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Purification and Characterization of a 1,2,4-Trihydroxybenzene 1,2-Dioxygenase from the Basidiomycete *Phanerochaete chrysosporium*

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1,2,4-Trihydroxybenzene (THB) is an intermediate in the *Phanerochaete chrysosporium* degradation of vanillate and aromatic pollutants. A *P. chrysosporium* intracellular enzyme able to oxidatively cleave the aromatic ring of THB was purified by ammonium sulfate precipitation, hydrophobic and ion-exchange chromatographies, and native gel electrophoresis. The native protein has a molecular mass of 90 kDa and a subunit mass of 45 kDa. The enzyme catalyzes an intradiol cleavage of the substrate aromatic ring to produce maleylacetate. $^{18}O_2$ incorporation studies demonstrate that molecular oxygen is a cosubstrate in the reaction. The enzyme exhibits high substrate specificity for THB; however, catechol cleavage occurs at approximately 20% of the optimal rate. THB dioxygenase catalyzes a key step in the degradation pathway of vanillate, an intermediate in lignin degradation. Maleylacetate, the product of THB cleavage, is reduced to β-ketoadipate by an NADPH-requiring enzyme present in partially purified extracts.

The white rot basidiomycete *Phanerochaete chrysosporium* degrades the plant cell wall polymer lignin (11, 15, 17, 24, 28, 34) and various aromatic pollutants (4, 22, 25, 26, 35, 39–41). Lignin peroxidase and manganese peroxidase secreted by the fungus catalyze lignin depolymerization, generating a wide variety of metabolic intermediates, including substituted benzoquinones, benzaldehydes, aromatic acids, and ring-opened fragments (15, 20, 38, 42, 43). Vanillate (4-hydroxy-3-methoxybenzoic acid) is an abundant product in the fungal degradation of wood (36). Quinone and hydroquinone intermediates are derived from vanillate and from lignin (9, 15, 29, 36, 44, 46) as well as from the fungal degradation of a variety of aromatic pollutants, including 2,4-dinitrotoluene (39), 2,4-dichlorophenol (40), 2,4,5-trichlorophenol (26), and 2,7-dichlorodibenzo-*p*-dioxin (41).

In contrast to the progress in our understanding of extracellular lignin depolymerization reactions, relatively little is known about the subsequent metabolic fate of these monomeric intermediates. Most studies on the degradation of aromatic intermediates such as vanillate have been carried out by using whole cultures to measure the release of the $^{14}CO_2$ from labeled substrates (2, 3, 8, 21). Vanillate hydroxylase (9, 46) and 1,2,4-trihydroxybenzene (THB) dioxygenase (7, 40, 41) activities have been detected in *P. chrysosporium* crude extracts. However, vanillate hydroxylase is the only enzyme involved in vanillate degradation by white rot fungi that has been purified (9). The enzyme catalyzes the oxidative decarboxylation of vanillate to 2-methoxy-1,4-dihydroxybenzene. Although metabolic studies suggest that this compound is demethoxylated to yield THB (2, 29), this reaction has not yet been demonstrated with cell-free systems.

Herein, we report the purification and characterization of the THB 1,2-dioxygenase from crude intracellular extracts of *P. chrysosporium*. We also describe the enzymatic reduction of maleylacetate to β-ketoadipate in the intracellular extracts.

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MATERIALS AND METHODS

Culture conditions. Stock cultures of *P. chrysosporium* OGC101 were maintained on slants as described previously (1). *P. chrysosporium* was grown from a conidial inoculum at 38°C in stationary culture (50 ml/1-liter Erlenmeyer flask) for 3 days (19). The medium used was described elsewhere (19, 30) and supplemented with 1% glucose, 12 mM ammonium tartrate, and 20 mM sodium-2,2-dimethylsuccinate (pH 4.5). Mycelial mats from two flasks were homogenized for 20 s in a blender and used to inoculate a 2-liter flask containing 1 liter of medium supplemented with 1% glucose, 12 mM ammonium tartrate, and 20 mM sodium acetate (pH 4.5) (18). Cultures were grown at 28°C on a rotary shaker (150 rpm) for 64 h. Vanillate (adjusted to pH 6, final concentration of 2 mM) was added to the cultures after 24 h of incubation (7, 46).

