Oxidation of Dibenzo-*p*-dioxin by Lignin Peroxidase from the Basidiomycete *Phanerochaete chrysosporium*†

Dinesh K. Joshi and Michael H. Gold*

Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291-1000

Received May 4, 1994; Revised Manuscript Received June 17, 1994®

ABSTRACT: Dibenzo-*p*-dioxin (**I**) was rapidly degraded in ligninolytic cultures of the basidiomycete *Phanerochaete chrysosporium*. Lignin peroxidase (LiP) oxidized **I** to generate the following products: catechol (**V**), dibenzo-*p*-dioxin-2,3-quinone (**VIII**), 2-hydroxy-5-(2-hydroxyphenoxy)-1,4-benzoquinone (**IX**), 4,5-dihydroxy-1,2-benzoquinone (**X**), 2-(2-hydroxyphenoxy)-1,4-benzoquinone (**XI**), 4-hydroxy-1,2-benzoquinone (**XII**), and 1,2-benzoquinone (**XIII**). Identical products were formed when the reaction was conducted under argon. No incorporation of ^{18}O into products was observed when the reaction was conducted under $^{18}O_2$. Oxidation of **I** in $H_2{}^{18}O$ resulted in incorporation of two atoms of ^{18}O into the quinone **VIII**. Nonenzymatic hydrolysis of the quinone (**VIII**) yielded catechol (**V**), **IX** and **X**. Hydrolysis of **VIII** in $H_2{}^{18}O$ resulted in incorporation of ^{18}O atoms into **IX** and **X**, whereas no incorporation of ^{18}O atoms into **V** was observed. These results are explained by mechanisms involving the one-electron oxidation of **I** by LiP to produce the corresponding cation radical. Nucleophilic attack of water on the cation radical generates a 2-hydroxydibenzo-*p*-dioxin radical, which is oxidized to a delocalized cation. The attack of water at position C-4a of the 2-hydroxydibenzo-*p*-dioxin cation, followed by oxidation and C–O–C bond cleavage, lead to formation of the quinone (**XI**), which undergoes 1,4-addition of water and cleavage of the second C–O–C bond to generate **V** and **XII**. Similarly, the attack of water on C-3 of the delocalized cation and subsequent oxidation generates the quinone **VIII**, which undergoes nonenzymatic 1,4-addition of water, followed by C–O–C bond cleavage to generate **IX**. **IX** also undergoes a water-addition reaction followed by C–O–C bond cleavage to generate **V** and **X**. Alternatively, the quinones **IX** and **XI** could undergo enzymatic oxidation of phenolic functions to generate their corresponding phenoxy radicals, which would be in resonance with carbon-centered radicals. Oxidation of these radicals to cations, with subsequent water attack and C–O–C bond cleavage, would yield 1,2-benzoquinone (**XIII**) and 2,5-dihydroxy-1,4-benzoquinone (**XIV**) from **IX**, and **XIII** and 2-hydroxy-1,4-benzoquinone (**XV**) from **XI**, respectively.

White-rot basidiomycetous fungi are primarily responsible for initiating the depolymerization of lignin, which is a key step in the earth's carbon cycle (Buswell & Odier, 1987; Gold et al., 1989; Higuchi, 1990; Kirk & Farrell, 1987). The best-studied lignin-degrading fungus, *Phanerochaete chrysosporium*, secretes two extracellular heme peroxidases—lignin peroxidase (LiP)[1] and manganese peroxidase (MnP)—which, along with an H_2O_2-generating system, are apparently major components of the organism's extracellular lignin degradative system (Gold et al., 1989; Hammel et al., 1993; Kirk & Farrell, 1987; Wariishi et al., 1991b).

Nucleotide sequences of several LiP cDNAs and genes (Gold & Alic, 1993), as well as the recently published LiP X-ray crystal structures (Edwards et al., 1993; Piontek et al., 1993; Poulos et al., 1993), demonstrate that important catalytic residues, including the proximal and the distal His, the distal Arg, and an H-bonded Asp, are all conserved within the heme pocket. LiP also shares mechanistic features with other plant and fungal peroxidases. Characterization of the formation and reactions of the oxidized intermediate LiP compounds I, II, and III indicates that the catalytic cycle of LiP is similar to that of horseradish peroxidase (Dunford & Stillman, 1976; Gold et al., 1989; Marquez et al., 1988; Renganathan & Gold, 1986). Yet LiP has several unique features including a very low pH optimum of ~3.0 (Renganathan et al., 1987; Tien et al., 1986) and an unusually high reactivity of compound II with H_2O_2 (Wariishi et al., 1991a). In particular, LiP is able to oxidize nonphenolic compounds with redox potentials beyond the reach of other peroxidases (Gold et al., 1989; Higuchi, 1990; Kirk & Farrell, 1987; Valli et al., 1992b; Schoemaker, 1990; Hammel et al., 1986).

Under ligninolytic conditions, *P. chrysosporium* also is capable of mineralizing a wide range of ^{14}C-labeled aromatic pollutants (Bumpus & Aust, 1987; Hammel, 1989; Joshi & Gold, 1993; Spadaro et al., 1992; Valli & Gold, 1991; Valli et al., 1992a,b). Recently, the pathways for the fungal degradation of 2,4-dichlorophenol (Valli & Gold, 1991), 2,4-dinitrotoluene (Valli et al., 1992a), 2,7-dichlorodibenzo-*p*-dioxin (Valli et al., 1992b), 2,4,5-trichlorophenol (Joshi & Gold, 1993), and anthracene (Hammel et al., 1991) have been elucidated, demonstrating that LiP and MnP as well as intracellular enzymes are involved in the degradative process.

Owing to their acute toxicity in animal tests, polychlorinated dibenzodioxins (PCDDs) have been recognized as environ-

† This work was supported by Grants MCB-9207997 from the National Science Foundation, R-82-1269 from the U.S. Environmental Protection Agency, and DE-FG06-92ER20093 from the U.S. Department of Energy, Division of Energy Biosciences.

* To whom correspondence should be addressed at the Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, P.O. Box 91000, Portland, OR 97291-1000. Telephone: 503-690-1076. Fax: 503-690-1464.

® Abstract published in *Advance ACS Abstracts,* August 15, 1994.

[1] Abbreviations: PCDDs, polychlorinated dibenzo-*p*-dioxins; LiP, lignin peroxidase; MnP, manganese peroxidase; HPLC, high-performance liquid chromatography; GC, gas chromatography; GCMS, gas chromatography–mass spectrometry.

0006-2960/94/0433-10969$04.50/0

mental hazards for several decades (Hansen, 1991; Rappe, 1980; Schwetz et al., 1973). The solubility of PCDDs decreases with the increasing number of chlorine atoms (Rappe, 1980). The insolubility of PCDDs and the formation of reactive intermediates during *in vivo* peroxidase-catalyzed reactions has complicated efforts to study the mechanistic aspects of their degradation. Since dibenzo-*p*-dioxin (**I**) is relatively more water soluble than its chlorinated derivatives, we examined its oxidation by LiP. In this report, we describe the reactions involved in the LiP oxidation of **I**.

