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Mechanism of β-Aryl Ether Dimeric Lignin Model Compound Oxidation by Lignin Peroxidase of *Phanerochaete chrysosporium*†

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ABSTRACT: Homogeneous lignin peroxidase (LiP) oxidized 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane, I, and 1-(4-ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1,3-dihydroxypropane, III, to yield four products from each substrate. The isolated products indicate that both the α,β and β-ether bonds were cleaved in the dimeric substrates or oxidized derivatives. LiP oxidized 1-(4-ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)phenoxy]-1,3-dihydroxypropane, II, to yield only two products, suggesting that the α,β bond of the substrate was cleaved. LiP oxidized 1-phenyl-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane, IV, to yield products that indicated that the β-ether bond was cleaved but the α,β bond remained intact. Cleavage of I in $H_2{}^{18}O$ resulted in 42% incorporation of ^{18}O at the 1-position of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane, X. Oxidation of the 1-[^{18}O]hydroxy-labeled dimer IIIa resulted in only 50% retention of the ^{18}O at the 1-position of the phenylglycerol X. Oxidation of the β-^{18}O-ether dimer resulted in 99% retention of the ^{18}O in the phenylglycerol product but only 85% of the ^{18}O in the phenolic counterpart product. Finally, when the 3-[^{18}O]hydroxy-labeled β-ether dimer IIIb was used as a substrate, the phenolic product XV retained 26% of the ^{18}O, suggesting that a rearrangement had taken place. These results are explained by mechanisms involving oxidation of either the phenyl (A) and/or the phenoxy (B) ring of the dimer to an aryl cation radical by the H_2O_2-oxidized enzyme. Oxidation of the A ring of I, II, or III to an aryl cation radical is followed by α,β cleavage, giving rise to a benzaldehyde and a C_6C_2 benzylic radical. The latter would be scavenged by O_2 to yield a glycolaldehyde and a phenol. The phenol would retain the ^{18}O label orginally in the β-ether oxygen of the substrate. Oxidation of the B ring in dimers I, III, or IV apparently is followed by intramolecular attack by the 1-hydroxyl on the methoxylated aryl cation radical carbon, releasing methanol and forming a benzodioxane radical transition state. This intermediate is hydrolyzed to yield a phenylglycerol and a catechol. Alternatively, oxidation of the B ring is followed by intramolecular attack of the 3-hydroxyl on the ether-linked B ring carbon, leading to a γ-aryl ether dimer. Radical cleavages of this intermediate yield a phenol and a benzaldehyde.

Lignin is a complex, optically inactive, and random phenylpropanoid polymer that comprises 20–30% of woody plants (Sarkanen, 1971; Crawford, 1981). Under nitrogen limitation, the white rot basidiomycete *Phanerochaete chrysosporium* efficiently degrades lignin during the secondary metabolic phase of growth (Kirk et al., 1978; Weinstein et al., 1980; Gold et al., 1982). Under ligninolytic conditions, *P. chrysosporium* produces at least two heme peroxidases (Gold et al., 1984; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985), one of which, lignin peroxidase (ligninase, diarylpropane oxygenase), has been purified to homogeneity (Gold et al.,

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1984; Tien & Kirk, 1984; Renganathan et al., 1985). Three different molecular forms of lignin peroxidase (LiP)[1] have recently been identified (Renganathan et al., 1985). The main isozymic form, LiP(II), has a $M_r \simeq 41\,000$ and is a glycoprotein with a 6% neutral carbohydrate content. All isozymic forms have a single iron protoporphyrin IX prosthetic group (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). Electronic absorption (Gold et al., 1984; Renganathan et al., 1985), EPR (Andersson et al., 1985), and resonance Raman data (Andersson et al., 1985; Kuila et al., 1985) suggest that the heme iron in the native protein is in the high-spin ferric state, with histidine coordinated as the fifth ligand and possibly a weakly bound water as a sixth ligand.

Initially this enzyme was characterized as an oxygenase, since under aerobic conditions one atom of dioxygen is incorporated into various products (Kuwahara et al., 1984; Gold et al., 1984; Tien & Kirk, 1984). However, our most recent studies indicate that oxygenation proceeds even under rigorously anaerobic conditions, with incorporation of ^{18}O from $H_2{}^{18}O$ into the products (Renganathan et al., 1986). In addition, we have characterized the LiP H_2O_2 reaction intermediates, compounds I–III, which are analogous to those of horseradish peroxidase (HRP) (Renganathan & Gold, 1986). There recent results demonstrate that the enzyme is, in fact, a peroxidase.

Since the diarylpropane dimeric model compound is oxidized by the fungus via a discrete cleavage pathway with readily identifiable products (Enoki & Gold, 1982; Nakatsubo et al., 1982), previous studies have concentrated on the enzymic cleavage of this substrate. However, since the arylglycerol–β–aryl ether linkage constitutes about 50% of the internuclear linkages in lignin, the oxidation of model compounds containing this linkage is of great interest. In early studies we conclusively demonstrated by gas chromatography–mass spectrometry (GCMS) analysis of reaction products that ligninolytic cultures of *P. chrysosporium* cleaved non-phenolic β-ether dimers or their oxidized derivatives at the α,β (Weinstein et al., 1980; Enoki et al., 1980) and β-ether bonds (Enoki et al., 1981a,b). Recently, on the basis of the products isolated from fungal cultures, two pathways for β-aryl ether dimer cleavage have been proposed (Umezawa et al., 1983a,b). Homogeneous LiP oxidizes β-aryl ether dimers to yield analogous products, indicating that this is the enzyme responsible for these cleavage reactions (Gold et al., 1984; Renganathan et al., 1985). However, detailed studies on the enzymic oxidation of β-aryl ether dimers have not been previously carried out. We have now studied the oxidation of a variety of structurally different β-ether models and ^{18}O-labeled compounds under anaerobic conditions. The results of this work enable us to propose mechanisms for both α,β and β-ether bond cleavage by this enzyme.

