Biochemistry **1999,** *38,* 3205−3210

Lignin and Mn Peroxidase-Catalyzed Oxidation of Phenolic Lignin Oligomers[†]

Lucia Banci,[‡] Simone Ciofi-Baffoni,[‡] and Ming Tien*[,§]

Department of Chemistry, University of Florence, Via G. Capponi 7, I-50121 Florence, Italy, and Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received September 3, 1998; Revised Manuscript Received December 9, 1998

ABSTRACT: The oxidation of phenolic oligomers by lignin and manganese peroxidases was studied by transient-state kinetic methods. The reactivity of peroxidase intermediates compound I and compound II was studied with the phenol guaiacol along with a β-O-4 phenolic dimer, trimer, and tetramer. Compound I of both peroxidases is much more reactive than compound II. The rate constants for these substrates with Mn peroxidase compound I range from 1.0×10^5 M^{-1} s^{-1} for guaiacol to 1.1×10^3 M^{-1} s^{-1} for the tetramer. Reactivity is much higher with lignin peroxidase compound I with rate constants ranging from 1.2×10^6 M^{-1}s^{-1} for guaiacol to 3.6×10^5 M^{-1} s^{-1} for the tetramer. Rate constants with compound II are much lower with Mn peroxidase exhibiting very little reactivity. The rate constants dramatically decreased with both peroxidases as the size of the substrate increased. The extent of the decrease was much more dramatic with Mn peroxidase, leading us to conclude that, despite its ability to oxidize phenols, Mn^{2+} is the only physiologically significant substrate. The rate decrease associated with increasing substrate size was more gradual with lignin peroxidase. These data indicate that whereas Mn peroxidase cannot efficiently directly oxidize the lignin polymer, lignin peroxidase is well suited for direct oxidation of polymeric lignin.

To degrade the aromatic polymer lignin, wood-destroying fungi secrete H_2O_2 and a large number of heme-containing peroxidase isozymes (*1*). These peroxidase isozymes, first discovered in the basidiomycetes *Phanerochaete chrysosporium* (*2*−*5*), catalyze the initial depolymerization of lignin. They fall into two distinct families; one family is the lignin peroxidase (LiP), and the other is the Mn peroxidase (MnP) family. Both enzymes follow the classical peroxidase mechanism (*6*) where ferric enzyme first reacts with H_2O_2 to form a two-electron-oxidized intermediate referred to as compound I. Compound I then returns to ferric enzyme by oxidizing two substrate molecules by one electron each. The enzyme becomes a one-electron-oxidized enzyme intermediate referred to as compound II during this process. The structures of both fungal peroxidases have been determined (*7*, *8*). They are homologous to each other and to other peroxidases of different classes, indicative of divergent evolution.

The distinguishing property of different peroxidases is the nature of reducing substrate. The nature of the substrates oxidized by these two fungal peroxidases has been a topic of great interest because it is still not apparent how turnover of the enzyme results in lignin depolymerization. Although the substrate of both LiP and MnP is polymeric lignin, the oxidizing activity of the heme active site has been proposed to be delivered through the action of low molecular weight substrates (*9*, *10*). The LiPs catalyze the oxidation of aromatic phenolic and nonphenolic substrates by one electron to yield the corresponding aryl cation radicals (*11*). The Mn peroxidases catalyze the oxidation of complexed Mn^{2+} to Mn^{3+} (*10*). Mn^{3+}, in turn, can oxidize a large number of phenolic substrates. For LiP, veratryl alcohol, also produced by ligninolytic fungi (*12*), has been proposed to act as a redox mediator; it is oxidized to the corresponding cation radical that in turn has been proposed to be a diffusible oxidant (*9*). The ability of the veratryl alcohol cation radical to diffuse and act at a distance has been questioned (*13*, *14*). In contrast, the role of mediators in MnP catalysis is well established (*9*). Mn^{2+} or phenolic compounds can serve as the reductant for compound I, forming compound II. However, compound II has an absolute requirement for Mn^{2+} as the reductant in MnP (*15*). The oxidation of Mn^{2+} by Mn peroxidases is dependent on the presence of organic acids, also produced by the fungus (*16*, *17*). Mn^{3+} is widely accepted as a diffusible oxidant, able to oxidize secondary substrates at a distance away from the active site of MnP.

