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Enzymatic Mechanisms Involved in Phenanthrene Degradation by the White Rot Fungus *Pleurotus ostreatus*

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The enzymatic mechanisms involved in the degradation of phenanthrene by the white rot fungus *Pleurotus ostreatus* were examined. Phase I metabolism (cytochrome P-450 monooxygenase and epoxide hydrolase) and phase II conjugation (glutathione *S*-transferase, aryl sulfotransferase, UDP-glucuronosyltransferase, and UDP-glucosyltransferase) enzyme activities were determined for mycelial extracts of *P. ostreatus*. Cytochrome P-450 was detected in both cytosolic and microsomal fractions at 0.16 and 0.38 nmol min^{-1} mg of $protein^{1}$, respectively. Both fractions oxidized [9,10-^{14}C]phenanthrene to phenanthrene *trans*-9,10-dihydrodiol. The cytochrome P-450 inhibitors 1-aminobenzotriazole (0.1 mM), SKF-525A (proadifen, 0.1 mM), and carbon monoxide inhibited the cytosolic and microsomal P-450s differently. Cytosolic and microsomal epoxide hydrolase activities, with phenanthrene 9,10-oxide as the substrate, were similar, with specific activities of 0.50 and 0.41 nmol min^{-1} mg of $protein^{-1}$, respectively. The epoxide hydrolase inhibitor cyclohexene oxide (5 mM) significantly inhibited the formation of phenanthrene *trans*-9,10-dihydrodiol in both fractions. The phase II enzyme 1-chloro-2,4-dinitrobenzene glutathione *S*-transferase was detected in the cytosolic fraction (4.16 nmol min^{-1} mg of $protein^{-1}$), whereas aryl adenosine-3′-phosphate-5′-phosphosulfate sulfotransferase (aryl PAPS sulfotransferase) UDP-glucuronosyltransferase, and UDP-glucosyltransferase had microsomal activities of 2.14, 4.25, and 4.21 nmol min^{-1} mg of $protein^{-1}$, respectively, with low activity in the cytosolic fraction. However, when *P. ostreatus* culture broth incubated with phenanthrene was screened for phase II metabolites, no sulfate, glutathione, glucoside, or glucuronide conjugates of phenanthrene metabolites were detected. These experiments indicate the involvement of cytochrome P-450 monooxygenase and epoxide hydrolase in the initial phase I oxidation of phenanthrene to form phenanthrene *trans*-9,10-dihydrodiol. Laccase and manganese-independent peroxidase were not involved in the initial oxidation of phenanthrene. Although *P. ostreatus* had phase II xenobiotic metabolizing enzymes, conjugation reactions were not important for the elimination of hydroxylated phenanthrene.

Polycyclic aromatic hydrocarbons (PAHs) are pollutants, many of which have toxic and carcinogenic properties (9). Ligninolytic fungi, which have the ability to degrade a wide variety of PAHs, have been evaluated for their potential in the remediation of contaminated sites (1, 6, 7, 11, 13, 19, 33, 44). Recently, we demonstrated the mineralization of PAHs, including phenanthrene, anthracene, and pyrene, by the white rot fungus *Pleurotus ostreatus* (2). We isolated and identified metabolic intermediates and suggested possible PAH degradation pathways (3, 4). On the basis of the metabolites formed from PAHs in culture broths of *P. ostreatus*, we speculated that the first steps in the biotransformation of PAHs were catalyzed by two phase I enzymes, cytochrome P-450 monooxygenase and epoxide hydrolase. We also suspected that *P. ostreatus* had the potential for phase II biotransformations (i.e., formation of sulfates, glucuronides, glucosides, or glutathione conjugates from phenolic derivatives of the PAHs), since we found water-soluble metabolites, which could be conjugation products, similar to those produced by *Cunninghamella elegans* (49) and other fungi (8, 28). Ring cleavage was also indicated, since 2,2′-diphenic acid and CO_2 were produced from phenanthrene (3). The ligninolytic enzymes may also be involved in PAH degradation by *P. ostreatus*, as was suggested by Hammel et al. (19–21) for *Phanerochaete chrysosporium.*

Phase I and phase II reactions are biochemical processes in higher animals that either modify the toxicity of xenobiotics or change them to water-soluble forms that are readily eliminated from the body (12). The most important phase I enzyme is cytochrome P-450 monooxygenase, which incorporates one atom of oxygen into the xenobiotic, producing an epoxide, and the other atom into water. Some epoxides are unstable and undergo further reactions, usually rearrangement to phenols or enzymatic hydration via another phase I enzyme, epoxide hydrolase, to *trans*-dihydrodiols (26, 35). The phase I metabolites can then act as substrates for phase II enzyme reactions, which are typically known as conjugation pathways. The addition of glutathione, sulfate, or sugar residues to the xenobiotics by phase II enzymes generally makes the compounds more water soluble, less toxic, and more easily eliminated from the body (8).