Materials and Methods

Enzyme. Lip(II) was purified from acetate-buffered agitated cultures of *P. chrysosporium* as previously described (Gold et al., 1984; Renganathan et al., 1985). The purified protein was electrophoretcally homogeneous and had an R_z value of ~5.0.

Enzyme Reactions. Model compound oxidations were carried out at 37 °C for 15 min in 1 mL of sodium succinate, pH 4.5, containing 5 μg of LiP and 0.02% substrate (added as a 10% solution in DMF). Reaction mixtures were evacuated and flushed with argon before the reaction was initiated by addition of similarly treated H_2O_2 to a final concentration of 100 μM. Following the reaction, mixtures were extracted with EtOAc (3 × 1 mL), dried over Na_2SO_4, evaporated under N_2, and silylated (BSTFA:pyridine, 2:1 v/v). Products were identified by capillary GC and GCMS. GCMS was performed at 70 eV on a VG analytical 7070E mass spectrometer fitted with an HP 5790A GC and a 15-m fused silica column (DB-5, J&W Science).

Preparation of Compounds. 1-(4-Ethoxy-3-methoxyphenyl)-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane (I) was prepared by a modification of a previous procedure (Katayama et al., 1981). (a) Ethyl 2-(2-methoxy-4-propylphenoxy)acetate (II) was prepared from 1-(4-hydroxy-3-methoxyphenyl)propane and ethyl 2-bromoacetate. (b) Condensation of 4-ethoxy-3-methoxybenzaldehyde and the above ester with lithium diisopropylamide (LDA), at −70 °C under N_2 in THF, for 1 h, yielded ethyl 3-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxy-4-propylphenoxy)-3-hydroxypropionate. (c) Reduction with $LiAlH_4$ in THF at room temperature under N_2 yielded I: MS m/z 390 (M^+); ^1H NMR (diacetate) ($CDCl_3$) δ 0.91 (3 H, t), 1.44 (3 H, t), 1.58 (2 H, m), 2.00, 2.02, 2.07 (6 H, 3 s), 2.51 (2 H, t), 3.77, 3.81, 3.85 (6 H, 3 s), 4.08 (2 H, q), 4.33 (2 H, m), 4.60 (1 H, m), 6.05 (1 H, dd), 6.6–7.0 (6 H, m).

1-(4-Ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-phenoxy]-1,3-dihydroxypropane (II) was prepared by condensing 4-ethylvanillin and ethyl 2-[4-(hydroxymethyl)phenoxy]acetate as described above: MS m/z 348 (M^+); ^1H NMR (triacetate) ($CDCl_3$) δ 1.44 (3 H, t), 1.97, 2.03, 2.09 (9 H, 3 s), 3.87 (3 H, s), 4.08 (2 H, q), 4.0–4.2 (2 H, m), 4.71 (1 H, m), 5.04 (2 H, s), 6.02 (1 H, d), 6.1–7.4 (7 H, m).

1-(4-Ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1,3-dihydroxypropane (III) was prepared by the above condensation method (Katayama et al., 1981) using 4-ethylvanillin and ethyl 2-[4-(hydroxymethyl)-2-methoxyphenoxy]acetate: MS m/z 378 (M^+); ^1H NMR (triacetate) ($CDCl_3$) δ 1.46 (3 H, t), 2.02, 2.07, 2.09 (9 H, 3 s), 3.80, 3.85 (6 H, 2 s), 4.07 (2 H, q), 4.0–4.4 (2 H, m), 4.70 (1 H, m), 5.04 (2 H, s), 6.03 (1 H, d), 6.7–7.1 (6 H, m).

1-(4-Ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1-[^{18}O]hydroxy-3-hydroxypropane (IIIa) was prepared as follows: (a) condensation of 1-(4-ethoxy-3-methoxyphenyl)-2-bromo-1-oxoethane with 3-methoxy-4-hydroxybenzyl alcohol as described previously (Enoki et al., 1980, 1981a); (b) hydroxymethylation using HCHO; (c) treatment with 5% HCl from Cl_2 gas in $H_2{}^{18}O$ (96%); (d) ketone oxygen exchange in 5% dioxane, dilute HCl, and $H_2{}^{18}O$; (e) treatment with $NaBH_4$ in $H_2{}^{18}O$, at O °C. MS (trimethylsilyl ether) m/z 596 (M^+). ^1H NMR (triacetate) was identical with that of the ^{16}O-containing compound. ^{18}O incorporation was 81 mol %.

1-(4-Ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1-hydroxy-3-[^{18}O]hydroxypropane (IIIb) was prepared as follows: (a) condensation of 1-(4-ethoxy-3-methoxyphenyl)-2-bromo-1-oxoethane with 3-methoxy-4-hydroxybenzyl alcohol as in Enoki et al. (1980, 1981a); (b) treatment with HCHO (paraformaldehyde) dissolved in $H_2{}^{18}O$ at 90 °C, then cooled on ice, precipitated, and filtered; (c) hydroxymethylation using $HCH^{18}O$ as in Enoki et al. (1980); (d) treatment with $NaBH_4$, in ethanol, at room temperature. NMR of IIIb was identical with that of III. MS (trimethylsilyl ether) m/z 596 (M^+, 2.4%), 298 (8.1%), 283 (10.3%), 253

[1] Abbreviations: LiP, lignin peroxidase; MS, mass spectrum; GCMS, gas chromatography–mass spectrometry; Me_4Si, tetramethylsilane; DMF, dimethylformamide; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; THF, tetrahydrofuran; FT, Fourier transform.