The activity of either LiP or MnP on more lignin-like substrates has not been well characterized. The proposed involvement of low molecular weight mediators would imply that these enzymes are less active with large, more bulky lignin-like substrates. The purpose of the present study is to investigate the role of substrate size on MnP and LiP catalysis. These kinetic studies will characterize the interaction between MnP or LiP and oligomeric lignin models. This, in turn, will allow us to assess the role of mediation in the

---

[†] This work was supported in part by a grant from the Department of Energy (DE-FG02-87ER13690) and by a grant from the European Community (project number CHRXCT940540). Financial support was also provided from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD program, CT95-0805, "OXEPI". Support from this agency does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

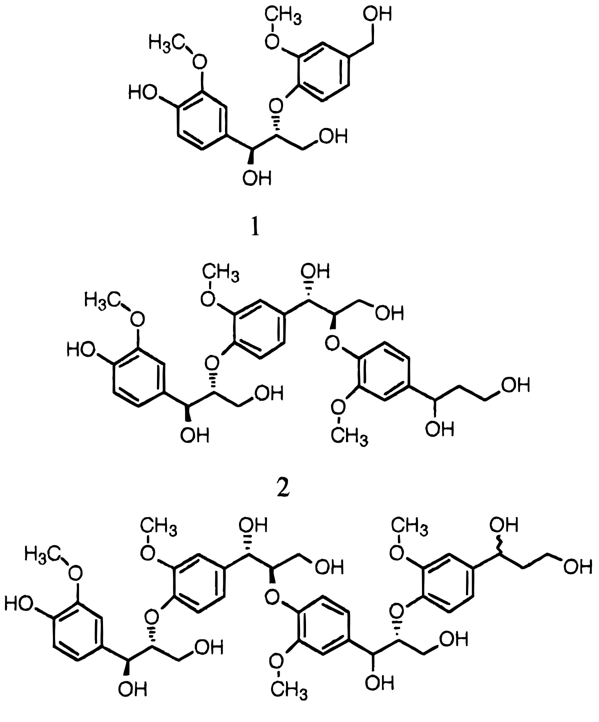
* Corresponding author: email, mxt3@psu.edu; telephone, 814-863-1165; fax, 814-863-8616.

[‡] University of Florence.

[§] The Pennsylvania State University.

10.1021/bi982139g CCC: $18.00

Chart 1



degradation of lignin. We describe the use of transient-state kinetics of MnP and LiP with a monomeric substrate, and newly synthesized dimeric, trimeric, and tetrameric substrates which contain only β-O-4 bonds between the units.

MATERIALS AND METHODS

Chemicals. H_2O_2 was purchased from Sigma Chemical Company. H_2O_2 solutions were prepared daily, and the concentration of H_2O_2 was determined spectrophotometrically at 240 nm using an extinction coefficient of 39.4 M^{-1} cm^{-1} (*18*). Guaiacol was purchased from Aldrich Chemical Co. The dimeric **1**, trimeric **2**, and tetrameric **3** phenolic β-O-4 lignin models, shown in Chart 1, were prepared and purified as previously described (*19*). The racemic dimeric compound **1** used in the kinetic measurements was diastereomerically pure, while the racemic trimeric compound **2** was a mixture of two diastereoisomers, inseparable by flash chromatography on silica gel. The relative configurations of the α,β-bonds are shown in Chart 1.

Preparation and Purification of MnP. The recombinant MnP (rMnP) was expressed in *Escherichia coli* strain BL21-(DE3)pLysS, as previously described (*20*). The preparation was purified by a modification of our previously described method (*21*) by two sequential DEAE columns in 50 mM Tris−Cl, pH 8.0. The enzyme was eluted with 400 and 300 mL, respectively for the first and the second column, of a linear 0−0.15 M $CaCl_2$ gradient in column buffer. The A_{407}/A_{280} ratio for purified MnP was in the 3−4 range, characteristic of highly purified MnP from *P. chrysosporium* (*10*). Protein samples for stopped-flow experiments were prepared by dialyzing the protein against distilled, deionized water. The enzyme was quantitated spectrophotometrically at 406 nm using an extinction coefficient of 127 mM^{-1} cm^{-1} (*21*).

Preparation of LiP. Wild-type fungal LiP isozyme H2 was isolated from strain BKM-F1767 and purified as previously described (*23*). The purified preparation exhibited an RZ ratio (A_{409}/A_{280} ratio) of at least 4. Protein samples for stopped-flow experiments were prepared by dialyzing the protein against distilled, deionized water. The concentration of LiP was spectrophotometrically determined at 409 nm using an extinction coefficient of 169 mM^{-1} cm^{-1} (22).