Until now, little has been known about the phase I and phase II enzymes involved in PAH metabolism by *P. ostreatus*. An earlier report (30) suggested the involvement of cytochrome P-450 in the hydroxylation of benzo[*a*]pyrene by *Pleurotus pulmonarius*. Studies of microbial cytochrome P-450 and epoxide hydrolase have concentrated on the metabolism of drugs (14), styrene, and its derivatives (22, 32), fungicides (16, 31), and PAHs (24, 40), and on biochemical and molecular genetics (25, 27, 34, 40, 42, 45). However, most of the data on phase I and phase II enzymes in drug metabolism have been obtained with

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mammalian systems, since these reactions result in toxification and/or detoxification of xenobiotics (12, 26, 35, 36, 38, 39).

In this work, we report on the enzymes involved in phenanthrene metabolism by *P. ostreatus* in the cytosolic and microsomal cell fractions as well as on the extracellular ligninolytic enzymes. An understanding of the enzymatic system involved in PAH degradation by *P. ostreatus* should make it more accessible for bioremediation and enable its use as an alternative model for xenobiotic metabolism. This study supports our hypothesis (3, 4) of the involvement of cytochrome P-450 and epoxide hydrolase in the initial hydroxylation of PAHs by *P. ostreatus.*

MATERIALS AND METHODS

Chemicals, radioisotopes, and enzymes. NADPH, NADH, flavin mononucleotide, flavin adenine dinucleotide, 1-naphthyl β-D-glucuronide, 1-naphthyl β-D-glucoside, 1-naphthylsulfate, [1-^{14}C]naphthol (7 mCi/mmol), 1-chloro-2,4-dinitrobenzene, glutathione (reduced form), PAPS (adenosine-3′-phosphate-5′-phosphosulfate) UDP-glucuronic acid, UDP-glucose, phenanthrene, phenanthrene 9,10-quinone, the cytochrome P-450 inhibitor 1-aminobenzotriazole (ABT), and all commercial enzymes used were purchased from Sigma Chemical Co. (St. Louis, Mo.). [9,10-^{14}C]phenanthrene (19.3 mCi/mmol) was purchased from Amersham/Searle (Arlington Heights, Ill.). 2,2′-Diphenic acid was purchased from Fluka AG (Buchs, Switzerland). SKF-525A (proadifen) was purchased from SmithKline Beecham (Philadelphia, Pa.). Phenanthrene 9,10-oxide and phenanthrene *trans*-9,10-dihydrodiol were synthesized by Peter P. Fu (National Center for Toxicological Research). All solvents were of high-performance liquid chromatography (HPLC) grade. All other chemicals were of the highest purity available.

Microorganisms and preparation of cell extracts. Stock cultures of *P. ostreatus* Florida f6 were maintained as described before (3, 18). The culture medium used was basidiomycetes rich medium (BRM), which contained the following per liter of culture: 0.6 g of L-asparagine, 1.0 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.5 g of yeast extract, 0.01 g of $FeSO_4 \cdot 7H_2O$, 2 mg of $ZnNO_3 \cdot 4H_2O$, 50 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 3 mg of $CaSO_4 \cdot 5H_2O$, 1% (wt/vol) D-glucose, and 1% (wt/vol) peptone (pH 5.5; Difco Laboratories, Detroit, Mich.). Water was added to 1 liter. Aliquots (10 ml) of the mycelium were used to inoculate 100 ml of BRM. All of the flasks were incubated at 28°C in the dark on a rotary shaker at 120 rpm.

Cell extracts were prepared from *P. ostreatus* cultures grown for 48 to 72 h in 2-liter Erlenmeyer flasks containing 700 ml of BRM. The fungus is in an active growth phase at this time point. Mycelia were harvested by filtration and frozen rapidly under liquid nitrogen. The frozen pellets were homogenized to a fine powder in a blender cup at 20-s intervals for 1 min. The powdered mycelium was resuspended in 50 mM potassium phosphate buffer (1:1 [wt/vol], pH 7.4) containing 10% (vol/vol) glycerol. The homogenate was filtered through cotton cloth, and the filtrate was centrifuged at 10,000 × *g* for 20 min and then ultracentrifuged at 105,000 × *g* for 90 min at 4°C. The microsomal pellet was resuspended in the same buffer. The 105,000 × *g* supernatant and pellet were analyzed for phase I and phase II enzymatic activities.

The UV absorption spectra of metabolites were obtained with a model 1040A diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, Calif.) and analyzed with a Hewlett-Packard model 300 computer. Radiolabeled metabolites eluting from the column were measured with a Radiomatic FLO-ONE model A-525A detector (Packard Instrument Co., Downers Grove, Ill.).

Thin-layer chromatography (TLC) of phenanthrene metabolites was performed on silica gel 60 F_{254} plates (20 by 20 cm, 0.25-mm thickness; E. Merck, Darmstadt, Germany). The metabolites were separated in benzene-ethanol (9:1 [vol/vol]). Compounds were observed on the chromatograms with a UV lamp. Radiolabeled chromatograms were exposed to X-ray film for 24 to 48 h. The film was developed, analyzed, and quantified by densitometry (Masterscan; Scananalytics, CSP, Inc., Billerica, Mass.). The radioactive bands on the TLCs were cut out and quantified by liquid scintillation spectrometry.

