H_2O_2 Recycling during Oxidation of the Arylglycerol β-Aryl Ether Lignin Structure by Lignin Peroxidase and Glyoxal Oxidase†

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ABSTRACT: Oxidative $C_\alpha-C_\beta$ cleavage of the arylglycerol β-aryl ether lignin model 1-(3,4-dimethoxyphenyl)-2-phenoxypropane-1,3-diol (**I**) by *Phanerochaete chrysosporium* lignin peroxidase in the presence of limiting H_2O_2 was enhanced 4−5-fold by glyoxal oxidase from the same fungus. Further investigation showed that each $C_\alpha-C_\beta$ cleavage reaction released 0.8−0.9 equiv of glycolaldehyde, a glyoxal oxidase substrate. The identification of glycolaldehyde was based on ^{13}C NMR spectrometry of reaction products obtained from β-, γ-, and β,γ-^{13}C-substituted **I**, and quantitation was based on an enzymatic NADH-linked assay. The oxidation of glycolaldehyde by glyoxal oxidase yielded 0.9 oxalate and 2.8 H_2O_2 per reaction, as shown by quantitation of oxalate as 2,3-dihydroxyquinoxaline after derivatization with 1,2-diaminobenzene and by quantitation of H_2O_2 in coupled spectrophotometric assays with veratryl alcohol and lignin peroxidase. These results suggest that the $C_\alpha-C_\beta$ cleavage of **I** by lignin peroxidase in the presence of glyoxal oxidase should regenerate as many as 3 H_2O_2. Calculations based on the observed enhancement of LiP-catalyzed $C_\alpha-C_\beta$ cleavage by glyoxal oxidase showed that approximately 2 H_2O_2 were actually regenerated per cleavage of **I** when both enzymes were present. The cleavage of arylglycerol β-aryl ether structures by ligninolytic enzymes thus recycles H_2O_2 to support subsequent cleavage reactions.

Lignin is a chemically recalcitrant biopolymer of phenylpropane structures that resists degradation by most organisms, but is nevertheless depolymerized rapidly by basidiomycetes that cause white rot of wood (Kirk & Farrell, 1987; Gold et al., 1989). The enzyme principally responsible for ligninolysis in many fungi is thought to be lignin peroxidase (LiP)[1] (Glenn et al., 1983; Tien & Kirk, 1983; Hammel et al., 1993), which oxidizes lignin structures by one electron, yielding cation radical intermediates that undergo spontaneous fission reactions (Hammel et al., 1985, 1986; Kersten et al., 1985; Schoemaker et al., 1985).

Studies with lignin model compounds have shown that LiP cleaves the predominant arylglycerol β-aryl ether (β-*O*-4) substructure of lignin, which accounts for about half of the total polymer, between C_α and C_β of its propyl side chain. The products of this fission reaction are a C_α-linked benzaldehyde, a phenol, and unknown one- or two-carbon fragments that contain C_β and C_γ (Figure 1) (Tien & Kirk, 1984; Kirk et al., 1986; Miki et al., 1986; Lundell et al., 1993). Tien and Kirk (1984), in examining the LiP-catalyzed cleavage of a modified β-*O*-4 model that carried a phenyl substituent rather than a hydroxyl at C_γ, found phenylacetaldehyde as a product and observed that the analogous $C_\beta-C_\gamma$ fragment from natural β-*O*-4 structures would be glycolaldehyde. More recently, glycolate, formaldehyde, and formate have been suggested as C_β/C_γ products that might be released during the enzymatic cleavage of β-*O*-4 lignin structures (Robert & Chen, 1989).

Evidence has accumulated that low molecular weight aldehydes and acids play important roles in the ligninolytic metabolism of white rot fungi. Acid chelators such as glycolate or oxalate are required for the activity of manganese peroxidases, which catalyze the oxidation of phenolic lignin structures (Glenn & Gold, 1985; Paszczynski et al., 1986; Wariishi et al., 1992; Kuan et al., 1993; Kuan & Tien, 1993). Aldehydes such as glycolaldehyde, glyoxal, glyoxylate, and formaldehyde are substrates for fungal glyoxal oxidase (GLOX), an enzyme that produces extracellular H_2O_2, which LiPs and manganese peroxidases require for oxidative turnover (Kersten & Kirk, 1987; Kersten, 1990; Kersten & Cullen, 1993). The $C_\beta-C_\gamma$ moiety released from lignin by the action of LiP may therefore be metabolically significant. Here we show that this fragment is the GLOX substrate glycolaldehyde. As a consequence, when LiP and GLOX act in tandem, the oxidative cleavage of β-*O*-4 structures *in vitro* regenerates H_2O_2 that supports subsequent cleavage reactions.