Transient-State Kinetic Studies. Reduction of MnP or LiP compound I to compound II by the phenolic substrates was monitored at 417 nm, the isosbestic wavelength between resting ferric enzyme and compound II (*16*). Reduction of compound II to ferric enzyme was monitored at 426 nm, the isosbestic wavelength between compound I and II. All reactions were at 28 °C. The stopped-flow apparatus, SX.18MV sequential stopped flow, SQ.1 variant containing a 1 cm light path, was obtained from Applied Photophysics. The reactions were studied under pseudo-first-order conditions with a four-syringe stopped-flow apparatus. MnP and LiP compound I and compound II were generated in the four-syringe stopped-flow apparatus. Their reaction with reducing substrates was followed by two sequential mixings in the stopped flow. The double mixing allows studies on unstable intermediates such as MnP and LiP compounds I and II. Resting ferric enzyme was first mixed with 1 equiv of H_2O_2 to form compound I. The solution was aged in the aging loop for 1.5 s to allow for the complete formation of compound I. The second push from the pneumatic rams forced the mixing of the resultant compound I with the content of a third syringe, which contained the reducing substrate of choice. Compound II was generated in two ways, either by reacting compound I with phenolic substrates or with ferrocyanide. Both methods yielded similar results. In experiments using ferrocyanide, the syringe containing the enzyme also contained 1 equiv of ferrocyanide, and the resultant reaction mixture, after mixing with H_2O_2, was allowed to age in the delay line for 2 s. Typically, 1.5 mM enzyme solution (concentration after mixing) was used with different substrate concentrations in 20 mM sodium tartrate at either pH 2.5 or 4.5 using MnP as enzyme and in 35 mM sodium tartrate at pH 3.5 using LiP. Each datum point was the average of three shots.

RESULTS

Reactions of MnP Compound I. Previous studies by Whitwam et al. (*21*) indicate that rMnP is kinetically similar to fungal wild-type MnP. The results of our experiments are in accord with this previous work. At pH 4.5, the optimum pH for steady-state turnover of MnP (*9*), the rMnP, as the native fungal enzyme, compound I reacts with Mn^{2+} at a rate too fast to be measured by a stopped-flow spectrophotometer. At the nonoptimal pH of 2.5, this rate constant is slowed to 5×10^4 M^{-1} s^{-1} in the fungal wild-type enzyme (*16*). For the recombinant MnP, we obtain a rate of 4.0×10^4 M^{-1} s^{-1}, which compares with 4.2×10^4 M^{-1} s^{-1} reported by Whitwam et al. (*21*).

The rate constants for the reaction of compound I of MnP with the phenolic substrates are much slower than with Mn^{2+}. The values measured at pH 4.5 for MnP compound I with a series of phenolic substrates with increasing size are reported in Table 1. Whereas at optimal pH 4.5 the rate constant for the reduction of compound I by Mn^{2+} is at least 10^7 M^{-1} s^{-1} (*16*), the rate constant for guaiacol is approximately 100

Table 1: Kinetic Parameters Obtained for the Reduction of LiP and MnP Compound I and MnP Compound II by a Series of Substrates

	k_{cpdI} (M^{-1} s^{-1})		k_{cpdII} (M^{-1} s^{-1})
substrate	MnP compd I[a]	LiP compd I[b]	MnP compd II[c]
guaiacol	1.0×10^5	1.2×10^6	186
dimer **1**	5.1×10^3	1.1×10^6	185
trimer **2**	3.9×10^3	6.6×10^5	155
tetramer **3**	1.1×10^3	3.6×10^5	71

[a] Assays are at pH 4.5 as described in Materials and Methods. [b] Assays are at pH 3.5 as described in Materials and Methods. [c] Assays are at pH 4.5 as described in Materials and Methods.

