Oxidative Cleavage of a Phenolic Diarylpropane Lignin Model Dimer by Manganese Peroxidase from *Phanerochaete chrysosporium*†

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ABSTRACT: In the presence of Mn^{II} and H_2O_2, homogeneous manganese peroxidase oxidized 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-methoxyphenyl)-1,3-dihydroxypropane (I) to yield 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-methoxyphenyl)-1-oxo-3-hydroxypropane (II), 2,6-dimethoxy-1,4-benzoquinone (III), 2,6-dimethoxy-1,4-dihydroxybenzene (IV), 1-(4-methoxyphenyl)-1-oxo-2-hydroxyethane (V), 1-(4-methoxyphenyl)-1,2-dihydroxyethane (VI), syringaldehyde (VIII), and 2-(4-methoxyphenyl)-3-hydroxypropanal (IX). Chemically prepared manganese(III) malonate catalyzed the same reactions. Oxidation of I in $H_2{}^{18}O$ under argon resulted in >80% incorporation of ^{18}O into the phenylglycol VI, the hydroquinone IV, and the quinone III. Oxidation of I in $H_2{}^{18}O$ under aerobic conditions resulted in 40% incorporation of ^{18}O into VI but no ^{18}O incorporation into V. Finally, oxidation of I under $^{18}O_2$ resulted in 89% and 28% incorporation of ^{18}O into V and VI, respectively. These results are explained by mechanisms involving the one-electron oxidation of the substrate I by enzyme-generated Mn^{III} to produce a phenoxy radical intermediate I′. Subsequent C_α–C_β bond cleavage of the radical intermediate yields syringaldehyde (VIII) and a C_6–C_2 benzylic radical. Syringaldehyde is oxidized by Mn^{III} in several steps to a cyclohexadiene cation intermediate I″, which is attacked by water to yield the benzoquinone III. The C_6–C_2 radical is scavenged by O_2 to form a peroxy radical that decomposes to V and VI. The C_6–C_2 radical is also oxidized by Mn^{III}, leading to the formation of VI. Alternatively, the radical I′ undergoes subsequent oxidation by Mn^{III} to yield a cyclohexadiene cation I″. Attack of the cation by water yields a triol that undergoes alkylphenyl cleavage to yield the phenylpropanal IX and the hydroquinone IV. The latter is oxidized by Mn^{III} to yield the quinone III. Finally, the cation intermediate I″ loses a proton to yield a quinone methide that rearranges to form the diarylpropanone II. In these reactions, Mn^{III} generated by manganese peroxidase catalyzes both formation of the substrate phenoxy radical and oxidation of carbon-centered radical intermediates, to yield reactive cations.

Lignin is a complex heterogeneous and random phenyl propanoid polymer that constitutes 20–30% of woody plants (Sarkanen & Ludwig, 1971). Since the biodegradation of cellulose is retarded by the presence of lignin (Crawford, 1981; Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989), the catabolism and potential utilization of this polymer are of enormous significance. White rot basidiomycetous fungi are primarily responsible for the initiation of the decomposition of lignin in wood (Crawford, 1981; Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989). When cultured under ligninolytic conditions, the white rot basidiomycete *Phanerochaete chrysosporium* produces two extracellular heme peroxidases (Gold et al., 1984, 1989; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985; Renganathan et al., 1985; Buswell & Odier, 1987; Kirk & Farrell, 1987) that, along with an H_2O_2-generating system (Kirk & Farrell, 1987), appear to be the major components of its lignin degradative system. The structure and mechanism of lignin peroxidase (ligninase) have been studied extensively (Gold et al., 1984, 1989; Kuwahara et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985; Renganathan & Gold, 1986; Buswell & Odier, 1987; Kirk & Farrell, 1987; Leisola et al., 1987; Tien, 1987). Manganese peroxidase (MnP)[1] has also been purified and characterized (Kuwahara et al., 1984; Glenn & Gold, 1985; Glenn et al., 1986; Paszczynski et al., 1986). This enzyme exists as a series of isozymes (p*I* = 4.2–4.9), contains one iron protoporphyrin IX prosthetic group, and is a glycoprotein of M_r ~46 000 (Kuwahara et al., 1984; Glenn & Gold, 1985; Glenn et al., 1986; Paszczynski et al., 1986; Leisola et al., 1987). A cDNA encoding an MnP isozyme has recently been isolated, and the nucleotide sequence has been determined (Pribnow et al., 1989). MnP catalyzes the H_2O_2- and Mn^{II}-dependent oxidation of a variety of phenols, amines, and organic dyes (Glenn & Gold, 1985; Glenn et al., 1986; Paszczynski et al., 1986; Wariishi et al., 1988; Gold et al., 1989), and its catalytic activity is stimulated by various organic acids such as lactate (Kuwahara et al., 1984; Glenn & Gold, 1985; Glenn et al., 1986).

Electronic absorption, EPR, and resonance Raman spectral evidence (Glenn & Gold, 1985; Mino et al., 1988; Wariishi et al., 1988; Gold et al., 1989) indicates that the heme iron in the native enzyme is in the high-spin pentacoordinate ferric state with histidine coordinated as the fifth ligand. Spectral and kinetic evidence (Wariishi et al., 1988, 1989) indicates that the H_2O_2-oxidized states (compounds I and II) and the catalytic cycle of MnP are similar to those of lignin and horseradish peroxidases. Most importantly, it has been demonstrated that MnP oxidizes Mn^{II} to Mn^{III} and that the Mn^{III}

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[1] Abbreviations: MnP, manganese peroxidase; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; GCMS, gas chromatography mass spectrometry; TMS, trimethylsilyl; MS, mass spectrum; FT-NMR, Fourier transform nuclear magnetic resonance.