MATERIALS AND METHODS

Enzymes. LiP isozyme H8 was purified from cultures of *Phanerochaete chrysosporium* (ATCC 24725) by quaternary aminoethyl ion exchange chromatography as described previously (Kirk et al., 1990). The preparation was free of detectable GLOX activity at the concentrations used in these experiments. Recombinant *P. chrysosporium* GLOX was produced in *Aspergillus nidulans* using an expression vector that contained the *P. chrysosporium* GLOX (glx-1c) gene (Kersten & Cullen, 1993; unpublished results). The crude enzyme was concentrated, dialyzed, and purified by ion-exchange chromatography (Kersten, 1990).

Substrates. An *erythro/threo* mixture of 1-(3,4-dimethoxyphenyl)-2-phenoxypropane-1,3-diol (**I**), labeled with ^{14}C in the dimethoxylated ring (0.2 mCi mmol^{-1}), was synthesized

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[1] Abbreviations: GLOX, glyoxal oxidase; LiP, lignin peroxidase.

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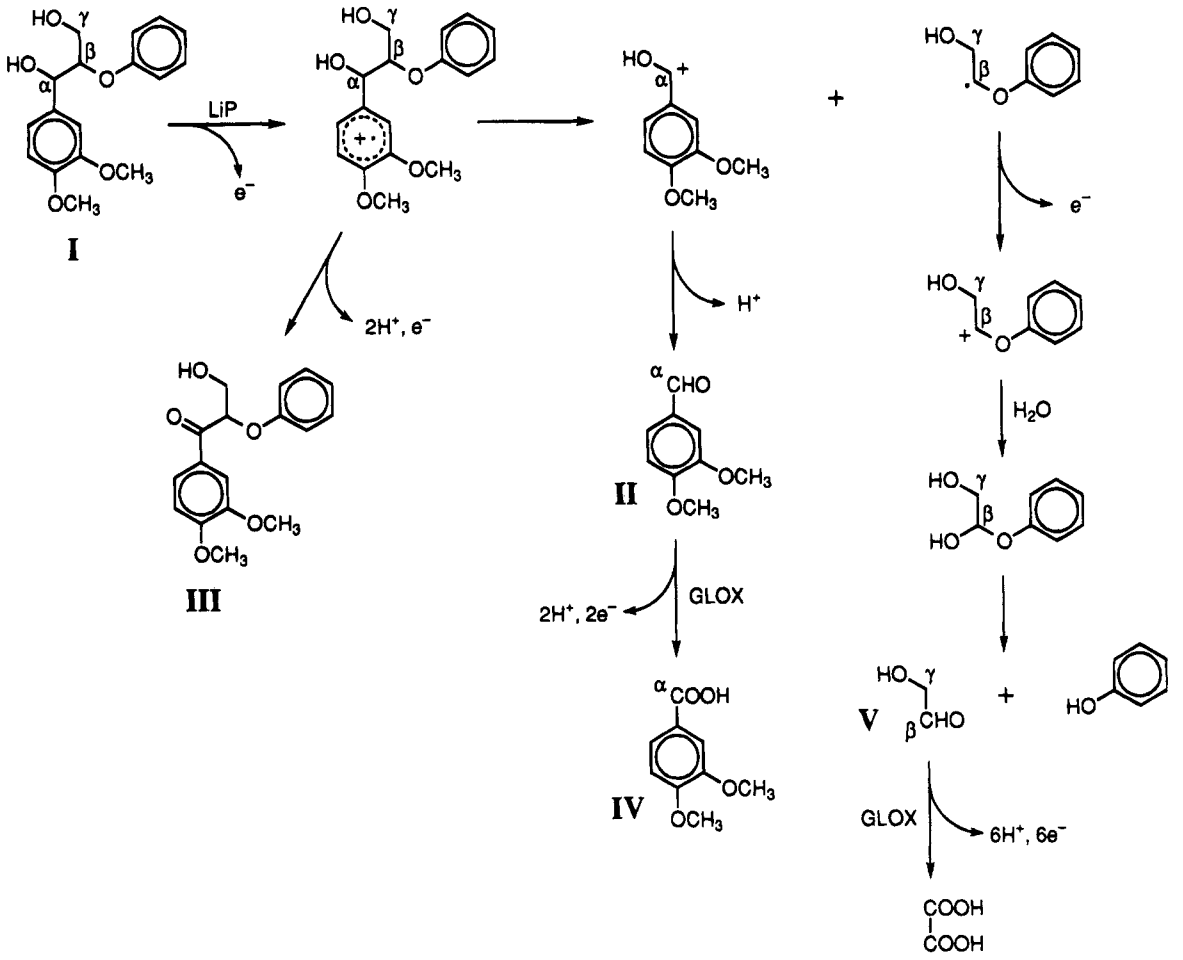

