Oxidation of 4-Methoxymandelic Acid by Lignin Peroxidase

MEDIATION BY VERATRAL ALCOHOL*

Ming Tien‡ and Dengbo Ma
From the Department of Biochemistry and Molecular Biology and Center for Biomolecular Structure and Function, The Pennsylvania State University, University Park, Pennsylvania 16802

The mechanism of veratral alcohol-mediated oxidation of 4-methoxymandelic acid by lignin peroxidase was studied by kinetic methods. For monomethoxylated substrates not directly oxidized by lignin peroxidase, veratral alcohol has been proposed to act as a redox mediator. Our previous study showed that stimulation of anisyl alcohol oxidation by veratral alcohol was not due to mediation but rather due to the requirement of veratral alcohol to complete the catalytic cycle. Anisyl alcohol can react with compound I but not with compound II. In contrast, veratral alcohol readily reduces compound II. We demonstrate in the present report that the oxidation of 4-methoxymandelic acid is mediated by veratral alcohol. Increasing veratral alcohol concentration in the presence of 2 mM 4-methoxymandelic acid resulted in increased oxidation of 4-methoxymandelic acid yielding anisaldehyde. This is in contrast to results obtained with anisyl alcohol where increased concentrations of veratral alcohol caused a decrease in product formation. ESR spectroscopy demonstrated that 4-methoxymandelic acid caused a decrease in the enzyme-bound veratral alcohol cation radical signal, which is consistent with its reaction at the active site of the enzyme.

To degrade the aromatic polymer lignin, the white-rot fungus Phanerochaete chrysosporium secretes H₂O₂ (1), and two families of H₂O₂-utilizing enzymes, the lignin peroxidase (LP)¹ and manganese peroxidases (MnP). The MnPs catalyze the oxidation of Mn²⁺ to Mn³⁺ (2) whereas the LPs catalyze the oxidation of phenolic and nonphenolic methoxylated aromatic substrates (3). The catalytic cycle of both peroxidases is similar to that of other peroxidases. The enzyme is first oxidized by H₂O₂ to form a two-electron-oxidized intermediate, compound I (4). Compound I then returns to the resting ferric enzyme by two sequential one-electron reduction steps producing two, one-electron-oxidized products. The one-electron oxidized species of the enzyme formed during turnover is referred to as compound II. Unique to these fungal peroxidases is the recalcitrant nature of their substrate (5). Both act on substrates that other peroxidases are not capable of oxidizing.

¹ The abbreviations used are: LP, lignin peroxidases; MnP, manganese peroxidase; 4-MMA, 4-methoxymandelic acid; ESR, electron spin resonance.

The role and mechanism by which the LP and MnP interact with lignin is yet to be elucidated. For both enzymes, the role of mediators has been proposed. With MnP, the mediation phenomenon is well characterized and widely accepted. Complexed Mn²⁺ (6, 7) is oxidized by MnP and diffuses away from the active site of the enzyme to oxidize lignin (8). In the case of LP, the role of mediators and the mechanism by which lignin is depolymerized by LP is still unknown. A key component of LP catalysis is veratral alcohol, a secondary metabolite also produced by ligninolytic cultures of P. chrysosporium (9). Harvey et al. (10) proposed that veratral alcohol is the mediator for LP as Mn²⁺ is the mediator for MnP. They proposed that the initial one-electron oxidation of veratral alcohol forms the cation radical which then diffuses away from the active site and oxidizes other substrates such as anisyl alcohol and 4-methoxymandelic acid (4-MMA). The mediation hypothesis explains the ability of LP to oxidize substrates such as anisyl alcohol and 4-MMA only in the presence of veratral alcohol (10). The mediation mechanism is also attractive as it accounts for how a large bulky enzyme can interact with large bulky insoluble substrates.

Harvey’s hypothesis was supported by the work of Khindaria et al. (11). They were able to generate the radical chemically and also enzymatically. Both forms were detected by ESR spectroscopy; the half-life was much longer for the enzyme-bound form (12). Candeias and Harvey (13) also generated the veratral cation radical by chemical means and characterized it by pulse radiolysis. They determined the lifetime of the radical and calculated that it was capable of diffusing up to 7 μm. However, the work of Candeias and Harvey (13) also showed that the veratral alcohol cation radical did not oxidize anisyl alcohol or 4-MMA. They postulated that the enzyme-bound radical could be more reactive or that it would have a longer half-life, thus allowing the radical to oxidize these monomethoxylated substrates.