(base ion), 207 (19.9%), 165 (9.7%). ^{18}O incorporation was 69 mol %.

1-Phenyl-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane (IV) was prepared as described in Katayama et al. (1981) by using benzaldehyde and ethyl 2-(2-methoxy-4-propylphenoxy)acetate in the condensation step: MS (trimethylsilyl ether) m/z 460 (M^+); ^1H NMR (diacetate) ($CDCl_3$) δ 0.92 (3 H, t), 1.56 (2 H, m), 1.99 (3 H, s), 2.11 (3 H, s), 2.52 (2 H, t), 3.78 (3 H, s), 4.0–4.2 (2 H, m), 4.60 (1 H, m), 6.10 (1 H, d), 6.6–6.8 (3 H), 7.3–7.4 (5 H).

1-(4-Ethoxy-3-methoxyphenyl)-2-(4,5-dimethyl-2-methoxyphenoxy)-1,3-dihydroxypropane 2-[^{18}O]ether (Va) and the corresponding ^{16}O-containing compound (V) were prepared as follows: (a) 3,4-Dimethylanisole was brominated with dioxane dibromide in ether at −10 °C (Schlegel et al., 1970) to give 2-bromo-4,5-dimethylanisole (VI). (b) VI was treated with n-BuLi and EtMgBr (Gilman et al., 1939) and exposed to $^{18}O_2$ (95% enriched) to yield 2-[^{18}O]hydroxy-4,5-dimethylanisole (VIIa): MS m/z 154 (M^+). (c) Condensation of VIIa with 1-(4-ethoxy-3-methoxyphenyl)-2-bromo-1-oxoethane was carried out. (d) Hydroxymethylation and reduction were carried out as described previously (Enoki et al., 1980). ^{18}O incorporation was 82 mol %. MS m/z 378 (M^+); ^1H NMR (diacetate) ($CDCl_3$) δ 1.44 (3 H, t), 2.04 (6 H, s), 2.08, 2.10, 2.16, 2.18 (6 H, 4 s), 3.77, 3.79, 3.85 (6 H, 3 s), 4.08 (2 H, q), 4.0–4.4 (2 H, m), 4.53 (1 H, m), 6.5–7.0 (5 H, m).

1,2-Dihydroxy-4,5-dimethylbenzene (VIII) was prepared by demethylating VII with trimethylsilyl iodide at room temperature for 2 days (Jung & Lyster, 1977): MS (trimethylsilyl ether) m/z 282 (M^+); ^1H NMR (diacetate) ($CDCl_3$) δ 2.22 (3 H, s), 2.25 (3 H, s), 2.26 (6 H, s), 6.92 (2 H, s).

1-Phenyl-1,2,3-trihydroxypropane (IX) was prepared as follows: (a) treatment of cinnamyl alcohol with m-chloroperoxybenzoic acid in CH_2Cl_2; (b) treatment with 0.1 M NH_2SO_4 (aqueous):dioxane (1:1) at room temperature for 1 h. MS (trimethylsilyl ether) m/z 369 ($M^+ - 15$), 179 (base ion).

1-(4-Ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (phenylglycerol) (X) was prepared as described previously (Enoki et al., 1981a).

3-(4-Ethoxy-3-methoxyphenyl)-2-(hydroxymethyl)-6-propyl-1,4-benzodioxane (XI) was prepared as follows: (a) demethylation of 2-methoxy-4-propylphenol (XII) with trimethylsilyl iodide as above to yield 4-propylcatechol (XIII); (b) coupling of 4-hydroxy-3-methoxycinnamyl alcohol and XIII by use of Ag_2O (Merlini et al., 1980); (c) ethylation using diazoethane. MS (trimethylsilyl ether) m/z 430 (M^+); ^1H NMR (acetate) ($CDCl_3$) δ 0.93 (3 H, t), 1.47 (2 H, t), 1.60 (2 H, m), 2.01 (3 H, s), 2.51 (2 H, t), 3.88 (3 H, s), 4.0–4.4 (2 H), 4.12 (2 H, q), 4.88 (1 H, d), 6.6–7.0 (6 H). ^1H NMR spectra were taken on a JEOL FX-90Q FT-NMR (internal standard Me_4Si).

Results

As shown in Figure 1, the β-aryl ether dimer I was oxidized by lignin peroxidase to yield four aromatic products: 4-ethoxy-3-methoxybenzaldehyde (XIV), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (X), 2-methoxy-4-propylphenol (XII), and 4-propylcatechol (XIII). Lignin peroxidase oxidized the β-aryl ether dimer III to produce four aromatic products: the benzaldehyde XIV, the triol X, 3-methoxy-4-hydroxybenzyl alcohol (XV), and 3,4-dihydroxybenzyl alcohol (XVI) (Figure 1). However, when 1-(4-ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)phenoxy]-1,3-dihydroxypropane (II) was used as a substrate, only two aromatic products were observed: XIV and 4-hydroxybenzyl alcohol (XVII) (Figure