MATERIALS AND METHODS

Chemicals. Dibenzo-*p*-dioxin (**I**) was obtained from Chem Service (West Chester, PA). Catechol (**V**), 2,5-dihydroxy-1,4-benzoquinone (**XIV**), 1-bromo-2,5-dimethoxybenzene, guaiacol, potassium iodate, and sodium periodate were obtained from Aldrich (Milwaukee, WI). 1,2,4-Trihydroxybenzene (**VI**) was obtained from Lancaster Synthesis (Windham, NH). Aromatic compounds obtained commercially were further purified by recrystallization.

Preparation of Substrates. Dibenzo-*p*-dioxin-2,3-quinone (**VIII**): Catechol was oxidized with potassium iodate as reported (Forsyth et al., 1960). The product (approximately 10% yield) was purified by silica gel column chromatography using chloroform:ethyl acetate (95:5) as the eluant.

2,3-Dihydroxydibenzo-*p*-dioxin (**II**): Sodium dithionite (45 mg) in water (10 mL) was added to a solution of dibenzo-*p*-dioxin-2,3-quinone (**VIII**) (25 mg) in methanol (20 mL), and the mixture was stirred at room temperature for 10 min. The mixture was extracted with ethyl acetate, dried over sodium sulfate, and evaporated under reduced pressure, and the product was purified by silica gel column chromatography using ethyl acetate:hexane (2:3) as the eluant.

2,2′,4,5-Tetrahydroxydiphenyl ether (**III**): A mixture of dibenzo-*p*-dioxin-2,3-quinone (**VIII**) (25 mg) and 6 N hydrochloric acid (4 mL) in methanol (30 mL) was stirred at room temperature for 10 min. The reaction mixture was brought to pH 7.0 with 3 N NaOH, treated with sodium dithionite (50 mg), stirred for an additional 5 min, and extracted with ethyl acetate. The organic phase was dried over sodium sulfate and evaporated under reduced pressure. The crude product mixture was derivatized with acetic anhydride and pyridine (2:1) and analyzed by GCMS. 2,3-Diacetoxydibenzo-*p*-dioxin, 2,2′,4,5-tetraacetoxydiphenyl ether, 1,2-diacetoxybenzene, and 1,2,4,5-tetraacetoxybenzene were produced in 58, 19, 7, and 1% yields, respectively. The products were separated by silica gel column chromatography using ethyl acetate and hexane (2:3) as the eluant.

2,2′,5-Trihydroxydiphenyl ether (**IV**) was prepared by coupling guaiacol and 1-bromo-2,5-dimethoxybenzene, followed by demethylation with $AlBr_3$ as described (Ungnade & Otey, 1951). The product was purified by silica gel column chromatography using ethyl acetate:hexane (2:3) as the eluent. 1,2,4,5-Tetrahydroxybenzene (**VII**) was prepared by the quantitative reduction of 2,5-dihydroxy-1,4-benzoquinone (**XIV**) using sodium dithionite as previously described (Joshi & Gold, 1993). 4,5-Dihydroxy-1,2-benzoquinone (**X**) and 1,2-benzoquinone (**XIII**) were prepared from 1,2,4,5-tetrahydroxybenzene (**VII**) and catechol (**V**), respectively, using 1 molar equiv of sodium periodate in water (Alder & Magnusson, 1959). The *o*-quinone products, compounds **X** and **XIII**, were converted to their phenazine derivatives as described (Valli et al., 1992b).

Fungal Degradation of Dibenzo-p-dioxins. P. chrysosporium strain OGC101 (Alic et al., 1987) was grown from a conidial inoculum at 38 °C in stationary culture (25 mL) as described (Gold et al., 1982) using a medium described previously (Gold et al., 1982; Kirk et al., 1978) with 2% glucose and 1.2 mM ammonium tartrate as the carbon and nitrogen sources, respectively, and with 20 mM sodium 2,2-dimethylsuccinate, pH 4.5, as the buffer. Cultures were incubated under air for 3 days, after which they were purged with O_2 every 3 days. After 6 days of incubation, the substrate in *N,N*-dimethylformamide (20 μL) was added to cultures to a final concentration of 25 μM. At the indicated intervals, an entire culture was homogenized in a blender (1 min), saturated with NaCl, and extracted with ethyl acetate (3 × 50 mL). The organic fraction was dried over sodium sulfate and evaporated under reduced pressure, and the disappearance of substrate was determined by GC analysis. The substrate also was added to uninoculated control cultures.

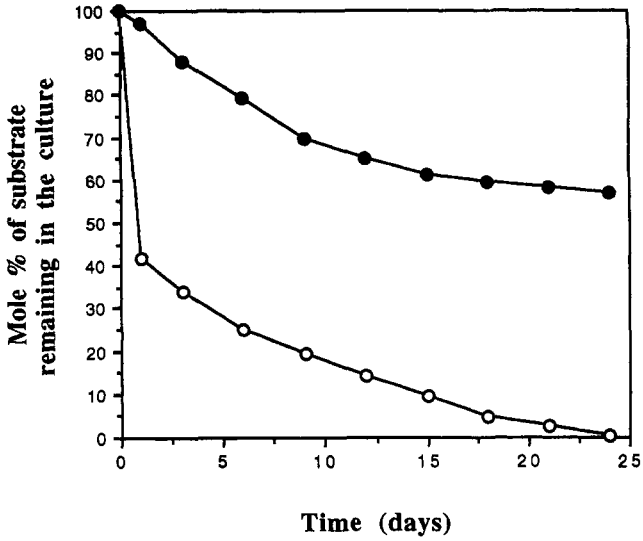

FIGURE 1: Comparison of *P. chrysosporium* degradation of dibenzo-*p*-dioxin (○) and 2,7-dichlorodibenzo-*p*-dioxin (●) under ligninolytic conditions. Stationary cultures containing 1.2 mM ammonium tartrate were inoculated with conidia and incubated for 6 days at 38 °C, after which the substrate was added. Flasks were purged with 100% O_2 periodically. The cultures were harvested, and the substrate remaining was determined as described in the text.

Enzymes. LiP isozyme II was purified from the extracellular medium of an acetate-buffered, agitated culture of *P. chrysosporium* as described previously (Gold et al., 1984; Wariishi & Gold, 1990). The LiP concentration was determined at 408 nm using an extinction coefficient of 133 mM^{-1} cm^{-1} (Gold et al., 1984).