Our previous work demonstrated that the veratral alcohol “mediation” phenomenon with anisyl alcohol was not due to redox mediation (14). The enhancement was due to the requirement of veratral alcohol to complete the catalytic cycle. Whereas most substrates can be oxidized by both compound I and II of LP, anisyl alcohol can only be oxidized by compound I (thus being stuck at compound II). Inclusion of veratral alcohol results in its oxidation by compound II allowing the enzyme to complete the catalytic cycle. In the present study, we demonstrate, to our surprise, that the oxidation of the other monomethoxylated substrate that Harvey and co-workers (13) characterized, 4-MMA, is mediated by veratral alcohol. Steady-state methods show that 4-MMA is oxidized by the enzyme-generated veratral alcohol cation radical. We also provide ESR spectroscopy data consistent with this where the ESR signal of the veratral alcohol cation radical is quenched by 4-MMA.

* This work was supported in part by United States Department of Energy Grant DE-FG02-87ER13690. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 814-863-1165; Fax: 814-863-8616; E-mail: mxt3@psu.edu.
Veratryl Alcohol Mediation

MATERIALS AND METHODS

**Enzyme Purification**—LP isozyme H1 (pI 4.7) was isolated from *P. chrysosporium* strain PSBL-1 as described previously (15). 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 LP was determined at 409 nm using an extinction coefficient of 169 mM m⁻¹ cm⁻¹ (16).

**Chemicals**—Hydrogen peroxide solutions were prepared daily and the concentration was determined using ε₄₃₄ nm = 39.4 M⁻¹ cm⁻¹ (17). Veratryl alcohol, veratraldehyde, 4-MMA, and anisaldehyde were purchased from Aldrich. Veratryl alcohol was vacuum-distilled before use; all other chemicals were used without further purification.

**Product Analysis by High Pressure Liquid Chromatography**—Reaction mixtures were sampled (100 μl) and added to 400 μl of methanol. A 10-μl aliquot of the resultant solution was injected onto a reverse phase C18 (Vydac™) column eluted at 0.5 ml/min with a 5–100% linear methanol gradient in water. Products were monitored at 280 nm and identified by comparison to retention times of authentic standards.

**Steady-state Kinetics**—The rate of veratraldehyde and anisaldehyde formation was determined spectrophotometrically. The absorption spectra of the alcohols and their corresponding aldehydes are shown in Fig. 1. Veratraldehyde formation was monitored at 330 nm (ε = 1.9 mM⁻¹ cm⁻¹) rather than at 310 nm as in previous studies since anisaldehyde is transparent at this wavelength. Anisaldehyde formation was determined spectrophotometrically (Fig. 2). For anisaldehyde formation, the absorbance increase at 300 nm (ε = 7.4 mM⁻¹ cm⁻¹) was determined by its absorbance at 330 nm. The substrates have no spectral contribution at 300 or 330 nm. The reaction mixtures contained 0.4 mM H₂O₂ and veratryl alcohol or varying concentrations (0–6 mM) of 4-MMA. Anisaldehyde formation at a constant veratryl alcohol concentration (0.4 mM) increased roughly proportional to the 4-MMA concentration, suggesting that veratryl alcohol oxidation did not proceed until most if not all of the 4-MMA is oxidized.

**Steady-state Kinetics**—Computer simulations of steady-state data using rate constants obtained from presteady-state studies were performed with the program KIMSIM (19), provided by Carl Frieden and Bruce Barshop (Washington University, St. Louis, MO).

RESULTS

**Steady-state Kinetics**—The effect of veratryl alcohol concentration on the rate of anisaldehyde formation from 4 mM 4-MMA was determined spectrophotometrically (Fig. 2). Formation of veratraldehyde and anisaldehyde was monitored at 330 nm. Anisaldehyde formation was determined spectrophotometrically at 300 nm. Anisaldehyde formation at a constant veratryl alcohol concentration (0.4 mM) increased roughly proportional to the 4-MMA concentration, suggesting that veratryl alcohol oxidation did not proceed until most if not all of the 4-MMA is oxidized.

**Simulation of Steady-state Kinetics**—Computer simulations of steady-state data using rate constants obtained from presteady-state studies were performed with the program KIMSIM (19), provided by Carl Frieden and Bruce Barshop (Washington University, St. Louis, MO).

FIG. 1. Absorption spectra of veratryl alcohol (VAC), 4-MMA, veratraldehyde (VAD), and anisaldehyde (AAD) in water.

