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Degradation of 2,7-Dichlorodibenzo-*p*-Dioxin by the Lignin-Degrading Basidiomycete *Phanerochaete chrysosporium*

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Under secondary metabolic conditions, the white-rot basidiomycete *Phanerochaete chrysosporium* degraded 2,7-dichlorodibenzo-*p*-dioxin (I). The pathway for the degradation of I was elucidated by the characterization of fungal metabolites and oxidation products generated by lignin peroxidase (LiP), manganese peroxidase (MnP), and crude intracellular cell-free extracts. The multistep pathway involves the degradation of I and subsequent intermediates by oxidation, reduction, and methylation reactions to yield the key intermediate 1,2,4-trihydroxybenzene (III). In the first step, the oxidative cleavage of the dioxin ring of I, catalyzed by LiP, generates 4-chloro-1,2-benzoquinone (V), 2-hydroxy-1,4-benzoquinone (VIII), and chloride. The intermediate V is then reduced to 1-chloro-3,4-dihydroxybenzene (II), and the latter is methylated to form 1-chloro-3,4-dimethoxybenzene (VI). VI in turn is oxidized by LiP to generate chloride and 2-methoxy-1,4-benzoquinone (VII), which is reduced to 2-methoxy-1,4-dihydroxybenzene (IV). IV is oxidized by either LiP or MnP to generate 4-hydroxy-1,2-benzoquinone, which is reduced to 1,2,4-trihydroxybenzene (III). The other aromatic product generated by the initial LiP-catalyzed cleavage of I is 2-hydroxy-1,4-benzoquinone (VIII). This intermediate is also generated during the LiP- or MnP-catalyzed oxidation of the intermediate chlorocatechol (II). VIII is also reduced to 1,2,4-trihydroxybenzene (III). The key intermediate III is ring cleaved by intracellular cell extracts to produce, after reduction, β-ketoadipic acid. In this pathway, initial oxidative cleavage of both C-O-C bonds in I by LiP generates two quinone products, 4-chloro-1,2-benzoquinone (V) and 2-hydroxy-1,4-benzoquinone (VIII). The former is recycled by reduction and methylation reactions to generate an intermediate which is also a substrate for peroxidase-catalyzed oxidation, leading to the removal of a second chlorine atom. Thus, this unique pathway results in the removal of both aromatic chlorines before aromatic ring cleavage takes place.

Owing to their acute toxicity in animal tests, polychlorinated dibenzo-*p*-dioxins (PCDDs) have been recognized as environmental hazards for several decades (25, 40, 43). PCDDs have been found as impurities in such industrial chemicals as 2,4,5-trichlorophenol and other chlorophenols (40). In addition, PCDDs have been identified in paper pulp mill effluents (25) and in ash generated by a variety of combustion processes (40). Since PCDDs are relatively chemically stable and lipophilic in nature (25, 40, 43), their accumulation in the food chain has been predicted and observed (3, 14, 29, 36). In addition, studies have concluded that these compounds are degraded very slowly by soil bacteria (9, 38, 41), probably increasing their environmental impact.

The white-rot basidiomycetous fungus *Phanerochaete chrysosporium* is capable of effectively degrading polymeric lignin and lignin model compounds (18, 23, 32, 54). Two extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), both of which occur as isozyme families, as well as an H_2O_2-generating system are thought to constitute the major components of this organism's extracellular lignin-degradative system (18, 23, 27, 32, 54). The degradation of a variety of environmentally persistent pollutants (5, 6, 10, 21, 28, 35) by this organism also has been reported. In several of these reports, the use of ^{14}C-labeled compounds demonstrated mineralization of the pollutant. However, until recently the pathways utilized by the fungus for the degradation of pollutants have not been elucidated. Recently, we reported the complete pathway for the degradation of 2,4-dichlorophenol (49) and for 2,4-dinitrotoluene (48) and suggested that LiP and MnP as well as intracellular enzymes are involved. Although the limited mineralization of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by *P. chrysosporium* has been reported (6), the pathways and mechanisms for the degradation of PCDDs have not been previously elucidated. In this report, we examine the degradation pathway of 2,7-dichlorodibenzo-*p*-dioxin (DCDD) by *P. chrysosporium*. We demonstrate the involvement of LiP and MnP as well as quinone reduction and methyl transferase reactions in this degradative pathway.

MATERIALS AND METHODS

Chemicals. DCDD (I) and 1-chloro-3,4-dihydroxybenzene (II) were obtained from AccuStandard Inc. (New Haven, Conn.) and Chemicals Procurement Laboratories Inc. (College Point, N.Y.), respectively. II was purified by preparative thin-layer chromatography (TLC; solvent, C_6H_6-ethyl acetate [4:1]) before use. 1,2,4-Trihydroxybenzene (III) and 1,4-dihydroxy-2-methoxybenzene (IV) were obtained from Lancaster Synthesis (Windham, N.H.). 1,2-Phenylenediamine and β-ketoadipic acid were obtained from Aldrich and Sigma, respectively.