Enzyme Reactions. Reaction mixtures (2 mL) consisted of LiP (10 μg/mL), substrate (0.2 mM), H_2O_2 (0.1 mM), and Tween 80 (0.1%) in 20 mM sodium succinate, pH 3.0. Veratryl alcohol (0.2 mM) was added to stimulate the reaction and to prevent enzyme inactivation (Wariishi & Gold, 1990). The enzyme and H_2O_2 were added twice, at 0 and 5 min, and the reaction was carried out at 28 °C for 10 min. Reaction products were reduced with sodium dithionite, extracted with ethyl acetate at pH 2, dried over sodium sulfate, evaporated under nitrogen, acetylated, and analyzed by GC or GCMS. Control reactions were conducted in the absence of enzyme or H_2O_2 or with boiled enzyme. 1,2-Benzoquinones were converted to their phenazine derivatives using 1,2-phenylenediamine in acetic acid and methanol prior to reduction as described (Valli et al., 1992b) and analyzed by GCMS.

^{18}O-Incorporation Studies. For experiments conducted in $H_2{}^{18}O$, reaction mixtures (0.5 mL) as described above were enriched with $H_2{}^{18}O$ (83%). $H_2{}^{18}O$ (97%) was obtained from Iso-Yeda Co., Ltd., Israel.

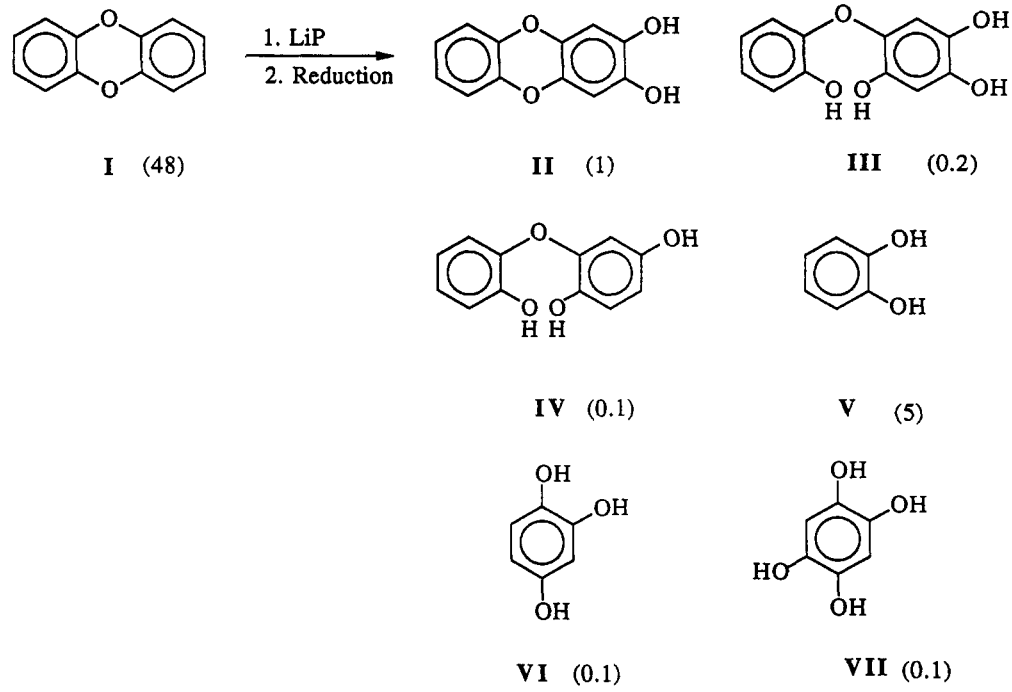

FIGURE 2: Products identified from the oxidation of dibenzo-*p*-dioxin (I) by LiP in the presence of H_2O_2, followed by reduction with sodium dithionite. Reaction conditions and identification of products were as described in the text. Mole percent yields of oxidation products and remaining substrate are indicated in parentheses.

For experiments conducted under $^{18}O_2$, the reaction vessel was evacuated, flushed with argon three times, and equilibrated with $^{18}O_2$ (99.1%; Isotec Inc., Miamisburg, OH) as described (Kuwahara et al., 1984; Renganathan et al., 1986). The reaction was initiated by the addition of O_2-free H_2O_2 via a syringe. In each case, the reaction mixture was reduced, derivatized, and analyzed as described above. The ^{18}O incorporation was determined by comparing intensities of the ion peaks.

Nonenzymatic Decay of Dibenzo-p-dioxin-2,3-quinone (VIII). The quinone (**VIII**) (0.2 mM) was incubated in 20 mM sodium succinate buffer, pH 3, for 10 min. The reaction mixture was reduced with sodium dithionite, extracted, dried over sodium sulfate, evaporated, and analyzed as described above. The quinone (**X**) was derivatized with 1,2-phenylenediamine prior to reduction as described above. The phenazine derivative of the quinone (**X**) was acetylated and analyzed by GCMS.

The above reaction also was performed by incubating dibenzo-*p*-dioxin-2,3-quinone (**VIII**) (0.2 mM) in 1% sulfuric acid for 3 min under air or under argon. The same hydrolysis reaction under air was conducted in 1% sulfuric acid enriched with 93 atom % $H_2{}^{18}O$. The products of all the reactions were reduced with sodium dithionite, acetylated, and analyzed as described above.

^{18}O-Isotope-Exchange Reaction between ^{18}O-Enriched Water and Quinone Oxygens. Freshly prepared 1,2-benzoquinone (**XIII**) was incubated in $H_2{}^{18}O$-enriched (90 atom %) 20 mM sodium succinate (pH 3) for 20 min. The product mixture was reduced, extracted, dried, and derivatized as described above. Similarly, freshly prepared 4,5-dihydroxy-1,2-benzoquinone (**X**) was incubated in $H_2{}^{18}O$-enriched (90 atom %) 1% sulfuric acid for 3 min.

Gas Chromatography and Mass Spectrometry. GCMS was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A gas chromatograph and a 30-m fused silica column (J & W Science, DB-5). GC analysis was performed on a similar gas chromatograph and column. The oven temperature was programmed from 70 to 320 °C at 10 °C/min. Products were identified by comparison of their retention times on GC and their mass fragmentation patterns with chemically prepared standards. Yields were quantitated on GC by using calibration curves obtained with standards using a flame ionization detector.

RESULTS

Fungal Degradation. Time courses for the degradation of dibenzo-*p*-dioxin (**I**) and 2,7-dichlorodibenzo-*p*-dioxin in cultures of *P. chrysosporium* are shown in Figure 1. The half-life of dibenzo-*p*-dioxin (**I**) in nitrogen-limited cultures was approximately 1 day, whereas only about 43% of 2,7-dichlorodibenzo-*p*-dioxin was degraded during the 24-day period of the experiment, confirming our earlier results (Valli et al., 1992b). The substrate was stable in uninoculated control cultures during the length of the experiment.