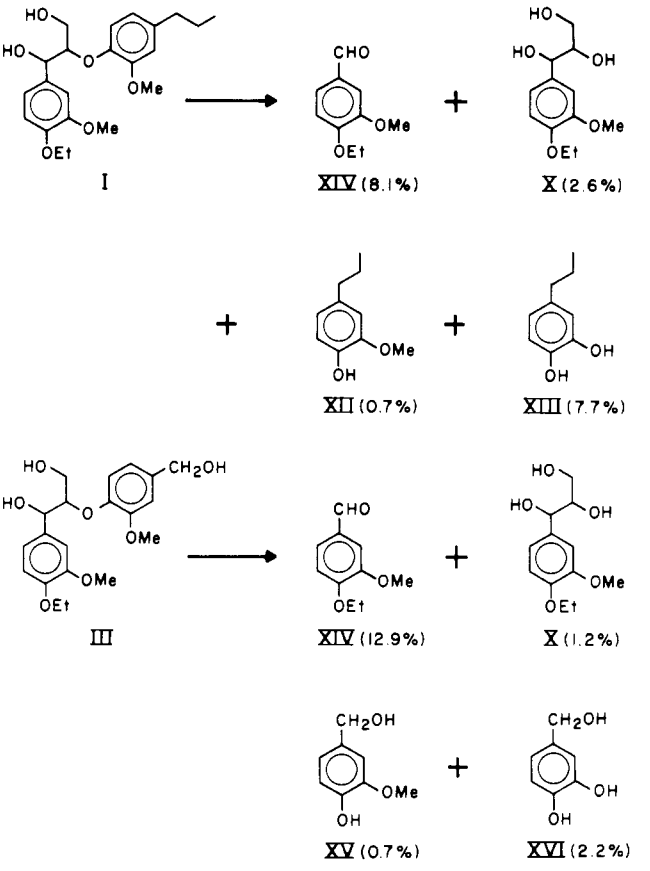

FIGURE 1: Oxidative cleavage of β-aryl ether dimers by homogeneous lignin peroxidase at both the α,β and β-ether bonds. Reactions were carried out and products were extracted and identified as described in the text. Numbers in parentheses refer to the amounts of remaining substrate or product formed, with respect to the amount of initial substrate. % = (mol of product formed/mol of initial substrate) × 100.

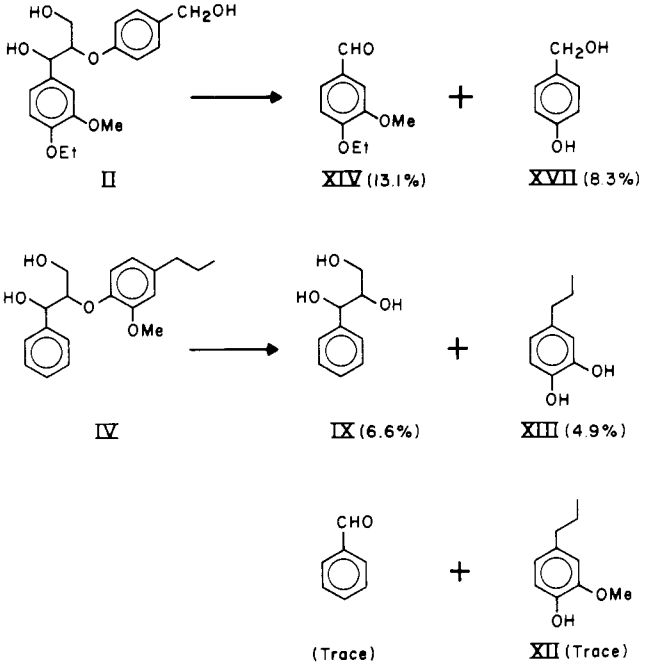

FIGURE 2: Oxidative cleavage of β-aryl ether dimers by homogeneous lignin peroxidase at either the α,β or β-ether bonds. Reactions were carried out and products were extracted and identified as described in the text. Numbers in parentheses refer to the amounts of remaining substrate or product formed, with respect to the amount of initial substrate. % = (mol of product formed/mol of initial substrate) × 100.

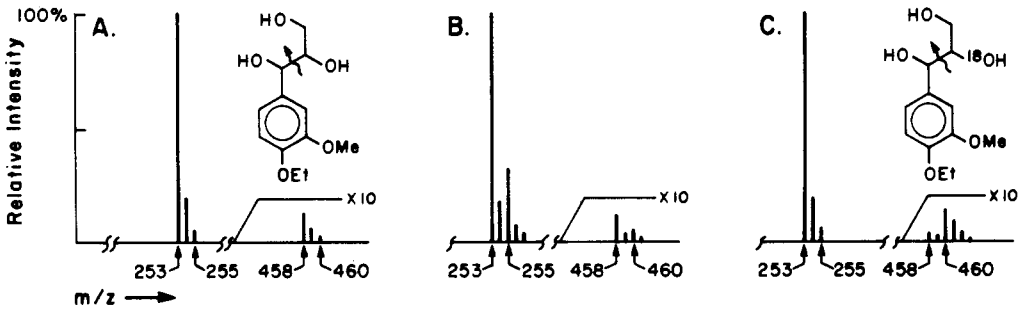

FIGURE 3: Portion of the MS of the trimethylsilyl-derivatized phenylglycerol X showing the molecular ion and benzyl alcohol fragment ion regions. (A) Synthetic ^{16}O-containing phenylglycerol X. (B) Product formed upon the oxidation of β-aryl ether dimer I in 50% enriched $H_2{}^{18}O$. (C) Product formed upon the oxidation of the β-[^{18}O]aryl ether dimer Va (82 mol %).