4-Chloro-1,2-benzoquinone (V) was prepared immediately prior to use from II (40 mg) by using 1 molar equivalent of $NaIO_4$ in water (4 ml) (room temperature, 1 min) (1). The reaction mixture was extracted with chloroform and dried over Na_2SO_4. The product (V) was separated on preparative TLC (solvent, $CHCl_3$-CH_3OH [99:1]). 1-Chloro-3,4-di-

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methoxybenzene (VI) was synthesized by methylation of II (50 mg) with excess iodomethane (5 equivalents) in DMF (*N,N'*-dimethylformamide) (5 ml) with a catalytic amount of NaH (room temperature, 3 h). Water was added, the reaction mixture was extracted with chloroform at pH 8, the organic phase was evaporated, and the product was purified by preparative TLC (solvent, C_6H_6-ethyl acetate [4:1]). 2-Methoxy-1,4-benzoquinone (VII) was prepared by oxidation of IV with silver oxide in ether as described previously (20). All other chemicals were reagent grade.

Culture conditions. *P. chrysosporium* OGC101 (2) was grown from a conidial inoculum at 38°C in stationary culture (20 ml) as described previously (11, 17). The medium used in this study was that described previously (17, 33) with 2% glucose and either 1.2 or 12 mM ammonium tartrate as the carbon and nitrogen sources, respectively. The medium was buffered with 20 mM sodium 2,2-dimethylsuccinate, pH 4.5. In addition, cultures for the metabolism of DCDD contained 0.1% Tween 80. Cultures were incubated under air for 3 days, after which they were purged with 99.9% O_2 every 3 days.

Metabolism of DCDD and metabolic intermediates. After 6 days of incubation, the substrates in DMF (100 μl) were added to cultures to a final concentration of 25 μM for I or 250 μM for the intermediates. After the indicated additional intervals, the cultures were acidified to pH 2 with HCl. Ethyl acetate (20 ml) was added immediately, and the mixture was stirred vigorously for 5 min and filtered through a Buchner funnel, and the organic layer was separated. The water layer was reextracted with ethyl acetate (two 10-ml portions). The combined organic fraction was washed with NaCl-saturated water (10 ml), dried over Na_2SO_4, and evaporated under reduced pressure. During this procedure, the extraction efficiency for I was estimated to be >95%. The products were analyzed by gas chromatography (GC) or GC-mass spectrometry (GC-MS) after derivatization. Acetylation was carried out in acetic anhydride-pyridine (2:1). For reductive acetylation, the reduction of the quinone products with sodium dithionite was carried out in the culture medium before extraction. The presence of an *o*-quinone group in V was confirmed after converting the product to a phenazine derivative by using 1,2-phenylenediamine in acetic acid-methanol (30). For high-pressure liquid chromatography (HPLC) analysis, the cell-free culture medium was centrifuged (5,000 × *g*, 5 min) in an Amicon Centricon 10 tube, and the filtrate was analyzed directly.

Enzymes. LiP and MnP were purified from the extracellular medium of an acetate-buffered agitated culture of *P. chrysosporium* as described before (15, 16, 51, 52). The LiP concentration was determined at 408 nm with an extinction coefficient of 133 mM^{-1} cm^{-1} (16). The MnP concentration was determined at 406 nm with an extinction coefficient of 129 mM^{-1} cm^{-1} (15). Glucose-6-phosphate dehydrogenase was obtained from Sigma.

Intracellular enzyme preparation. Six-day-old cells grown under nitrogen-limiting conditions were filtered and washed with ice-cold 0.5% NaCl. The cells (10 g [wet weight]) were ground with 15 g of acid-washed sand with a mortar and pestle. Subsequently, 50 mM sodium phosphate buffer (pH 7.0) (25 ml) was added, and the mixture was stirred at 4°C for 15 min. The crude extract was centrifuged at 15,000 × *g*, and the supernatant was concentrated by ultrafiltration with an Amicon PM-10 membrane. The final protein concentration was determined to be 1 mg/ml (44).

Enzyme reactions. For DCDD oxidations, LiP reaction mixtures (10 ml) consisted of enzyme (5 μg/ml), substrate (10 μM), H_2O_2 (100 μM), and Tween 80 (0.1%) in 20 mM sodium succinate, pH 3.0. The same reaction mixture composition was used for the oxidation of metabolic intermediates; however, the volume of the reaction mixture was 1 ml, the substrate concentration was 100 μM, and no Tween 80 was added. To avoid enzyme inactivation (46, 50, 52), veratryl alcohol (100 μM) was added to reaction mixtures as indicated.