**FIG. 2. The effect of varying veratryl alcohol concentration on 4-MMA oxidation.** Reaction mixtures contained 0.4 mM H₂O₂, 0.05 μM LP, 4 mM 4-MMA, and varying concentrations of veratryl alcohol in 50 mM sodium tartrate, pH 3.5. The rates of anisaldehyde (open circles) and veratraldehyde (closed circles) formation were determined as described under "Materials and Methods."
all of the 4-MMA was first oxidized.

**Presteady-state Kinetics**—The reactivity of 4-MMA with compound I and II of LP was studied with a three-syringe stopped-flow technique under pseudo-first order reaction conditions. Compound I was generated in the stopped-flow by mixing one equivalent of LP with H₂O₂ and aged for 4 s before mixing with 4-MMA. Similar to results obtained with anisyl alcohol (14), 4-MMA reacts with compound I but much more slowly than veratryl alcohol (Fig. 5). A rate constant of 4.3 \( \times 10^3 \) M⁻¹ s⁻¹ was calculated from the slope for the reaction of compound I with 4-MMA. Reactions of compound II with other reductants always exhibit a hyperbolic concentration dependence. We were not able to observe this with 4-MMA at higher concentrations (data not shown). This suggests that 4-MMA has little or no reactivity with compound II. When extremely high concentrations are required to observe a reaction, a low level contaminant could account for the reaction; this we have observed with anisyl alcohol (14).

**ESR Spectroscopy**—The ESR spectrum of the veratryl alcohol cation radical is shown in Fig. 6. The radical was generated by enzymatic incubations quenched with HNO₃. Stoichiometric amounts of 25 μM enzyme was incubated with H₂O₂ in the presence of 2 mM veratryl alcohol and varying concentrations of 4-MMA. The ESR signal intensity was determined by double integration and calculated as described previously (12). Spectrum A is an incubation without 4-MMA; a value of 7.3 μM was calculated for the cation radical. Addition of 0.5 mM 4-MMA decreased the signal intensity by 53%. Addition of 1 mM 4-MMA resulted in an 85% decrease in signal intensity.

**DISCUSSION**

Veratryl alcohol was first detected in ligninolytic cultures of *P. chrysosporium* by Lundquist and Kirk in 1978 (9), long before the discovery of LP (20, 21). It was not until the discovery of LP that a role for veratryl alcohol in lignin degradation was first proposed. Veratryl alcohol not only protects the enzyme from H₂O₂-dependent inactivation (22) but also serves as a convenient assay substrate for the enzyme (23). The most debated role is that of a redox mediator between LP and polymeric lignin. This role was initially formulated from data showing the ability of veratryl alcohol to enhance the oxidation of recalcitrant compounds that are not directly oxidized by the enzyme. Lignin (24), anisyl alcohol (10), 4-MMA (10), chloropromazine (25), and guaiacol (26) are only oxidized by LP if veratryl alcohol is included in the reaction mixture. Although the mediation mechanism provided a suitable explanation for the phenomenon and provided a model for how a large bulky enzyme could degrade an insoluble large bulky polymer, there were chemical arguments against the model.
Despite its proposed existence as a stable mediator able to participate in oxidation reactions at a distance, there was no direct proof of the radical for many years. Khindaria et al. (11) were the first to detect the veratryl alcohol cation radical by ESR spectroscopy. They first generated and characterized the species by oxidation with Ce(IV) in 10% HNO₃. After establishing the ESR parameters, these workers were then able to detect the enzyme-bound radical during steady-state turnover of LP at pH 3.5. At this pH, the radical was not detected when generated by Ce(IV) oxidation indicating that the radical is not stable free in solution. Khindaria et al. (12) proposed that the enzyme extends the half-life of the radical.

Candeias and Harvey (13) also characterized the veratryl alcohol cation radical. They generated the species with thallium (II) and studied it by pulse radiolysis. The spectral and kinetic properties of the radical determined by Candeias and Harvey (13) did not totally agree with those of Khindaria et al. (12). Nevertheless, these workers determined that the half-life of the radical was approximately 60 ms in solution thus allowing it to diffuse a distance of 7 mm. This would allow for it to act as a diffusible oxidant. Khindaria et al. (12) determined a half-life of 0.57 ms resulting in a much lower diffusion radius. Regardless, Candeias and Harvey (13) demonstrated that the radical was capable of diffusing far enough to react with the polymeric dye Poly R-478. Surprisingly, no reaction was observed with either anisyl alcohol or 4-MMA. Candeias and Harvey (13) rationalized that the enzyme could serve to increase the reactivity of the radical (with positively charged residues) thus allowing the veratryl alcohol cation radical to oxidize both anisyl alcohol and 4-MMA. Alternatively, they indicated that if the enzyme extended the half-life of the cation radical, the probability of reaction would be increased.