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in turn oxidizes the organic substrates (Glenn & Gold, 1985; Glenn et al., 1986; Paszczynski et al., 1986; Wariishi et al., 1988). Transient-state kinetic analysis (Wariishi et al., 1989) has confirmed that Mn^{II}/Mn^{III} acts as a redox couple rather than as an enzyme binding activator. Kinetic (Wariishi et al., 1989) and spectroscopic evidence (Glenn & Gold, 1985; Glenn et al., 1986; Wariishi et al., 1988) indicates that organic acids such as lactate chelate Mn^{III}, thereby facilitating its release from the manganese–enzyme complex. In addition, chelation by organic acids also stabilizes the Mn^{III} at a relatively high redox potential (0.9–1.2 V) (Waters & Littler, 1965; Demmber et al., 1980). Preliminary studies on the substrate specificity of the enzyme system have also been reported (Kuwahara et al., 1984; Glenn & Gold, 1985; Glenn et al., 1986; Paszczynski et al., 1986). These studies have utilized simple phenols, amines, and dyes. Since ~15% of the phenylpropanoid units of native lignin are free phenols (Sarkanen & Ludwig, 1971), it has been assumed that the oxidation of these phenolic units by MnP results in polymeric degradation. Herein, we report the mechanism of oxidative cleavage of a free phenolic lignin substructure by MnP. These results imply a role for the enzyme in the degradation of lignin.

Materials and Methods

Manganese peroxidase isozyme I was purified from the extracellular medium of an acetate-buffered agitated culture of *P. chrysosporium* strain OGC101 (Alic et al., 1987) as previously reported (Glenn & Gold, 1985; Wariishi et al., 1989). The purified enzyme was electrophoretically homogeneous and had a p*I* of 4.9.

Enzyme Reactions. Model compound oxidations were carried out at 37 °C for 30 min in 1 mL of 50 mM sodium lactate, pH 4.5 (citrate, malonate, or oxalate was substituted for lactate as indicated), containing MnP (5 μg), substrate (0.2 mM), and $MnSO_4$ (0.5 mM). Reactions were initiated by adding H_2O_2 (0.2 mM).

^{18}O Incorporation Experiments. For experiments conducted under $^{18}O_2$, reaction vessels contained enzyme, $MnSO_4$, 50 mM sodium lactate, and substrate in one compartment and H_2O_2 in the other. The vessels were evacuated, flushed with scrubbed argon (3×), and equilibrated with $^{18}O_2$ (95%, Monsanto Research Corp.) as previously described (Kuwahara et al., 1984; Renganathan et al., 1986) after which the contents were mixed.

For experiments conducted in $H_2{}^{18}O$, reaction mixtures were enriched with $H_2{}^{18}O$ (60%) and incubated under 100% O_2 or argon as previously described (Renganathan et al., 1986).

Oxidation of the Phenolic Diarylpropane I by Mn^{III}–Malonate Complex. Mn^{III}–malonate complex (4 mM stock solution) was prepared by dissolving manganese(III) acetate (Aldrich) in 0.1 M sodium malonate immediately prior to use. Reaction mixtures (1.6 mL) contained substrate (I) (0.2 mM), Mn^{III}-malonate (0.2 or 2.0 mM) in 50 mM sodium malonate buffer, pH 4.5. Reactions were carried out for 5 min at room temperature. The reactions were saturated with NaCl, extracted with EtOAc (3 × 1 mL), dried over Na_2SO_4, evaporated with N_2, and analyzed either directly or following derivatization (BSTFA:pyridine 2:1 v/v). GCMS was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP5790A GC and a 25-m fused silica column (DB-5, J & W Science). ^1H NMR spectra were determined with a JEOL FX90Q-FT-NMR with chemical shifts expressed as parts per million (δ) downfield from an internal standard of tetramethylsilane.

Preparation of Compounds. 1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(4-methoxyphenyl)-1,3-dihydroxypropane (I) was synthesized by the condensation of *o*-benzylsyringaldehyde and the methyl ester of methoxyphenylacetic acid followed by debenzylation and reduction of the resultant methyl ester as previously described (Nakatsubo & Higuchi, 1975). MS (*m/z*) (tri-TMS ether) 550 (M^+, 0.8%), 535 (1.2), 485 (9.9), 385 (5.8), 327 (100), 297 (11.2), 223 (27.5), 134 (26.0). ^1H NMR ($CDCl_3$) δ 3.05–3.12 (C_β 1 H, m), 3.74–3.77 (C_γ 2 H, m), 3.80 [Ar(B)OCH_3 3 H, s], 3.83 [Ar(A)OCH_3 6 H, s], 4.90 (C_α 1 H, d), 6.47 [Ar(B) 2 H, s], 6.89 [Ar(B) 2 H, d], 7.17 [Ar(A) 2 H, d].

