Thiol-mediated Oxidation of Nonphenolic Lignin Model Compounds by Manganese Peroxidase of *Phanerochaete chrysosporium*

(Received for publication, March 22, 1989)

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In the presence of MnIII, H2O2, and glutathione (GSH), manganese peroxidase oxidized veratryl alcohol (I) to veratraldehyde (IV). Anisyl alcohol (II) and benzyl alcohol (III) were also oxidized by this system to their corresponding aldehydes (V and VI). In the presence of GSH, chemically prepared MnIII or γ-irradiation also catalyzed the oxidation of I, II, and III to IV, V, and VI, respectively. GSH and dithiothreitol rapidly reduced MnII to MnIII in the absence of aromatic substrates and the dithiothreitol was oxidized to its disulfide (4,5-dihydroxy-1,2-dithiane). These results indicate that the thiol is oxidized by enzyme-generated MnIII to a thyl radical. The latter abstracts a hydrogen from the substrate, forming a benzylic radical which reacts with another thyl radical to yield an intermediate which decomposes to the benzyaldehyde product.

In the presence of MnIII, GSH, and H2O2, manganese peroxidase also oxidized 1-(4-ethoxy-3-methoxyphenyl)-2-(4'-hydroxymethyl-2'-methoxyphenyl)-1,3-dihydroxypropane (XII) to yield vanillyl alcohol (VII), vanillin (VIII), 1-(4-ethoxy-3-methoxyphenyl)-1,3-dihydroxypropane (XVI), 1-(4-ethoxy-3-methoxyphenyl)-1-ox-o-3-hydroxypropane (XIX), and several C8 oxidized dimeric products. Abstraction of the C8 (A ring) hydrogen of the dimer (XII) yields a benzylic radical, leading to C8 oxygen ether cleavage. The resultant intermediates yield the ketone (XIX) and vanillyl alcohol (VII) or vanillin (VIII). Alternatively, benzylic radical formation at the C6 position (B ring) leads to radical cleavage, yielding a quinone methide and a C8 radical, which yield vanillin and the 1,3-diol (XVI), respectively. In these reactions, MnIII oxidizes a thiol to a thyl radical which subsequently abstracts a hydrogen from the substrate to form a benzylic radical. The latter undergoes nonenzymatic reactions to yield the final products.

Lignin, the most abundant renewable aromatic polymer, constitutes 20–30% of woody plants (1). Since the biodegradation of cellulose is retarded by the presence of lignin, the catabolism and utilization of this polymer are of enormous significance (2–5). White rot basidiomycete Phanerochaete chrysosporium produces two heme peroxidases (3–10) which along with an H2O2 generating system (3) appear to be the major components of its lignin degradative system. The structure and mechanism of lignin peroxidase have been studied extensively (3–5, 7, 8, 11–13). Manganese peroxidase (MnP) has also been purified and characterized (5, 6, 9, 13–15). The enzyme exists as a series of isozymes (13), contains one iron protoporphyrin IX prosthetic group (9), and is a glycoprotein of M, ~46,000 (6, 9, 15). MnP catalyzes the H2O2- and MnIII-dependent oxidation of a variety of phenols, amines, and organic dyes (5, 9, 14–16).

Electronic absorption (5, 9, 16), EPR, and resonance Raman spectral evidence (17) indicates that the heme environment of native MnP has features which are similar to those of other plant peroxidases (18). The nucleotide sequence of a cDNA encoding an MnP isozyme has been determined and confirms the presence of a proximal and distal histidine at the active center of the enzyme (19). In addition, spectral and kinetic evidence (16, 20) indicates that the H2O2-oxidized states (compounds I and II) and the catalytic cycle of MnP are similar to those of lignin and horseradish peroxidases (11, 18). Most importantly, it has been demonstrated that MnP oxidizes MnII to MnIII and that the MnIII in turn oxidizes monomeric phenols (9, 14–16) and phenolic lignin dimers (21) via the formation of a phenoxy radical. Transient state kinetic analysis (20) has confirmed that MnIII/MnIII acts as a redox couple rather than as an enzyme binding activator. Chelation by certain organic acids such as lactate and malonate stabilizes the MnIII at a high redox potential (0.9–1.2 V) facilitating the oxidation of organic substrates (22, 23).