For the oxidation of DCDD, MnP reaction mixtures (10 ml) consisted of enzyme (2 μg/ml), substrate (10 μM), $MnSO_4$ (200 μM), H_2O_2 (200 μM), and Tween 80 (0.1%) in 50 mM sodium malonate, pH 4.5. Again, the same reaction mixture composition was used for the oxidation of metabolic intermediates; however, the volume of the mixture was 1 ml, the substrate concentration was 100 μM, and no Tween 80 was added.

Both LiP and MnP reactions were conducted at 25°C under O_2 for 10 min. Reaction mixtures were extracted with ethyl acetate at pH 2, dried over Na_2SO_4, evaporated under N_2, and analyzed by GC or GC-MS after derivatization. For reductive acetylation, sodium dithionite was added to the reaction mixture before the extraction. For HPLC analysis, the reaction mixture was filtered through a Centricon (Amicon Centricon 10) filter, and the filtrate was analyzed directly. Control reactions were conducted in the absence of either enzyme or H_2O_2.

Chloride release during the oxidation of I by LiP was examined by a modification of a previously described method (13). Owing to the low solubility of I in water, the reaction was conducted in a 500-ml reaction mixture with the same concentrations of reactants as above except that the Tween 80 was omitted. The AgCl precipitate was collected in a glass tube after centrifugation at 5,000 × *g*, dried over P_2O_5 under reduced pressure, and weighed. Chloride release during the oxidation of II by LiP and MnP and of VI by LiP was measured in the same manner. However, the reaction mixtures (200 ml) contained the substrates at a concentration of 100 μM. Chloride release was also measured by a colorimetric method (24).

LiP oxidations of DCDD (I) were also carried out under $^{18}O_2$ or argon as described before (37). For these reactions, 20-ml flasks contained 10 ml of reaction mixture as described above without the H_2O_2. The flasks were evacuated, flushed three times with scrubbed argon, and equilibrated with $^{18}O_2$ (95%; Monsanto Research Corp.). H_2O_2 was introduced with a microsyringe to initiate the reaction. Anaerobic reactions were conducted in the same manner except that the flasks were equilibrated with argon.

Ring cleavage of 1,2,4-trihydroxybenzene. The reaction mixture (10 ml) contained 1 ml of concentrated cell extract, 1,2,4-trihydroxybenzene (25 μmol), NADPH (10 μmol), glucose-6-phosphate (25 μmol), and glucose-6-phosphate dehydrogenase (15 U) in 50 mM sodium phosphate, pH 7.0. The reaction mixture was stirred slowly at 28°C for 30 min as described previously (7). At the end of the reaction, the pH was adjusted to 2.0 and the mixture was immediately extracted with ethyl acetate. The organic fraction was washed with water, dried over sodium sulfate, and evaporated. Trimethylsilylation of the product was carried out in bis (*N,O*-trimethylsilyl)trifluoroacetimide-pyridine (2:1). Control reactions were conducted with boiled extracts.

GC-MS. GC-MS 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 gel column (DB-5; J & W Science). The oven temperature was programmed from 80 to 320°C at 10°C/min. Quantitation of products was

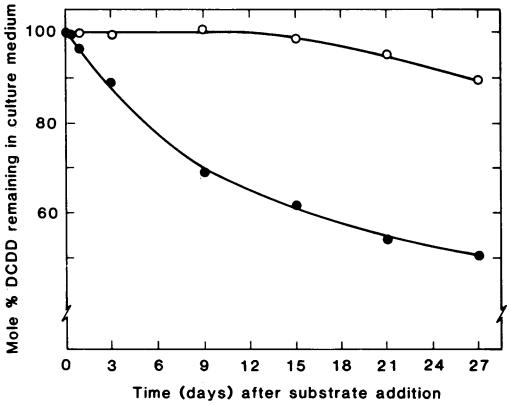

FIG. 1. Effect of nitrogen concentration on the degradation of DCDD. Stationary cultures containing 1.2 (●) or 12 (○) mM ammonium tartrate were inoculated with conidia and incubated for 6 days at 38°C, after which the substrate was added. Flasks were purged with O_2 periodically. The cultures were harvested, and the substrate remaining was determined as described in the text.

carried out on an HP 5890 series II gas chromatograph equipped with the same column and a flame ionization detector. Products were also analyzed by HPLC with an HP LiChrospher 100 RP-8 column and a linear gradient from 30% methanol in water (isocratic for 8 min) to 100% methanol (15 to 18 min) at a flow rate of 1.0 ml/min. The absorbance detector was operated at either 254 or 280 nm. Products were identified by comparison of their retention times on GC and HPLC and of mass fragmentation patterns with chemically prepared standards. Yields were quantitated on GC and/or HPLC by using calibration curves obtained with standards.