Enzymatic Oxidation of Dibenzo-p-dioxin (I). Oxidation of dibenzo-*p*-dioxin (**I**) by LiP, followed by chemical reduction, yielded 2,3-dihydroxydibenzo-*p*-dioxin (**II**), 2,2′,4,5-tetrahydroxydiphenyl ether (**III**), 2,2′,5-trihydroxydiphenyl ether (**IV**), catechol (**V**), 1,2,4-trihydroxybenzene (**VI**), and 1,2,4,5-tetrahydroxybenzene (**VII**). When the products of the reaction were analyzed without subsequent reduction, 4,5-dihydroxy-1,2-benzoquinone (**X**) and 1,2-benzoquinone (**XIII**) also were identified. The yields of the products formed are shown in Figure 2. These yields are underestimates, owing to the polymerization of quinones. All products were identified by comparing their GC retention times and mass spectra with those of chemically prepared standards (Table 1). The same product pattern was obtained when the reaction was conducted under either aerobic or anaerobic conditions. No products were obtained when the reaction was performed in the absence of either enzyme or H_2O_2 or with boiled enzyme.

^{18}O-Incorporation Studies. When substrate **I** was incubated with LiP under $^{18}O_2$ in $H_2{}^{16}O$, no incorporation of labeled oxygen into any of the products was observed. When the reaction was conducted in $H_2{}^{18}O$, one (15.5 atom %, *m/z* 112)

Table 1: Mass Spectra of Substrate (Dibenzo-*p*-dioxin) and Derivatives of Enzyme Oxidation Products[a]

substrate or metabolite	GC retention time (min)	mass spectrum *m/z* (rel int)
dibenzo-*p*-dioxin (**I**)	12.49	184 (100), 155 (7.29), 128 (34.78), 102 (13.69), 92 (19.90), 76 (6.62), 63 (15.76)
2,3-diacetoxydibenzo-*p*-dioxin	20.53	300 (6.2), 258 (8.98), 216 (100), 187 (9.95), 171 (1.89), 142 (2.24), 131 (2.79), 102 (1.67), 92 (1.71), 77 (9.81)
2,2′,4,5-tetraacetoxydiphenyl ether	22.12	402 (3.8), 360 (17.15), 318 (37.25), 276 (84.71), 234 (100), 215 (4.41), 187 (2.79), 161 (2.73), 141 (4.39), 126 (11.85), 110 (20.39), 93 (9.11)
2,2′,5-triacetoxydiphenyl ether	17.24	344 (2.64), 302 (41.29), 260 (71.38), 218 (100), 199 (2.02), 171 (3.26), 147 (2.22), 125 (8.65), 110 (31.86), 94 (36.88)
1,2-diacetoxybenzene	9.79	194 (3.41), 152 (26.82), 110 (100), 92 (1.39), 81 (5.81), 63 (3.30)
1,2,4-triacetoxybenzene	14.56	252 (2.15), 210 (13.41), 168 (30.61), 126 (100), 97 (3.52), 69 (2.36), 55 (2.35)
1,2,4,5-tetraacetoxybenzene	17.58	310 (10.5), 268 (20.1), 226 (69.3), 184 (84.1), 142 (100), 113 (11.1), 69 (20.2)
1,2-benzoquinone (phenazine derivative)	11.98	180 (100), 153 (9.75), 127 (4.55), 110 (3.70), 102 (9.15), 90 (7.63), 76 (18.37), 63 (8.69)
4,5-diacetoxy-1,2-benzoquinone (phenazine derivative)	21.90	296 (14.2), 254 (33.5), 212 (100), 183 (13.8), 166 (8.1), 102 (8.9)

[a] Products identified from the oxidation of dibenzo-*p*-dioxin by LiP. Reaction conditions and analysis were as described in the text. In all cases, the retention time and mass spectra of standard compounds were essentially identical to those of the oxidation products.

Table 2: Relative Intensities (%) of the Molecular Ion Region of Mass Spectra of Products of Enzymatic Oxidations and Nonenzymatic Reactions[a]

A. Enzymatic Oxidation of Dibenzo-*p*-dioxin (**I**) product	*m/z*	$^{18}O_2$[b]	$H_2{}^{18}O$[c]	$H_2{}^{16}O$
2,3-dihydroxydibenzo-*p*-dioxin (**II**)	216	100	7.3	100
	218	0.8	6.7	0.9
	220	0	100	0
catechol (**V**)	110	100	100	100
	112	0.7	23.1	0.7
	114	0	55.0	0

B. Nonenzymatic Decay of Dibenzo-*p*-dioxin-2,3-quinone[d] product	*m/z*	$H_2{}^{18}O$[d]	$H_2{}^{16}O$
2,3-dihydroxydibenzo-*p*-dioxin (**II**)	216	100	100
	218	1.0	0.9
	220	0	0
2,2′,4,5-tetrahydroxydiphenyl ether (**III**)	234	6.18	100
	236	67.33	2.3
	238	100	0
	240	58.83	0
catechol (**V**)	110	100	100
	112	0.8	0.7
	114	0	0
1,2,4,5-tetrahydroxybenzene (**VII**)	142	2.76	100
	144	13.67	0.3
	146	68.62	0
	148	100	0
	150	32.09	0

C. Isotope-Exchange Reaction between $H_2{}^{18}O$ and Quinone Oxygens product	*m/z*	$H_2{}^{18}O$	$H_2{}^{16}O$
catechol (**V**)[e]	110	100	100
	112	21.1	0.83
	114	3.1	0
1,2,4,5-tetrahydroxybenzene (**VII**)[f]	142	100	100
	144	90.3	0.31
	146	30.7	0

[a] Reaction conditions and analysis were as described in the text. [b] Reaction under 99.1% ^{18}O-enriched O_2. [c] Reaction in 83% $H_2{}^{18}O$-containing buffer. [d] Reaction in 93% $H_2{}^{18}O$-containing 1% sulfuric acid. [e] Reaction under 90% $H_2{}^{18}O$-containing buffer. [f] Reaction under 90% $H_2{}^{18}O$-containing 1% sulfuric acid.

or two (36.7 atom %, *m/z* 114) atoms of ^{18}O were incorporated in catechol (Table 2). Similarly, compound **II** incorporated one (6.0 atom %, *m/z* 218) or two (89.4 atom %, *m/z* 220) atoms of ^{18}O.

Nonenzymatic Decay of Dibenzo-p-dioxin-2,3-quinone (VIII). When dibenzo-*p*-dioxin-2,3-quinone (**VIII**) was incubated in 20 mM succinate buffer (pH 3) for 20 min followed by reduction, 2,2′,4,5-tetrahydroxydiphenyl ether (**III**) (1%), catechol (**V**) (3%), and 1,2,4,5-tetrahydroxybenzene (**VII**) (trace) were obtained. The hydrolysis of **VIII** occurred more rapidly in 1% sulfuric acid, wherein 2,2′,4,5-tetrahydroxydiphenyl ether (**III**) (6%), catechol (**V**) (10%), and 1,2,4,5-tetrahydroxybenzene (**VII**) (0.5%) were formed after 3 min. Derivatization of the product mixture with 1,2-phenylenediamine, prior to reduction with sodium dithionite, yielded the phenazine derivative of 4,5-dihydroxy-1,2-benzoquinone (**X**).