The one-electron oxidation of phenols (14, 16, 24) and thiol (24–26) to phenoxy or thyl radicals by MnIII and other transition metals has been well studied. In contrast, MnIII complexes with organic acids such as malonate apparently are not capable of easily oxidizing most nonphenolic lignin model compounds such as veratryl alcohol under normal physiological conditions (5, 6, 9, 21). In contrast, a recent report by Forrester et al. (27) claims that nonphenolic lignin model compounds are oxidized directly by MnIII-pyrophosphate in the presence of glutathione via the initial formation of aryl cation radicals. However, since the mechanism of Forrester et al. (27) appeared unlikely, we have re-examined the oxidation of nonphenolic lignin models by MnP-generated and chemically prepared MnIII in the presence of thiols. Herein, we demonstrate that in this system, MnIII oxidizes thiol C8 radicals which in turn react with the lignin models to form carbon-centered radicals. The latter undergo a variety of reactions to yield the final products.

*This work was supported by Grants DMB 8607279 from the National Science Foundation and DE-FG06-86ER 13550 from the U. S. Department of Energy. 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.

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1 The abbreviations used are: MnP, manganese peroxidase; DTT, dithiothreitol; DTE, dithioerythritol; GCMS, gas chromatography mass spectrometry.
**Materials and Methods**

MnP enzyme I was purified from the extracellular medium of an acetic acid-biodegraded agitated culture of *P. chrysosporium* strain OGC 101 (29) as previously reported (9, 20, 23). The purified enzyme was electrophoretically homogeneous and had a pI of 4.9.

**Enzyme Reactions**—Aromatic compound oxidations were carried out at 37 °C for 30 min in 1 ml of 50 mM sodium-malonate, pH 4.5, containing aromatic substrate (0.2 mM), GSH (5.0 mM) (DTE, DTT, or Cys were substituted where indicated), MnSO₄ (0.5 mM) and enzyme (5.0 μg) under anaerobic conditions or as indicated. Anaerobic conditions were obtained as described (6, 29). Reactions were initiated by adding H₂O₂ (0.2 mM).

**Oxidation of Aromatic Substrates by Mn²⁺-Manganese Complex in the Presence of GSH**—Mn²⁺ (0.1 mM stock solution) was prepared by dissolving MnCl₂-acetate (Aldrich) in 0.1 M sodium-malonate immediately prior to use. Mn²⁺-malonate showed absorption maxima at 270 and 460 nm. Reaction mixtures (1.6 ml) contained aromatic substrate (0.2 mM), GSH (5.0 mM), and MnCl₂-malanate (2.0 mM) in 50 mM Na-malonate buffer, pH 4.5. Reactions were carried out at 37 °C for 5 min under anaerobic conditions.

**γ-Irradiation**—Reaction mixtures (1.0 ml) contained aromatic substrate (0.2 mM) and GSH (5.0 mM) in 10 mM Na-phosphate buffer, pH 7.2. Samples were irradiated under anaerobic conditions for 6 h with a 60Co γ-ray source (21.2 Krad/h).

**Kinetic and Spectral Analyses**—Veratryl alcohol oxidations were carried out in 2 ml of 50 mM Na-malonate, pH 3.1–6.1, containing veratryl alcohol (0.1 mM), enzyme (2 μg), MnSO₄ (0.1 mM), and GSH (5.0 mM). Reactions were initiated by adding H₂O₂ (0.1 mM) using a microsyringe, conducted under argon or air as indicated, and monitored at 310 nm. Lactate, oxalate, or pyrophosphate replaced malonate, and DTT, DTE, or Cys replaced GSH where indicated. Mn²⁺-malonate oxidations were carried out in 2 ml of 50 mM Na-malonate, pH 4.5, containing MnSO₄ (0.1 mM), enzyme (5 μg), GSH (0.1 mM), and H₂O₂ (0.1 mM) under anaerobic conditions. Mn²⁺-malonate formation was monitored at 270 nm.