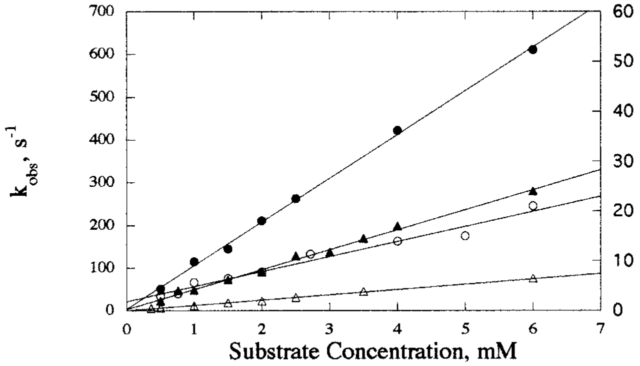


FIGURE 1: Reduction of MnP compound I to compound II by guaiacol and β-O-4 lignin model compounds. Reaction mixtures contained 1.5 μM MnP in 20 mM sodium tartrate at pH 4.5 and 28 °C. Compound I was mixed with increasing concentrations of guaiacol (closed circles), dimer **1** (open circles), trimer **2** (closed triangles), and tetramer **3** (open triangles). Two scales are used for k_{obs}. The left axis (up to 700 s^{-1}) is used for guaiacol and the right axis is used for the dimer, trimer and tetramer. Each datum point is the mean of three kinetic traces. Error bars represent the standard error of the mean (the error bars are not visible because they are less than the radius of the point drawn in the figure).

times slower and easily measured by the stopped flow. At pH 4.5, a linear relationship between k_{obs} and substrate concentration was observed up to 6 mM concentration of guaiacol and the dimeric, trimeric, and tetrameric substrates (Figure 1). The second-order rate constants, k_{cpdI}, for the reduction of compound I by guaiacol and the dimeric, trimeric, and tetrameric substrates were calculated from the slopes of k_{obs} as function of substrate concentration (Table 1). No saturation was observed in all experiments using higher concentrations of the substrates (data not shown).

Reactions of MnP Compound II. Reactions of guaiacol, the dimeric, trimeric, and tetrameric substrates with compound II of MnP were studied at pH 4.5 and followed at 426 nm, the isosbestic point between resting enzyme and compound I. The reaction was much slower than that of compound I, but again there is a linear relationship between the pseudo-first-order rate constant, k_{obs}, and substrate concentration between 0 and 3.5 mM for guaiacol and the dimeric, trimeric, and tetrameric substrates (Figure 2). Again, no saturation was observed for substrate concentration up to 4 mM. The second-order rate constants for the studied substrates are reported in Table 1.

Reaction of LiP Compound I. The reactivity of guaiacol and the dimeric, trimeric, and tetrameric substrates with LiP compound I was also studied by transient-state techniques. Compound I was generated in the stopped-flow apparatus with 1 equiv of H_2O_2 and let to react with each lignin model within 1.5 s. The rate for the reaction of compound I (yielding compound II) is linearly proportional to the concentrations of each lignin model molecule between 0 and 0.3 mM at the optimal pH of 3.5 for the LiP protein (Figure 3). No saturation is observed for substrate concentrations up 1 mM (data not shown). The rate constants, k_{cpd}I, for the reaction of LiP compound I are shown in Table 1. All of the rate dependencies as function of substrate concentration showed a nonzero intercept, which suggests reaction reversibility. The rate limits at zero substrate concentration vary from 4 s^{-1} for the trimeric compound to 17 s^{-1} for the tetrameric compound.

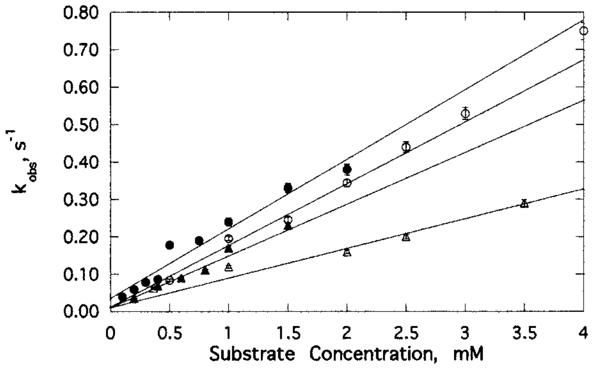


FIGURE 2: Reduction of MnP compound II to resting enzyme by guaiacol and β-O-4 lignin model compounds. Incubations contained 1.5 μM MnP in 20 mM sodium tartrate at pH 4.5 and 28 °C. Compound II was mixed with increasing concentrations of guaiacol (closed circles), dimer **1** (open circles), trimer **2** (closed triangles), and tetramer **3** (open triangles). Each datum point is the mean of three kinetic traces. Error bars represent the standard error of the mean (some error bars are not visible due to their low value).