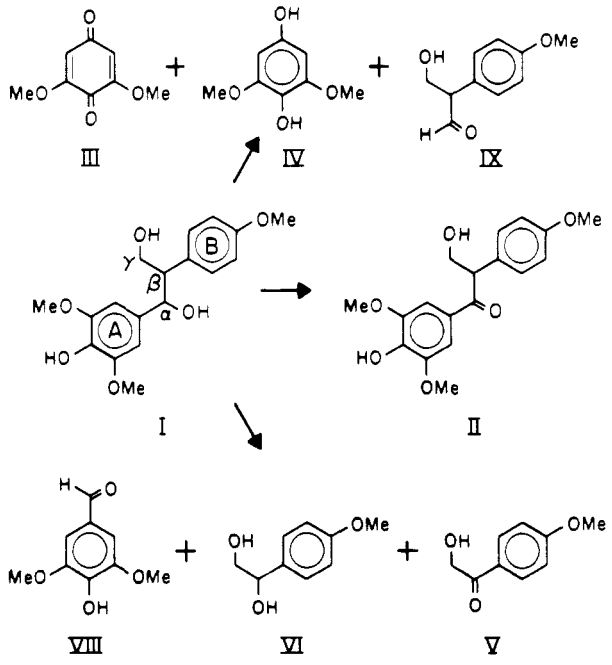

FIGURE 1: Structures of the substrate I and manganese peroxidase produced products II–VI, VIII, and IX identified in these studies. Reactions were conducted and products isolated and analyzed as described in the text.

1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(4-methoxyphenyl)-1-oxo-3-hydroxypropane (II) was prepared by the oxidation of I using 1 equiv of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in dioxane (room temperature, 16 h) (Feiser, 1982). MS (*m/z*) (di-TMS ether) 476 (M^+, 3.3%), 461 (6.2), 386 (10.0), 342 (26.1), 253 (100), 223 (7.9), 134 (20.6).

2,6-Dimethoxy-1,4-benzoquinone (III) was prepared from 2,6-dimethoxyphenol in nitric acid (50% in EtOH) (40 °C, 5 h) as previously described (Baker, 1941).

2,6-Dimethoxy-1,4-dihydroxybenzene (IV) was prepared by the reduction of III using sodium dithionite in water. MS (*m/z*) (di-TMS ether) 314 (M^+, 100%), 299 (11.8), 284 (87.3), 269 (3.9), 254 (30.2), 224 (5.0), 112 (7.2).

1-(4-Methoxyphenyl)-1-oxo-2-hydroxyethane (V), 1-(4-methoxyphenyl)-1,2-dihydroxyethane (VI), and 1-(3,4-diethoxyphenyl)-2-(4-methoxyphenyl)-1,3-dihydroxypropane (VII) were prepared as previously reported (Enoki & Gold, 1982). V: MS (*m/z*) (TMS ether) 238 (M^+, 2.2%), 223 (38.2), 207 (19.2), 149 (2.5), 135 (100), 121 (12.5). VI: MS (*m/z*) (di-TMS ether) 312 (M^+, 1.3%), 297 (9.3), 222 (6.2), 209 (100), 150 (5.9), 117 (14.8).

Syringaldehyde (VIII), MS (*m/z*) (TMS ether) 254 (M^+, 42.1%), was obtained from Aldrich and recrystallized before use. All other chemicals were of reagent grade.

Results

As shown in Figure 1, the phenolic diarylpropane I was oxidized by MnP under aerobic conditions to yield seven identifiable products. The diarylpropanone II, benzoquinone III, hydroquinone IV, phenylketol V, phenylglycol VI, and

Table I: Yield of Products from the Oxidation of I

substrate	oxidant	conditions[a]	products[b] (mol % of starting substrate) I	II	III	IV	V	VI	VIII	IX
I	MnP/Mn^{II}	O_2	14	36	11	2.1	1.6	2.6	1.8	8
I	MnP/Mn^{II}	Ar	12	38	12	2.4	0	5.0	2.0	10
IV	MnP/Mn^{II}	O_2	–	–	82	10	–	–	–	–
VIII	MnP/Mn^{II}	O_2	–	–	8.0	T[c]	–	–	83	–
I	Mn^{III}	O_2	18	42	6.5	3.2	1.4	2.8	2.5	7

[a] O_2 = aerobic, under 100% molecular oxygen; Ar = anaerobic, under argon. [b] Products were identified and quantitated as described in the text. [c] T = trace.

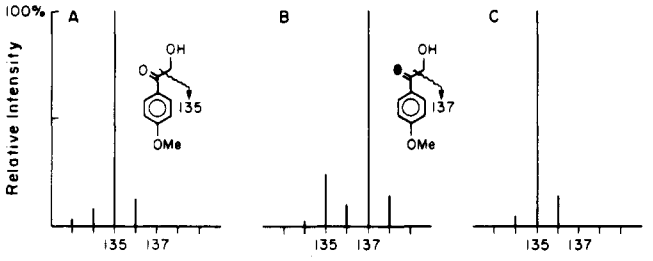

FIGURE 2: Portion of the MS of the phenylketol V showing the anisyl aldehyde radical fragment ion region. (A) Product formed during the oxidation of I under $^{16}O_2$ and $H_2{}^{16}O$. (B) Product formed under $^{18}O_2$ in $H_2{}^{16}O$. (C) Product formed under $^{16}O_2$ and $H_2{}^{18}O$. (●) Oxygen derived from O_2.