**Chemicals and Preparation of Compounds**—H₂O₂ and the reduced thiols GSH, DTT, DTE, and Cys were obtained from Sigma. 3,4-Dimethoxybenzyl alcohol (veratryl alcohol) (I), 4-methoxybenzyl alcohol (II), and benzyl alcohol (II), the corresponding aldehydes veratraldehyde (IV), anisyl alcohol (V), and benzaldehyde (VI), 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol) (VII), and the corresponding aldehyde vanillin (VIII) were all obtained from Aldrich. All other chemicals were reagent grade.

1,2-Bis(3,4-dimethoxyphenyl)-1,2-dihydroxyethane (X) was prepared by microcrystalline condensation as described previously (30): (a) veratraldehyde/KCN in 80% EtOH, reflux 6 h; (b) NaBH₄ in EtOH, room temperature, 16 h. 1,2-Bis(3,4-dimethoxyphenyl)-1,2-dihydroxyethane (IX) was prepared by the oxidation of XVI using one equivalent of 2,3-dichloro-5,6-dicyano-1,4-dibenzoquinone (32). MS m/z XIV (di TMS ether) 520 (M⁺, 67), 483 (5), 297 (2), 252 (100), 225 (3), 239 (22), 206 (6), 147 (4), 73 (35). MS m/z XV (di TMS ether) 476 (M⁺, 5%), 431 (5), 428 (4), 315 (26), 293 (29), 209 (21), 179 (89), 152 (24), 151 (25), 73 (100).

1-(4-Ethoxy-3-methoxyphenyl)-1,3-dihydroxypropene (XVI) was prepared by the condensation of 4-ethoxy-3-methoxybenzaldehyde and ethyl bromocetate in benzene with zinc powder (reflux, 30 min), followed by treatment with 10% H₂SO₄ in benzene (0 °C) and by reduction with LiAlH₄ in THF (~30 °C, 1 h) (35). 1-(4-Ethoxy-3-methoxyphenyl)-2,1-dihydroxypropane (XVIII) were all obtained but the yields were ~50% of starting substrate.

**RESULTS**

**Oxidation of Aryl Alcohols by the MnP/Mn³⁺/Thiol System**—As shown in Table I, veratryl alcohol (I), anisyl alcohol (II), and benzyl alcohol (III) were oxidized almost quantitatively by MnP/Mn³⁺ in the presence of GSH under anaerobic conditions to yield the corresponding aldehydes IV, V, and VI and coupled dimers IX, X, and XI. No products were obtained when the reactions were carried out in the absence of enzyme, GSH, Mn³⁺, or H₂O₂. Identical products were obtained if lactate or oxalate replaced the malonate buffer or if DTT, DTE, or Cys replaced the glutathione. Under aerobic conditions, the same products were obtained and no veratryl alcohol ring cleavage was observed.

In the presence of thiol, under anaerobic conditions, chemically prepared Mn²⁺-malonate was also capable of oxidizing

**Table I** Products obtained from the oxidation of substituted benzyl alcohols

| Oxidant         | Substrate                | Products (mol % of starting substrate) | Coupled dimers | IX | X | XI |
|-----------------|--------------------------|----------------------------------------|----------------|----|---|----|
| MnP/Mn³⁺/GSH    | Veratryl alcohol         | 96                                     | T              | T  | T | T  |
|                 | Anisyl alcohol           | 95                                     | T              | T  | T | T  |
|                 | Benzyl alcohol           | 90                                     | T              | T  | T | T  |
| Mn²⁺/GSH        | Veratryl alcohol         | 92                                     | T              | T  | T | T  |
|                 | Anisyl alcohol           | 91                                     | T              | T  | T | T  |
|                 | Benzyl alcohol           | 95                                     | T              | T  | T | T  |
| GSH/γ-irradiation| Veratryl alcohol         | 85                                     | T              | T  | T | T  |
|                 | Anisyl alcohol           | 80                                     | T              | T  | T | T  |
|                 | Benzyl alcohol           | 80                                     | T              | T  | T | T  |

*Valid, Aald, and Bald-veratraldehyde, anisaldheyde, and benzaldheyde, respectively.

*T = trace.*

1-(4-Ethoxy-3-methoxyphenyl)-2-(4'-formyl-2'-methoxyphenox)-1,3-dihydroxypropane (XIV) was prepared as follows: (a) the formyl group of methyl(4-formyl-2-methoxyphenox)acetate was protected by forming the dimethyl acetal derivative using methyl orthoformate and p-toluene sulfonic acid (32). (b) The dimethyl acetal derivative was condensed with 4-ethoxy-3-methoxybenzaldehyde (32, 33).