Table I: Oxidation of Specifically ^{18}O-Labeled β-Aryl Ether Dimers by Lignin Peroxidase

	incorporation into product (mol %)[a]				
substrates[b]	1-[^{18}O]hydroxytriol Xa	2-[^{18}O]-hydroxytriol Xb	3-[^{18}O]hydroxytriol Xc	phenol XIIa	phenol XVa
β-aryl ether I, $H_2{}^{18}O$	42				
1-[^{18}O]hydroxy-β-aryl ether IIIa	50				
2-[^{18}O]aryl ether Va		99		85	
3-[^{18}O]hydroxy-β-aryl ether IIIb			98		26

[a] Mol % incorporation = mol % ^{18}O in product/mol % ^{18}O in substrate. [b] ^{18}O-Labeled substrates or unlabeled substrate and $H_2{}^{18}O$ were oxidized with LiP as described in the text. Substrates and products were extracted, dried, silylated, and analyzed by GCMS as described in the text.

2). Neither the triol X nor a catechol were detected.

With 1-phenyl-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane (IV) as the substrate (Figure 2), two products were identified conclusively: 4-propylcatechol (XII) and 1-phenyl-1,2,3-trihydroxypropane. A third product, 1-phenyl-3-(2-methoxy-4-propylphenoxy)-1,2-dihydroxypropane (XVIII) (not shown in Figure 2), was identified only by its mass spectrum: MS (trimethylsilyl ether) m/z 460 (M^+, 6.4%), 281 (12.7%), 238 (30.9%), 206 (7.6%), 191 (25.5%), 179 (base ion), 165 (21.5%). Benzaldehyde and 2-methoxy-4-propylphenol (XII) were obtained only in trace amounts.

^{18}O Studies. Cleavage of the β-aryl ether dimer I in $H_2{}^{18}O$ (50% enriched) under anaerobic conditions resulted in 42% incorporation of the ^{18}O into the 1-position of the phenylglycerol X as determined from the molecular ion and benzyl alcohol fragment ion peaks (Figure 3, Table I). No ^{18}O incorporation into the 2-position of the phenylglycerol X was detected. Similar observations have been made with intact cultures of *P. chrysosporium* (Umezawa & Higuchi, 1984). Although ^{18}O incorporation was also found in the carbonyl oxygen of the benzaldehyde XIV and in the phenolic hydroxyls of the catechol XIII, these incorporations were probably the result of exchange between the aldehyde or the phenolic hydroxyls and H_2O.

When 1-(4-ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenyl]-1-[^{18}O]hydroxy-3-hydroxypropane (IIIa) (81 mol % excess) was used as a substrate, the 1-[^{18}O]hydroxyphenylglycerol Xa product contained only 41 mol % excess of ^{18}O in the 1-position; i.e., 50% of the ^{18}O was lost (Table I). In addition, no incorporation of ^{18}O into the phenolic hydroxyl of vanillyl alcohol (XV) was detected.

When the β-^{18}O-ether dimer Va (82 mol % excess) was used as a substrate, the product 1-(4-ethoxy-3-methoxyphenyl)-2-[^{18}O]hydroxy-1,3-dihydroxypropane (Xa) contained 81 mol % ^{18}O; i.e., it retained 99% of the ^{18}O (Figure 3, Table I). In contrast, 2-hydroxy-4,5-dimethylanisole (VII) contained 85 mol % ^{18}O. When 2-[^{18}O]hydroxy-4,5-dimethylanisole (VIIIa) was used as a substrate for the enzyme, the recovered phenol completely retained the label under the assay conditions.

Finally, when the 3-[^{18}O]hydroxy β-ether dimer IIIb was used as a substrate, the phenolic product XII retained 26% of the ^{18}O and the phenylglycerol Xc retained 98% of the ^{18}O label (Table I).

Discussion

Recently, lignin peroxidase, a heme-containing, H_2O_2-requiring enzyme, has been purified from the extracellular medium of ligninolytic cultures of the white rot basidiomycete *P. chrysosporium* (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). The homogeneous enzyme oxidizes a variety of lignin model compounds (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985) including diarylpropanes, β-aryl ether dimers, phenylpropanes, and phenylpropenes. The enzyme cleaves the α,β bond of diarylpropanes, generating a benzaldehyde and either a phenylglycol or phenylketol (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). The H_2O_2-oxidized enzyme initiates a one-electron oxidation of the α-phenyl ring of the substrate to form an aryl cation radical (Renganathan et al., 1986; Renganathan & Gold, 1986; Schoemaker et al., 1985; Kersten et al., 1985). Subsequent α,β cleavage of this cation radical produces a benzaldehyde and a C_6C_2 benzylic radical (Hammel et al., 1985; Renganathan et al., 1986). The latter would be scavenged by molecular oxygen to produce phenylglycol or phenylketol products (Kersten et al., 1985; Renganathan et al., 1986; Schoemaker et al., 1985). Recently, we have characterized the oxidized states of LiP as being similar to compounds I and II of HRP (Renganathan & Gold, 1986). Involvement of a benzene cation radical in the LiP-catalyzed oxidation of dimethoxybenzene has also been demonstrated (Kersten et al., 1985). Like chloroperoxidase (Geigert et al., 1983), LiP can dehydrogenate some benzyl alcohols. This reaction appears to proceed through two single-electron oxidation steps, possibly with an aryl cation radical intermediate (Renganathan & Gold, 1986; Tien et al., 1986), as observed for dimethoxybenzene (Kersten et al., 1985).