Table II: Incorporation of ^{18}O (%) into Products during the Oxidation of the Diarylpropane I

products	m/z	conditions $^{18}O_2/H_2{}^{16}O$	$Ar/H_2{}^{18}O$	$^{16}O_2/H_2{}^{18}O$
III	168/170	2.5[a]	83.4	76.6
IV	314/316	2.2	86.6	78.5
V	135/137	88.8	ND[b]	0
VI	209/211	28.4	89.3	42.7

[a] Percent incorporation of ^{18}O = ^{18}O content of product/^{18}O content of the O_2 or H_2O in the reaction mixture. Products IV–VI were silylated derivatives. Products were isolated, silylated, and analyzed as described in the text. [b] ND = V was not detected when the reactions were conducted under anaerobic conditions.

syringaldehyde VIII were identified by comparison of their retention times on GC and by comparison of their MS spectra with those of chemically synthesized standards. GCMS data alone indicated the occurrence of 2-(4-methoxyphenyl)-3-hydroxypropanal (IX) and the unsaturated carbonyls 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-methoxyphenyl)-1-oxo-2-propene (X) and 2-(4-methoxyphenyl)-2-propen-1-al (XI). IX: MS (*m/z*) (TMS ether) 252 (M^+, 37.5%), 237 (5.4), 223 (50.8), 222 (100), 192 (11.4), 162 (30.0), 148 (8.8), 134 (72.2), 121 (86.5). X: MS (*m/z*) (TMS ether) 386 (M^+, 62.0), 371 (11.3), 355 (17.5), 253 (100), 241 (10.0), 133 (35.1). XI: MS (*m/z*) 162 (M^+, 54.6%), 133 (100%), 118 (10.2). The formation of similar α,β unsaturated carbonyls during mass spectrometric analysis has been reported (Kawai et al., 1988). To confirm that the α,β unsaturated carbonyl X could be derived from the diarylpropanone II, the latter was purified preparatively by TLC using solvent systems a (benzene:acetone, 1:1) and b (chloroform:methanol, 95:5), silylated, and analyzed by GCMS as above. In addition to II, a trace amount of X was observed.

The propanal IX was also purified from the reaction mixture by TLC without derivatization by using solvent systems a and b and analyzed by direct inlet mass spectrometry. IX: MS (*m/z*) 180 (M^+, 30.3%), 162 (11.5), 133 (13.4), 121 (100), 91 (6.9), 78 (13.5), 77 (12.2). When this underivatized fraction was analyzed by GCMS, a peak corresponding to the α,β unsaturated carbonyl XI was seen, indicating that H_2O was readily lost during thermal treatment in the GC.

Yield of products (Table I) was determined by using the GC with FID detection and 4,4′-dimethoxybenzhydrol as an internal standard. When the enzyme-catalyzed reaction was conducted under anaerobic conditions, the phenylketol product (V) was not detected and approximately twice as much phenylglycol VI was obtained. Identical products were found when the reaction was conducted in lactate, citrate, malonate, or oxalate buffers. No products were obtained when the reaction was conducted in the absence of either enzyme, H_2O_2, or Mn^{II}.

MnP could not catalyze the oxidation of either the phenylglycol VI or the nonphenolic diarylpropane VII under similar conditions in lactate, citrate, malonate, or oxalate buffers.

Oxidation of Monomeric Phenols by MnP. MnP readily catalyzed the quantitative conversion of 2,6-dimethoxy-1,4-dihydroxybenzene (IV) to 2,6-dimethoxy-1,4-benzoquinone (III) (Table I). When syringaldehyde (VIII) was used as the substrate, the benzoquinone III and a trace of the hydroquinone were found as oxidation products (Table I). The presence of oxygen did not affect the products obtained from substrates IV and VIII. The oxidation of syringaldehyde (VIII) was considerably slower than the oxidation of syringic acid under the same conditions (data not shown).

^{18}O Studies. Figure 2 and Table II show the anisyl aldehyde ion fragment for the phenylketol V formed under a variety of conditions. V formed under $^{16}O_2$ and in $H_2{}^{16}O$ exhibited a

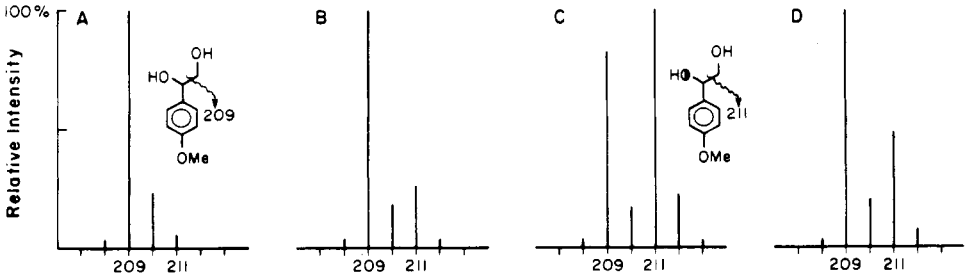

FIGURE 3: Portion of the MS of the TMS ether derivative of the phenylglycol VI showing the anisyl alcohol radical fragment ion regions. (A) Product formed during the oxidation of I under $^{16}O_2$ in $H_2{}^{16}O$. (B) Product formed under $^{18}O_2$ in $H_2{}^{16}O$. (C) Product formed under argon and in $H_2{}^{18}O$. (D) Product formed under $^{16}O_2$ and $H_2{}^{18}O$. (◑) Oxygen derived from H_2O.