RESULTS

A time course for the degradation of DCDD (I) in cultures of *P. chrysosporium* is shown in Fig. 1. After a 27-day incubation period, only ~50% of the substrate remained in nitrogen-limited (1.2 mM ammonium tartrate) cultures, while ~90% of the substrate remained in nitrogen-sufficient cultures. Subsequent metabolic studies were performed under nitrogen-limited conditions.

Metabolism of DCDD and metabolic intermediates. Products and yields obtained from the fungal metabolism of DCDD (I) and various intermediates are shown in Fig. 2. Two aromatic products were identified from the fungal metabolism of I: 1-chloro-3,4-dihydroxybenzene (II) and 1,2,4-trihydroxybenzene (III). For II, mass spectrum *m/z* (relative intensity [%] shown in parentheses) (diacetyl ester), 230 (3), 228 (M^+, 8), 188 (7), 186 (19), 146 (36), 144 (100); GC retention time, 6.8 min. For III, mass spectrum *m/z* (relative intensity, %) (triacetyl ester), 252 (M^+, 7), 210 (21), 168 (52), 126 (100); GC retention time, 9.1 min.

1-Chloro-3,4-dihydroxybenzene (II) was also added exogenously to *P. chrysosporium* cultures. Three aromatic products were identified as metabolites of II: 1,2,4-trihydroxybenzene (III), 4-chloro-1,2-benzoquinone (V), and 1-chloro-3,4-dimethoxybenzene (VI). For V, mass spectrum *m/z* (relative intensity, %) (phenazine derivative), 216 (34), 214 (M^+, 100), 179 (32), 152 (13), 107 (15), 75 (20); GC retention time, 10.5 min. For VI, mass spectrum *m/z* (relative intensity, %), 174 (31), 172 (M^+, 100), 159 (19), 157 (67), 131 (16), 129 (39), 93 (62), 79 (54), 65 (72); GC retention time, 4.6 min. The retention times of underivatized II, III, V, and VI on HPLC were 2.91, 0.98, 4.35, and 14.8 min, respectively.

The metabolites 4-chloro-1,2-benzoquinone (V) and 1-chloro-3,4-dimethoxybenzene (VI) were added to fungal cultures. From 4-chloro-1,2-benzoquinone (V), the metabolites chlorocatechol (II), trihydroxybenzene (III), and 1-chloro-3,4-dimethoxybenzene (VI) were identified. In ad-

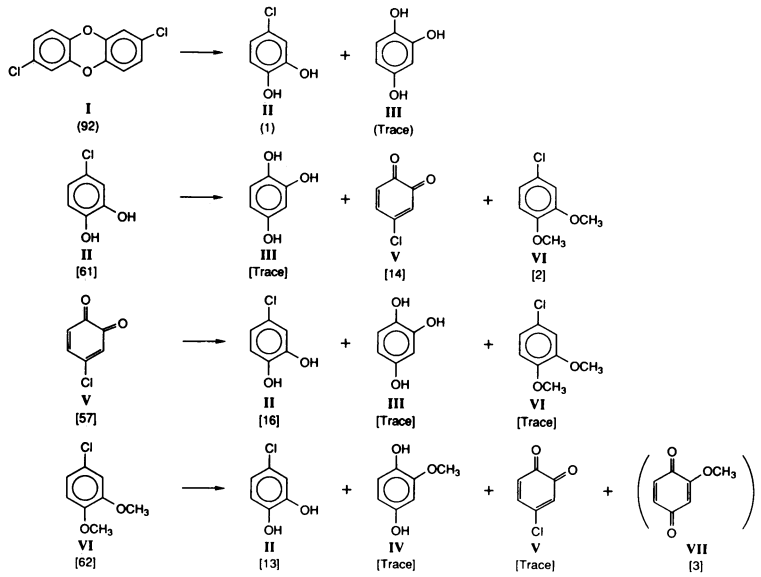

FIG. 2. Metabolites identified from the fungal degradation of DCDD and pathway intermediates. Cultures were incubated and extracted and products were analyzed as described in the text. Moles percent yields of the compounds for incubations of 1 h (values in brackets) and 48 h (values in parentheses) are listed below each compound. The formation of the product shown in parentheses at bottom right was suggested only by HPLC.