Our recent studies (unpublished results) show that the LiP(II) isozyme only dehydrogenates benzyl alcohols with at

least two alkoxy substitutions on the ring. Benzyl alcohols with less than two ring alkoxy groups or with ring electron-withdrawing substituents, such as chloro, bromo, or nitro groups, are not substrates. Electron-donating groups appear to be necessary for the formation and/or stabilization of the aryl cation radical (Walling et al., 1984).

A number of reports have demonstrated that ligninolytic cultures of *P. chrysosporium* are capable of oxidizing β-aryl ether dimers to produce benzaldehydes, phenylglycerols, and phenoxyethanol (Enoki et al., 1980, 1981a,b; Umezawa et al., 1983a,b; Umezawa & Higuchi, 1984, 1985a), as well as products of ring-opening reactions (Umezawa & Higuchi, 1985b). This suggested that β-aryl ether dimers are oxidized by several different pathways. Since LiP can carry out several of these reactions, a detailed study of the oxidation of β-aryl ether dimers by this enzyme was initiated.

The β-aryl ether I is oxidized by LiP to yield four products: the benzaldehyde XIV, the propylphenol XII, the phenylglycerol X, and the catechol XIII. Most of the benzaldehyde XIV and the propylphenol XII probably arise through the α,β cleavage of the dimer via a pathway similar to that found for the oxidation of diarylpropanes by this enzyme (Renganathan et al., 1986; Schoemaker et al., 1985). It was previously suggested that the phenylglycerol is formed through β-O-4 ether bond hydrolysis (Enoki et al., 1981a). This is the first report of catechol formation by the fungal culture or enzyme oxidation of a β-aryl ether dimer. Oxidation of the β-aryl ether dimer III yields four analogous products, again indicating both α,β bond and β-O-4 ether bond cleavage. Isolation of the catechols XIII, XVI, and VIII as products of the phenoxy (B) ring suggests that LiP is capable of demethoxylating β-aryl ether dimers and probably the lignin polymer as well. Previous work indicated that the enzyme is capable of demethoxylating dimethoxybenzene (Kersten et al., 1985). Oxidation of the β-aryl ether dimer II containing only one alkoxy substituent on the B ring yields only two products, the benzaldehyde XIV and the 4-hydroxybenzyl alcohol XVII. Neither the phenylglycerol nor a catechol are obtained. This indicates that the dimer is cleaved only at the α,β bond.

In contrast, oxidation of the β-aryl ether dimer IV containing no alkoxy groups on the phenyl (A) ring yields three different products: the phenylglycerol IX, the catechol XIII, and the γ-aryl ether XVIII (identified by an examination of the MS only); only traces of benzaldehyde and the phenol are obtained. This indicates that this dimer is oxidized without significant cleavage of the α,β bond.

These observations can be explained in terms of the formation of an aryl cation radical on either the A or B ring of β-aryl ether dimers (Figure 4). The minimum requirement for the enzymatic formation of an aryl cation appears to be two alkoxy ring substituents. Depending on their alkoxy substituents, both the A and B rings of a β-aryl ether dimer potentially can be oxidized to an aryl cation radical. In the case of models I and III, both the A and B rings contain two alkoxy substituents and hence both rings can be oxidized to the corresponding aryl cation radical. In the case of dimer II, only the A ring has the required alkoxy substituents and thus only this ring can form an aryl cation radical. In contrast, in dimer IV, only the B ring can form an aryl cation radical.

Formation of an Aryl Cation Radical on Ring A. Initial formation of an aryl cation radical on ring A of dimers I, II, or III would be followed by α,β bond cleavage, as first proposed for diarylpropane dimers (Enoki & Gold, 1982; Nakatsubo et al., 1982; Renganathan et al., 1986; Schoemaker et al., 1985), giving rise to the benzaldehyde XIV and a phenoxy C_2 radical intermediate (Figure 4). The latter would be scavenged by O_2 under aerobic conditions to form a phenoxyglycol, which would break down to form a phenol and glycolaldehyde. Under anaerobic conditions, the phenoxy C_2 radical would probably be oxidized to a carbonium ion by the oxidized enzyme, which would be attacked by H_2O to yield the same products (Figure 4, pathway a).

^{18}O Labeling Experiments. Oxidation of the β-aryl ether I in $H_2{}^{18}O$ resulted in 42% incorporation of the ^{18}O into the 1-position of the phenylglycerol X. A similar result has been reported with intact fungal cultures (Umezawa & Higuchi, 1984) with no explanation for this observation. No incorporation of ^{18}O from $H_2{}^{18}O$ was found in the phenolic oxygen of the product XII. In addition, when the 1-[^{18}O]hydroxy-β-aryl ether dimer IIIa was used as a substrate, the phenylglycerol product Xa only contained 50 mol % of the label; i.e., 50% of the label was lost. Neither the 1-hydroxyl of III nor that of X are easily exchangeable with the solvent.

Oxidation of 2-^{18}O-labeled 1-(4-ethoxy-3-methoxyphenyl)-2-(4,5-dimethyl-2-methoxyphenoxy)-1,3-dihydroxypropane (Va) resulted in 99 mol % retention of the label in the phenylglycerol X but only 85% retention of the label in 2-[^{18}O]hydroxy-4,5-dimethylanisole (VIIa). No ^{18}O was found in the 1-position of the phenylglycerol X. Finally, oxidation of the 3-[^{18}O]hydroxy-β-aryl ether dimer IIIb resulted in 98 mol % retention of the label in the triol Xc and 26 mol % retention of the label in the phenol XV (Table I).