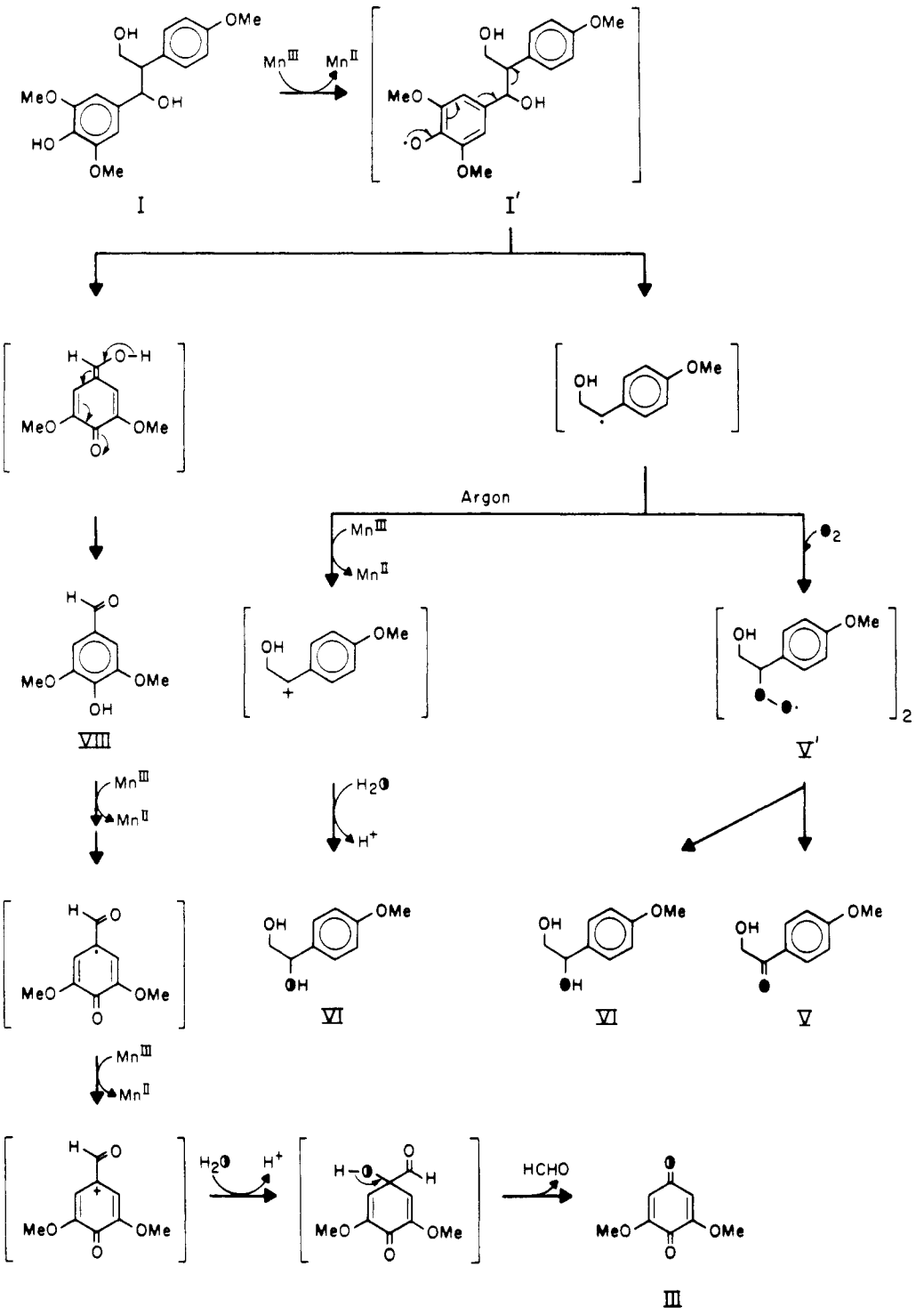

FIGURE 4: Proposed pathways for the C_α–C_β cleavage of the substrate I by manganese peroxidase. (●) Oxygen derived from O_2. (◑) Oxygen derived from H_2O.

fragment ion peak at m/z 135. When V was formed under $^{18}O_2$ and in $H_2{}^{16}O$, 88% incorporation of ^{18}O occurred at the carbonyl position. When V was formed under $^{16}O_2$ and in $H_2{}^{18}O$, ^{18}O was not incorporated.

Figure 3 and Table II show the anisyl alcohol ion fragment for the phenylglycol VI formed under a variety of conditions. VI formed under $^{16}O_2$ and in $H_2{}^{16}O$ exhibited a fragment ion peak at m/z 209. When VI was formed under argon and in $H_2{}^{18}O$, 89% incorporation of ^{18}O was found at the 1-position. In contrast, under $^{16}O_2$ and in $H_2{}^{18}O$, only 42% incorporation of ^{18}O was detected at the 1-position of VI. Finally, when VI was formed under $^{18}O_2$ but in $H_2{}^{16}O$, approximately 28% incorporation of ^{18}O was found at the 1-position. In all cases the M + 4 fragment ion was not detected, indicating that only a single atom of oxygen was incorporated into products V and VI. Table II also shows the ^{18}O content of the benzoquinone III and hydroquinone IV products. The benzoquinone and hydroquinone each contained ~1 atom of ^{18}O when those products were formed in $H_2{}^{18}O$ under either aerobic or anaerobic conditions. In contrast, when either III or IV was formed under $^{18}O_2$ but in $H_2{}^{16}O$, no significant oxygen incorporation was detected. ^{18}O incorporation was not detected for the diarylpropanone II, syringaldehyde (VIII), or the phenylpropanal IX.