FIGURE 1: Products and proposed intermediates resulting from the oxidation of β-*O*-4 model **I** by LiP and GLOX.

from [*ring*-^{14}C]guaiacol, acetic acid, phenol, and formaldehyde by published methods (Landucci et al., 1981; Kirk et al., 1986). ^{1}H NMR ($CDCl_3$) δ 3.55−3.95 (2H, m, -$C_\gamma H_2$), 3.86 (6H, 2s, Ar-OCH_3), 4.37−4.44 (1H, m, -$C_\beta H$), 4.98−5.04 (1H, d, -$C_\alpha H$), 6.78−7.32 (8H, m, Ar*H*). ^{13}C NMR ($CDCl_3$) δ 61.2, 61.6 (C_γ); 73.8 (C_α); 82.0, 83.0 (C_β). MS (*m/z*, rel int) 304 (M^+, 1), 176 (25), 166 (100), 165 (5), 151 (2), 139 (15), 120 (8). **I** substituted with ^{13}C at various positions was prepared by the same procedure, using [2-^{13}C]-acetic acid (99+ atom %) for substitution at C_β, [^{13}C]-formaldehyde (99+ atom %) for substitution at C_γ, and both precursors for disubstitution. Spectral data for the isotope-enriched compounds were the same as those found for natural abundance **I**, except that NMR signal splittings and MS mass increases consistent with ^{13}C substitution were observed.

Effect of GLOX on the LiP-Catalyzed C_α−C_β Cleavage of I. Reaction mixtures (1.0 mL) contained [^{14}C]**I** (250 μM, 1.1×10^5 dpm), LiP (0.6 μM), and H_2O_2 (50 or 100 μM) in potassium 2,2-dimethylsuccinate buffer (25 mM, pH 4.5) at room temperature. Where indicated, GLOX (0.7 μM) was included. The H_2O_2 was delivered to the stirred reaction vial over a period of 8 h with a syringe pump. Half of each enzyme was added to the reaction at the outset, and the other half was delivered from a separate syringe over 8 h. Once reagent addition was complete, the reaction mixture was allowed to stir for an additional 8 h to ensure completion. The yields of oxidation products were then determined by reversed-phase HPLC analysis on a 10 μm particle size Vydac C18 column. The column was eluted at 1 mL min^{-1} and ambient temperature with 25% aqueous methanol for 10 min, followed by a 28-min linear gradient to 60% methanol. Fractions (1 mL) were collected and assayed for ^{14}C by scintillation counting.

Identification of Glycolaldehyde as a Product of C_α−C_β Cleavage. [^{13}C]**I** (1.6 mM) in 2.0 mL of potassium phosphate buffer (50 mM, pH 3.0, room temperature) was reacted with LiP (1 μM) and H_2O_2 (3 mM, added gradually in 10 portions). The sample was then applied to a 1 mL column of C18 reversed-phase resin (LC18, Supelco), which had been conditioned beforehand with 2 mL of methanol and then with 2 mL of pH 3.0 phosphate buffer. Hydrophilic products were eluted from the column with phosphate buffer and were analyzed for C_β- or C_γ-containing material by ^{13}C NMR spectrometry.

^{13}C NMR data were obtained on a Bruker AMX360 spectrometer, utilizing a 5-mm multinuclear probe tuned to 90.6 MHz. Samples were in an H_2O/D_2O mixture that contained a trace of methanol from the preceding reversed phase separation. The samples were analyzed at 300 K in 5-mm tubes, and acquisitions consisting of $2.5-3.0 \times 10^5$ transients were obtained. DEPT (distortionless enhancement by polarization transfer) spectra were acquired with a standard Bruker microprogram. A line broadening of 0.75 Hz was applied prior to Fourier transformation. Chemical shifts were determined relative to methanol, which was assigned a shift of 49 ppm relative to tetramethylsilane.