Formation of an Aryl Cation Radical on Ring B. Initial formation of an aryl cation radical on ring B of dimers I and III or sole oxidation of ring B of dimer IV could lead to at least two cleavage pathways. The predominant pathway appears to be intramolecular attack of the ring B cation radical by the 1-hydroxyl function, forming a benzodioxane type of intermediate with elimination of methanol (Figure 4, pathway b). Such a cation radical intermediate could be attacked by water at three positions (Figure 4). Attack of H_2O at the 3-position of this benzodioxane radical would explain the incorporation of ^{18}O from $H_2{}^{18}O$ into the 1-position of the phenylglycerol X. It would also explain the loss of ^{18}O from the 1-position during the conversion of the 1-[^{18}O]hydroxy-β-aryl ether IIIa to the phenylglycerol Xa in reactions conducted in $H_2{}^{16}O$. The attack of H_2O on the B ring of the dioxane radical would produce the observed catechol product. The O-methoxy phenols VII, XII, and XV are not demethoxylated by LiP, indicating that the demethoxylation observed occurs on the dimer itself by the nucleophilic attack of the 1-hydroxyl on the B ring carbon bearing the methoxyl function. Incubation of the benzodioxane dimer XI with the enzyme gave the predicted products, the benzaldehyde XIV, the phenylglycerol X, and the propyl catechol XIII (data not shown).

Our present data indicate that the major fraction of the phenol product is generated via α,β cleavage. For example, phenol is a major product of the cleavage of dimer II but only a minor product of the cleavage of dimer IV. However, when the 2-[^{18}O]ether dimer Va was used as the substrate, the phenylglycerol retained all of the ^{18}O while the phenol VII retained only 85% of the ^{18}O. Since any phenol generated by the α,β cleavage of a β-ether dimer should retain 100% of the ^{18}O originally found in the dimer, this loss indicates that some of the phenol VII was generated by a different pathway. Since no ^{18}O from $H_2{}^{18}O$ was incorporated into the phenolic oxygen of VII, the phenol probably did not arise from direct hydrolysis of the β-O-ether bond. This was confirmed by an examination of the oxidation of the dimer IV, which is not cleaved at the

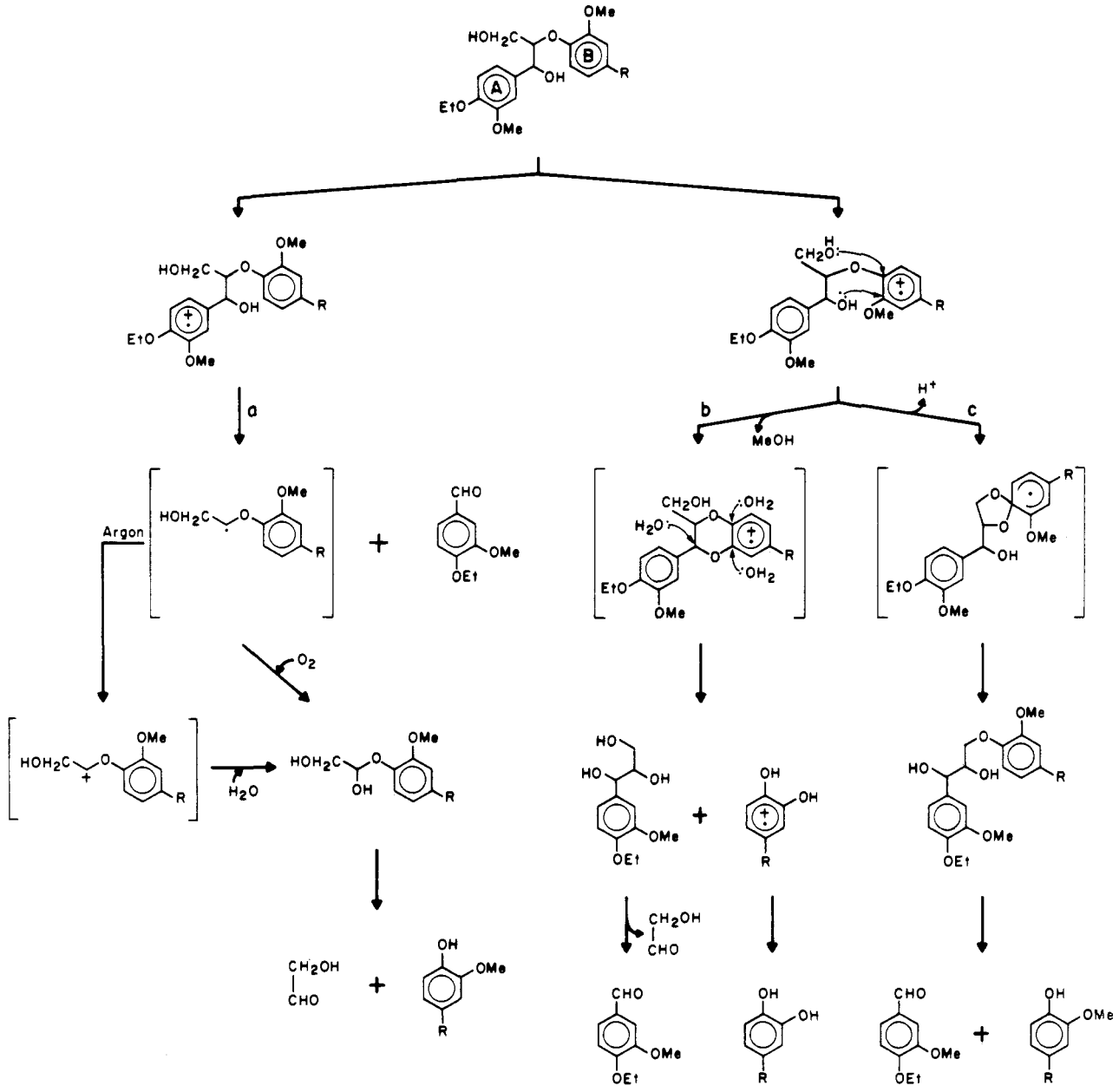

FIGURE 4: Proposed pathways for the oxidative cleavage of β-aryl ether dimers under argon by the lignin peroxidase from *P. chrysosporium*. R = $-CH_2OH$, $-CH_2CH_2CH_3$.