Preparation of enzyme extracts. Mycelial pellets of *P. chrysosporium* were harvested by filtration and washed successively with ice-cold water, 0.5% (wt/vol) NaCl solution, and extraction buffer (50 mM sodium phosphate [pH 7.0]). One flask yielded approximately 10 g of mycelium, which was stored at −80°C. All steps were carried out at 4°C. Routinely, 20 g of frozen cells was broken by grinding with a mortar and pestle in 200 ml of extraction buffer containing 0.004% phenylmethylsulfonyl fluoride (4% [wt/vol] stock solution in 2-propanol) and 25 g of sand. The homogenate was centrifuged at 16,000 × *g* for 30 min. The supernatant was subjected to centrifugation at 190,000 × *g* for 30 min to produce a clarified extract.

Purification of the THB 1,2-dioxygenase. The clarified extract was fractionated by sequential additions of solid ammonium sulfate, with each addition followed by centrifugation at 12,000 × *g* for 30 min. The dioxygenase activity precipitated between 45 and 65% ammonium sulfate saturation and was redissolved in extraction buffer containing 10% (wt/vol) glycerol and 0.004% phenylmethylsulfonyl fluoride (buffer A) and stored at −80°C.

(i) Chromatography on phenyl-Sepharose. A column (2.5 by 7 cm) of phenyl-Sepharose CL-4B was equilibrated with extraction buffer containing 0.5 M ammonium sulfate. The protein extract in 0.5 M ammonium sulfate was applied to the column at a ratio of about 1 ml (containing approximately 7 mg of protein) per 1.7 ml of resin. The column was washed with 120 ml of equilibration buffer, after which protein was eluted with a linear gradient (200-ml total volume) of decreasing ammonium sulfate concentration from 0.5 to 0 M. The fractions containing dioxygenase activity were pooled and concentrated by precipitation with 65% ammonium sulfate. The precipitate was redissolved in 5 ml of buffer A.

(ii) Chromatography on Mono Q. The phenyl-Sepharose fraction was desalted by passage through a Sephadex G-25 column equilibrated with 10 mM sodium phosphate (pH 7.0) (buffer B). Two milliliters of protein extract (3.6 mg of total protein) was loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer B at room temperature. Unbound protein was washed off with 5 ml of buffer B, and the column was eluted with a linear two-phase gradient of increasing concentration of sodium phosphate (pH 7.0). The phosphate concentration was increased from 10 to 83.5 mM over 10 ml and then to 98.2 mM over 5 ml. For sodium phosphate concentrations of 10, 83.5, and 98.2 mM, the corresponding ionic strengths are 0.018, 0.147, and 0.173. Active fractions were pooled and concentrated by ultrafiltration.

(iii) Native PAGE. Native polyacrylamide gel electrophoresis (PAGE) was carried out in a 4-7.5% discontinuous gel system (Tris-glycine buffer), containing 10% (wt/vol) glycerol but otherwise as described previously (14), at a constant current of 35 mA at 4°C for 5 h. Dioxygenase activity was detected directly in the gel by incubating slices in 100 mM sodium phosphate (pH 6.0) (assay buffer) and measuring THB-dependent oxygen consumption. The area containing the dioxygenase activity was cut out, and the enzyme was eluted from the gel by using a model 433 Electro-Eluter (Bio-Rad Laboratories).

The native molecular mass of the dioxygenase was determined by size exclusion chromatography on a Sephacryl 200 HR column (1.5 by 45 cm) preequilibrated with buffer C (10 mM sodium phosphate [pH 7.0], 150 mM NaCl) at a flow rate of 0.4 ml/min. Sodium dodecyl sulfate (SDS)-PAGE was carried out in a 12% Tris-glycine gel system by the method of Laemmli (32). Proteins were visualized in gels by silver staining.

Enzyme assays. Dioxygenase activity was assayed polarographically by using a Clark O_2 electrode (Rank, Cambridge, Great Britain). The reaction mixture (2 ml) contained air-saturated assay buffer plus substrate (100 μM) and enzyme fraction. Reactions were started by addition of substrate in water and reaction mixtures incubated at 30°C. The electrode was calibrated as described previously (37).

Maleylacetate reductase activity was assayed spectrophotometrically by measuring NADPH oxidation at 340 nm. The incubation conditions were the same as those for the dioxygenase except that the reaction volume was 1 ml and contained NADPH (100 μM). Protein concentrations were determined as described previously (6).