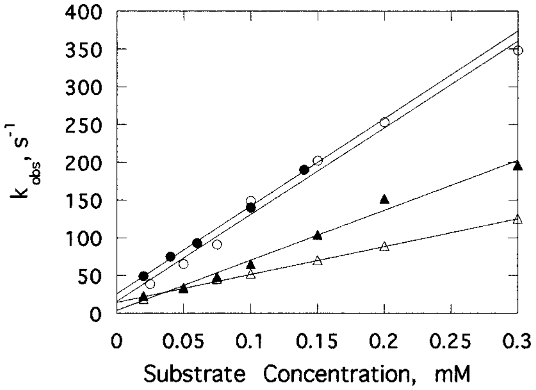


FIGURE 3: Reduction of LiP compound I to compound II by guaiacol and β-O-4 lignin model compounds. Incubations contained 1.5 μM LiP in 35 mM sodium tartrate at pH 3.5 and 28 °C. Compound I was mixed with increasing concentrations of guaiacol (closed circles), dimer **1** (closed squares), trimer **2** (open circles), and tetramer **3** (open squares). Each datum point is the mean of three kinetic traces. Error bars represent the standard error of the mean (the standard error was very small and not visible on the graph).

Reaction of LiP Compound II. The observed rates for the compound II reduction at pH 3.5 by the dimeric, trimeric, and tetrameric lignin models exhibit a hyperbolic dependence upon substrate concentration (Figure 4). This behavior is similar to that already reported (*24*) for the reaction of compound II with the monomeric substrate guaiacol. It is consistent with a two-step mechanism shown below where S is the substrate and LiP II is lignin peroxidase compound II.

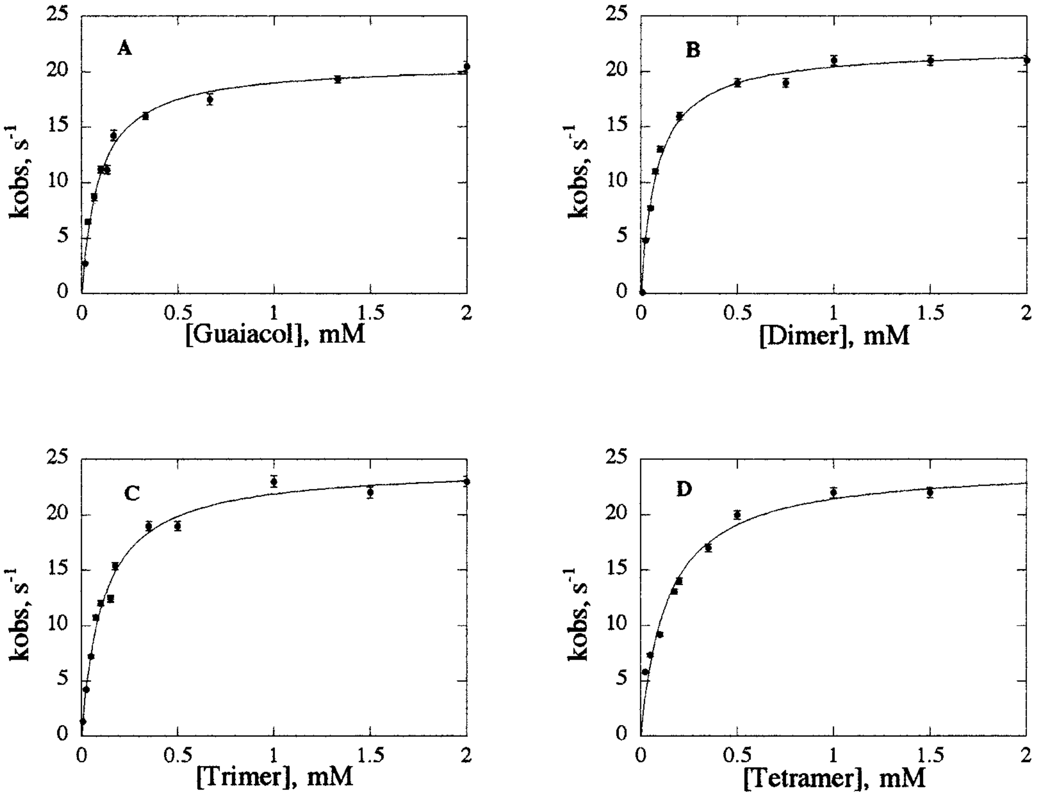


FIGURE 4: Reduction of LiP compound II to resting enzyme by guaiacol and β-O-4 lignin model compounds. Incubations contained 1.5 μM LiP in 35 mM sodium tartrate at pH 3.5 at 28 °C. Compound II was mixed with increasing concentrations of guaiacol (A), dimer **1** (B), trimer **2** (C), and tetramer **3** (D). Each datum point is the mean of three kinetic traces. Error bars represent the standard error of the mean (some error bars are not visible due to their low value).