Oxidation of I by Mn^{III}. The phenolic diarylpropane I was oxidized by chemically prepared Mn^{III} (in the presence of

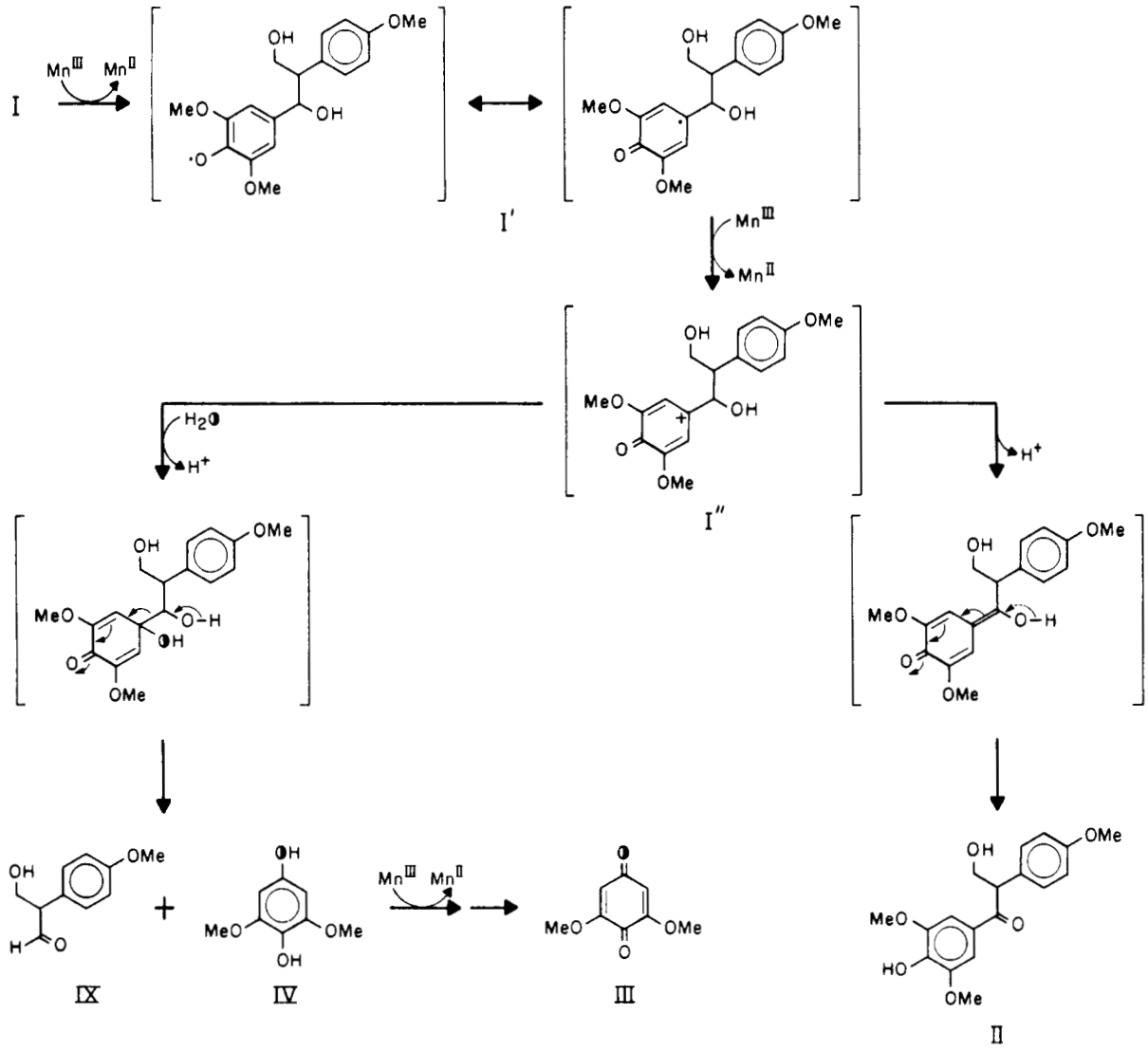

FIGURE 5: Proposed pathways for alkyl phenyl cleavage and C_α oxidation of I by manganese peroxidase.

malonate, lactate, citrate, or oxalate). Table I shows the yields produced when 10 equiv of Mn^{III} was added to I in sodium malonate, pH 4.5. The products were identical with those produced by the enzyme system. Only trace amounts of oxidation products were formed when 1 equiv of Mn^{III} was used.