Reaction product analysis. Reactions were stopped by acidification to pH 2 with HCl. Mixtures were saturated with NaCl and extracted three times with an equal volume of ethyl acetate. The organic phases were combined, dried over Na_2SO_4, evaporated under a stream of nitrogen, derivatized with bis(trimethylsilyl)trifluoroacetamide-pyridine (2/1 [vol/vol]), and analyzed by gas chromatography (GC)-mass spectroscopy (MS) as described previously (38). Sodium borohydride reduction of possible product aldehydes was carried out in methanol after two ethyl acetate extractions. Ethylation was performed with ethyl iodide and 1,8-diazabicyclo[5.4.0]undec-7-ene. All substrates and reaction products were identified by their GC retention times and mass spectra. Maleylacetate also was converted to β-ketoadipate and compared with a standard.

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 column (DB-5; J&W Science). The oven temperature was programmed from 70 to 320°C at 10°C/min.

High-pressure liquid chromatography (HPLC) analysis was carried out with an HP LiChrospher 100 RP-18 column, using a linear gradient of methanol (0 to 20%, 15 ml) in water at a flow rate of 1 ml/min. Retention times for THB and 2-hydroxy-1,4-benzoquinone were 3.3 and 8.6 min, respectively.

$^{18}O_2$ incorporation experiments. Incubation mixtures (2 ml) were evacuated and flushed with argon three times and equilibrated with $^{18}O_2$ as described previously (31). Reactions were started by adding THB under anaerobic conditions, and reaction mixtures were incubated for 10 min. Reaction products were extracted and analyzed as described above.

Chemicals. THB was obtained from Lancaster Synthesis Inc. (Windham, N.H.) and purified by recrystallization. 2,5-Dihydroxy-1,4-benzoquinone was obtained from Aldrich. 1,2,4,5-Tetrahydroxybenzene and 5-chloro-1,2,4-trihydroxybenzene were prepared as previously reported (26). $^{18}O_2$ (99.1 atom% ^{18}O) was from Isotec, Inc. (Miamisburg, Ohio). β-Ketoadipate was obtained from Sigma. 1,8-Diazabicyclo[5.4.0]undec-7-ene was obtained from Aldrich. All other chemicals were reagent grade.

RESULTS

Enzymatic oxidation of THB. Crude extracts from *P. chrysosporium*, grown in the presence of vanillate, catalyzed aromatic ring cleavage of THB with concomitant consumption of molecular oxygen. Reactions were carried out at pH 6 to minimize substrate autoxidation to the corresponding quinone which occurs at higher pH (13). The amount of substrate remaining after the reaction was determined by HPLC. Crude cell extracts, but not purified fractions, consumed some oxygen in the absence of added substrate. The rate of oxygen consumption was corrected for autooxidation of the substrate and endogenous O_2 uptake by the cell extract. A typical trace of O_2 consumption by the Mono Q-purified protein fraction is shown in Fig. 1. The ratio of O_2 consumption to THB oxidation was approximately 1:1. The rate of oxygen consumption was directly proportional to the amount of protein added to the incubation mixture (Fig. 1, inset). Boiled protein was inactive.

Identification of maleylacetate. Reaction products were analyzed by GC-MS. Three different product peaks, all having identical mass spectra, were separated by GC (Table 1). The ratio of the three peaks was dependent on the extraction pH and the length of time between reaction and analysis. The mass spectral patterns are consistent with maleylacetate, and the three peaks probably represent different stereoisomers. Under the conditions used, all of the substrate was converted to maleylacetate.

Aromatic ring cleavage can occur via either *ortho* or *meta* cleavage with the concomitant formation of a dicarboxylic acid or a semialdehyde, respectively. The trimethylsilyl (TMS) derivatives of both of these products would have the same molecular mass. However, treatment of the reaction products with sodium borohydride followed by silylation or ethylation did not cause a change in the molecular mass, which would be