When dibenzo-*p*-dioxin-2,3-quinone (**VIII**) was hydrolyzed in $H_2{}^{18}O$-enriched 1% sulfuric acid followed by reduction, 1,2,4,5-tetrahydroxybenzene (**VII**) incorporated one (6.3 atom %, *m/z* 144), two (31.65 atom %, *m/z* 146), three (46.14 atom %, *m/z* 148), or four (14.84 atom %, *m/z* 150) atoms of ^{18}O (Table 2). Similarly, 2,2′,4,5-tetrahydroxydiphenyl ether (**III**) incorporated one (29.5 atom %, *m/z* 236), two (43.3 atom %, *m/z* 238), or three (24.8 atom %, *m/z* 240) atoms of ^{18}O. However, no incorporation of ^{18}O into 2,3-dihydroxydibenzo-*p*-dioxin (**II**) or catechol (**V**) was observed.

Isotope-Exchange Reactions between ^{18}O-Enriched Water and Quinone Oxygens. When 1,2-benzoquinone was incubated in $H_2{}^{18}O$, incorporation of one (20 atom %, *m/z* 112) or two (2.7 atom %, *m/z* 114) atoms of ^{18}O (Table 2) into the quinone was observed. When 4,5-dihydroxy-1,2-benzoquinone (**X**) was incubated in $H_2{}^{18}O$, incorporation of one (44.4 atom %, *m/z* 144) or two (15.2 atom %, *m/z* 146) atoms of ^{18}O was observed.

DISCUSSION

Under ligninolytic conditions, *P. chrysosporium* is capable of mineralizing many persistent aromatic pollutants (Bumpus & Aust, 1987; Hammel, 1989; Joshi & Gold, 1993; Spadaro et al., 1992; Valli & Gold, 1991; Valli et al., 1992a,b). Recently, the *P. chrysosporium* degradative pathways for the pollutants 2,4-dichlorophenol (Valli & Gold, 1991), 2,4-dinitrotoluene (Valli et al., 1992a), 2,7-dichlorodibenzo-*p*-dioxin (Valli et al., 1992b), 2,4,5-trichlorophenol (Joshi & Gold, 1993), and anthracene (Hammel et al., 1991) have been examined. These studies demonstrate that both LiP and MnP play important roles in the *P. chrysosporium* degradation of these pollutants. To our knowledge *P. chrysosporium* and presumably other white-rot fungi are the only microorganisms

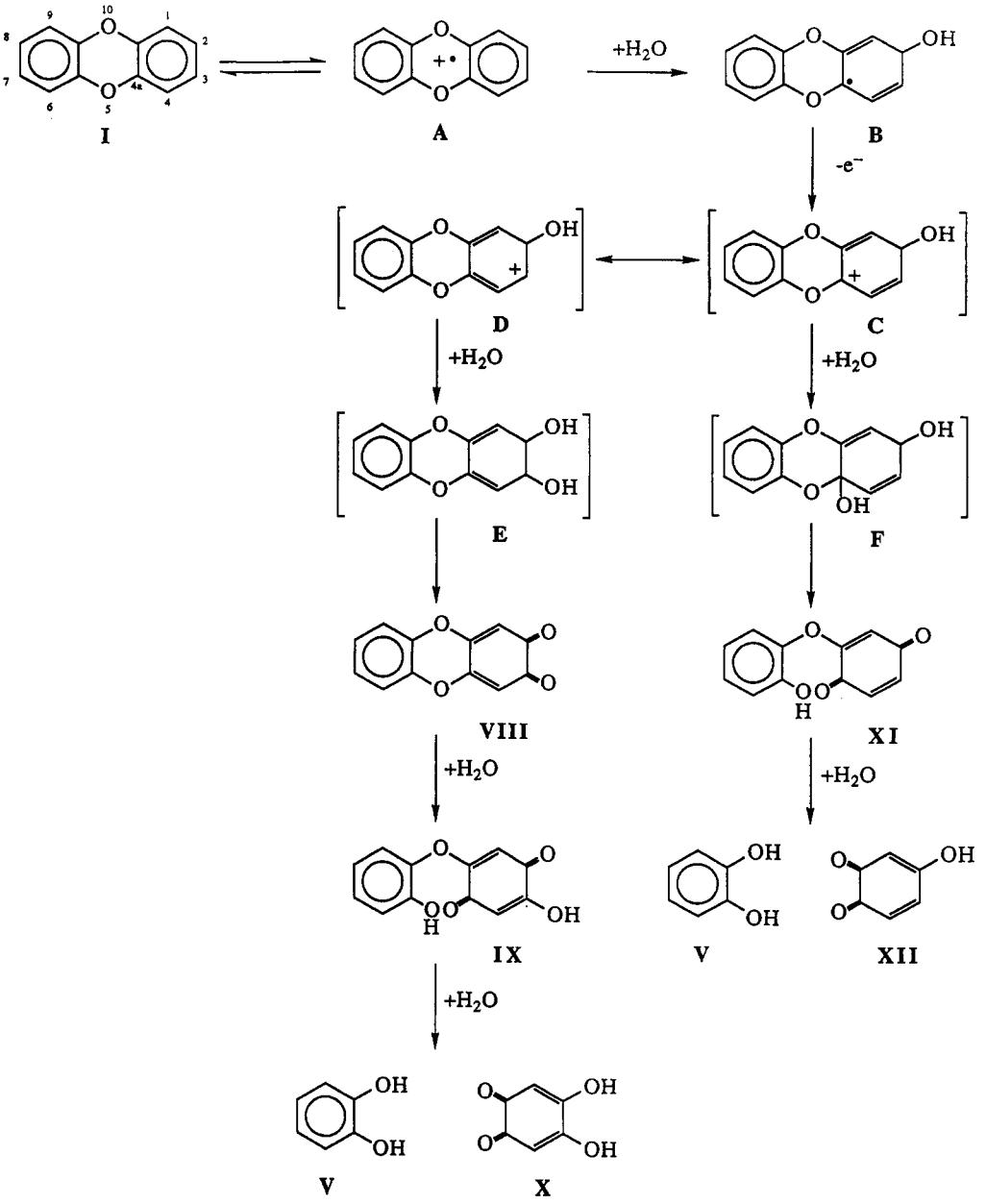

FIGURE 3: Proposed mechanism for lignin peroxidase-catalyzed oxidation of dibenzo-*p*-dioxin (**I**), showing the involvement of water addition to quinone intermediates.

known to degrade polychorinated dibenzo-*p*-dioxin pollutants (Valli et al., 1992b). Thus a study of the mechanisms involved in these degradations may have practical significance.