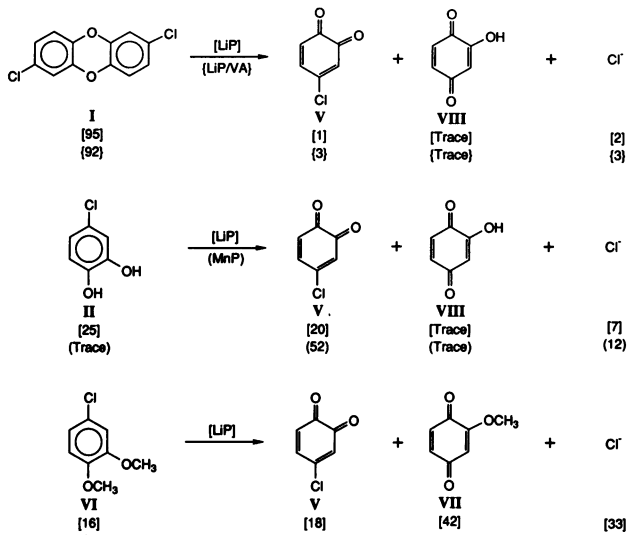

FIG. 3. Products identified from the oxidation of DCDD and several intermediates by purified LiP and MnP. Reaction conditions were as described in the text. Moles percent yields from the reaction with LiP and MnP are indicated in brackets and in parentheses, respectively, below each compound. Yields shown in braces were obtained when veratryl alcohol (VA) was added to the LiP reaction mixture.

dition, chlorocatechol (II), 1,4-dihydroxy-2-methoxybenzene (IV), and 4-chloro-1,2-benzoquinone (V) were identified as metabolites of VI (Fig. 2). For IV, mass spectrum *m/z* (relative intensity, %) (diacetyl ester), 224 (M^+, 9), 182 (39), 140 (100), 125 (46), 69 (11); GC and HPLC retention times, 7.9 and 2.09 min, respectively. The formation of 2-methoxy-1,4-benzoquinone (VII) from VI was suggested by HPLC analysis (retention time, 2.62 min).

Enzymatic oxidation of substrates and metabolic intermediates. DCDD (I) was slowly oxidized by LiP to yield 4-chloro-1,2-benzoquinone (V) and 2-hydroxy-1,4-benzoquinone (VIII). V was identified as its phenazine derivative, and the unstable intermediate VIII was identified as 1,2,4-trihydroxybenzene (III) after reductive acetylation. HPLC analysis revealed a peak cochromatographing with authentic V (retention time, 4.35 min) and another peak at 1.65 min, suggesting that the enzymatic oxidation products were the two benzoquinones V and VIII. Release of a small amount of Cl^- was also observed (Fig. 3). When veratryl alcohol was added to the reaction mixture, the product yields increased (Fig. 3), but no additional products from DCDD were observed. The reductive acetylation of 4-hydroxy-1,2-benzoquinone would also generate trihydroxybenzene (III). However, the phenazine derivative of the *o*-quinone was not observed. No reaction products were obtained if either enzyme or H_2O_2 was absent from the reaction mixture. As expected, DCDD (I) was not oxidized by MnP.

When LiP oxidation of DCDD (I) was conducted under $^{18}O_2$, no ^{18}O incorporation into the products V (phenazine derivative) or III (reductive acetylation derivative of VIII) was observed. When the oxidation of I was conducted under argon, identical products and yields were obtained.

Both LiP and MnP oxidized 1-chloro-3,4-dihydroxybenzene (II) to 4-chloro-1,2-benzoquinone (V) and 2-hydroxy-1,4-benzoquinone (VIII). These products were identified as described above. Release of Cl^- was also observed (Fig. 3). LiP but not MnP oxidized 1-chloro-3,4-dimethoxybenzene (VI) to the *o*-quinone (V) and 2-methoxy-1,4-benzoquinone (VII) (Fig. 3). VII was identified by GC-MS as 2-methoxy-1,4-dihydroxybenzene (IV) after reductive acetylation and by HPLC via coelution with the chemically prepared standard (VII) (retention time, 2.62 min). The release of Cl^- was also observed in this reaction. The yields of quinone products obtained in these reactions are probably underestimates because of their propensity for polymerization. The yield of chloride released during the oxidation of I may also be an underestimate, because the amount of Cl^- released is at the lower limit of detection by the method used.

When the oxidation of 1,2,4-trihydroxybenzene was carried out with crude cell extract in the presence of NADPH and an NADPH-generating system, β-ketoadipic acid (IX) was detected. For IX, mass spectrum *m/z* (relative intensity, %) (tri-trimethylsilyl derivative), 376 (M^+, 15.4), 361 (73), 317 (15.4), 286 (38.5), 259 (15.4), 231 (38.5), 169 (88.5), 147 (65.4), 125 (15.4), 73 (100). Under the basic conditions used in derivatization, β-ketoadipic acid exists in its enolic form; hence, we obtained the tri-trimethylsilyl derivative of β-ketoadipic acid for both the experimentally produced and standard compounds.