(c) Reduction with LiAlH₄ in THF, room temperature, followed by treatment with HCl and Xylene (34). 1-(4-Ethoxy-3-methoxyphenyl)-2-(4'-formyl-2'-methoxyphenox)-1-oxo-3-hydroxypropane (XV) was prepared by the oxidation of XIV with 2,3-dichloro-5,6-dicyano-1,4-dibenzoquinone (34). MS m/z XV (di TMS ether) 520 (M⁺, 67), 509 (3), 297 (2), 252 (100), 225 (3), 239 (22), 206 (6), 147 (4), 73 (35). MS m/z XV (di TMS ether) 476 (M⁺, 5%), 431 (5), 428 (4), 315 (26), 293 (29), 209 (21), 179 (89), 152 (24), 151 (25), 73 (100).

1-(4-Ethoxy-3-methoxyphenyl)-1,3-dihydroxypropane (XVI) was prepared by the condensation of 4-ethoxy-3-methoxybenzaldehyde and ethyl bromocetate in benzene with zinc powder (reflux, 30 min), followed by treatment with 10% H₂SO₄ in benzene (0 °C) and by reduction with LiAlH₄ in THF (~30 °C, 1 h) (35). 1-(4-Ethoxy-3-methoxyphenyl)-2,1-dihydroxypropane (XVIII) were all obtained but the yields were ~50% of starting substrate.

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these substituted benzyl alcohols to the corresponding aldehyde and coupled dimer products (Table I). When the Mn"-malonate or Mn"-pyrophosphate reaction was carried out in the absence of thiol at either pH 4.5 or 3.0, no products were observed.

In order to test the possibility that Mn"-generated thyl radicals (24-26) were involved in the oxidation of the benzyl alcohols I, II, and III, we examined the oxidation of I, II, and III in a γ-irradiation system consisting of substrates, GSH, and buffer. The generation of thyl radicals from thiols by γ-irradiation has previously been well studied (37, 38). As shown in Table I, when the alcohols were irradiated in the presence of GSH under anaerobic conditions, the same aldehyde and coupled dimer products were obtained. Furthermore, in the absence of GSH, γ-irradiation did not lead to oxidation of the substrates.

Since the formation of the coupled dimer (IX) was negligible, the initial rate of veratryl alcohol oxidation could be followed spectrophotometrically by measuring the rate of formation of veratraldehyde at 310 nm (7, 8, 10) (Table II). Under anaerobic conditions GSH, DTE, and DTT were all effective thiols. However, activity was considerably reduced when cysteine was used as the thiol. In the presence of GSH, both malonate and lactate were effective as Mn" chelators. Activity was considerably reduced when oxalate or pyrophosphate were substituted for malonate. Very low activity was detected when succinate was unable to form an Mn" complex (23). With all thiols and organic acids used, the initial rate of veratraldehyde formation was ~2-fold greater under anaerobic conditions than under aerobic conditions.

Oxidation of Thiol by Mn"—As shown in Fig. 1A, Mn"-malonate was effectively reduced by GSH in the absence of substituted benzyl alcohols. Addition of 10 equivalents of GSH to Mn"-malonate resulted in the rapid formation of a featureless spectrum between 250-500 nm, characteristic of Mn" organic acid complexes (Fig. 1A) (9, 14). In contrast, in the absence of thiol, less than 10% of the Mn"-malonate was reduced upon addition of 10 equivalents of veratryl alcohol (data not shown). Furthermore, Fig. 1B shows that in the absence of veratryl alcohol, Mn"-malonate accumulation in the enzyme system is suppressed by the addition of thiol. The kinetic curve obtained in the presence of 1.0 mM GSH (Fig. 1B) suggests that there is an initial burst of Mn"-malonate followed by a plateau when the rate of Mn"-malonate formation approximates the rate of Mn" reduction. After Mn"-malonate was reduced by DTT (1,4-dimercapto-2,3-dihydroxybutane), the mixtures were extracted with chloroform, derivatized, and analyzed by GCMS. The MS spectrum of the products demonstrated the formation of 4,5-dihydroxy-1,2-dithiane which contains an intramolecular disulfide bond. MS m/z (di TMS ether) 296 (M+, 13%), 203 (9), 180 (59), 147 (25), 116 (100), 101 (24), 73 (92). These results indicate that the Mn"-malonate is capable of effectively oxidizing the thiol to a thyl radical which subsequently undergoes radical coupling to form an intramolecular disulfide bond.