The results in Figure 1 show that, under nutrient nitrogen-limiting conditions, *P. chrysosporium* degrades dibenzo-*p*-dioxin (**I**) considerably faster than 2,7-dichlorodibenzo-*p*-dioxin (Valli et al., 1992b), suggesting that chlorine substitution on the aromatic ring retards the degradation rate. This decrease in degradation rate is probably due to two factors. First, the solubility of chlorinated dibenzo-*p*-dioxins decreases with an increasing number of ring chlorine substituents (Rappe, 1980). In addition, chlorine substitution on the aromatic ring raises the redox potential, making the substrate more resistant to oxidation. We have selected dibenzo-*p*-dioxin (**I**) to study the mechanistic aspects of dioxin oxidation because **I** is sufficiently soluble in water and is a relatively good LiP substrate.

Lignin peroxidase catalyzes the oxidation of a variety of nonphenolic aromatic compounds to their corresponding cation radicals (Gold et al., 1989; Higuchi, 1990; Kirk & Farrell, 1987; Kersten et al., 1985; Schoemaker, 1990; Hammel et al., 1986). Indeed, the LiP oxidation of dibenzo-*p*-dioxin (**I**) to its cation radical has been demonstrated by electron spin resonance spectroscopy (Hammel et al., 1986). However, no degradation products were identified in that study. The cation radical of dibenzo-*p*-dioxin also has been generated chemically and electrochemically and identified by electron spin resonance spectroscopy (Tomita & Ueda, 1964; Shine & Shade, 1974; Cauquis & Maurey-Mey, 1972).

The LiP-catalyzed oxidation of dibenzo-*p*-dioxin (**I**), followed by chemical reduction of the quinone products, yields the following products: 2,3-dihydroxydibenzo-*p*-dioxin (**II**), 2,2′,4,5-tetrahydroxydiphenyl ether (**III**), 2,2′,5-trihydroxydiphenyl ether (**IV**), catechol (**V**), 1,2,4-trihydroxybenzene (**VI**), and 1,2,4,5-tetrahydroxybenzene (**VII**) (Figure 2). The nature of the products generated by the oxidation of dibenzo-*p*-dioxin (**I**) suggests the initial formation of an aryl cation radical, which undergoes further reactions with water to form the variety of products observed in Figure 3.

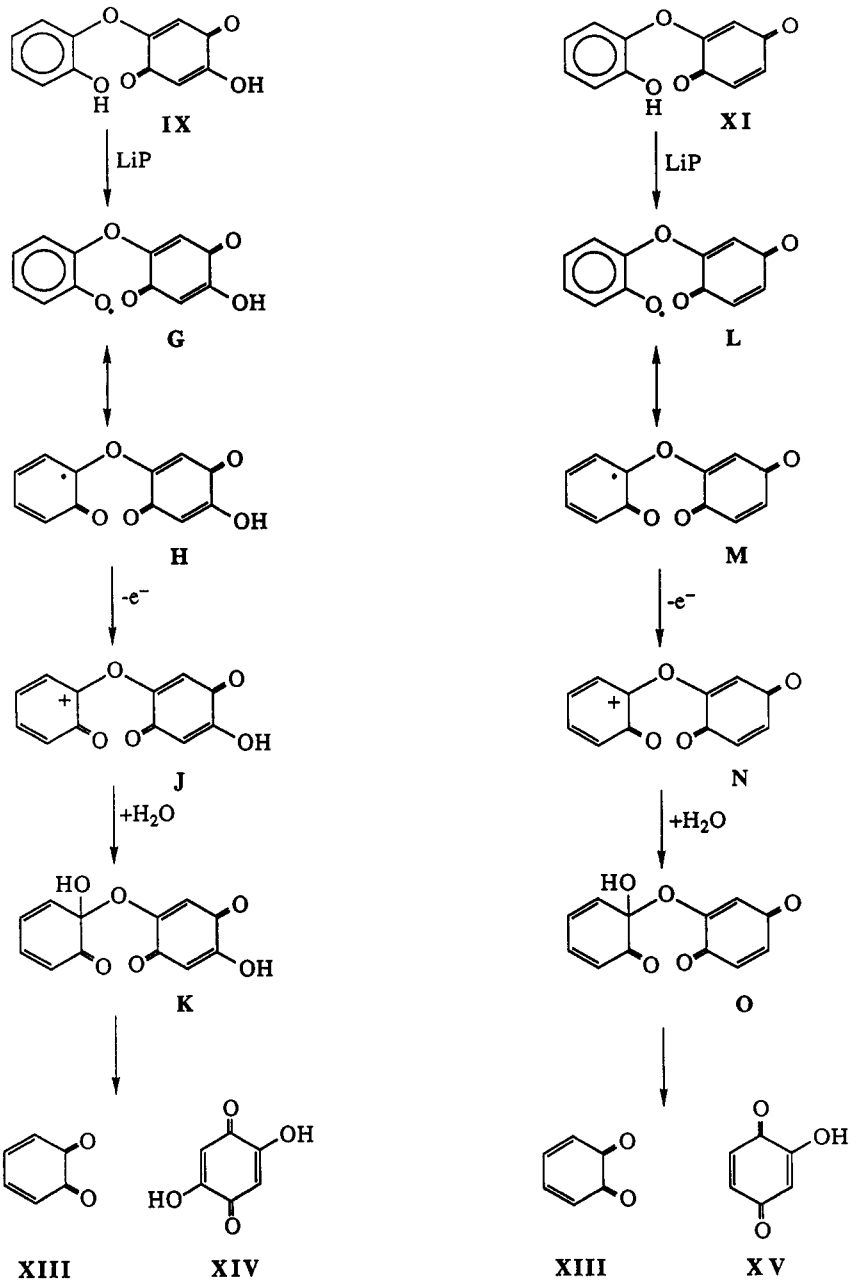

FIGURE 4: Proposed mechanism for lignin peroxidase-catalyzed cleavage of C–O–C bonds in the quinones **IX** and **XI**, showing the possible further oxidation of phenolic intermediates.

As shown in Figure 3, the initial nucleophilic attack of H_2O at position C-2 of the dibenzo-*p*-dioxin cation radical (**A**) would generate a delocalized 2-hydroxydibenzo-*p*-dioxin radical (**B**), which loses another electron, either by a disproportionation reaction or by LiP oxidation, to form the delocalized cation **C** or **D**. The attack of water on cation **C**, followed by cleavage of the C–O–C bond, would generate 2-(2-hydroxyphenoxy)-1,4-benzoquinone (**XI**), which was identified as 2,2′,5-trihydroxydiphenyl ether (**IV**). Quinones are known to undergo 1,4-addition reactions with water (Finley, 1974). Our results suggest that the quinone **XI** undergoes a spontaneous addition of water with subsequent cleavage of the remaining C–O–C bond to form catechol (**V**) and 4-hydroxy-1,2-benzoquinone (**XII**). The quinone **XII** was identified as 1,2,4-trihydroxybenzene (**VI**) after reductive acetylation. Catechol was identified as its acetylated derivative.