Enzyme assays. The reduced cytochrome P-450 carbon monoxide difference spectrum was determined as described by Omura and Sato (37), by using an extinction coefficient of 9.1 mM^{-1} cm^{-1} (37). The cytochrome P-450 monooxygenase activity was determined with [9,10-^{14}C]phenanthrene as the substrate and by separating metabolites by HPLC or TLC. The 1-ml assay solution contained cytosolic or microsomal fractions (200 to 800 μg of protein) and 10 μg of [9,10-^{14}C]phenanthrene in 50 mM phosphate buffer (pH 7.4). The reaction mixtures were incubated at 30°C overnight, extracted with ethyl acetate, dried over anhydrous sodium sulfate, and evaporated to dryness in vacuo at 40°C. The residues were redissolved in methanol for TLC and HPLC and compared with residues from controls. Phenanthrene and its metabolites were resolved by reversed-phase HPLC. An Ultrasphere C_{18} octadecylsilane column (25 cm by 4.6 mm inside diameter; Altex Scientific, Berkeley, Calif.), with a 40-min linear gradient of water-methanol (50:50 to 5:95 [vol/vol]) at a flow rate of 1 ml/min, was used to separate neutral metabolites. Acidic metabolites were separated under the same HPLC conditions, except that 1% (vol/vol) acetic acid was added to the mobile phase. The inhibition of cytochrome P-450 activity was determined with the same cytochrome P-450 monooxygenase inhibitors used for whole-cell experiments (3), i.e., 0.1 mM ABT, 0.1 mM SKF-525A, or carbon monoxide, which was bubbled through the reaction mixture for 20 s. The decrease in the amount of phenanthrene *trans*-9,10 dihydrodiol produced was measured by either HPLC or TLC.

Epoxide hydrolase activity was determined by incubating cytosolic and microsomal fractions with phenanthrene 9,10-oxide. One unit is the amount of enzyme that converts 1 nmol of phenanthrene 9,10-oxide to phenanthrene *trans*-9,10-dihydrodiol per min. The 1-ml assay solution contained the cell fractions (200 to 800 μg of protein) and 10 μg of phenanthrene 9,10-oxide in 50 mM phosphate buffer (pH 7.4). The reaction mixtures were incubated at 30°C for 15 to 30 min, extracted with ethyl acetate, and analyzed for phenanthrene *trans*-9,10-dihydrodiol by HPLC. Inhibition of cytosolic and microsomal epoxide hydrolases was determined by adding the epoxide hydrolase inhibitor cyclohexene oxide (0.1, 1, and 5 mM) (39) to the reaction mixture. The decrease in the amount of phenanthrene *trans*-9,10-dihydrodiol produced was measured by HPLC.

Aryl sulfotransferase activity was determined with [1-^{14}C]α-naphthol as the substrate by the method of Leakey et al. (29) with the following modifications. The 500-μl incubation mixture contained 1 mM [1-^{14}C]α-naphthol (0.1 μCi μmol^{-1}), 50 or 100 μM PAPS, and cell extract (100 to 500 μg of protein) and was incubated at 37°C for 15 min to 2 h. The aqueous layer was washed twice with 2 ml of chloroform. Control mixtures contained the same ingredients except that either boiled cell extract was used or PAPS was eliminated. Portions of the washed aqueous phase were counted in a Packard Tri-Carb 2000CA liquid scintillation analyzer (Packard Instrument Company, Meriden, Conn.). One unit of aryl sulfotransferase is defined as that amount of enzyme required to convert 1 nmol of 1-naphthol to 1-naphthylsulfate per min under the assay conditions.

UDP-glucuronosyltransferase and UDP-glucosyltransferase activities were assayed by the same procedure as that used for aryl sulfotransferase, except that PAPS was replaced by 1 mM UDP-glucuronic acid or 1 mM UDP-glucose, respectively. Glutathione *S*-transferase activity was determined as described by Habig et al. (17). The reaction mixture consisted of the substrate, 1 mM 1-chloro-2,4-dinitrobenzene, 1 mM reduced glutathione, and 40 to 400 μg of protein in 1 ml of 50 mM phosphate buffer (pH 6.5). Formation of the glutathione conjugate was monitored spectrophotometrically by measuring absorption at 340 nm for 10 min with a UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Columbia, Md.) and by using an extinction coefficient of 9.6 mM^{-1} cm^{-1} (17).

Protocatechuate 3,4-dioxygenase activity was determined as described by Whittaker et al. (46) and Wojtas-Wasilewska et al. (47, 48) by measuring the reduction in absorbance at 290 nm at 25°C. Catalase and glucose-6-phosphatase were determined by standard methods (15, 23).

Protein concentrations were determined with a Coomassie brilliant blue protein assay reagent kit (Pierce, Rockford, Ill.) with bovine serum albumin as the standard.

Metabolites from ligninolytic activity. (i) Whole-cell experiments. *P. ostreatus* was grown in the presence of phenanthrene, phenanthrene *trans*-9,10-dihydrodiol, phenanthrene 9,10-quinone, or 2,2′-diphenic acid (2.5 mg/100 ml) as described previously (3). After 14 days of incubation, the cultures were extracted with ethyl acetate and analyzed by HPLC (3).