Discussion

As shown in eq 1–4, spectral and kinetic studies indicate that Mn^{II}/Mn^{III} acts as an obligatory redox couple in the MnP catalytic mechanism (Glenn & Gold, 1985; Glenn et al., 1986; Wariishi et al., 1988). These experiments have demonstrated

$$MnP + H_2O_2 \rightarrow MnPI + H_2O \quad (1)$$

$$MnPI + Mn^{II} \rightarrow MnPII + Mn^{III} \quad (2)$$

$$MnPI + AH \rightarrow MnPII + A^\bullet \quad (2')$$

$$MnPII + Mn^{II} \rightarrow MnP + Mn^{III} \quad (3)$$

$$Mn^{III} + AH \rightarrow Mn^{II} + A^\bullet \quad (4)$$

that although Mn^{II} and a variety of phenols are capable of reducing the oxidized enzyme intermediate manganese peroxidase compound I (MnPI) (eq 2, 2'), only Mn^{II} is capable of reducing manganese peroxidase compound II (MnPII) at a kinetically significant rate (eq 3) (Wariishi et al., 1988, 1989). These results indicated that in the absence of Mn^{II} the enzyme cannot complete its catalytic cycle. Kinetic analysis also indicated that certain organic acids such as lactate and malonate facilitate the release of Mn^{III} from its complex with the oxidized enzyme intermediates MnPI and MnPII by forming an Mn^{III}–organic acid complex (Wariishi et al., 1989). These Mn^{III}–organic acid complexes are relatively stable, yet have a high redox potential (~1.0 V) (Waters & Littler, 1965; Demmber et al., 1980; Glenn et al., 1986). Studies on the oxidation of a variety of organic compounds by Mn^{III} have been reported (Waters & Littler, 1965; Arndt, 1981; Glenn et al., 1986), although several of those reactions were conducted under nonphysiological conditions (e.g., organic solvents, high temperature).

Previous work has shown that MnP is capable of oxidizing monomeric phenols (Glenn & Gold, 1985; Glenn et al., 1986; Paszczynski et al., 1986); however, the oxidiation of these simple substrates does not model the oxidative reactions involved in the cleavage of the lignin polymer. Since the mechanism of the MnP-catalyzed oxidation of phenolic lignin substructures had not been examined thoroughly, a study of the oxidation of the phenolic diarylpropane I was undertaken. This particular dimer was chosen because during its oxidation ring condensation via radical coupling is inhibited by the presence of a methoxy group at the 5-position of ring A (Figure 1).

The phenolic diarylpropane I was readily oxidized by MnP to produce a variety of products (Figure 1, Table I). Al-

kyl–phenyl bond cleavage produced the benzoquinone III, hydroquinone IV, and phenylpropanal IX. C_α–C_β bond cleavage produced syringaldehyde (VIII), the phenylketol V, and the phenylglycol VI. Finally, C_α oxidation yielded the diarylpropanone II. Exogenously added syringaldehyde was oxidized by MnP to produce the benzoquinone III. The nonphenolic substrates VI and VII were not oxidized by MnP under these conditions, suggesting that the critical step in the MnP-catalyzed reaction is the oxidation of the phenol to a phenoxy radical by enzyme-generated Mn^{III}. In addition, I was oxidized by chemically prepared Mn^{III} to produce identical products. Identification of these oxidation products in addition to the results of ^{18}O incorporation studies suggests several mechanisms for the MnP-catalyzed oxidation of I.

Mechanism of C_α–C_β Bond Cleavage of the Diarylpropane I. Oxidation of I to a phenoxy radical I′ by enzyme-generated Mn^{III} would result in C_α–C_β bond cleavage, giving rise to a quinone methide and a C_6–C_2 radical (Figure 4). The quinone methide would undergo rearrangement to yield syringaldehyde (VIII). Subsequently, VIII would be oxidized by Mn^{III} to form the corresponding phenoxy radical (Figure 4). Radical rearrangement would result in a cyclohexadienyl radical that also would be oxidized by Mn^{III} to form the corresponding cation (Heiba et al., 1969). The cation is susceptible to attack by water to yield the quinone III and formaldehyde. One atom of ^{18}O from water is incorporated into the benzoquinone (Table II) as predicted by this pathway. Syringic acid is oxidized by MnP to produce quinone III and hydroquinone IV; however, no evidence for its formation was obtained in this study. The C_6–C_2 radical would be scavenged by O_2 under aerobic conditions to form peroxy radical V′. The peroxy radical V′ could decompose via a tetraoxide intermediate as described previously (Ingold, 1969; Renganathan et al., 1986) to form the phenylketol V and phenylglycol VI (Figure 4). The observations that the phenylketol V was formed only under aerobic conditions and that one atom of molecular oxygen was incorporated into V support this mechanism. The observation of 28% incorporation of ^{18}O from $^{18}O_2$ at the 1-position of the phenylglycol VI also supports this mechanism. Alternatively, homolytic cleavage of the hydroperoxide of V′ could lead to the formation of the phenylketol V. Under anaerobic conditions, the C_6–C_2 radical would be oxidized by Mn^{III} to a carbonium ion, which would be attacked by H_2O to yield the phenylglycol VI. The oxidation of a benzylic radical to a benzyl cation by Mn^{III} has been reported (Heiba et al., 1969). The observations (Table II) that one atom of oxygen from water was incorporated into the phenylglycol under anaerobic conditions but that only 43% incorporation of oxygen from water occurred at the 1-position of the phenylglycol VI under aerobic conditions, where both pathways are operative, support these dual mechanistic pathways. The ratio of phenylketol V and phenylglycol VI formed was found to be 1:1.6 (MnP oxidation) and 1:2 (Mn^{III} oxidation) (Table II). In addition, only 28% incorporation of ^{18}O from $^{18}O_2$ into the glycol VI under aerobic conditions was observed. These observations suggest that even under aerobic conditions the C_6–C_2 radical may be attacked by Mn^{III} at a significant rate.