The attack of H_2O at position C-3 of the cation intermediate **D** (Figure 3) and further oxidation would lead to the formation of dibenzo-*p*-dioxin-2,3-quinone (**VIII**), presumably via the dihydrodiol intermediate (**E**). The quinone **VIII** was identified as 2,3-dihydroxydibenzo-*p*-dioxin (**II**) after reductive acetylation. Attempts by other researchers to identify this quinone as a product of the reaction of the dibenzo-*p*-dioxin cation radical (**A**) with water were unsuccessful (Shine & Shade, 1974; Cauquis & Maurey-Mey, 1972). The dibenzo-*p*-dioxin-2,3-quinone (**VIII**) would undergo addition of water (at position C-4a), followed by cleavage of the first C–O–C bond, to generate 2-hydroxy-5-(2-hydroxyphenoxy)-1,4-benzoquinone (**IX**). This product also was identified after reductive acetylation as 2,2′,4,5-tetrahydroxydiphenyl ether (**III**). The quinone **IX** also undergoes an addition reaction with a second molecule of H_2O, followed by cleavage of the second C–O–C bond, to form catechol (**V**) and 4,5-dihydroxy-1,2-benzoquinone (**X**). The quinone **X** was identified both as its phenazine derivative and as 1,2,4,5-tetrahydroxybenzene (**VII**) after reductive acetylation. Catechol was identified as its acetylated product. The direct observation of the quinones

VIII and XI indicates that the attack of water on the 2-hydroxydibenzo-*p*-dioxin cation is occurring at both the C-3 and C-4a positions. The relative yields of these quinones (VIII and XI) depends on the contribution of structures C and D to the resonance hybrid.

We have confirmed the mechanism discussed above by conducting the reaction in ^{18}O-enriched water. Eighty-nine percent incorporation of two atoms of ^{18}O (*m/z* 220) from labeled water into 2,3-dihydroxydibenzo-*p*-dioxin (II) suggests that both quinone oxygens in compound VIII are derived from water. When the reaction was performed under argon, identical products were obtained. Furthermore, when the reaction was conducted under $^{18}O_2$, no incorporation of ^{18}O in the products was observed. These results suggest that the cleavage of C–O–C bonds occurs via the nucleophilic attack of water on the cation rather than via the scavenging of a radical by molecular oxygen.

Formation of considerable amounts of unlabeled catechol (V) (47.8%) during the LiP oxidation of dibenzo-*p*-dioxin (I) in ^{18}O-labeled water supports a mechanism whereby the quinones VIII and XI undergo nonenzymatic reaction with H_2O. To further confirm the mechanism of the nonenzymatic reaction of the quinone VIII with water, we chemically synthesized dibenzo-*p*-dioxin-2,3-quinone (VIII). When the quinone VIII was hydrolyzed in succinate buffer (pH 3), catechol (V), 2-hydroxy-5-(2-hydroxyphenoxy)-1,4-benzoquinone (IX), and 4,5-dihydroxy-1,2-benzoquinone (X) were formed. The quinone X was identified as its phenazine derivative and also as 1,2,4,5-tetrahydroxybenzene (VII) after reductive acetylation, whereas the quinone IX was identified as 2,2′,4,5-tetrahydroxydiphenyl ether (III) after reductive acetylation. Detection of 2-hydroxy-5-(2-hydroxyphenoxy)-1,4-benzoquinone (IX) suggests that dibenzo-*p*-dioxin-2,3-quinone (VIII) undergoes stepwise cleavage of C–O–C bonds to form catechol (V) and 4,5-dihydroxy-1,2-benzoquinone (X). Cauquis and co-workers reported the formation of 2,5-dihydroxy-1,4-benzoquinone (XIV) from the reaction of the dibenzo-*p*-dioxin cation radical with water (Cauquis & Maurey-Mey, 1972). Under the conditions used in that study, the initially formed 4,5-dihydroxy-1,2-benzoquinone (X) may have undergone rapid isomerization to form the thermodynamically more stable 2,5-dihydroxy-1,4-benzoquinone (XIV). We also have observed that the hydrolysis reaction is catalyzed by acid. When the reaction was performed in 1% sulfuric acid, the yields of the short-lived quinone X were improved, suggesting that the polymerization of quinone X occurs over time. When the hydrolysis of the quinone VIII was conducted in $H_2{}^{18}O$, incorporation of ^{18}O atoms was observed in both quinones IX and X, suggesting that the quinone oxygens are derived from water. Excess incorporation of labeled oxygens in quinones IX and X is presumably due to the rapid exchange reaction between ^{18}O-enriched water and quinone oxygens as shown by the isotope-exchange reaction between the quinone X oxygen and water. However, no exchange of labeled oxygens was observed with the quinone VIII.

An alternative mechanism for the oxidation of dibenzo-*p*-dioxin is shown in Figure 4. In this mechanism, the quinones IX and XI undergo enzymatic oxidation of the phenolic function to generate their corresponding phenoxy radicals, G or L (Figure 4). Oxidation of the intermediate IX generates the phenoxy radical, G (Figure 4), which is in resonance with the carbon-centered radical, H. Further oxidation of this radical, followed by attack of H_2O on the cation, results in the cleavage of the C–O–C bond and generation of 1,2-benzoquinone (XIII) and 2,5-dihydroxy-1,4-benzoquinone (XIV). Previously, we proposed this mechanism for the LiP oxidation of 2,7-dichlorodibenzo-*p*-dioxin (Valli et al., 1992b).

Similarly, 2-(2-hydroxyphenoxy)-1,4-benzoquinone (XI) can undergo enzymatic oxidation to generate the phenoxy radical L, which is in resonance with the carbon-centered radical M (Figure 4). Further oxidation of this radical with subsequent attack of H_2O on the cation results in the cleavage of the C–O–C bond to generate 1,2-benzoquinone (XIII) and 2-hydroxy-1,4-benzoquinone (XV).

The phenoxy radical mechanism predicts the incorporation of one ^{18}O atom from labeled water into 1,2-benzoquinone. However, catechol (V) derived from the nonenzymatic reaction of the quinones IX and XI with water would be expected to undergo subsequent oxidation by LiP, resulting in the formation of 1,2-benzoquinone, with incorporation of ^{18}O from labeled water. Incorporation of ^{18}O due to quinone isotope exchange reactions also is expected. Thus, it is difficult to differentiate the phenoxy radical mechanism from these other possibilities.

On the basis of the products detected during LiP oxidation of dibenzo-*p*-dioxin (I), we conclude that the reaction is initiated by the one-electron oxidation of dibenzo-*p*-dioxin (I) to its corresponding cation radical. The cation radical undergoes reactions with water to yield various quinone intermediates (VIII, IX, or XI). The phenoxyquinone intermediates may undergo nonenzymatic as well as enzymatic reactions. MnP efficiently oxidizes phenols (Wariishi et al., 1989; Tuor et al., 1992). Thus in culture, MnP is likely to catalyze the oxidation of these phenoxyquinone intermediates, accelerating the degradative process.

