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Toluene-4-Monooxygenase, a Three-Component Enzyme System That Catalyzes the Oxidation of Toluene to *p*-Cresol in *Pseudomonas mendocina* KR1

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***Pseudomonas mendocina* KR1 grows on toluene as a sole carbon and energy source. A multicomponent oxygenase was partially purified from toluene-grown cells and separated into three protein components. The reconstituted enzyme system, in the presence of NADH and Fe^{2+}, oxidized toluene to *p*-cresol as the first detectable product. Experiments with *p*-deuterotoluene led to the isolation of *p*-cresol which retained 68% of the deuterium initially present in the parent molecule. When the reconstituted enzyme system was incubated with toluene in the presence of $^{18}O_2$, the oxygen in *p*-cresol was shown to be derived from molecular oxygen. The results demonstrate that *P. mendocina* KR1 initiates degradation of toluene by a multicomponent enzyme system which has been designated toluene-4-monooxygenase.**

Several small aromatic hydrocarbons can be utilized by bacteria as sole carbon and energy sources for growth. Degradation of these hydrocarbons is initiated by either oxidation of an alkyl side group (26, 36) or direct attack on the aromatic ring (1, 9, 11, 12, 20). Toluene has been used by a number of laboratories as a model compound to study the metabolism of aromatic hydrocarbons. When utilized as a sole carbon and energy source, toluene can be metabolized via oxidation of the methyl group to yield benzyl alcohol as the first metabolite (30, 36). This type of oxidation is catalyzed by enzymes of the TOL pathway. Toluene may also be degraded by enzymes of the TOD pathway, in which initial attack is by dioxygenation of the aromatic nucleus at the 2,3 position (11, 25, 38). In this case, the first detectable product is (+)-*cis*-1(*S*),2(*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol). Recently, toluene has been shown to be oxidized by whole cells of organisms which grow on toluene to *o*-cresol (33) and *p*-cresol (35a) as initial metabolites.

A bacterium which grows on toluene as a sole carbon and energy source has been isolated and identified in our laboratory as *Pseudomonas mendocina* KR1 (31a). After growth with toluene, whole cells of this organism oxidize *p*-cresol at a rapid rate. We now report the separation into three protein components of the enzyme system responsible for the conversion of toluene to *p*-cresol. The reconstituted enzyme system catalyzed oxidation of toluene to *p*-cresol by an NAD(P)H-dependent monooxygenation reaction in which one atom of molecular oxygen is incorporated into the hydroxyl group of *p*-cresol. The reaction proceeded with a concomitant NIH shift, which suggests that toluene-3,4-oxide is the first initial oxidation product.

(A preliminary report of this work has been presented [35a].)

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MATERIALS AND METHODS

Chemicals and reagents. The following materials were obtained from the sources indicated. Plastic-backed silica gel $60F_{254}$ sheets for thin-layer chromatography were from EM Reagents, Darmstadt, Federal Republic of Germany; DE-52 DEAE-cellulose was from Whatman Ltd., Maidstone, Kent, England; Sephacryl S-200, SI-17, was from Pharmacia Fine Chemicals, Piscataway, N.J.; NADH, NADPH, flavin adenine dinucleotide (FAD), DL-6-methyl-5,6,7,8-tetrahydropterine dihydrochloride, DNase I, Bis-Tris propane, Trizma base (Tris), *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonate (HEPES), morpholine propane sulfonate (MOPS), dithiothreitol (DTT), acrylamide, and *N*,*N*,*N*',*N*'-tetramethylethylenediamine were from Sigma Chemical Co., Rockford, Ill.; 2-(*N*-morpholine)ethanesulfate was from U.S. Biochemical Corp., Cleveland, Ohio; sodium dodecyl sulfate (SDS) was from Fisher Scientific, Fairlawn, N.J.; 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine-HCl, *o*-cresol, *m*-cresol, *p*-cresol, *p*-hydroxybenzyl alcohol, 4-methylcatechol, deuterium oxide, and *p*-bromotoluene were from Aldrich Chemical Co., Milwaukee, Wis.; β-mercaptoethanol was from J. T. Baker Chemical Co., Phillipsburg, N.J.; $^{18}O_2$ was from Stohler/KOR Stable Isotopes, Cambridge, Mass.; [^{14}C]toluene was from Pathfinders Laboratories Inc., St. Louis, Mo.; dialysis tubing with a molecular weight cutoff of 12,000 to 14,000 was from Spectrum Medical Ind., Inc., Los Angeles, Calif.; Bio-Rad protein reagent was from Bio-Rad Laboratories, Richmond, Calif. All other chemicals were of the highest purity commercially available.