(ii) Cell-free experiments. *P. ostreatus* cultures were filtered through glass wool after 5 days of incubation; the filtrate was analyzed for the ligninolytic enzymes laccase and manganese-independent peroxidase (2). The filtrate, which contained both activities, was incubated with phenanthrene or phenanthrene *trans*-9,10-dihydrodiol overnight at 30°C, at pH 7.5, in the dark. Each of the reactions was performed in the presence or absence of H_2O_2 for peroxidase activity or 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate), which is suspected to be a laccase mediator (5). At the end of the incubation period, the reaction mixtures were extracted with ethyl acetate and the metabolites were analyzed by HPLC.

The commercially available laccase from *Pyricularia oryzae* and that purified from *P. ostreatus* were also incubated with phenanthrene, phenanthrene *trans*-9,10-dihydrodiol, or phenanthrene 9,10-quinone overnight, in the presence or absence of H_2O_2, at 30°C and pH 6.5. The reaction mixtures were extracted and analyzed by HPLC.

Deconjugation experiments. *P. ostreatus* was grown in the presence of ^{14}C-phenanthrene (1 μCi) and 2.5 mg of unlabeled phenanthrene per 100 ml of BRM. The deconjugation experiments were based on the methods of Cerniglia and Gibson (10) and Casillas et al. (8). After 5 days, the mycelium was removed and the filtrate was extracted with 6 equal volumes of ethyl acetate. Each of the aqueous and solvent-extracted phases was divided into four portions. The aqueous phase was bubbled with N_2 to remove residual ethyl acetate. The first sample was incubated with 500 U of β-D-glucuronidase (type VII-A, from *Escherichia coli*) in 0.2 M acetate buffer (pH 5.5). The second sample was incubated with 250 U of β-D-glucosidase (from almonds) in 0.2 M acetate buffer (pH 5.0). The third sample was incubated with 10 U of arylsulfatase (type VI, from *Aerobacter aerogenes*) in 0.1 M phosphate buffer (pH 7.4) and 12.5 μmol of D-saccharic acid 1,4-lactone. The latter compound is an effective inhibitor of the β-D-glucuronidase present in arylsulfatase preparations (10). The fourth sample was a control and contained no enzymes. The reaction mixtures were incubated overnight at 37°C. Samples were then extracted with 3 volumes of ethyl acetate and prepared

TABLE 1. Activities of marker enzymes and of phase I and phase II enzymes in the cytosolic (105,000 × *g*) and microsomal (105,000 × *g*) fractions of *P. ostreatus*

Enzyme[a]	Activity (nmol min^{-1} mg^{-1}) in fraction[b]	
	Cytosolic	Microsomal
Marker enzymes		
Glucose-6-phosphatase	5.50 ± 0.71	41.5 ± 7.62
Catalase	0.85 ± 0.05	ND[c]
Phase I enzymes		
Cytochrome P-450		
A_{450} from CO difference spectrum	0.16 ± 0.03	0.38 ± 0.16
A_{420} from CO difference spectrum	3.60 ± 0.04	3.95 ± 0.12
Epoxide hydrolase	0.50 ± 0.35	0.41 ± 0.16
Phase II enzymes		
Glutathione *S*-transferase	4.16 ± 0.14	ND
Sulfotransferase	0.66 ± 0.04	2.14 ± 0.09
UDP-glucuronosyltransferase	0.26 ± 0.03	4.25 ± 0.17
UDP-glucosyltransferase	0.96 ± 0.10	4.21 ± 0.22

[a] A_{450}, absorbance at 450 nm.
[b] Data represent means ± standard deviations for three experiments.
[c] ND, not detected.

for HPLC analysis. Radioactivity remaining in the aqueous phase after ethyl acetate extraction was counted by liquid scintillation.

RESULTS

The phase I and phase II enzymatic activities in the cytosolic and microsomal fractions of *P. ostreatus* mycelia are summarized in Table 1. Microsomal and cytosolic marker enzyme activities (glucose-6-phosphatase and catalase, respectively) were used to indicate adequate separation between cytosolic and microsomal fractions.

Phase I metabolism. (i) Cytochrome P-450 monooxygenase. Cytochrome P-450 monooxygenase was hypothesized (3) to be the initial oxidative enzyme attacking the aromatic ring. Carbon monoxide difference spectra of both the cytosolic and microsomal fractions showed peaks at 450 nm, but each also had a major peak at 420 nm (Fig. 1), suggesting that significant degradation of the cytochrome P-450 had occurred. Another possible explanation is that other heme proteins may have masked the peak at 450 nm. The concentrations calculated from the carbon monoxide difference spectra for the cytosolic and microsomal P-450s (0.16 and 0.38 nmol min^{-1} mg of $protein^{-1}$, respectively) and P-420s (3.6 and 3.95 nmol min^{-1} mg of protein, respectively) were similar (Table 1). Both fractions oxidized [^{14}C]phenanthrene to [^{14}C]phenanthrene *trans*-9,10-dihydrodiol. The phenanthrene *trans*-9,10-dihydrodiol was identified by comparison of the R_f value to that of the synthetic compound by TLC (Fig. 2).