As we have shown (Valli et al., 1992b), similar mechanisms are involved in the oxidation of 2,7-dichlorodibenzo-*p*-dioxin by LiP, including an initial enzyme-catalyzed one-electron oxidation to generate a cation radical. However, the nature of the final products is determined by the chlorine substitution pattern on the substrate. In the case of asymmetrically substituted chlorinated dibenzo-*p*-dioxin isomers, the initial attack of water on the corresponding cation radical could occur on either of the aromatic rings, leading to the formation of a large variety of products. We are continuing to study the mechanisms involved in the fungal degradation of polychlorinated dibenzo-*p*-dioxins.

REFERENCES

Alder, E., & Magnusson, R. (1959) *Acta Chem. Scand. 12*, 505–519.

Alic, M., Letzring, C., & Gold, M. H. (1987) *Appl. Environ. Microbiol. 53*, 1464–1469.

Bumpus, J. A., & Aust, S. D. (1987) *Bioessays 6*, 166–170.

Buswell, J. A., & Odier, E. (1987) *Crit. Rev. Biotechnol. 6*, 1–60.

Cauquis, G., & Maurey-Mey, M. (1972) *Bull. Soc. Chim. Fr.*, 3588–3593.

Dunford, H. B., & Stillman, J. S. (1976) *Coord. Chem. Rev. 19*, 187–251.

Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H., & Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 750–754.

Finley, K. T. (1974) in *The Chemistry of the Quinonoid Compounds, Part 2* (Patai, S., Ed.) pp 877–1144, John Wiley & Sons, London.

Forsyth, W. G. C., Quesnel, V. C., & Roberts, J. B. (1960) *Biochim. Biophys. Acta 37*, 322–326.

Gold, M. H., & Alic, M. (1993) *Microbiol. Rev. 57*, 605–622.

Gold, M. H., Mayfield, M. B., Cheng, T. M., Krisnangkura, K., Enoki, A., Shimada, M., & Glenn, J. K. (1982) *Arch. Microbiol. 132*, 115–122.

Gold, M. H., Kuwahara, M., Chiu, A. A., & Glenn, J. K. (1984) *Arch. Biochem. Biophys. 234*, 353–362.

Gold, M. H., Wariishi, H., & Valli, K. (1989) in *Biocatalysis in Agricultural Biotechnology* (Whitaker, J. R., & Sonnet, P. E., Eds.) ACS Symposium Series 389, pp 127–140, American Chemical Society, Washington, DC.
Hammel, K. E. (1989) *Enzyme Microb. Technol. 11*, 776–777.
Hammel, K. E., Kalyanaraman, B., & Kirk, T. K. (1986) *J. Biol. Chem. 261*, 16948–16952.
Hammel, K. E., Green, B., & Gai, W. Z. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10605–10608.
Hammel, K. E., Jensen, K. A., Jr., Mozuch, M. D., Landucci, L. L., Tien, M., & Pease, E. A. (1993) *J. Biol. Chem. 268*, 12274–12281.
Hansen, D. J. (1991) *Chem. Eng. News 69* (32), 7–14.
Higuchi, T. (1990) *Wood Sci. Technol. 24*, 23–63.
Joshi, D. K., & Gold, M. H. (1993) *Appl. Environ. Microbiol. 59*, 1779–1785.
Kersten, P. J., Tien, M., Kalyanaraman, B., & Kirk, T. K. (1985) *J. Biol. Chem. 260*, 2609–2612.
Kirk, T. K., & Farrell, R. L. (1987) *Annu. Rev. Microbiol. 41*, 465–505.
Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., & Zeikus, J. G. (1978) *Arch. Microbiol. 177*, 277–285.
Kuwahara, M., Glenn, J. K., Morgan, M. A., & Gold, M. H. (1984) *FEBS Lett. 169*, 247–250.
Marquez, L., Wariishi, H., Dunford, H. B., & Gold, M. H. (1988) *J. Biol. Chem. 263*, 10549–10552.
Piontek, K., Glumoff, T., & Winterhalter, K. (1993) *FEBS Lett. 315*, 119–124.
Poulos, T. L., Edwards, S., Wariishi, H., & Gold, M. H. (1993) *J. Biol. Chem. 268*, 4429–4440.
Rappe, C. (1980) in *The Handbook for Environmental Chemistry* (Hutzinger, O., Ed.) pp 157–179, Springer-Verlag KG, Berlin.
Renganathan, V., & Gold, M. H. (1986) *Biochemistry 25*, 1626–1631.
Renganathan, V., Miki, K., & Gold, M. H. (1986) *Arch. Biochem. Biophys. 246*, 155–161.
Renganathan, V., Miki, K., & Gold, M. H. (1987) *Biochemistry 26*, 5127–5132.
Schoemaker, H. E. (1990) *Recl. Trav. Chim. Pays-Bas 109*, 255–272.
Schwetz, B. A., Norris, J. M., Sparschu, G. L., Rowe, V. K., Gehring, P. J., Emerson, J. L., & Gerbig, C. G. (1973) in *Chlorodioxins—Origin and Fate* (Blair, E. H., Ed.) ACS Symposium Series 120, pp 55–69, American Chemical Society, Washington, DC.
Shine, H. J., & Shade, L. R. (1974) *J. Heterocycl. Chem. 11*, 139–141.
Spadaro, J. T., Gold, M. H., & Renganathan, V. (1992) *Appl. Environ. Microbiol. 58*, 2397–2401.
Tien, M., Kirk, T. K., Bull, C., & Fee, J. A. (1986) *J. Biol. Chem. 261*, 1687–1693.
Tomita, M., & Ueda, S. (1964) *Chem. Pharm. Bull. 12*, 33–40.
Tuor, U., Wariishi, H., Schoemaker, H. E., & Gold, M. H. (1992) *Biochemisty 31*, 4986–4995.
Ungnade, H. E., & Otey, F. H. (1951) *J. Org. Chem. 16*, 70–72.
Valli, K., & Gold, M. H. (1991) *J. Bacteriol. 173*, 345–352.
Valli, K., Brock, B. J., Joshi, D. K., & Gold, M. H. (1992a) *Appl. Environ. Microbiol. 58*, 221–228.
Valli, K., Wariishi, H., & Gold, M. H. (1992b) *J. Bacteriol. 174*, 2131–2137.
Wariishi, H., & Gold, M. H. (1990) *J. Biol. Chem. 265*, 2070–2077.
Wariishi, H., Valli, K., & Gold, M. H. (1989) *Biochemistry 28*, 6017–6023.
Wariishi, H., Huang, H., Dunford, H. B., & Gold, M. H. (1991a) *J. Biol. Chem. 266*, 20694–20699.
Wariishi, H., Valli, K., & Gold, M. H. (1991b) *Biochem. Biophys. Res. Commun. 176*, 269–275.