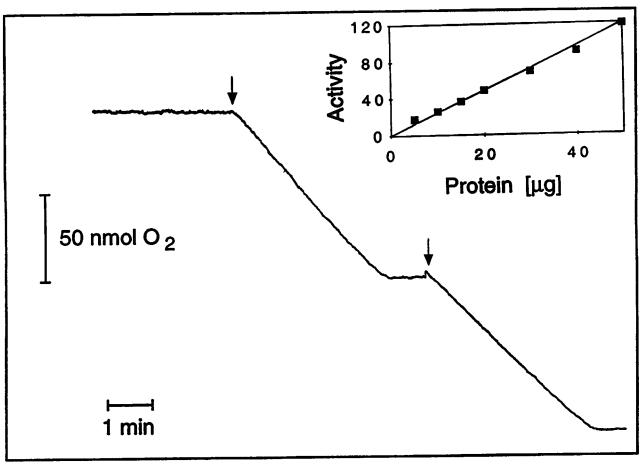

FIG. 1. Oxygen consumption during the incubation of THB with protein extract. The incubation mixture contained 15 μg of Mono Q-purified protein fraction. Consumption of oxygen was recorded with a Clark oxygen electrode. At the time points indicated, 100 nmol of THB was added to the incubation mixture. (Inset) Dependence of the rate of oxygen consumption on the amount of enzyme present in the incubation mixture.

expected from a semialdehyde derived from *meta* cleavage, confirming the formation of maleylacetate via *ortho* cleavage.

Purification of the THB 1,2-dioxygenase. Table 2 summarizes the purification procedure. The clarified extract was fractionated with ammonium sulfate, chromatographed on phenyl-Sepharose, and then chromatographed on a Mono Q column. The Mono Q column chromatogram is shown in Fig. 2. Several Mono Q column runs were pooled and concentrated prior to the native gel electrophoresis step. SDS-PAGE indicates that the purity of the dioxygenase preparation was greater than 96% following native gel electrophoresis (Fig. 3). The overall enzyme yield was 7% with a concomitant 1,680-fold purification. The specific activity of the purified enzyme for the cleavage of THB was 33.6 μmol min^{-1} mg^{-1}.

Molecular mass of the dioxygenase. The molecular mass of the native dioxygenase was determined to be 90 kDa by gel exclusion chromatography. The purified protein contained a single subunit with a mass of 45 kDa as determined by SDS-PAGE (Fig. 3).

Incorporation of $^{18}O_2$ into maleylacetate. To establish the origin of the oxygen used by the dioxygenase, $^{18}O_2$ was used as the substrate in the reactions. The molecular mass of maleylacetate was greater by four mass units than with reactions carried out with $^{16}O_2$ (Table 1). In control reactions that were evacuated and flushed only with argon, no maleylacetate was formed and all of the THB was recovered. These results

TABLE 1. Mass spectral patterns and GC retention times of THB, its substrate analogs, and their reaction products

Compound[a]	Retention time(s) (min)	Mass spectrum *m/z* (relative intensity)
THB	10.24	342 (100.00), 327 (19.86), 254 (6.19), 239 (56.04), 179 (3.60), 147 (6.25), 133 (2.41), 73 (98.00)
Maleylacetate	11.85, 12.15, 12.56	374 (3.27), 359 (20.17), 315 (2.70), 257 (100.00), 241 (2.43), 197 (3.41), 147 (50.28), 123 (7.05), 95 (9.35), 73 (98.00)
Maleylacetate[b]	11.85, 12.15, 12.56	378 (4.70), 363 (20.10), 317 (3.10), 259 (100.00), 241 (3.00), 147 (38.70), 125 (9.11), 97 (12.81), 73 (86.00)
Maleylacetate[c]	16.40	242 (3.72), 214 (0.62), 197 (9.95), 154 (41.37), 140 (71.87), 127 (74.87), 98 (79.50), 84 (100.00), 58 (89.75)
β-Ketoadipate	12.34	376 (11.71), 361 (59.13), 317 (11.14), 286 (22.70), 259 (17.51), 243 (14.44), 231 (43.47), 169 (86.38), 147 (73.20), 125 (15.51), 97 (6.07), 73 (100.00)
Catechol	5.56	254 (89.00), 239 (22.50), 166 (21.20), 151 (25.00), 147 (24.11), 136 (20.21), 91 (12.43), 73 (100.00)
Muconate	8.50	286 (6.01), 271 (20.50), 196 (8.11), 169 (100.00), 147 (61.00), 128 (7.31), 73 (64.51)
5-Chloro-1,2,4-trihydroxybenzene	11.43	378 (40.10), 376 (100.00), 363 (6.90), 361 (13.50), 275 (18.10), 273 (39.90), 237 (62.30), 179 (33.80), 147 (28.50), 93 (6.10), 73 (94.31)
3-Chloro-4-hydroxymuconate	11.90	410 (1.2), 408 (3.91), 395 (6.00), 393 (10.12), 293 (46.13), 291 (100.00), 175 (11.71), 147 (21.51), 73 (80.40)
1,2,4,5-Tetrahydroxybenzene	11.11	430 (100.00), 415 (11.56), 355 (5.10), 342 (8.32), 254 (4.99), 147 (41.58), 131 (4.54), 73 (98.00)
3,4-Dihydroxymuconate	13.15	462 (4.13), 447 (11.34), 403 (3.60), 345 (84.51), 247 (12.90), 175 (22.62), 147 (27.31), 73 (100.00)