$$\text{LiP II} + \text{S} \overset{K_D}{\rightleftharpoons} \text{LiP II}\cdot\text{S} \rightarrow \text{LiP} + \text{S}^{+\bullet}$$

In this mechanism, k is the rate-limiting step, and the binding of S to compound II is at rapid equilibrium. The observed rate constants can be calculated by using the following equation:

$$k_{\text{obs}} = \frac{k[\text{S}]}{K_{\text{D}} + [\text{S}]} \tag{1}$$

In this equation, k is essentially determined by the electron-transfer rate, k_{ET}. From this relationship, the rate of compound II reduction and the K_{D} can be calculated by fitting the data using the Levenberg–Marquardt algorithm. The lines drawn in Figure 4 are the best fits obtained from eq 1 with the parameters reported in Table 2.

Table 2: Kinetic Parameters Obtained for the Reduction of LiP Compound II by Phenolic Substrates[a]

substrate	K_{D} (μM) ± SE[b]	k (s^{-1}) ± SE
guaiacol	64 ± 0.01	21 ± 0.58
dimer **1**	93 ± 0.01	23 ± 0.56
trimer **2**	116 ± 0.01	24 ± 0.60
tetramer **3**	136 ± 0.02	24 ± 0.99

[a] Assays are as described in Materials and Methods. [b] SE = standard error.

DISCUSSION

For reasons of convenience, most of the kinetic studies on LiP and MnP have been on either monomeric or dimeric substructural models of lignin. The activity of LiP and MnP on more lignin-like substrates has not been well characterized, mainly because these larger models are more difficult to synthesize and their solubility properties are not ideal. Thus, the ability of LiP and MnP to interact and oxidize the lignin polymer has not been well characterized. Furthermore, studies have suggested that neither one of these enzymes interacts directly with the lignin polymer. Srebotnik et al. (*25*) demonstrated the ability of fungi to degrade woody biomass despite the inability of enzymes larger than 17 000 Da to penetrate the wood matrix. Furthermore, the proposed involvement of low molecular weight mediators would imply that these enzymes are less active with polymeric lignin. To test this hypothesis, the purpose of the present study was to characterize the reactivity of both LiP and MnP with larger, more bulky lignin-like substrates. Although wood-degrading fungi oxidize both nonphenolic and phenolic substituents, phenolic substituents are the substrate of choice for both LiP (*24*) and MnP (*3, 5*). Furthermore, sound lignin still contains a high percentage of phenolic substituents, and their degradation may be more physiologically significant. Thus, we have characterized the oxidation of β-O-4 phenolic lignin model compounds of different sizes.

The oxidation of phenolic β-O-4 lignin model compounds has been characterized with both laccase (*26*) and MnP (*27*) only on dimeric substrates. These studies did not provide kinetic analyses of the reaction; they characterized the products formed from the one-electron oxidation of these substrates. The products from both MnP- and laccase-catalyzed oxidation of the β-O-4 substrates were shown to be similar; the predominant reaction is oxidation of C_α yielding the carbonyl. The further oxidation of the carbonyl product was proposed to lead to Ca−Cb cleavage (*27*). Although our focus here is on kinetics, we nevertheless have used 1H NMR to show that similar Ca−Cb cleavage products are formed from the oxidation of the dimeric, trimeric and tetrameric substrates by MnP (data not shown).

For both LiP and MnP, the kinetic constants indicate that both enzymes more readily oxidize the smaller lignin-like substrates. However, the decrease in rate as substrate size increases is much more dramatic with MnP. Furthermore, the activity of MnP compound II with these phenolic substrates is exceedingly low. As a trend, the rate of compound II is usually 10 times slow than that of compound I of most peroxidases. This was not the case with MnP compound II with phenolic substrates where the rates are 25 to 300 times slower than reactions of compound I. Despite its ability to directly oxidize phenolic substrates, our results suggest that MnP does not directly interact with polymeric lignin in vivo. A more probable mechanism is that they directly oxidize Mn^{2+} at the active site. The oxidized trivalent Mn, in turn, acts as a diffusible oxidant. These results reaffirm why MnP requires Mn^{2+} to complete its catalytic cycle. It also supports previous observations on the requirement for Mn^{2+} in the oxidation of monomeric and dimeric phenolic lignin models (*10*).