The pH dependence from pH 3.1-6.1 for the oxidation of veratryl alcohol by MnP/Mn"/GSH is shown in Fig. 2. Activity increased with increasing pH. Fig. 2 also shows the pH dependence of the lignin peroxidase-catalyzed oxidation of veratryl alcohol (39). Here, activity increased with decreasing pH.

Oxidation of β-Aryl Ether Lignin Model Compounds—As shown in Fig. 3, the non-phenolic β-vanillyl alcohol ether (XII) was oxidized by the MnP/Mn"/GSH system under anaerobic conditions to yield the dimeric ketone (XIII), the β-vanillin ether dimer (XIV), the phenylpropane-1,3-diol (XVI), the phenylpropane-1-oxo-3-ol (XIX), vanillyl alcohol (VIII), and vanillin (VII). The isomers of XVI, the phenylpropane-2,3-diol (XVIII), and the phenylpropane-1,2-diol (XVII) were not found.

The MnP/Mn"/GSH system also oxidized the nonphenolic β-vanillin dimer (XIV) under anaerobic conditions to yield the dicarbonyl dimer (XV), the phenyl propane-1-oxo-3-ol (XIX), and vanillin (VIII) (Fig. 3). Finally, the phenylpropane-1,3-diol (XVI) was readily oxidized to the phenylpropane-1-oxo-3-ol (XIX) by the enzyme system. No products were obtained when the reactions were conducted in the absence of either GSH, Mn", H₂O₂, or enzyme.

When the substrates XII, XIV, and XVI were irradiated under anaerobic conditions in the presence of GSH, the same products were obtained as with the enzyme system (Fig. 3). In the absence of GSH, no products were obtained and substrate was recovered nearly quantitatively after irradiation for 6 h.

Oxidation of Substrates under Aerobic Conditions—Product analysis revealed that with all of the substrates examined using each of the three systems ((i) MnP/Mn"/thiol; (ii) Mn"/thiol; (iii) γ-irradiation/thiol), identical products were obtained under aerobic conditions but in reduced yield (~50% of anaerobic samples).

**DISCUSSION**

Spectral and kinetic studies have indicated that the principal function of MnP is the oxidation of Mn" to Mn" via a typical peroxidase catalytic cycle (9, 14, 16, 18, 20). The enzymatically generated Mn" in turn oxidizes a variety of organic substrates (9, 14–16, 20, 21). A number of organic acids are capable of forming complexes with Mn" (9, 16, 20–22, 25). For example, malonate and lactate chelate Mn" to form distorted octahedral complexes, containing two water molecules (23). These complexes are relatively stable in aqueous solution but still possess a high redox potential (0.9–1.2 V) (22, 23). We have recently demonstrated that chelation of Mn" by organic acids also facilitates its release from the enzyme-Mn complex (20). Mn"-organic acid complexes are well-studied one-electron oxidants which are capable of oxidizing numerous substrates including phenols and thiols (9, 14, 22, 24, 25). We recently reported that MnP catalyzes C₆H₅C₆H₅ and alkylphenyl bond cleavage of a phenolic diarylpropane dimer and that these cleavage reactions are initiated by the oxidation of the dimer to a phenoxyl radical by enzymatically generated Mn" (21). Nonphenolic lignin models are not oxidized by either the MnP/Mn"-malonate system or by Mn"-malonate complexes (21) under physiological conditions. In