[a] Unless otherwise indicated, all compounds were TMS derivatives. The substrate and its respective product(s) are grouped together.
[b] Incubation was carried out in the presence of $^{18}O_2$.
[c] Reaction product was derivatized with 1,8-diazabicyclo[5.4.0]undec-7-ene and ethyliodide.

TABLE 2. Purification of the THB 1,2-dioxygenase

Step	Total vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Yield (%)	Purification (fold)
Clarified extract	395	0.9	355	6.23	0.02	100	1
45–64% $(NH_4)_2SO_4$ fractionation	21	7.6	160	5.28	0.03	85	2
Phenyl-Sepharose	18	1.8	32	5.54	0.17	89	9
Mono Q	3	0.2	0.6	1.34	2.23	22	111
Native PAGE	1.5	0.008	0.012	0.41	33.6	7	1,680

[a] One unit equals 1 μmol of THB cleaved per min.

indicate that molecular oxygen is the source for both oxygen atoms incorporated into the product.

Substrate specificity. Several different polyphenolic compounds were tested as substrates with both the crude extract and the purified dioxygenase (Mono Q fraction). Table 3 shows the various substrates used and the relative rates of ring cleavage. Only THB and catechol were cleaved significantly by both the crude extract and the Mono Q fraction. 1,2,4,5-Tetrahydroxybenzene and 5-chloro-1,2,4-trihydroxybenzene were cleaved in trace amounts by the crude extract but not by the Mono Q fraction. Table 1 shows the mass spectra of the reaction products. Resorcinol, *p*-hydroquinone, pyrogallol, 2-methoxy-1,4-dihydroxybenzene, and protocatechuate were not cleaved by the 45 to 65% ammonium sulfate fraction. In all incubations in which no cleavage occurred, all of the substrate was recovered as measured by GC analysis.

Conversion of maleylacetate to β-ketoadipic acid. In the presence of NADPH, a product different from maleylacetate was detected in incubations with clarified extract. This product was identified as β-ketoadipate by GC-MS using authentic β-ketoadipate as a standard. The formation of β-ketoadipate was dependent on the presence of NADPH. Maleylacetate obtained from the incubation of THB with the dioxygenase and purified by extraction also was converted to β-ketoadipate by the clarified extract. The activity required NADPH, and the stoichiometry of the reduction was 1 mol of NADPH consumed to 1 mol of β-ketoadipate formed. Boiled extract was inactive.

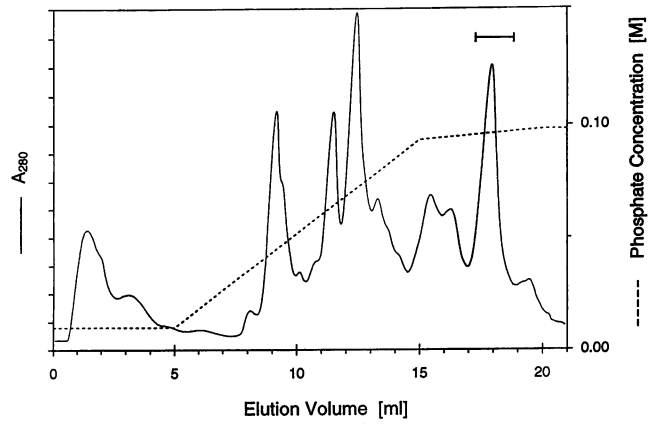

FIG. 2. Mono Q ion-exchange chromatography of the dioxygenase. The bar at the upper right indicates the location of the dioxygenase activity.

DISCUSSION

White rot basidiomycetes are the only organisms known to efficiently degrade the plant cell wall polymer lignin (11, 15, 17, 22, 24, 28, 43). Recently, white rot fungi also have been shown to degrade a wide range of aromatic pollutants, including nitrotoluenes, polychlorinated phenols, polychlorinated dibenzo-*p*-dioxins, and polynuclear aromatics (4, 22, 25, 26, 35, 39–41). Like lignin, these aromatic pollutants are converted to substituted benzoquinones, hydroquinones, and other intermediates (26, 39–41). The subsequent degradation of the monoaromatic intermediates derived from both lignin and aromatic pollutants is not well understood.