α,β bond. Significant hydrolysis of the β-ether bond of this dimer would have produced larger amounts of phenol.

We recently demonstrated the rearrangement of a monomeric aryl ether 2-(3-methoxy-4-propylphenoxy)-1,3-dihydroxypropane to 3-(2-methoxy-4-propylphenoxy)-1,2-dihydroxypropane and proposed that the rearrangement is initiated through an aryl cation radical (Miki et al., 1986). Similar rearrangement of the β-aryl ether, initiated by intramolecular attack of the γ-hydroxyl on the β-O-ether carbon of the B ring, would result in a γ-O-aryl ether (Figure 4, pathway c). Subsequent oxidation of the A ring to an aryl cation radical might result in radical cleavages between the α,β and γ-O-ether bonds to yield a benzaldehyde, an acetaldehyde, and a phenoxy radical. Disproportionation of the latter would yield the phenol (Figure 4). Oxidation of the 3-[^{18}O]hydroxy-β-aryl ether IIIb generates a phenol that retains 26% of the ^{18}O as predicted from the proposed mechanisms. Umezawa and Higuchi (1985b), using a 2-^{18}O-aryl ether with intact cultures, observed complete retention of the label in the phenolic product whereas we obtained only 85% retention. In the former work, incubation with intact cultures was done under O_2 which, as we have recently observed, severely inhibits rearrangement. The loss of 15% of the ^{18}O label from β-^{18}O-ether oxygen in its conversion to a phenolic oxygen and incorporation of 26% of the label from the 3-[^{18}O]-hydroxy-β-aryl ether into the phenol is probably due to the rearrangement of the β-O-4 ether to a γ-O-4 ether.

In summary, we have obtained evidence of three pathways for the LiP-catalyzed oxidation of β-aryl ethers under anaerobic conditions. The initial step in the oxidation appears to be the formation of a benzene cation radical, on either the A or B ring. Formation of an aryl cation radical with LiP(II) requires two alkoxy substituents on the ring. If an aryl cation radical is formed on the A ring, subsequent α,β cleavage results in the formation of a benzaldehyde and a phenol. Most, although apparently not all, of the phenolic product arises via this pathway. Formation of the aryl cation radical on the B ring results in intramolecular attack by the 1-hydroxyl, releasing methanol and possibly forming a benzodioxane radical intermediate. Subsequent hydrolysis explains the incorporation

of ^{18}O from $H_2{}^{18}O$ into the 1-position of the phenylglycerol and the formation of catechol. A mechanism for the generation of phenol via 3-hydroxyl attack on the aryl cation followed by rearrangement and cleavage has also been proposed. An initial obligatory B ring opening reaction has been proposed previously for the formation of the phenylglycerol (Umezawa & Higuchi, 1985b). However, the benzodioxane pathway indicates that aromatic ring opening is not a prerequisite to the formation of the phenylglycerol.

With the available data, it is difficult to assess the relative contributions to the oxidations of β-aryl ether dimers by pathways resulting from either A ring or B ring oxidation. Further studies on the oxidation of β-aryl ether dimers are planned in order to elucidate completely the mechanism of oxidation of these compounds by lignin peroxidase.

Registry No. I, 103258-51-1; II, 103258-52-2; III, 103258-53-3; IIIa, 103258-54-4; IIIb, 103258-55-5; IV, 103258-56-6; V, 103258-57-7; Va, 103258-58-8; VI, 33500-88-8; VII, 7771-25-7; VIIa, 103258-59-9; VIII, 2785-74-2; IX, 63157-81-3; X, 77891-26-0; XI, 103258-60-2; XII, 2785-87-7; XIII, 2525-02-2; XIV, 120-25-2; XV, 498-00-0; XVI, 3897-89-0; XVII, 623-05-2; XVIII, 103258-61-3; LiP, 42613-30-9; $BrCH_2CO_2Et$, 105-36-2; p-$HOCH_2C_6H_4OCH_2CO_2Et$, 103258-64-6; 1-(4-hydroxy-3-methoxyphenyl)propane, 2785-87-7; 4-ethylvanillin, 121-32-4; 3,4-dimethylanisole, 4685-47-6; lignin, 9005-53-2; ethyl 2-(2-methoxy-4-propylphenoxy)acetate, 103258-62-4; 4-ethoxy-3-methoxybenzaldehyde, 120-25-2; ethyl 3-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxy-4-propylphenoxy)-3-hydroxypropionate, 103258-63-5; ethyl 2-[4-(hydroxymethyl)-2-methoxyphenoxy]acetate, 103258-65-7; 1-(4-ethoxy-3-methoxyphenyl)-2-bromo-1-oxoethane, 84159-64-8; 4-hydroxy-3-methoxycinnamyl alcohol, 458-35-5.

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