, resulting in its own oxidation (Fig. 8B). The rate constant for this reaction of the veratryl alcohol cation radical and guaiacol must be near the diffusion limit. The results from the inhibition studies can only be explained by mediation of guaiacol oxidation by veratryl alcohol.

There is still the question of why guaiacol oxidation does not proceed in a linear fashion and that compound III accumulates during turnover. Harvey and Palmer (23) were the first to observe such a phenomenon. They attributed the inhibition to the inability of guaiacol to convert compound III to ferric enzyme. Our results are in agreement, in part. Although we showed that guaiacol can partly convert compound III to resting enzyme, guaiacol is not as effective as veratryl alcohol in this process (8). The oxidation of veratryl alcohol very efficiently results in the conversion of compound III to resting enzyme (26, 27) by a mechanism that is yet to be defined. Formation of compound III during turnover would explain the decreased rate of guaiacol oxidation at high H₂O₂ concentrations. Compound III is readily formed with lignin peroxidase at high H₂O₂ concentrations (10). However, this does not completely explain the decrease in the yield of tetraguaiacol at high H₂O₂ concentrations. One would predict that the yield would remain constant even with decreased rates. This decreased yield appears to involve superoxide. The addition of tetranitromethane, a superoxide scavenger and an agent capable of converting compound III to ferric enzyme (8), also increased the yield of tetraguaiacol. Superoxide has been shown to be pro-

![Fig. 7. Conversion of lignin peroxidase compound III to ferric enzyme (solid line). Compound III was generated by adding 40 eq of H₂O₂ to native enzyme (maximum at 409 nm) resulting in the formation of compound III (maxima at 419, 543, and 575 nm). Guaiacol (50 eq) was then added to this compound III preparation (Fig. 7). The resultant spectrum shows the conversion of compound III to the native state.](image-url)

![Fig. 8. Reaction scheme of lignin peroxidase (LP) with guaiacol (Gu) and H₂O₂ in the absence (A) and presence (B) of veratryl alcohol (VA). Rate constants and references are as follows: k₁, 5.8 × 10⁶ M⁻¹ s⁻¹ (15), k₂, 1.5 × 10⁵ M⁻¹ s⁻¹ (15), k₃, 1.2 × 10⁶ M⁻¹ s⁻¹ (this work), k₄, 16 s⁻¹ (15), and k₅, 17 s⁻¹ (this work). The K₅₇₆ values for veratryl alcohol and guaiacol are 280 μM (14) and 64 μM (this work), respectively.](image-url)
duced during lignin peroxidase turnover (28). The product tetraguaiacol is not extremely stable (29), and thus, the observed decrease in yield might be due to the possible involvement of superoxide in the decomposition of tetraguaiacol.

Many roles have been proposed for veratryl alcohol in lignin biodegradation. Veratryl alcohol has been shown to protect lignin peroxidase from H$_2$O$_2$-dependent inactivation (11, 30); it has been shown to prevent compound III accumulation (9); and it has been proposed to act as a redox mediator in lignin depolymerization (12). Strong arguments can be made for the physiological significance of the first two roles, whereas the latter role as a redox mediator has been contested. Although our studies here demonstrated the ability of veratryl alcohol to mediate the oxidation of a phenol, we doubt that this is physiologically significant. No veratryl alcohol is bound to lignin peroxidase upon purification from fungal cultures. Therefore, veratryl alcohol cannot be viewed as a tightly bound prosthetic group. In addition, the veratryl alcohol concentration in fungal cultures is below its $K_m$ for lignin peroxidase. This indicates that under physiological conditions, the enzyme is not saturated with veratryl alcohol and that phenolic substrates would be just as readily oxidized directly by the enzyme as they would be by the veratryl alcohol cation radical. Furthermore, there are also no data indicating that the recently detected veratryl alcohol cation radical is stable and diffusible (31). Thus, it does not seem likely that veratryl alcohol can be acting at a distance as a diffusible oxidant. Consequently, the mediation of phenol oxidation would have to occur near or at the enzyme active site. Our results here are more consistent with a physiological role of veratryl alcohol protecting the enzyme from H$_2$O$_2$-dependent inactivation and serving as a substrate for compound II, where more recalcitrant substrates, such as those found in lignin, may not be able to serve such a role (15).

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