Stoichiometry of LiP-Catalyzed Glycolaldehyde Production. Reaction mixtures (3.0 mL) contained [^{14}C]**I** (250 μM,

Table 1: Effect of GLOX on the Stoichiometry of **I** Cleavage by LiP

enzyme(s)	H_2O_2 added (μM)	**II** produced (μM)	**IV** produced (μM)	**II** + **IV** produced per H_2O_2 added (equiv)	**II** oxidized to **IV** (equiv)
LiP	50	15	0	0.30 (*n*)[a]	0
LiP + GLOX	50	48	21	1.38 (*v*)	0.30 (*y*)
LiP	100	36	0	0.36 (*n*)	0
LiP + GLOX	100	106	35	1.41 (*v*)	0.25 (*y*)

[a] Italicized letters indicate variables used for the derivation of eqs 2 and 3 (see Discussion).

3.3 × 10^5 dpm) and LiP (0.6 μM) in potassium 2,2-dimethylsuccinate buffer (25 mM, pH 4.5) at room temperature. Oxidations were driven with successive 250 μM additions of H_2O_2 until no further C_α-carbonyl formation was observable spectrophotometrically at 308 nm. Samples were taken after the first addition of 250 μM H_2O_2 and after the reaction was complete (1 mM H_2O_2). They were assayed for glycolaldehyde with yeast NADH-dependent alcohol dehydrogenase (Sigma) as previously described (Goedde & Langenbeck, 1974) and for veratraldehyde by HPLC as outlined above. The ratio of the glycolaldehyde yield to the veratraldehyde yield was taken as the number of glycolaldehydes produced per $C_\alpha-C_\beta$ cleavage of **I**.

Pathway of Glycolaldehyde Oxidation by GLOX. The GLOX-catalyzed oxidation of glycolaldehyde, glyoxal, and glyoxylate was monitored spectrophotometrically at 308 nm in coupled reactions with LiP and veratryl alcohol. H_2O_2 production was inferred from the veratraldehyde yield (ϵ_{308nm} = 9.3 mM^{-1} cm^{-1}), making use of the observation that each H_2O_2 consumed by LiP oxidizes 1 veratryl alcohol to veratraldehyde (Tien et al., 1986). The assay mixtures (1.0 mL, room temperature) contained potassium 2,2-dimethylsuccinate buffer (50 mM, pH 4.5), veratryl alcohol (1.0 mM), GLOX (0.04 μM for glyoxylate, 0.3 μM for glyoxal, 0.4 μM for glycolaldehyde), GLOX substrate (35−110 μM), and a small quantity of H_2O_2 (5 μM) to activate the GLOX. A small portion (≤20%) of the veratraldehyde produced in these assays resulted from a low veratryl alcohol oxidase activity exhibited by GLOX. This contribution was subtracted to obtain the final results.

Oxalate formed by the action of GLOX on glycolaldehyde was determined after derivatization with 1,2-diaminobenzene to give 2,3-dihydroxyquinoxaline (McWhinney et al., 1986). Treated samples were analyzed by reversed-phase HPLC on a 5 μm particle size ODS column (Alltech) that was eluted with 0.4 M aqueous ammonium acetate/methanol, 85:15, at 1 mL min^{-1} and ambient temperature. The eluate was monitored spectrophotometrically at 312 nm, and the integrated product peak was quantitated against an authentic oxalate standard that had been derivatized by the same procedure.

RESULTS

Effect of GLOX on the Stoichiometry of Lignin Model **I** *Cleavage by LiP.* Experiments with [^{14}C]**I** showed that LiP without GLOX oxidized it to give labeled veratraldehyde (**II**) and 1-(3,4-dimethoxyphenyl)-3-hydroxy-2-phenoxypropan-1-one (**III**), as observed previously with similar model substrates (Figures 1 and 2) (Tien & Kirk, 1984; Kirk et al., 1986; Miki et al., 1986). In the presence of GLOX, an additional $C_\alpha-C_\beta$ cleavage product, veratric acid (**IV**) was formed. **IV** was identified by collection of the HPLC peak, methylation with diazomethane, and GC/MS analysis of the ester: mass spectrum *m/z* (rel int) 196 (M^+, 100), 181 (4), 165 (34), 122 (6), 121 (7). Further investigation showed that GLOX exhibited a low veratraldehyde oxidase activity, from which we conclude that **IV** was formed from **II** after the cleavage of **I** had already occurred. The HPLC results showed that 25−30% of the **II** initially formed during the cleavage of **I** was oxidized further to **IV** (Table 1).