DISCUSSION

White-rot basidiomycetous fungi are primarily responsible for the initial depolymerization of lignin in wood (18, 23, 27, 32, 54). The best-studied white-rot fungus, *P. chrysosporium*, degrades lignin during secondary metabolic (idiophasic) growth (8, 17, 18, 32, 33). Under ligninolytic conditions, *P. chrysosporium* secretes two heme peroxidases (LiP and MnP) in addition to an H_2O_2-generating system (18, 32). These two peroxidases appear to be primarily responsible for the oxidative depolymerization of this heterogeneous, random phenylpropanoid polymer (4, 18, 23, 27, 32, 54). Other studies have demonstrated that *P. chrysosporium* is capable of mineralizing many persistent environmental pollutants (5, 6, 10, 21, 28, 35, 49), including, to a limited extent, TCDD (6). However, the *P. chrysosporium* degradative pathway for PCDDs and the enzyme(s) involved have not been studied. Recently, the *P. chrysosporium* degradative pathways for the pollutants 2,4-dichlorophenol and 2,4-dinitrotoluene were elucidated (48, 49), demonstrating that oxidative, reductive, and methylation reactions are involved and that both LiP and MnP play important roles.

While very slow degradation of TCDD has been reported for a number of bacteria and traces of polar metabolites are generated, for the most part these pathways have not been elucidated (9). The slow cometabolism of mono-, di-, and trichlorodioxins by a biphenyl-utilizing *Beijerinkia* strain has been reported (34). In this system, dioxygenation leading to dihydrodiols appears to be an early step in the pathway. Oxidative ring cleavage of dibenzo-*p*-dioxin has also been reported recently (26). In mammalian systems, the accumulation of [^{14}C]TCDD in liver and fatty tissues has been observed, but no metabolites have been found (41). The mammalian metabolism of less-chlorinated PCDDs is initiated by ring hydroxylation via epoxidation (47). Since the 2 and 3 positions are chlorinated in TCDD, the hydroxylation reaction is less likely to occur (40, 47).

In this study, the fungal degradative pathway for DCDD, including C—O—C bond cleavage, is reported for the first time, and the involvement of the *P. chrysosporium* lignin degradation system is demonstrated. The position and number of chlorine substituents on the dibenzo-*p*-dioxin skeleton affect its solubility and toxicity (40, 43). Among the 75 possible PCDDs, TCDD is the most toxic and the best

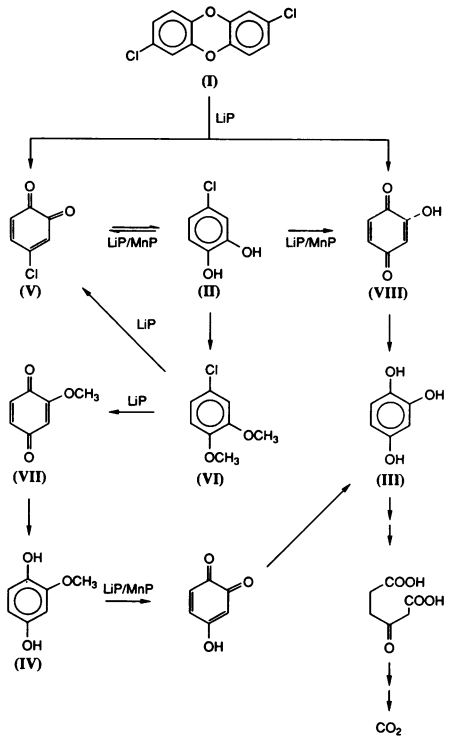

FIG. 4. Proposed pathway for the degradation of DCDD by *P. chrysosporium*.

studied. DCDD was selected for our initial metabolic study because of its increased solubility in H_2O (25, 40) and its reduced toxicity (43). In addition, we expected that this isomer would generate fewer metabolic products.

Our results demonstrate that *P. chrysosporium* extensively degrades DCDD (I) only under nutrient nitrogen-limiting conditions (Fig. 1), suggesting that the lignin-degradative system is involved. Sequential identification of the primary metabolites produced during DCDD degradation and subsequent identification of the secondary metabolites after addition of synthesized primary metabolites to cultures have enabled us to propose a pathway for the degradation of DCDD (Fig. 4).