Vanillate has been identified as a key aromatic intermediate in the degradation of wood by *P. chrysosporium* (36). Vanillate is decarboxylated to form methoxyhydroquinone, which is apparently converted to THB (2, 3, 8, 29). We have shown that THB is an intermediate in the degradation of several environmental pollutants by *P. chrysosporium* (39, 41). Thus, the degradative pathways for both lignin and aromatic pollutants may merge at or before the formation of THB (Fig. 4). Ring cleavage of THB has been described in crude cell extracts from *P. chrysosporium* mycelia that degrade vanillate and several aromatic pollutants (7, 39, 41). This finding suggests that precursors are taken up by the hyphal cells. Following ring cleavage, the resulting dicarboxylic acid would be further metabolized.

Although lignin peroxidase-catalyzed ring cleavage has been demonstrated in our laboratory and elsewhere (24, 33), intramolecular dioxygenases are the enzymes commonly used to cleave aromatic rings (23). The majority of these enzymes have been purified from prokaryotes (12, 13, 16). Catechol, protocatechuate, homogentisate, and their substituted derivatives have been identified as substrates for ring cleavage (5, 16). Catabolism of aromatic compounds in eukaryotes usually proceeds with catechol or protocatechuate as an intermediate (23).

We describe here the identification of the intracellular enzyme activity responsible for the cleavage of THB by *P. chrysosporium*. The enzyme was purified from fungal cells grown in the presence of vanillate. The addition of vanillate to the culture medium results in a two- to threefold increase in dioxygenase activity, confirming previous results (7, 46).

GC-MS analysis demonstrates that maleylacetate is the ring-cleaved product. We observed three GC peaks for the TMS derivative of maleylacetate. All three peaks have identical mass spectra which are consistent with a maleylacetate derivative, suggesting they represent three different stereoisomer derivatives. Maleylacetate can undergo keto-enol tautomerization leading to *cis* and *trans* isomers with respect to the C-2 double bond. For TMS derivatives, *cis-trans* isomerization could have occurred at the C-2 double bond. Additional

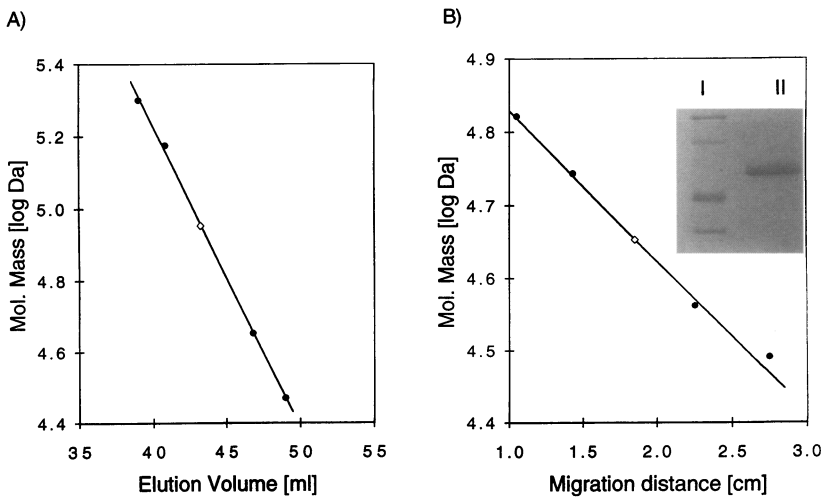

FIG. 3. (A) Molecular mass determination of the dioxygenase by gel exclusion chromatography. The molecular (mol.) mass standards are sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), egg ovalbumin (45 kDa), and bovine erythrocyte carbonic anhydrase (29 kDa). ◇, molecular mass of the THB dioxygenase. (B) Molecular mass determination of the dioxygenase subunit on SDS-PAGE. The molecular mass standards are bovine serum albumin (66.3 kDa), bovine liver glutamic dehydrogenase (55.4 kDa), porcine muscle lactate dehydrogenase (36.5 kDa), and bovine erythrocyte carbonic anhydrase (31 kDa). ◇, molecular mass of the THB dioxygenase. (Inset) Stained gel. Lane I, protein standards; lane II, purified dioxygenase.

conformers may have been trapped, owing to decreased free rotation about C-C single bonds (e.g., the C-3 single bond) due to the bulkiness of the derivatizing TMS groups. These isomers apparently were resolved into three GC peaks. All three isomers of maleylacetate (detectable as TMS derivatives) were completely converted to β-ketoadipate when incubated with extracts containing maleylacetate reductase. If boiled extract was used or NADPH was omitted from the incubation, the same three isomers were recovered after extraction and trimethylsilylation.