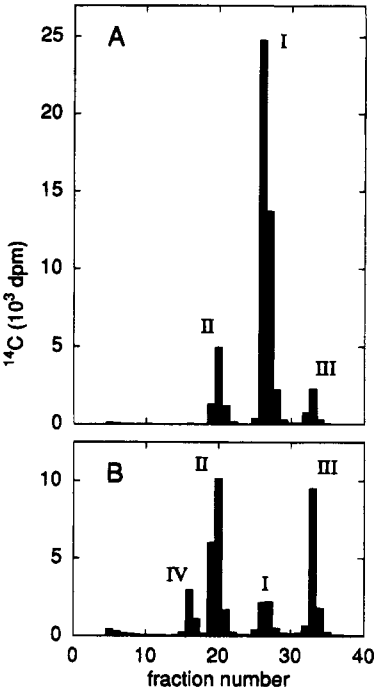

FIGURE 2: HPLC separation and radiochemical detection of products from the oxidation of [^{14}C]**I**. (A) Oxidized with LiP. (B) Oxidized with LiP and GLOX.

In the absence of GLOX, the yield of **II**, the expected $C_\alpha-C_\beta$ cleavage product, was equivalent to 30−36% of the H_2O_2 supplied (Table 1). With GLOX present, oxidation was markedly enhanced and the total yield of $C_\alpha-C_\beta$ cleavage products was about 140% of the H_2O_2 initially supplied (Figure 2; Table 1). The participation of GLOX thus amplified the LiP-catalyzed $C_\alpha-C_\beta$ cleavage of **I** 4−5-fold. The simplest explanation for this result appeared to be that the oxidative cleavage of **I** released a GLOX substrate.

Identification of the Two-Carbon Fragment. Experiments with [^{13}C]**I** showed that the $C_\beta-C_\gamma$ fragment released during cleavage was glycolaldehyde (**V**, Figure 1), as shown by ^{13}C NMR spectrometry of the polar, water-soluble fraction of the reaction products (Figure 3). The enzymatic cleavage of β-^{13}C-labeled **I** gave a single polar product in which C_β exhibited a chemical shift at 89.8 ppm. A DEPT spectrum (not shown) was consistent with the presence of a methine hydrogen at C_β, and a spiking experiment with authentic glycolaldehyde confirmed that the 89.8 ppm chemical shift

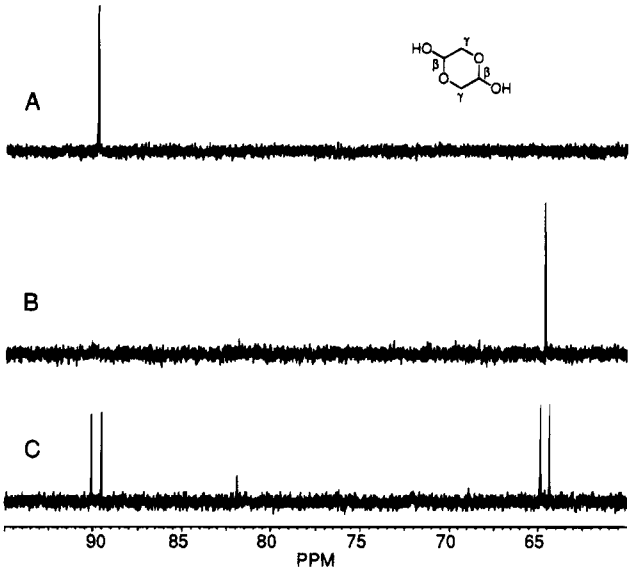

FIGURE 3: ^{13}C NMR spectra of glycolaldehyde released during the LiP-catalyzed oxidation of ^{13}C-substituted **I**. (A) With [β-^{13}C]**I**. (B) With [γ-^{13}C]**I**. (C) With [β,γ-^{13}C]**I**.

was identical to that exhibited by C_β of the glycolaldehyde dimer in H_2O. The analogous experiment with γ-^{13}C-labeled **I** gave a single polar product in which C_γ exhibited a chemical shift at 64.6 ppm. A DEPT experiment (not shown) showed the presence of two methylene hydrogens, and a spiking experiment with glycolaldehyde confirmed the chemical shift assignment. The results were confirmed in an experiment with β,γ-^{13}C-labeled **I**, which gave a single polar reaction product that exhibited two doublets centered at 89.8 and 64.6 ppm (J = 48 Hz).