The cytochrome P-450 inhibitors ABT and SKF-525A, which inhibit phenanthrene degradation in whole-mycelium experiments (3), were used at 0.1 mM for the in vitro inhibition of phenanthrene *trans*-9,10-dihydrodiol formation due to cytosolic and microsomal cytochrome P-450 oxidation activity. Carbon monoxide was also used as an inhibitor. All three inhibitors affected both cell fractions. ABT inhibited the cytosolic activity by 25.6% and the microsomal activity by 96.7%. SKF-525A inhibited the cytosolic and microsomal activities by 88.4 and 82.2%, respectively. Carbon monoxide was less inhibitory (0% in the cytosol and 21% in the microsomes) (Fig. 2 and Table 2).

Phase I metabolism. (ii) Epoxide hydrolase. The formation of *trans*-dihydrodiol metabolites of PAH degradation by *P. ostreatus* (3, 4), and those of phenanthrene in particular, also suggested the involvement of epoxide hydrolase. Since the epoxide intermediate was not found, phenanthrene 9,10-oxide was synthesized for this study. Epoxide hydrolase activity in the presence of phenanthrene 9,10-oxide was found in almost equal amounts in the cytosolic and microsomal fractions (Table 3). The phenanthrene *trans*-9,10-dihydrodiol product from the epoxide hydrolase reaction was isolated and identified by HPLC based on a retention time and UV spectral properties identical to those of the authentic compound (Fig. 3). Addition of the epoxide hydrolase inhibitor cyclohexene oxide resulted in higher inhibition with increasing inhibitor concentrations (Table 3).

The ligninolytic system. The extracellular ligninolytic system was also suspected of involvement in PAH metabolism by *P. ostreatus*. Laccase and manganese-independent peroxidase were detected in the extracellular filtrate of *P. ostreatus*. This filtrate was incubated with phenanthrene or phenanthrene *trans*-9,10-dihydrodiol to determine products of initial oxidation or ring cleavage, respectively, due to extracellular enzymes. In addition, the laccase mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS; 1 mM) (5) was added to one set of experimental tubes. Hydrogen peroxide was added to another set to test for peroxidase activity. Purified laccase from *P. ostreatus* was also incubated with phenanthrene in the presence and absence of ABTS or cotton stalk extract. None of the experiments revealed any metabolic products. In contrast, whole-mycelium replacement culture experiments revealed the production of phenanthrene *trans*-9,10-dihydrodiol and 2,2′-diphenic acid from phenanthrene, 2,2′-diphenic acid from ei-

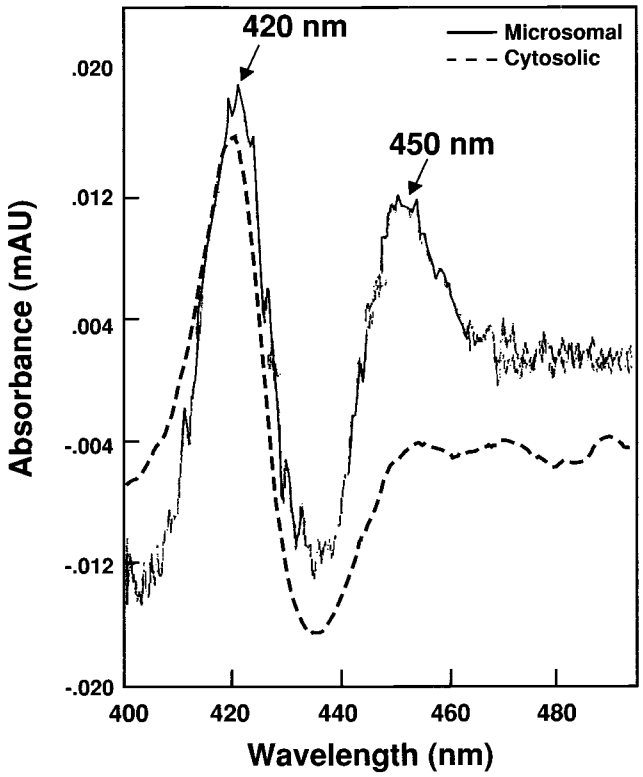

FIG. 1. Reduced carbon monoxide difference spectra of the cytosolic and microsomal fractions of *P. ostreatus*.