The first step in the pathway is the oxidative cleavage of the dioxin ring of DCDD (I), resulting in the formation of the two aromatic products 4-chloro-1,2-benzoquinone (V) and 2-hydroxy-1,4-benzoquinone (VIII) and suggesting the involvement of LiP. These products were analyzed in fungal cultures as their reduced derivatives chlorocatechol (II) and trihydroxybenzene (III). The results of enzyme studies confirm that LiP oxidizes DCDD to generate 4-chloro-1,2-benzoquinone (V) and 2-hydroxy-1,4-benzoquinone (VIII), with the release of chloride (Fig. 3). Enzyme-catalyzed C—O—C bond cleavage of PCDDs has not been observed previously (5, 9, 21). This reaction is catalyzed by LiP alone, albeit at a slow rate. The addition of veratryl alcohol facilitates the formation of quinones from I (Fig. 3), confirming the direct involvement of LiP. The enhancement of LiP reactions by veratryl alcohol has been observed previously (18, 23, 32, 39, 46, 50).

One of the initial enzymatic oxidation products from I, 4-chloro-1,2-benzoquinone (V), is not a substrate for either LiP or MnP. However, *P. chrysosporium* cultures convert the chlorobenzoquinone (V) to chlorocatechol (II), the trihydroxybenzene (III), and 1-chloro-3,4-dimethoxybenzene (VI). III and VI also were identified as fungal metabolites from chlorocatechol (II) (Fig. 2). Both LiP and MnP oxidize chlorocatechol (II) to 4-chloro-1,2-benzoquinone (V) and 2-hydroxybenzoquinone (VIII), with the release of Cl^- (Fig. 3). However, under the conditions used, MnP is the more effective catalyst. These observations suggest that the intermediate 4-chloro-1,2-benzoquinone (V) is taken up and reduced to chlorocatechol (II), which is subsequently methylated to produce 1-chloro-3,4-dimethoxybenzene (VI). Some of the chlorocatechol may be exported for oxidation to 2-hydroxy-1,4-benzoquinone (VIII) (Fig. 4). Both the reduction of chlorobenzoquinones to chlorohydroquinones and the subsequent methylation of the hydroquinone to the dimethoxybenzene by *P. chrysosporium* have been described previously (49). The 2-hydroxy-1,4-benzoquinone (VIII) is probably reduced intracellularly to 1,2,4-trihydroxybenzene (III), as recently proposed (48).

1-Chloro-3,4-dimethoxybenzene (VI) is oxidized by *P. chrysosporium* cultures and by LiP but not by MnP to 2-methoxy-1,4-benzoquinone (VII) and 4-chloro-1,2-benzoquinone (V). This result demonstrates that LiP is capable of oxidizing both 1-chloro-3,4-dimethoxybenzene and 2-chloro-1,4-dimethoxybenzene (49), although at a slower rate than that for the oxidation of 1,4-dimethoxybenzene (31). In fungal cultures, both quinone products VII and V are reduced to their corresponding hydroquinones II and IV (Fig. 2 and 3). 1,4-Dihydroxy-2-methoxybenzene (IV) has been shown to be a substrate for both LiP and MnP (48). It has also been shown that the fungus metabolizes IV to trihydroxybenzene (III) via 4-hydroxy-1,2-benzoquinone (48). 2-Methoxy-1,4-benzoquinone (VII) is a major product of the oxidation of 1-chloro-3,4-dimethoxybenzene (VI) by LiP, whereas 2-methoxy-1,4-benzoquinone (VII) and its corresponding hydroquinone IV are very minor products in fungal cultures (Fig. 2 and 3). This suggests that the reduction and subsequent metabolism of 2-methoxybenzoquinone (VII) proceed rapidly. This is not unexpected, because 2-methoxy-1,4-benzoquinone can arise from the oxidative decarboxylation of the lignin metabolite vanillic acid (8, 12). 2-Methoxy-1,4-benzoquinone is also a product of the LiP-catalyzed oxidation of the *P. chrysosporium* secondary metabolite veratryl alcohol (20).

The other initial oxidation product of the LiP-catalyzed oxidation of I is 2-hydroxy-1,4-benzoquinone (VIII). The product would be reduced to 1,2,4-trihydroxybenzene (III). The trihydroxybenzene (III) generated during the metabolism of monomeric products arising from both rings of I is in turn ring cleaved, presumably by an intracellular dioxygenase, to produce, after subsequent reduction, β-ketoadipic acid. The likely intermediate, maleylacetic acid, not identified in this crude system owing to its presumed instability, was probably reduced to yield β-ketoadipic acid (7, 45).