Stoichiometry of Glycolaldehyde Production. The glycolaldehyde released during LiP-catalyzed C_α–C_β cleavage was determined in NADH-linked assays with alcohol dehydrogenase (Goedde & Langenbeck, 1974). Although alcohol dehydrogenase is not specific for glycolaldehyde, this approach was feasible in the present case because other simple aldehydes or alcohols that might obscure the analysis were not produced when **I** was oxidized by LiP (Figure 3). The data showed that the release of glycolaldehyde from **I** during C_α–C_β cleavage was nearly quantitative: 0.8–0.9 equiv was found per equivalent of **II** produced.

Determination of H_2O_2 Produced from Glycolaldehyde by GLOX. Since glycolaldehyde is a GLOX substrate (Kersten, 1990), the foregoing results indicate that β-*O*-4 structures in lignin are a potential source of H_2O_2 when both LiP and GLOX are present. However, they do not establish the magnitude of this contribution, which depends in part on the pathway for glycolaldehyde oxidation by GLOX. One possibility is that glycolaldehyde is oxidized to glycolate (OHC-CH_2OH → HOOC-CH_2OH), which is not a GLOX substrate, in a reaction that yields 1 H_2O_2. Alternatively, glycolaldehyde might be oxidized to glyoxal, which is a GLOX substrate. The product of glyoxal oxidation would probably be glyoxylate, also a substrate, which in turn would presumably yield oxalate. This pathway (OHC-CH_2OH → OHC-CHO → OHC-COOH → HOOC-COOH) would theoretically yield 3 H_2O_2.

To distinguish between these possibilities, glycolaldehyde was oxidized with GLOX, and the reaction mixture was analyzed by HPLC for oxalate after derivatization with 1,2-diaminobenzene. Each equivalent of glycolaldehyde yielded 0.9 equiv of oxalate (as 2,3-dihydroxyquinoxaline) after enzymatic oxidation (Figure 4), which establishes that the longer of the two pathways is the correct one. Assays of GLOX-catalyzed H_2O_2 production from the pathway intermediates confirmed this conclusion. In coupled assays with LiP and veratryl alcohol, GLOX generated 0.9 equiv of H_2O_2 from glyoxylate, 1.8 equiv of H_2O_2 from glyoxal, and 2.8 equiv of H_2O_2 from glycolaldehyde (Figure 5).

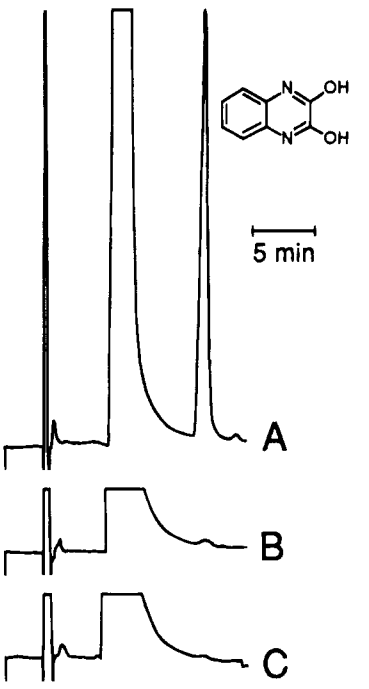

FIGURE 4: HPLC analysis of oxalate (as 2,3-dihydroxyquinoxaline) obtained from the action of GLOX on glycolaldehyde. (A) Complete reaction. (B) Reaction lacking glycolaldehyde and GLOX. (C) Reaction lacking LiP and GLOX. The large truncated peak eluting before 2,3-dihydroxyquinoxaline is unreacted 1,2-diaminobenzene.