Our studies with anisyl alcohol clearly showed that its oxidation by LP is not mediated by veratryl alcohol (14). We found that anisyl alcohol is not oxidized by compound II. Compound I, in contrast, is able to readily oxidize anisyl alcohol. Thus with only anisyl alcohol, the enzyme would be stuck at the compound II intermediate. The addition of veratryl alcohol, which is readily oxidized by compound II would allow the enzyme to complete the catalytic cycle. We showed that this mechanism would predict stimulation of anisyl alcohol oxidation at low veratryl alcohol concentrations. This is because reactions with compound II to complete the catalytic cycle (enhancement) would offset the effect of veratryl alcohol reacting with compound I (inhibitory). At higher concentrations of veratryl alcohol, the rate of anisyl alcohol oxidation should decrease because it would more effectively compete with anisyl alcohol at compound I. Consistent with the proposed mechanism, our data show that an optimal rate of anisyl alcohol oxidation is observed at 0.1 mM veratryl alcohol (14).

Two recent studies have shown that veratryl alcohol enhanced oxidation of a second substrate is due to mediation. Goodwin et al. (25) demonstrated veratryl alcohol-mediated oxidation of chlorpromazine; Koduri and Tien (26) showed...
Veratryl Alcohol Mediation

The proposed mechanism for mediation of these substrates is shown in Fig. 7. Kinetic simulation of mediation reveals two unique aspects which are born out by the data: (i) veratryl alcohol oxidation does not occur until the secondary substrate is depleted (lag phase kinetic traces) and (ii) the effect of veratryl alcohol on the oxidation of the secondary substrate exhibits saturation kinetics. Both of these properties are observed with chloropromazine (25) and guaiacol (26). These properties differ from those observed with anisyl alcohol where no lag phases are observed and an optimal rate is observed at low veratryl alcohol concentrations.

Results obtained here with 4-MMA oxidation by LP resemble those obtained with guaiacol and chloropromazine oxidation. The addition of 4-MMA caused lag phase kinetics on veratryl alcohol oxidation (Fig. 4) and saturation kinetics were observed when the veratryl alcohol concentration was varied (Fig. 2). These two results clearly point to mediation of 4-MMA oxidation by veratryl alcohol. Mediation is also consistent with the ESR results. The veratryl alcohol cation radical generated by the veratryl alcohol cation radical. The elimination of the β-carboxyl group as CO₂ would drive the reaction forward (Fig. 7B). Candeias and Harvey (13) suggested that the lack of reactivity between the veratryl alcohol cation radical and 4-MMA in solution can be explained by the short half-life of the cation radical. Our kinetic simulations, described below agree with this conclusion. We simulated the effect of 4-MMA on veratraldehyde formation (similar to data shown in Fig. 4) with a short-lived and a long-lived veratryl alcohol cation radical. The mechanism used for this simulation is shown in Fig. 8A. Khindaria et al. (12) measured the half-life of the cation radical in solution and enzyme-bound to be 0.57 ms and 370 ms, respectively. Candeias and Harvey (13) estimated the rate constant between the veratryl alcohol cation radical with 4-MMA to be 5 × 10⁴ m⁻¹ s⁻¹ (we used 9 × 10⁴ m⁻¹ s⁻¹). With a k₃ of 17 s⁻¹ for LP and a half-life of 370 ms for the enzyme-bound cation radical, the simulations shown in Fig. 8B were obtained which resemble the real data shown in Fig. 4. When the half-life of the cation radical is decreased to 0.57 from 370 ms, no inhibition of veratraldehyde formation is observed (Fig. 8B), indicating that its half-life was too short for reactions with 4-MMA.