Mechanism of Alkyl Phenyl Cleavage. Oxidation of I by enzyme-generated Mn^{III} would yield the phenoxy radical I′. The alternative resonance form of I′, a cyclohexyldienyl radical (Figure 5), would be oxidized by Mn^{III} to form the corresponding cation (I″) (Heiba et al., 1969). Attack by H_2O would yield the triol intermediate, which readily cleaves at the alkyl–phenyl bond to produce the phenylpropanal IX and the hydroquinone IV. The latter is readily oxidized by Mn^{III} to produce the quinone III. The observation (Table II) that one atom of ^{18}O from water is incorporated into both the quinone III and hydroquinone IV supports this mechanism. The hydroquinone IV is readily oxidized by MnP to form the quinone III (Table I). A phenoxy radical is an intermediate in that reaction (Bridge & Porter, 1958). Finally, loss of an α-proton by the cation intermediate I″ (Figure 5) would result in a quinone methide that rearranges to form the diarylpropanone II. In this case, as predicted, no incorporation of ^{18}O from molecular oxygen or water was seen.

Thus, the phenolic lignin model diarylpropane I is oxidatively cleaved by MnP-generated Mn^{III}. In all of the oxidative mechanisms described in the present study, the initial formation of a phenoxy radical is followed by radical rearrangements and other nonenzymatic reactions. The reactions described herein are similar to but not identical with those reported previously with laccase-catalyzed reactions (Kawai et al., 1987, 1988; Wariishi et al., 1987). The MnP system apparently differs from laccase due to the ability of Mn^{III} to readily oxidize carbon-centered radicals to the corresponding cations, which are subsequently attacked by H_2O.

As we reported earlier, MnP preferentially oxidizes Mn^{II} to Mn^{III}, and the Mn^{III} in turn oxidizes phenolic substrates. This system is advantageous because the Mn^{III} chelated by organic acids can diffuse from the enzyme and oxidize the relatively inaccessible, insoluble lignin polymer. The results reported herein indicate some of the oxidative mechanisms that are likely to be involved in the MnP-catalyzed oxidation of lignin. The oxidation of diols and ketols (Zonis & Kornilova, 1950; Zonis, 1954; Arndt, 1981) and olefins (Bush & Finkbeiner, 1968; Heiba et al., 1968; Arndt, 1981) by Mn^{III} complexes has been reported previously. Studies on the oxidation of these compounds and of polymeric lignin by MnP and Mn^{III} are in progress.

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Registry No. I, 121013-64-7; I methyl ester, benzylated precursor, 121013-65-8; II, 121013-66-9; III, 530-55-2; IV, 15233-65-5; V, 4136-21-4; VI, 13603-63-9; VII, 83459-30-7; VIII, 134-96-3; VIII *O*-benzyl derivative, 6527-32-8; IX, 121013-67-0; Mn, 7439-96-5; methoxyphenolacetic acid methyl ester, 23786-14-3; lignin, 9005-53-2; peroxidase, 9003-99-0.

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Development of a Novel Photoreactive Calmodulin Derivative: Cross-Linking of Purified Adenylate Cyclase from Bovine Brain†

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ABSTRACT: A novel photoreactive calmodulin (CaM) derivative was developed and used to label the purified CaM-sensitive adenylate cyclase from bovine cortex. ^{125}I-CaM was conjugated with the heterobifunctional cross-linking agent *p*-nitrophenyl 3-diazopyruvate (DAPpNP). Spectral data indicated that diazopyruvoyl (DAP) groups were incorporated into the CaM molecule. Iodo-CaM–DAPs behaved like native CaM with respect to (1) Ca^{2+}-dependent enhanced mobility on sodium dodecyl sulfate–polyacrylamide gels and (2) Ca^{2+}-dependent stimulation of adenylate cyclase activity. ^{125}I-CaM–DAP photochemically cross-linked to CaM-binding proteins in a manner that was both Ca^{2+} dependent and CaM specific. Photolysis of forskolin–agarose-purified adenylate cyclase from bovine cortex with ^{125}I-CaM–DAP produced a single cross-linked product which migrates on sodium dodecyl sulfate–polyacrylamide gels with an apparent molecular weight of approximately 140 000.

Calmodulin (CaM) mediates a wide variety of calcium-dependent cellular processes, including cyclic nucleotide metabolism [reviewed by Stoclet et al. (1987)]. Its action as a regulator of brain cyclic AMP (cAMP) phosphodiesterase was discovered by Cheung (1970) and Kakiuchi et al. (1970), and it was subsequently found to regulate the adenylate cyclase activity from brain (Brostrom et al., 1975; Cheung et al., 1975). CaM appears to mediate the calcium-dependent stimulation of adenylate cyclase by interacting directly with the catalytic subunit of this enzyme (Smigel, 1986; Minocherhomjee et al., 1987). While GTP or GTP-binding protein subunits are not required for the stimulation of adenylate cyclase by CaM (Heideman et al., 1982; Seamon & Daly,

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