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**TABLE I**

**Effects of thiols and organic acids on the oxidation of veratryl alcohol by manganese peroxidase**

| Thiol | Organic acid | Initial rate | Anaerobic | Aerobic |
|------|--------------|-------------|-----------|---------|
| GSH  | Malonate     | 6.7         | 3.0       |         |
| DTE  | Malonate     | 5.9         | 2.2       |         |
| DTT  | Malonate     | 5.1         | 2.5       |         |
| Cys  | Malonate     | 1.3         | 0.5       |         |
| GSH  | Lactate      | 1.3         | 0.5       |         |
| GSH  | Oxalate      | 1.1         | 0.5       |         |
| GSH  | Succinate    | 0.01        | 0.0       |         |
| GSH  | Pyrophosphate* | 1.4   | 0.6       |         |

*50 mM Na-pyrophosphate was dissolved in 50 mM Na-succinate buffer, pH 4.5.
Thiyl Radical Involvement in Mn Peroxidase Reactions

Fig. 1. Reduction of Mn\textsuperscript{III}-malonate by GSH. A, reaction mixtures contained Mn\textsuperscript{III}-malonate (0.1 mM) in 50 Na-malate buffer, pH 4.5, to which the indicated equivalents of GSH were added. Spectra were recorded 10 s after the addition of GSH. B, Accumulation of Mn\textsuperscript{III}-malonate in the presence of various amounts of GSH. Reaction mixtures contained Mn\textsuperscript{III} (0.1 mM), GSH (as indicated), MnP (5.0 \mu g) in 50 mM Na-malate buffer, pH 4.5. Reactions were initiated by adding 0.1 mM H\textsubscript{2}O\textsubscript{2} and conducted under anaerobic conditions. Mn\textsuperscript{III}-malonate formation was monitored at 270 nm.

Fig. 2. pH profile for veratryl alcohol oxidation by MnP in the presence of GSH (C) and by lignin peroxidase (\Phi). For MnP, reaction mixtures contained enzyme (1 \mu g/ml), Mn\textsuperscript{II} (0.1 mM), veratryl alcohol (0.1 mM), GSH (5.0 mM), and H\textsubscript{2}O\textsubscript{2} (0.1 mM) in 50 mM Na-malate buffer, pH 3.1-6.1. Reactions were conducted under anaerobic conditions. The profile for lignin peroxidase was previously obtained in our laboratory (39).

contrast, lignin peroxidase catalyzes the one-electron oxidation of non-phenolic lignin model compounds to form aryl cation radicals which subsequently undergo a variety of non-enzymatic reactions including C\textsubscript{6}-C\textsubscript{9} cleavage and ring cleavage (3-5, 11, 12, 29, 33).

Recently, the oxidation of veratryl alcohol and nonphenolic \beta-aryl-ether-type lignin dimers by MnP in the presence of thiols has been reported (27). In that report (27) the authors claim that in the presence of thiols the Mn\textsuperscript{III}-pyrophosphate complex is capable of oxidizing aromatic substrates to their corresponding aryl cation radicals and that GSH stimulates the reaction by reducing oxygen to superoxide. We considered this to be an unlikely mechanism (27) for several reasons. (a) Aryl cation radicals of lignin models readily undergo C\textsubscript{6}-C\textsubscript{9} cleavage in reactions involving LiP or other single electron oxidizing agents (3-5, 12); yet C\textsubscript{6}-C\textsubscript{9} cleavage was not observed in the MnP/Mn\textsuperscript{II}/thiol system (27). (b) Stimulation by thiol was attributed to the production of superoxide radical; however, superoxide dismutase did not significantly affect the reaction rate (27). (c) The redox potential of Mn\textsuperscript{III}-pyrophosphate (~0.6 V at pH 4.5) (40) is less than the redox potential of Mn\textsuperscript{III}-malonate or lactate (22, 23). Therefore, the oxidation to aryl cation radicals of veratryl alcohol and other dimethoxy benzenes with higher redox potentials (41) by Mn\textsuperscript{III}-pyrophosphate is not energetically favorable.

To clarify the mechanism of the MnP-catalyzed thiol-mediated oxidation of nonphenolic lignin models, we have re-examined the reaction using spectroscopic, kinetic, and product analyses.