Whereas the binding site for Mn^{2+} in MnP has been clearly identified (*8*, *28*, *29*), little is known about the substrate-binding site for LiP. The enzyme does not crystallize with the substrate veratryl alcohol bound (*7*). Our attempts to identify the substrate-binding site by NMR spectroscopy have not been successful. The location of this binding site is important in revealing whether the lignin polymer can directly interact with LiP or degrade lignin through the action of low molecular weight mediators. Molecular dynamic simulations, performed by Du and Loew (*30*), suggested two binding sites for LiP. One site is found at the heme cavity, as previously suggested by Poulos et al. (*31*), as the binding site for veratryl alcohol. The other site is near the C-terminus in the primary structure and is more surface exposed than the putative veratryl alcohol-binding site. Indeed, LiP and MnP differ from CcP structure (*32*) in having an extra C-terminal segment that spans over the surface of the enzyme. Du and Loew (*30*) suggested that these sites could be the binding sites for different substrates. The site near the heme accommodates small substrates. Lignin, being polymeric, cannot bind in this site but was suggested to interact with the more accessible site near the C-terminus.

Saturation kinetics is not observed for the reactions of compound I of both LiP and MnP. This indicates that $K_D \gg [S]$ for all the S concentrations used in the present study. Equation 1 can then be rewritten as $k_{obs} = k_{ET}[S]/K_D$. The rate constants are therefore a function of the dissociation constant K_D (between the enzyme reactive intermediate (compound I or II) and substrate) and the rate of the electron transfer within this complex (k_{ET}):

$$k_{2nd} = K_D^{-1} k_{ET} \tag{2}$$

where k_{2nd} is the second-order rate constant of the reaction (k_{cpdI} or k_{cpdII}). In the Marcus equation of electron transfer, the rate of electron transfer depends on the thermodynamic driving force ΔE (*33*−*35*):

$$k_{ET} = k_0 \exp(-\Delta G^{\ddagger}/RT)$$

$$\Delta G^{\ddagger} = \lambda(1 - F\,\Delta E/\lambda)^2/4$$

where $\Delta G^{\ddagger}$ is the activation free energy, k_0 is the rate of activationless electron transfer in the enzyme−substrate complex, λ is the reorganization energy, F and R are the Faraday and gas constants, respectively, and T is the absolute temperature. The observed reduction in the second-order rate constants with increasing the substrate size indicates a less favorable interaction between the enzyme and the substrates as they become bulkier rather than a more difficult electron transfer. This is because the parameters determining $\Delta G^{\ddagger}$, λ, and ΔE, do not vary when only the size of the substrate is changed, if the same binding site is assumed for all the substrates. A less favorable interaction between enzyme and substrate could influence the electron transfer limit rate k_0 at zero activation energy that depends on the distance between the redox centers. Consequently, the rate of the reactions involving LiP compound I will be determined by the dissociation constant of the enzyme−substrate complex and, consequently, by the rate of activationless electron transfer in the enzyme−substrate complex, k_0. The kinetic constants for the reaction of LiP compound II to yield resting enzyme, measured in the present work, are in agreement with the previous conclusions. Indeed, the observed saturation kinetics, which is caused by the slowest electron-transfer rate of the catalytic cycle (k), does not change with increasing the size of the lignin model (Table 2). This suggests that the distance between compound II and the substrate within the complex, and therefore the electron-transfer pathway, does not change by increasing the size of the substrate. Thus, the increase in the thermodynamic parameter, dissociation constant K_D, as the substrate size increases reflects the more unfavorable interaction between bulky substrate and LiP. For LiP, the results indicate a preference for low molecular weight substrates by compound I. However, for the reaction of compound II, the rate-limiting step in catalysis, all of the phenolic substrates show a rate limit of approximately 22 s^{-1}, and the K_D values for the substrates exhibit small incremental increases as the size of the substrate increases. These results suggest that LiP is equally adept at oxidizing small or large aromatic substrates and that the differences observed here do not preclude the ability of LiP to function as an enzyme that directly interacts with the lignin polymer.

One can make some minimal estimates on the nature of the aromatic binding sites for both MnP and LiP. Whereas the Mn-binding site in MnP is defined by the heme 6-propionate group, Glu35, Glu39, and Asp179 (*28*, *29*), none of these three residues appear to be involved in the reaction of the enzyme with phenolic substrates. No information is available concerning the possible binding site between MnP and phenolic substrates. However, our results obtained using as substrate the different molecular weight lignin models show that the reduction of MnP compound I is much more sensitive to substrate size than LiP. This would suggest that the phenolic substrate-binding site for LiP is much more exposed and accessible than is the corresponding site for MnP. Further studies are ongoing to map the nature of the LiP active site using the corresponding nonphenolic aromatic substrates described here.