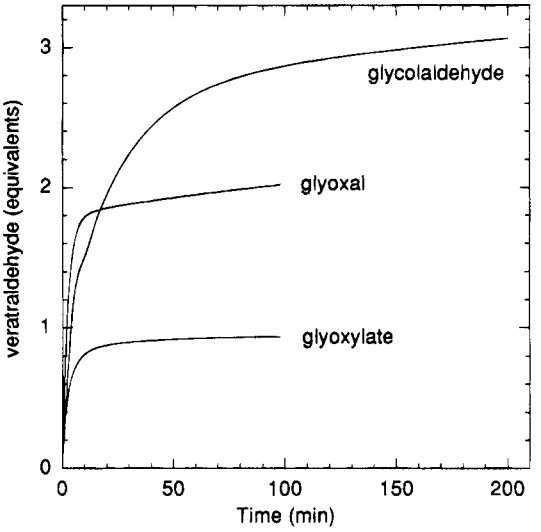

FIGURE 5: Oxidation of veratryl alcohol to veratraldehyde by LiP, using H_2O_2 generated by GLOX from limiting quantities of glyoxylate, glyoxal, and glycolaldehyde. Each veratraldehyde produced indicates the generation of 1 H_2O_2. The slow oxidation rate that persisted late in the assays was due to the veratryl alcohol oxidase activity of GLOX. This activity is less evident in the glyoxylate experiment, which contained 0.04 μM GLOX, than in the other two, which contained 0.3–0.4 μM GLOX.

DISCUSSION

Pathway of **I** *Cleavage by LiP.* LiP ionizes nonphenolic lignin structures to give aryl cation radicals, which subsequently undergo $C_\alpha-C_\beta$ fission and other nonenzymatic reactions (Hammel et al., 1985, 1986; Kersten et al., 1985; Schoemaker et al., 1985; Kirk et al., 1986). The $C_\alpha-C_\beta$ cleavage of **I** by this mechanism probably yields an α-hydroxydimethoxybenzyl carbonium ion and a C_β-centered 2-phenoxyethanol radical. Our finding that $C_\alpha-C_\beta$ cleavage and glycolaldehyde production occurred roughly in a 1:1 ratio is most easily explained by further oxidation of the C_β-centered radical to give a carbonium ion, which then reacts with H_2O to give a hemiacetal, which in turn decomposes to release phenol and glycolaldehyde (Figure 1).

Oxidations by GLOX. GLOX oxidized glycolaldehyde by the route that maximizes H_2O_2 production. The end product, oxalate, was not further oxidized under our conditions and appears not to be a GLOX substrate. Our results suggest that the $C_\beta-C_\gamma$ moiety of the lignin side chain may be one source of the oxalate that accumulates in wood being degraded by many white rot fungi (Eriksson et al., 1990). However, it is also evident that at least one of these fungi, *P. chrysosporium*, produces GLOX substrates such as methylglyoxal and glyoxal *de novo* without requiring lignin structures as precursors (Kersten & Kirk, 1987). These substrates allow *P. chrysosporium* to produce extracellular H_2O_2 in the absence of lignin. Glyoxal is a likely source of the oxalate that can be detected in *P. chrysosporium* cultures grown without lignin (Wariishi et al., 1992; Kuan & Tien, 1993).

The veratryl alcohol and veratraldehyde oxidase activities of GLOX were much lower than the activities exhibited toward glycolaldehyde, glyoxal, or glyoxylate. We observed that these aryl oxidase activities required oxygen, but polarographic stoichiometries were not obtainable because of the long reaction times required. For purposes of calculation (see below) we have assumed that one O_2 is consumed and one H_2O_2 is produced for each veratryl alcohol or veratraldehyde oxidized. Both aryl oxidase activities increased upon storage of the enzyme in frozen buffered solution, and it is not presently clear whether they are natural or artifactual properties of GLOX. Aryl alcohol oxidase activity is exhibited by another enzyme that overlaps partially with GLOX in substrate specificity, the galactose oxidase of the fungus *Dactylium dendroides* (Kosman, 1984).

Coupling between LiP and GLOX via Glycolaldehyde. GLOX amplified the LiP-catalyzed $C_\alpha-C_\beta$ cleavage of **I** significantly, which indicates that GLOX used the released glycolaldehyde to regenerate H_2O_2 for use by LiP. To assess the extent of this H_2O_2 recycling, we modeled the oxidation of **I** by LiP and GLOX as an iteration of oxidative cleavage steps and H_2O_2 regeneration steps. The total number of $C_\alpha-C_\beta$ cleavage reactions obtained for each H_2O_2 initially supplied is a quantity, v, that can be expressed in terms of three variables: n, the proportion of LiP-catalyzed **I** oxidations that result in $C_\alpha-C_\beta$ cleavage (as opposed to ketone **III** formation or any other oxidative reaction); x, the number of H_2O_2 molecules produced per $C_\alpha-C_\beta$ cleavage reaction due to the release and GLOX-catalyzed oxidation of glycolaldehyde; and y, the number of H_2O_2 molecules produced per $C_\alpha-C_\beta$ cleavage reaction due to the oxidation of veratraldehyde by GLOX. An expression for v was derived as follows.