As shown in Tables I and II, veratryl alcohol, anisyl alcohol, and benzyl alcohol were oxidized by (i) MnP/Mn\textsuperscript{II}/thiol, (ii) Mn\textsuperscript{III}-malonate/thiol, and (iii) \gamma\textsuperscript{-}irradiation in the presence of thiol. The redox potential of these reactions. The addition of aromatic methoxy groups lowers the redox potential of methoxy benzenes (41); for example, the redox potentials of monomethoxy and dimethoxy benzenes have been determined to be ~1.76 and 1.34-1.45 V, respectively (41). Thus, the oxidation of methoxy benzenes to aryl cation radicals by peroxidases is facilitated by the addition of methoxy groups (33, 42). For example, lignin peroxidase easily oxidizes veratryl alcohol, but oxidizes anisyl alcohol slowly, and is not capable of oxidizing benzyl alcohol. It is therefore unlikely that the reactions reported herein proceed through a cation radical intermediate.

The results in Fig. 1A indicate that Mn\textsuperscript{III} is rapidly reduced to Mn\textsuperscript{II} in the presence of GSH. Furthermore, accumulation of Mn\textsuperscript{II} in the enzyme system is suppressed in the presence of GSH (Fig. 1B). Finally, reduction of Mn\textsuperscript{III} with DTT yields

\( \text{K. Valli, H. Wariishi, and M. H. Gold, unpublished results.} \)
an intramolecular disulfide product. All of these results indicate that the MnIII oxidizes the thiol to a thiyl radical which undergoes radical coupling to form the disulfide. The oxidation of thiols to thiyl radicals by MnIII and other transition metals has been reported previously (23-26, 43). The existence of free thiyl radicals has been confirmed by electron spin resonance spectroscopy (44, 45).

As further proof of the involvement of thiyl radicals, we utilized γ-irradiation in the presence of thiols as a source of these radicals. Generation of thiyl radicals from thiols by γ-irradiation via .OH mediation has been well established (37, 38, 46). Table I shows that the thiol/γ-irradiation system oxidizes all of the benzyl alcohols to yield the same products as the MnP/MnIII/thiol system. None of the reactions occurred in the absence of thiol. These results also suggest that the enzyme-generated MnIII oxidizes thiols to thiyl radicals which, in turn, mediate the dehydrogenation of the substituted benzyl alcohols. Hydrogen abstraction from active hydrogen donors by thiyl radicals to yield carbon-centered radicals has been previously reported (47, 48).

The initial rate of the reactions conducted under anaerobic conditions is twice the reaction rate under aerobic conditions (Table II). As described previously (26), molecular oxygen reacts with thiyl radicals to form superoxide anion, thereby lowering the effective concentration of the reactive thiyl radicals. These results contradict the proposal by Forrester et al. (27) that thiols stimulate the oxidation by forming superoxide radical. In addition, in contrast to the results reported by Forrester et al. (27), Table II shows that malonate and lactate stimulate the oxidation of veratryl alcohol in these systems more effectively than either oxalate or pyrophosphate. The results in Fig. 2, demonstrating that activity increases with increasing pH, suggest that protonation of the thiol inhibits its oxidation (26).

Mechanism of Benzyl Alcohol Oxidation—Oxidation of thiol by MnIII generates a thiyl radical, which in turn can abstract a benzylic hydrogen from benzyl alcohol (47, 48) to form a benzylic radical. Under anaerobic conditions, the benzylic radical probably couples with another thiyl radical to produce an unstable thiohemiacetal (Fig. 4). The latter would decompose to form a free thiol and a benzaldehyde. The formation of stable GSH conjugates from thiyl radicals has been reported previously (45, 49). Radical coupling of two benzylic radicals would yield the coupled dimers IX, X, and XI, which were observed in this study. Although these coupled dimers are obtained in only trace amounts, their formation strongly supports this mechanism. Under aerobic conditions the benzylic radical could be scavenged by molecular oxygen or a hydroperoxy radical to yield a peroxo intermediate which would decompose to yield the benzaldehyde product (50).

Formation of the dimeric α-carbonyl (XIII) and the β-vanillin ether (XIV) from the β-vanillyl alcohol ether (XII) (Fig. 3) and the benzylic oxidation of XIV and XVI to yield XV and XIX, respectively (Fig. 3) can be explained in a similar manner.