Two ring cleavage mechanisms have been described for dioxygenases (23). Enzymes that cleave the aromatic ring between two adjacent hydroxy groups (*ortho* cleavage) produce dicarboxylic acids, whereas enzymes that cleave outside the two hydroxy groups (*meta* cleavage) yield semialdehydes (5, 16, 23). The TMS derivatives of the theoretical reaction products from THB generated by either cleavage mechanism would have the same molecular mass. Therefore, the dioxygenase reaction product was treated with sodium borohydride followed by ethylation or silylation. This procedure selectively reduces the nonderivatizable aldehyde function to derivatizable alcohol groups. Reduction of the semialdehyde produces an additional alcohol group, and the molecular mass of the derivatized product should increase. In contrast, the molecular mass of a dicarboxylic acid would remain unchanged by treatment with sodium borohydride. The mass of the derivatized reaction product from THB does not change by this treatment, confirming the presence of the dicarboxylic acid and *ortho* cleavage.

TABLE 3. Substrate specificity of THB dioxygenase[a]

Substrate	Relative activity (%)	
	45–65% $(NH_4)_2SO_4$ fraction	Mono Q fraction
THB	100	100
Catechol (1,2-dihydroxybenzene)	20	20
1,2,4,5-Tetrahydroxybenzene	<1	0
5-Chloro-1,2,4-trihydroxybenzene	<1	0
Hydroquinone (1,4-dihydroxybenzene)	0	ND[b]
Resorcinol (1,3-dihydroxybenzene)	0	ND
Pyrogallol (1,2,3-trihydroxybenzene)	0	ND
2-Methoxy-1,4-dihydroxybenzene	0	ND
Protocatechuate (3,4-dihydroxybenzoic acid)	0	ND

[a] Incubations were carried out as described in the text for 30 min at 30°C; the substrate concentration was 100 μM; 1 mg and 15 μg of protein were used from the ammonium sulfate and Mono Q fractions, respectively; analysis by GC-MS was as described in the text.

[b] ND, not determined.

Intermolecular dioxygenases require two acceptor substrates, whereas intramolecular dioxygenases utilize only one substrate as the oxygen acceptor (23). $^{18}O_2$ incorporation experiments demonstrate that molecular oxygen is the cosubstrate of the THB dioxygenase. Furthermore, the increase in the molecular mass of maleylacetate by four mass units indicates incorporation of two atoms of ^{18}O into the product. Since the enzyme does not require an additional cosubstrate other than oxygen and since the ratio of oxygen consumption to THB cleavage is approximately 1:1, the enzyme appears to incorporate both atoms of oxygen into maleylacetate.

Lignin degradation results in a multitude of substituted aromatic intermediates. Further metabolism requires ring cleavage of these compounds, via either an enzyme with broad substrate specificity or conversion of the intermediates into a limited number of substrates for ring cleavage. Since it was not known whether *P. chrysosporium* produces several dioxygenases with different substrate specificities or one enzyme with a broad substrate range, we tested both the 45 to 65% ammonium sulfate fraction and the Mono Q-purified extract for their ability to cleave various polyphenolic monomers. Only THB and catechol were substrates for ring cleavage by these fractions, indicating that THB dioxygenase has a narrow substrate specificity. 1,2,4,5-Tetrahydroxybenzene and 5-chloro-1,2,4-trihydroxybenzene were cleaved in trace amounts by the ammonium sulfate fraction but not by the further-purified Mono Q fraction. This finding suggests that an additional enzyme, which did not survive extraction or purification, is required for