For each H_2O_2 that is supplied to a reaction mixture containing LiP, GLOX, and excess **I**, n $C_\alpha-C_\beta$ cleavage reactions (measured as **II** and **IV** production) occur. The release and oxidation of glycolaldehyde then results in the regeneration of nx new H_2O_2, and the action of GLOX on some of the **II** formed during cleavage gives an additional ny H_2O_2. The total H_2O_2 regenerated following n $C_\alpha-C_\beta$ cleavage reactions is accordingly $n(x + y)$.

This regenerated H_2O_2 can support another cycle of the process just outlined, by cleaving additional **I** to give $n^2(x + y)$ equivalents of $C_\alpha-C_\beta$ cleavage products and glycolaldehyde, which in turn lead to the production of $n^2(x + y)^2$ H_2O_2. Reiteration of this model shows that v, the final number of $C_\alpha-C_\beta$ cleavage reactions per H_2O_2 initially supplied, is expressed by the following series:

$$v = [n + n^2(x + y) + n^3(x + y)^2 + n^4(x + y)^3 + ...] \quad (1)$$

If $n(x + y) < 1$, the series converges to

$$v = \frac{n}{1 - (n[x + y])} \quad (2)$$

and a finite $C_\alpha-C_\beta$ cleavage yield is obtainable from any starting amount of H_2O_2. It is noteworthy that if $n(x + y) \geq 1$, eq 1 does not converge, and $C_\alpha-C_\beta$ cleavage can continue indefinitely, independently of the amount of H_2O_2 initially added.

To check the hypothesis that LiP and GLOX regenerated H_2O_2 from glycolaldehyde when they acted together on **I**, we calculated x for the coupled reaction, using values for v, n, and y that were determined from LiP reaction stoichiometries (Table 1). Rearrangement of eq 2 gives

$$x = \frac{v - n}{vn} - y \quad (3)$$

The use of eq 3 requires some assumptions. First, n was necessarily determined in experiments without GLOX, and its values are therefore valid only if GLOX does not affect the efficiency of $C_\alpha-C_\beta$ cleavage by LiP; *i.e,* LiP and GLOX must act independently. There is presently no evidence for a direct interaction between the two enzymes that would alter the product profile of LiP-catalyzed reactions, but the possibility has not been ruled out. Second, the determination of n and y in endpoint assays assumes that the values for these variables remain constant during the entire course of the reaction. It appears that this assumption is approximately correct, because the values for n and y do not differ grossly between the 50 and 100 μM H_2O_2 experiments (Table 1).

Substitution of the Table 1 values into eq 3 yields x = 2.3 H_2O_2 regenerated per $C_\alpha-C_\beta$ cleavage for the experiment with 50 μM initial H_2O_2, and x = 1.8 H_2O_2 regenerated per cleavage for the experiment with 100 μM initial H_2O_2. These values, although short of the theoretical expectation (3 H_2O_2), are consistent with the hypothesis that LiP and GLOX couple *in vitro*, using released glycolaldehyde to regenerate H_2O_2.

In vivo, the situation is more complicated. The efficiency of coupling between LiP and GLOX is a function of n, the probability that any given H_2O_2 molecule will support cleavage of a β-*O*-4 lignin unit. In decaying wood, a variety

of factors are likely to decrease n. These include the availability of other LiP substrates that do not contain β-*O*-4 linkages but can compete for H_2O_2, as well as the presence of metal ions or other peroxidases that can react with H_2O_2 (Kirk & Farrell, 1987; Srebotnik et al., 1988; Eriksson et al., 1990). On the other hand, C_α ketones analogous to **III** appear not to be formed in appreciable quantities when LiP oxidizes polymeric lignin *in vitro* (Hammel et al., 1993). Any reduction in this nonproductive reaction *in vivo* would increase n. It is questionable whether the H_2O_2 regeneration cycle we have described ever reaches the break-even point, but it may in any case contribute a significant portion of the oxidizing equivalents needed for ligninolysis.

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