Mechanism of β-Ether Cleavage—The β-vanillyl alcohol ether dimer (XII) has two benzylic hydrogens available for abstraction by thiyl radicals. Abstraction of the C₄ (A ring) hydrogen yields a benzylic radical, leading to C₄ oxygen ether bond cleavage. This results in the formation of the unstable

![Fig. 3. Products obtained from the oxidation of the nonphenolic substrates XII, XIV, and XVI by both the MnP/MnIII/GSH and the γ-irradiation/GSH systems. Reactions were conducted and analyzed as described in the text.](image)

![Fig. 4. Proposed mechanism for benzyl alcohol oxidation by the MnP/MnIII/thiol (RSH) and MnIII/thiol systems. Ar = aromatic.](image)
Thyl Radical Involvement in Mn Peroxidase Reactions

**FIG. 5.** Proposed mechanisms for β-aryl ether cleavage of the nonphenolic β-aryl ether dimer (XII) by MnP/Mn\textsuperscript{II}/thiol.

phenylpropene and phenoxy radical intermediates (Fig. 5, left). The phenylpropene would be converted to the phenylpropene-1-oxo-3-ol (XIX). The phenoxy radical could abstract a hydrogen from GSH as previously proposed (43) to yield vanillyl alcohol (VII) and another thiyl radical (Fig. 5). When the reaction was conducted in D\textsubscript{2}O, no deuterium was incorporated into the vanillyl alcohol (data not shown), suggesting that the phenolic hydrogen derived from GSH. The phenoxy radical of vanillyl alcohol may also be oxidized to vanillin via the transient formation of a quinone methide intermediate (21, 51).

Alternatively, when the benzylic radical is formed at the C\textsubscript{5} (ring B) (Fig. 5, right), the ensuing radical cleavage yields a quinone methide and a C\textsubscript{3} radical intermediate. The quinone methide spontaneously rearranges to vanillin. The C\textsubscript{3} radical may abstract a proton from GSH to generate the phenylpropane-1,3-diol (XVI). The fact that when the reaction was conducted in D\textsubscript{2}O, no deuterium was incorporated into the diol (XVI) supports this mechanism. Exogenous phenylpropane-1,3-diol was oxidized to the corresponding ketone (XIX) by the enzyme system (Figs. 3 and 5).

The results reported here and previously can be summarized in the following reactions:

\[
\begin{align*}
\text{Mn}^{\text{III}} + \text{RSH} &\rightarrow \text{Mn}^{\text{II}} + \text{RS} - + \text{H}^+ \quad (1) \\
\text{RS} - + \text{AH} &\rightarrow \text{A} - + \text{RSH} \quad (2) \\
\text{A} - &\rightarrow \text{further nonenzymic reactions} \quad (3) \\
2\text{RS} - &\rightarrow \text{RSSR} \quad (4)
\end{align*}
\]

where RSH and AH represent thiol and nonphenolic aromatic substrates, respectively. The key steps in the system are the oxidation of thiols to thiyl radicals by enzymically generated Mn\textsuperscript{III} (reaction 1) and hydrogen abstraction at the α-carbon by thiyl radicals to form a benzylic radical (reaction 2). These results preclude formation of an aryl cation radical as proposed by Forrester et al. (27).

Product and kinetic analyses (Tables I and II) demonstrate that molecular oxygen is not required for these reactions. Indeed, oxygen probably inhibits the reaction by competing for the thiyl radical (26) (reactions 5 and 6):

\[
\begin{align*}
\text{RS} - + \text{O}_2 &\rightarrow \text{RSO}_2^- \quad (5) \\
\text{RSO}_2^- + \text{RSH} &\rightarrow \text{RSSR} + \text{H}^+ + \text{O}_2^- \quad (6)
\end{align*}
\]

Although nonphenolic β-aryl ether lignin dimers were effectively cleaved at the C\textsubscript{5}-O bond subsequent to the thiol-mediated formation of benzylic radicals, it seems unlikely that extracellular thiols could play a significant role in the process of lignin degradation by white rot fungi since (a) lignin degradation is greatly stimulated under aerobic conditions, and (b) there is no evidence for free thiols in the extracellular culture media of several white rot fungi. Nevertheless, the effective thiol-mediated degradation of dimeric model compounds and of polymeric lignin (27) by MnP suggests that this system may have potential applications in the degradation of industrial lignins. Further studies on the degradation of polymeric lignin are in progress.

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