In conclusion, we have shown that 4-MMA oxidation is mediated by veratryl alcohol. This work supports the hypothesis put forth by Candeias and Harvey (13) suggesting that extending the half-life of the radical can increase the reaction yield. However, our results still do not support the ability of veratryl alcohol to act as a diffusible oxidant. The cation radical is too short-lived when free in solution to oxidize other substrates. The results of Khindaria et al. (12) and ours clearly show that it must be stabilized to participate in electron transfer reactions with other aromatic substrates. Nevertheless, the list of substrates for which veratryl alcohol serves as an electron-transfer agent continues to grow. It therefore, would not be surprising to find that it serves such a role with lignin. Furthermore, as suggested by Shoemaker et al. (27), other mechanisms may be operating in fungal cultures to stabilize the cation radical and allow it to act as a diffusible oxidant.

Acknowledgments—We are indebted to Aditya Khindaria and Steven D. Aust of the Utah State University for performing ESR experiments.

REFERENCES
1. Forney, L. J., Reddy, C. A., Tien, M., and Aust, S. D. (1982) J. Biol. Chem. 257, 11455–11462
2. Renganathan, V., and Gold, M. H. (1986) Biochemistry 25, 1626–1631
3. Tien, M. (1987) Crit. Rev. Microbiol. 15, 141–168
4. Chance, B. (1952) Arch. Biochem. Biophys. 36, 416–424
5. Kirk, T. K., Tien, M., Kersten, P. J., Mozuch, M. D., and Kalyanaraman, B. (1992) Biochem. J. 286, 279–287
6. Chance, B., and Tien, M. (1965) Arch. Biochem. Biophys. 114, 242–245
7. Kuan, L.-C., and Tien, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1242–1246
8. Wariishi, H., Vally, K., and Gold, M. H. (1991) Biochem. Biophys. Res. Commun. 176, 269–275
9. Lundquist, K., and Kirk, T. K. (1978) Phytochemistry 17, 1676
10. Harvey, P. J., Shoemaker, H. E., and Palmer, J. M. (1986) FEBS Lett. 195, 242–246
11. Khindaria, A., Grover, T. A., and Aust, S. D. (1995) Biochemistry 34, 6020–6025
12. Khindaria, A., Yamazaki, I., and Aust, S. D. (1996) Biochemistry 35, 6416–6424
13. Candeias, L. P., and Harvey, P. J. (1995) J. Biol. Chem. 270, 16745–16748
14. Koduri, R. S., and Tien, M. (1994) Biochemistry 33, 4225–4230
15. Tien, M., and Myer, S. B. (1996) Appl. Environ. Microbiol. 62, 2540–2544
16. Tien, M., Kirk, T. K., Bull, C., and Fee, J. A. (1986) J. Biol. Chem. 261, 1687–1693
17. Nelson, D. P., and Kiesow, L. A. (1972) Anal. Biochem. 49, 474–478
18. Morrisey, J. B. (1976) in Spin Labelling, Theory and Applications (Berliner, L. J., ed) pp. 274–338, Academic Press, New York
19. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) Anal. Biochem. 130, 134–145
20. Tien, M., and Kirk, T. K. (1983) Science 221, 661–663
21. Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983) Biochem. Biophys. Res. Commun. 114, 1077–1083
22. Tonon, F., and Odier, E. (1988) Appl. Environ. Microbiol. 56, 466–472
23. Tien, M., and Kirk, T. K. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2280–2284
24. Hammel, K. E., Jensen, K. A., Mozuch, M. D., Landucci, L. L., Tien, M., and Pease, E. A. (1993) J. Biol. Chem. 268, 12274–12281
25. Goodwin, D. G., Aust, S. D., and Grover, T. A. (1995) Biochemistry 34, 5060–5065
26. Koduri, R. S., and Tien, M. (1995) J. Biol. Chem. 270, 22254–22258
27. Schoemaker, H. E., Lundell, T. K., Floris, R., Glumoff, T., Winterhalter, K. H., and Piontek, K. (1994) Bioorganic & Medicinal Chemistry 2, 509–519
28. Hammel, K., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985) J. Biol. Chem. 260, 8348–8353
29. Schmidt, H. W. H., Haemmerli, S. D., Schoemaker, H. E., and Leisola, M. S. A. (1989) Biochemistry 28, 1776–1783
Oxidation of 4-Methoxymandelic Acid by Lignin Peroxidase: MEDIATION BY VERATRYL ALCOHOL
Ming Tien and Dengbo Ma

J. Biol. Chem. 1997, 272:8912-8917.
doi: 10.1074/jbc.272.14.8912

Access the most updated version of this article at http://www.jbc.org/content/272/14/8912

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 12 of which can be accessed free at http://www.jbc.org/content/272/14/8912.full.html#ref-list-1