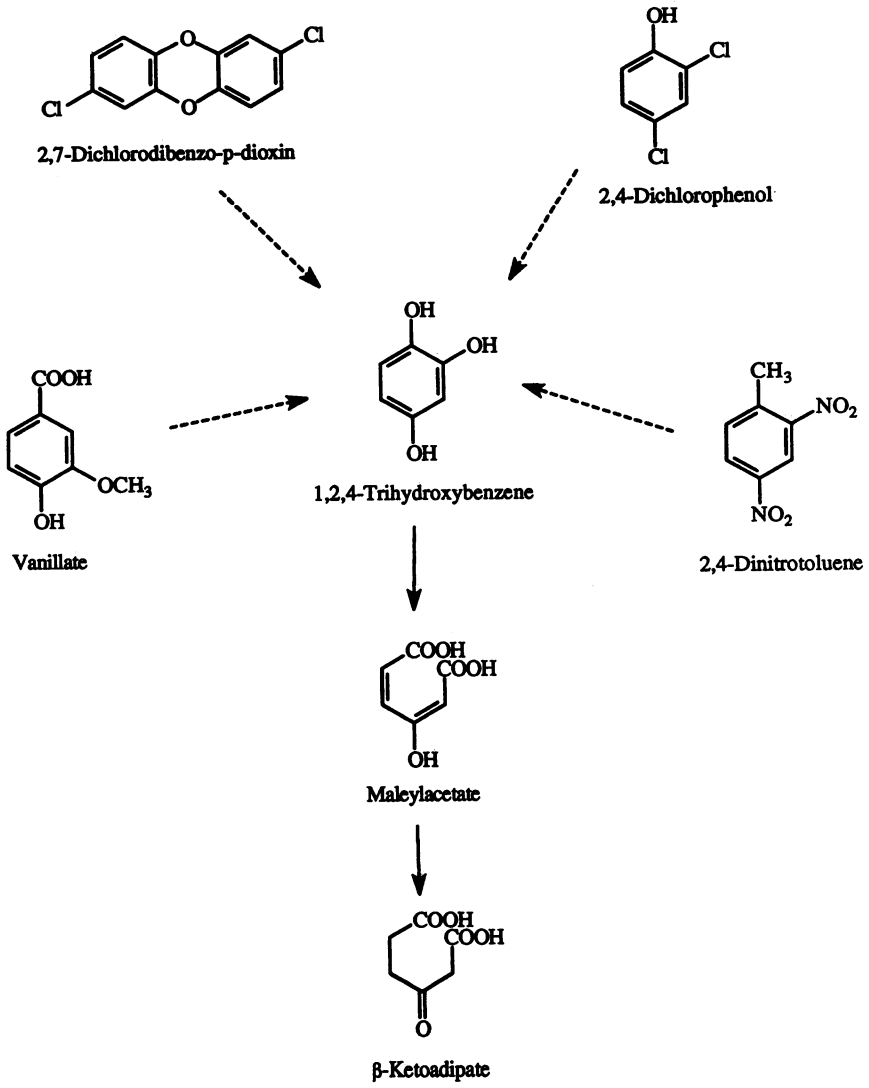

FIG. 4. Convergent pathways for the degradation of vanillate and toxic pollutants by *P. chrysosporium*. Dashed arrows indicate that these compounds are metabolic precursors of THB.

the oxidation of these compounds. Although protocatechuate-3,4-dioxygenase has been reported previously in the white rot fungi *Pleurotus ostreatus* and *Chaetomium piluliferum* (44, 45), we were unable to detect this enzyme activity in *P. chrysosporium* extracts.

The *P. chrysosporium* extracellular lignin-degrading system is expressed during secondary metabolism which is triggered by nutrient nitrogen limitation (11, 15, 17, 28). Since the THB dioxygenase described here was purified from cells grown under primary metabolic conditions, the regulation of this dioxygenase appears to be different from that of the lignin-degrading peroxidases. Vanillate hydroxylase, an enzyme that catalyzes the decarboxylation of vanillate, also is present under nitrogen-sufficient conditions (46). In addition, a mutant strain of *P. chrysosporium* that is unable to express the extracellular lignin-degrading enzymes still decarboxylates vanillate (19). These results indicate that the intracellular vanillate degradation pathway is regulated via mechanisms distinct from those regulating the lignin-degrading peroxidases. However, even under nitrogen-limited conditions, sufficient dioxygenase and vanillate hydroxylase (15) are available for the degradation of intermediates generated during lignin breakdown.

Purification of THB dioxygenase will enable us to study its structure and function on the molecular level. It also will facilitate the isolation and characterization of its encoding gene and subsequent studies on regulation of gene expression.

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