Mechanism of C—O—C bond cleavage. The nature of the products identified from the LiP-catalyzed oxidation of DCDD (I)—4-chloro-1,2-benzoquinone (V), 2-hydroxy-1,4-benzoquinone (VIII), and chloride—and our understanding of previous LiP-catalyzed reactions enable us to propose a mechanism for this reaction. When the reaction of I was conducted under argon, identical products and yields were obtained as in the reaction under oxygen. Furthermore, when the reaction was conducted under $^{18}O_2$, no incorporation of ^{18}O into the aromatic products was obtained. These observations strongly suggest that C—O—C bond cleavage occurs via the nucleophilic attack of water on a cation rather than via the scavenging of a radical by molecular O_2. The first step in this proposed mechanism is the one-electron

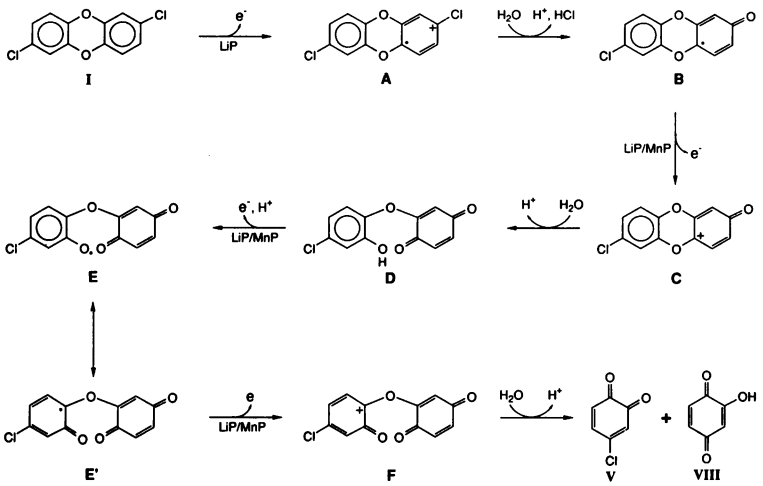

FIG. 5. Proposed mechanism for the lignin peroxidase-catalyzed dioxin ring cleavage of DCDD.

oxidation of I by the oxidized enzyme intermediate LiP compound I, resulting in the formation of the aryl cation radical A (Fig. 5). The LiP oxidation of a variety of nonphenolic aromatics to their corresponding aryl cation radicals has been well established (18, 27, 31, 32, 42). Indeed, the LiP oxidation of the related nonchlorinated compound dibenzo-*p*-dioxin to a cation radical has been reported (22). However, DCDD is considerably less soluble than dibenzo-*p*-dioxin (22). Furthermore, chloride is a much better leaving group than hydrogen; therefore, the aryl cation radical A is probably short-lived. For these reasons, the cation radical A was not detected during the oxidation of DCDD by LiP (22).

Attack of H_2O at the cation would result in the loss of chloride and the formation of the carbon-centered radical intermediate B (Fig. 5). One-electron oxidation of B by LiP or MnP would result in the formation of the cation intermediate C. Evidence for the oxidation of aromatic carbon-centered radicals to cations by LiP and MnP has been reported previously (32, 53). Attack of H_2O on intermediate C would lead to the first C—O—C bond cleavage and the formation of the quinone intermediate D. Subsequent oxidation of the phenolic function of D would generate the phenoxy radical E, which is in resonance with the carbon-centered radical E'. Oxidation of E' by either LiP or MnP would yield the cation F. Finally, attack of H_2O on the cation F would result in the cleavage of the second C—O—C bond and generation of 4-chloro-1,2-benzoquinone (V) and 2-hydroxy-1,4-benzoquinone (VIII). The identification of these aromatic products and chloride in the reactions, in addition to the lack of incorporation of ^{18}O from molecular oxygen, lends support for this proposed pathway. Owing to the low solubility of the substrate in aqueous solution and to the low yield of products, it was not possible to test for ^{18}O incorporation from $H_2{}^{18}O$.

In the metabolic pathway, both chlorine atoms are removed from the aromatic rings as chloride before ring cleavage takes place. This is advantageous because the possible formation of toxic chlorinated aliphatics is avoided. Indeed, peroxidase-catalyzed oxidative dechlorination followed by reduction of the quinone products results in the introduction of phenolic groups, which facilitate subsequent ring opening. Similar peroxidase-catalyzed oxidative dechlorinations of polychlorinated phenols have been reported (24, 49).

The mammalian metabolism of PCDDs is initiated by hydroxylation via epoxidation (47). Since TCDD and other highly chlorinated dioxins do not have an available 3-position for hydroxylation, they are relatively resistant to mammalian degradation (19, 47). In contrast, *P. chrysosporium* initiates C—O—C bond cleavage via one-electron oxidations catalyzed by LiP. Therefore, DCDD is effectively degraded and even TCDD is mineralized in this system (6). We propose that a similar metabolic pathway is probably involved in the fungal degradation of TCDD and have initiated studies to examine its metabolism.

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