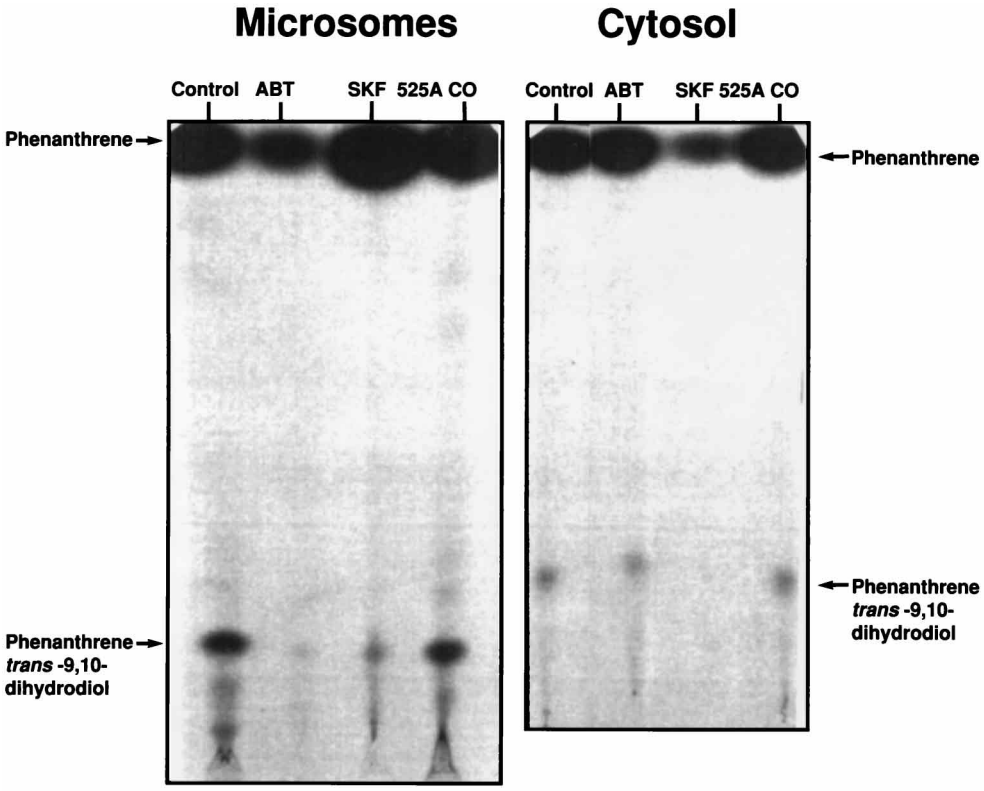

FIG. 2. TLC autoradiogram of [9,10-^{14}C]phenanthrene oxidation by microsomal and cytosolic fractions from *P. ostreatus* with and without the cytochrome P-450 inhibitors ABT, SKF-525A, and carbon monoxide.

ther phenanthrene *trans*-9,10-dihydrodiol or phenanthrene 9,10-quinone, and the disappearance of 2,2′-diphenic acid when it was added by itself.

Ring cleavage activity—protocatechuate 3,4-dioxygenase. Earlier, we showed that *P. ostreatus* metabolizes phenanthrene to the ring cleavage product 2,2′-diphenic acid (3). Since this metabolite is an *ortho* cleavage product, protocatechuate 3,4-dioxygenase activity was determined. We found a decrease in absorbance at 290 nm in the presence of protocatechuate with the cytosolic enzyme only, indicating an enzymatic transformation of protocatechuic acid to β-carboxy-*cis*,*cis*-muconic acid (data not shown).

Phase II enzymatic activities. Phase II enzymes, which are able to detoxify PAHs by conjugating their derivatives to water-soluble compounds such as glucosides, sulfates, and glutathiones, were also determined in the cell extracts of *P. ostreatus*. 1-Chloro-2,4-dinitrobenzene glutathione *S*-transferase was found in the cytosol with a specific activity of 4.16 nmol min^{-1}

TABLE 2. Inhibition of P-450 monooxygenase in the cytosolic and microsomal fractions of *P. ostreatus* by different cytochrome P-450 inhibitors

Mycelial fraction	Inhibitor	Concn (mM)	P-450 activity[a] (nmol min^{-1} mg^{-1})	% Inhibition
Cytosolic (105,000 × *g* supernatant)	Control		0.043 ± 0.011	
	1-Aminobenzotriazole	0.1	0.032 ± 0.006	25.6
	SKF-515A	0.1	0.005 ± 0.005	88.4
	Carbon monoxide	—[b]	0.044 ± 0.015	0
Microsomal (105,000 × *g* pellet)	Control		0.152 ± 0.041	
	1-Aminobenzotriazole	0.1	0.005 ± 0.005	96.7
	SKF-525A	0.1	0.027 ± 0.004	82.2
	Carbon monoxide	—	0.120 ± 0.025	21.0

[a] The P-450 activity is expressed as the amount of [9,10-^{14}C]phenanthrene *trans*-9,10-dihydrodiol formed from [9,10-^{14}C]phenanthrene per incubation as determined by the TLC assay. The activity was compared with that of the control (without inhibitor). Data represent means ± standard deviations for three experiments.

[b] —, bubbled for 20 s.

TABLE 3. Inhibition of epoxide hydrolase in the cytosolic and microsomal fractions of *P. ostreatus* by different concentrations of inhibitor

Mycelial fraction	Inhibitor	Concn (mM)	Epoxide hydrolase activity[a] (nmol min^{-1} mg^{-1})	% Inhibition
Cytosolic (105,000 × *g* supernatant)	Control		0.50 ± 0.35	
	Cyclohexene oxide	0.1	0.50 ± 0.22	0
	Cyclohexene oxide	1.0	0.25 ± 0.15	50.0
	Cyclohexene oxide	5.0	0.14 ± 0.01	72.0
Microsomal (105,000 × *g* pellet)	Control		0.41 ± 0.16	
	Cyclohexene oxide	0.1	0.40 ± 0.08	0
	Cyclohexene oxide	1.0	0.22 ± 0.04	46.3
	Cyclohexene oxide	5.0	0.08 ± 0.01	80.4

[a] The epoxide hydrolase activity is expressed as the amount of phenanthrene *trans*-9,10-dihydrodiol formed from phenanthrene 9,10-oxide as determined by the HPLC assay. The activity was compared with that of the control (without inhibitor). Data represent means ± standard deviations for three experiments.