REFERENCES

1. Kirk, T. K., and Farrell, R. L. (1987) *Annu. Rev. Microbiol. 41*, 465−505.
2. Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983) *Biochem. Biophys. Res. Commun. 114*, 1077−1083.
3. Glenn, J. K., and Gold, M. H. (1985) *Arch. Biochem. Biophys. 242*, 329−341.
4. Tien, M., and Kirk, T. K. (1983) *Science 221*, 661−663.
5. Paszczynski, A., Huynh, V. B., and Crawford, R. (1985) *FEMS Microbiol. Lett. 29*, 37−41.
6. Chance, B. (1949) *J. Biol. Chem. 179*, 1311−1330.
7. Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H., and Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 750−754.
8. Sundaramoorthy, M., Kishi, K., Gold, M. H., and Poulos, T. L. (1994) *J. Biol. Chem. 269*, 32759−32767.
9. Harvey, P. J., Schoemaker, H. E., and Palmer, J. M. (1986) *FEBS Lett. 195*, 242−246.
10. Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986) *Arch. Biochem. Biophys. 251*, 688−696.
11. Kersten, P. J., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985) *J. Biol. Chem. 260*, 2609−2612.
12. Lundquist, K., and Kirk, T. K. (1978) *Phytochemistry 17*, 1676.
13. Koduri, R. S., and Tien, M. (1994) *Biochemistry 33*, 4225−4230.
14. Tien, M., and Ma, D. (1997) *J. Biol. Chem. 272*, 8912−8917.
15. Wariishi, H., Akileswaran, L., and Gold, M. H. (1988) *Biochemistry 27*, 5365−5370.
16. Kuan, I.-C., Johnson, K. A., and Tien, M. (1993) *J. Biol. Chem. 268*, 20064−20070.
17. Kuan, I.-C., and Tien, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1242−1246.
18. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474−478.
19. Ciofi-Baffoni, S., Banci, L., and Brandi, A. (1998) *J. Chem. Soc., Perkin Trans. 1*, 3207−3217.
20. Whitwam, R. E., Gazarian, I. G., and Tien, M. (1995) *Biochem. Biophys. Res. Commun. 216*, 1013−1017.
21. Whitwam, R., and Tien, M. (1996) *Arch. Biochem. Biophys 333*, 439−446.
22. Millis, C. D., Cai, D., Stankovic, M. T., and Tien, M. (1989) *Biochemistry 28*, 8484−8489.
23. Tien, M., and Kirk, T. K. (1988) *Methods Enzymol. 161*, 238−249.
24. Koduri, R. S., and Tien, M. (1995) *J. Biol. Chem. 270*, 22254−22258.
25. Kawai, S., Higuchi, T., Nabeta, K., and Okuyama, H. (1989) in *Biotechnology in Pulp and Paper Manufacture* (Kirk, T. K., and Chang, H., Eds.) pp 359−365, Butterworths, Stoneham.
26. Tuor, U., Wariishi, H., Schoemaker, H. E., and Gold, M. H. (1992) *Biochemistry 31*, 4986−4995.
27. Srebotnik, E., Messner, K., and Foisner, R. (1988) *Appl. Environ. Microbiol. 54*, 2608−2614.
28. Kusters-van Someren, M., Kishi, K., Lundell, T., and Gold, M. H. (1995) *Biochemistry 34*, 10620−10627.
29. Whitwam, R. E., Brown, K. R., Musick, M., Natan, M. J., and Tien, M. (1997) *Biochemistry 36*, 9766−9773.
30. Du, P., and Loew, G. H. (1993) in *Plant Peroxidases: Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C., and Greppin, H. Eds.) pp 27−30, University of Geneva, Switzerland.
31. Poulos, T. L., Edwards, S. L., Wariishi, H., and Gold, M. H. (1993) *J. Biol. Chem. 268*, 4429−4440
32. Poulos, T. L., and Kraut, J. (1980) *J. Biol. Chem. 255*, 8199−8205.
33. Marcus, R. A. (1964) *Annu. Rev. Phys. Chem. 15*, 155−196.
34. Marcus, R. A., and Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265−275.
35. Canters, G. W., and van de Kamp, M. (1992) *Curr. Biol. 2*, 859−869.

BI982139G