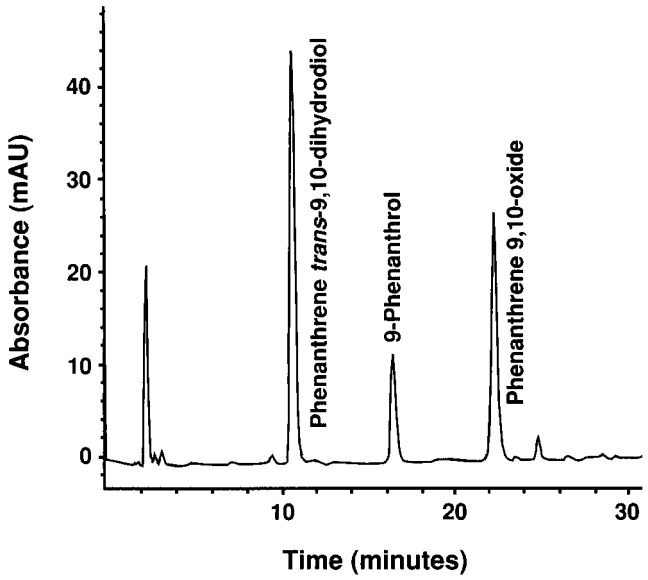

FIG. 3. HPLC elution profile of phenanthrene 9,10-oxide metabolism by the cytosolic fraction from *P. ostreatus.*

mg of protein^{-1}. The aryl PAPS sulfotransferase, UDP-glucuronosyltransferase, and UDP-glucosyltransferase exhibited major microsomal activities of 2.14, 4.25, and 4.21 nmol min^{-1} mg of protein^{-1}, respectively, and minor cytosolic activities of 0.66, 0.26, and 0.96 nmol min^{-1} mg of protein^{-1}, respectively.

A deconjugation experiment to assess the relationship between the phase II conjugation enzymes and the unidentified water-soluble and ethyl acetate-extractable metabolites was conducted with arylsulfatase, β-D-glucuronidase, and β-D-glucosidase. The HPLC analysis of the water-soluble and organic solvent-soluble phases after treatment with deconjugation enzymes, however, did not show any evidence of deconjugation products (data not shown).

DISCUSSION

The cytochrome P-450 monooxygenase system has been suggested (3, 4) to play a role in the initial oxidation of PAHs by *P. ostreatus*. Identification of phase I metabolites, mycelial cytochrome P-450 inhibition studies, and $^{18}O_2$ incorporation experiments support this suggestion (2–4). Cytochrome P-450 found in *Pleurotus pulmonarius* is able to oxidize benzo-[*a*]pyrene (30). In our study, both microsomal and cytosolic cell fractions contained P-450 activity and were able to oxidize phenanthrene to phenanthrene *trans*-9,10-dihydrodiol in vitro. Spectral detection of cytochrome P-450 in both cytosolic and microsomal fractions also provided strong evidence that it is important for PAH oxidation by *P. ostreatus.*

The xenobiotic-oxidizing cytochrome P-450s in mammals are usually membrane bound (12, 38). Both enzyme fractions in *P. ostreatus* were able to oxidize [^{14}C]phenanthrene and produce [^{14}C]phenanthrene *trans*-9,10-dihydrodiol in vitro, although the metabolism of phenanthrene occurred at a lower rate in the cytosolic fraction than in the microsomal fraction. It may be that in the intact mycelium, only the microsomal enzyme is active in PAH oxidation. The cytosolic system may also have the ability to oxidize PAHs because of its broad specificity but does not act on the hydrophobic molecules, since they are not bioavailable.

The soluble and particulate fractions appeared to be different in their responses to cytochrome P-450 inhibitors. ABT inhibited the microsomal system more than the cytosolic one. On the other hand, carbon monoxide was less effective than its microsomal counterpart and SKF-525A inhibited the cytosolic P-450 similarly to its microsomal counterpart. It should be noted that many mammalian cells have multiple cytochrome P-450 isoenzymes (12, 38).

Epoxide hydrolase was also found in both cell fractions. How-

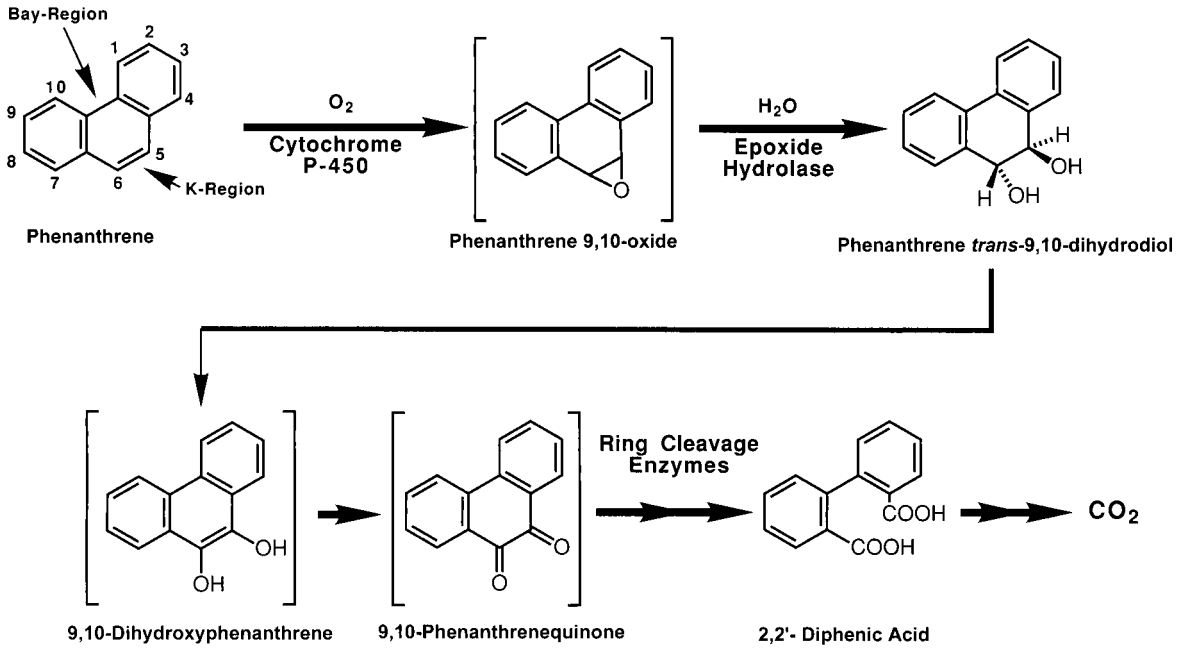

FIG. 4. Proposed pathway for phenanthrene degradation by *P. ostreatus.*

ever, in contrast to cytochrome P-450, both cytosolic and microsomal epoxide hydrolases are active in xenobiotic metabolism (25, 26, 35, 36, 39, 41). Cyclohexene oxide inhibited the microsomal and cytosolic epoxide hydrolases by similar amounts.

The results of this study provide supporting evidence for our previous suggestion (3, 4) that *P. ostreatus* uses a pathway with components common to both ligninolytic and nonligninolytic fungi to degrade PAHs. *P. ostreatus* initially oxidized the 9,10 positions of phenanthrene via cytochrome P-450 monooxygenation, followed by epoxide hydrolase activity to produce phenanthrene *trans*-9,10-dihydrodiol. These metabolic steps occurred in both N-limited and N-sufficient culture media and are similar to those in nonligninolytic fungi, such as *C. elegans* (9, 11, 43), and N-sufficient cultures of *Phanerochaete chrysosporium* (44). The stereospecificity of the PAH *trans*-dihydrodiols produced by *P. ostreatus* has been compared to that produced by the ligninolytic fungus *Phanerochaete chrysosporium*, the nonligninolytic fungus *C. elegans*, and mammalian systems (3, 4). The enantiomers of PAH *trans*-dihydrodiols produced by *P. ostreatus* were similar to those produced by *C. elegans* and opposite to those produced by *Phanerochaete chrysosporium* (44).

The ring cleavage metabolite 2,2′-diphenic acid is formed from phenanthrene (3) via either phenanthrene *trans*-9,10-dihydrodiol or phenanthrene 9,10-quinone. 2,2′-Diphenic acid was found to be produced by manganese peroxidase and lignin peroxidase activities in *Phanerochaete chrysosporium* (19, 20). The results presented here suggest that the importance of the ligninolytic system of *P. ostreatus* in the initial oxidation steps is limited and that the predominant activity is effected by phase I enzymes. The involvement of the ligninolytic system in the ring cleavage mechanism of *P. ostreatus* is not yet clear. $^{14}CO_2$ evolution experiments with [^{14}C]phenanthrene and [^{14}C]catechol, using *P. ostreatus* I88, which is a low laccase producer (data not shown), showed mineralization of both compounds, but the $^{14}CO_2$ levels were lower than those produced with a high-laccase-producing *P. ostreatus* strain. In contrast, pure laccase or extracellular filtrates from *P. ostreatus* culture media did not reveal any metabolic products during incubation with phenanthrene under different experimental conditions. Therefore, either laccase or cytosolic enzymes may be responsible for the ring cleavage activity. Indeed, protocatechuate 3,4-dioxygenase, which exhibits *ortho* ring opening activity, was found in this study. The ability to open and mineralize the aromatic ring is characteristic of ligninolytic fungi but not of nonligninolytic fungi (11, 19). The degradation pathway of phenanthrene by *P. ostreatus* is presented schematically in Fig. 4. In the figure, we indicate the enzymatic activities that may be involved in the degradation mechanism.

Unlike the results with nonligninolytic fungi (8, 10, 49), we could not find conjugation products in *P. ostreatus* cultures despite the presence of the conjugation enzymes aryl PAPS sulfotransferase, UDP-glucuronosyltransferase, UDP-glucosyltransferase, and glutathione *S*-transferase. These enzymes may have other functions.

This study clarifies a part of the enzymatic mechanism of PAH degradation and its location in the white rot fungus *P. ostreatus*. We were able to detect the activity of cytochrome P-450 in the initial oxidation of PAHs by *P. ostreatus*, followed by epoxide hydrolase activity to produce PAH *trans*-dihydrodiols. Ring cleavage of 2,2′-diphenic acid may be intracellular, via protocatechuate 3,4-dioxygenase or another intradiol ring cleavage dioxygenase, or it may be due to laccase activity.

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