Bacterium and culture conditions. *P. mendocina* KR1 is the organism described earlier by Richardson and Gibson (31a). A stock culture was stored at −70°C in Luria broth medium containing (per liter) 10 g of tryptone, 5.0 g of yeast extract, 5 g of NaCl, and 250 ml of glycerol. The organism was grown in mineral salts medium (34) or on mineral salts medium agar (2.0%) plates. Toluene was supplied in the vapor phase as previously described (10). Large quantities of cells used in enzyme purification studies were grown with forced aeration at 25°C in 12-liter cultures in a New Brunswick Microferm fermentor. Toluene was supplied to the culture by sparging with toluene-saturated air. Cells were

harvested by centrifugation in a Sharples air-driven centrifuge and frozen at −20°C until used.

Analytical methods. High-performance liquid chromatography (HPLC) of substrates and metabolites was conducted with a Waters Associates 600A solvent delivery system and 440 absorbance detector. Analysis of *p*-deuterotoluene was conducted with a Zorbax 5μ ODS column (6.2 by 250 mm). The solvent used was MeOH-H_2O (90:10) at a flow rate of 1.0 ml/min. Reverse-phase chromatography of toluene-4-monooxygenase metabolites was carried out with the same column in CH_3CN-H_2O (35:65) containing 1.0% acetic acid. The flow rate was 1.0 ml/min. Normal-phase chromatography was conducted with a Spherisorb 5μ CN column (4.6 by 250 mm). The solvent used was hexane-CH_2Cl_2 (85:15) at a flow rate of 1.2 ml/min. HPLC of proteins was conducted with a Milton Roy minipump and an HR10/10 column packed with SI-17 anion-exchange resin. The flow rate was 1.0 ml/min. As indicated elsewhere in the text, an LKB G-4000 SW molecular sieve column (7.5 by 300 mm) was used for HPLC separations. This system utilized a Tracor 950 solvent delivery system and a Tracor 970A absorbance detector. Absorption spectra were recorded on a Beckman 25 or Aminco DW-2 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer 137 spectrophotometer. Mass spectra were recorded on a Du Pont 21-491 double-focusing, low-resolution spectrometer. Proton magnetic resonance spectra and carbon magnetic resonance were recorded on either a Varian FT-80A or Nicolet NT360 spectrometer. Scintillation counting was conducted on a Beckman LS6800 scintillation counter. Polyacrylamide gel electrophoresis was performed as described by Laemmli, in the presence of 0.1% SDS (27). Proteins were denatured by dilution with 0.1% SDS and 1.0% 2-mercaptoethanol and incubated at 100°C for 5 min prior to electrophoresis.

Synthesis of *p*-deuterotoluene. *p*-Deuterotoluene was synthesized by formation of the Grignard reagent from *p*-bromotoluene, followed by quenching with deuterium oxide. Crude *p*-deuterotoluene, obtained by distillation, was collected at 115°C and redistilled. The product was identical to authentic toluene when analyzed by HPLC, gas chromatography, absorption, and infrared spectrophotometry. Analysis of the product by proton magnetic resonance indicated that it was 100% deuterated at the *para* position. However, careful mass spectral analysis with a low ionizing voltage (−12 eV) revealed that the product contained 79% deuterium. The position of the deuterium was confirmed by integration of the C3 and C5 carbon magnetic resonance signals. Deuterium shifts these signals in the labeled compound, allowing the ratio of labeled and unlabeled compounds to be quantitated. The synthesized compound was estimated to contain 79% deuterium at the *para* position.

Enzyme assays. Toluene-4-monooxygenase activity was assayed by measuring the formation of *p*-[^{14}C]cresol from [^{14}C]toluene. Assays conducted to locate separated components during purification procedures utilized [^{14}C]toluene (28.5 μCi/μmol) dissolved in dimethylformamide (DMF). The standard assay contained 100 nmol of $FeSO_4$, 1.1 nmol of FAD, 400 nmol of NADH, and 7.7 nmol of [^{14}C]toluene in 20 μl of DMF. Appropriate amounts of protein and 50 mM Bis-Tris propane HCl (pH 6.8; BTP buffer) were added to a final volume of 400 μl. Reactions were initiated by addition of [^{14}C]toluene. After a 5-min incubation period, 25 μl was applied to a piece of plastic-backed silica (15 cm by 20 cm) to absorb the polar *p*-[^{14}C]cresol and the sample was allowed to air dry in a fume hood for 20 min to remove the volatile [^{14}C]toluene. The amount of *p*-[^{14}C]cresol formed was quantitated by scintillation counting. For assays conducted to determine the specific activity of each component, a saturating concentration of toluene was required. [^{14}C]toluene was diluted with unlabeled toluene to a specific activity of 2.12 μCi/μmol, and 100 nmol of toluene was added to the reaction mixture. The reactions contained saturating concentrations of two toluene-4-monooxygenase components, and the third component was added in various concentrations. Specific activities are reported as milliunits (mU) per milligram of protein. A milliunit is the amount of enzyme required to form 1.0 nmol of *p*-cresol from toluene in 1 min. Protein concentrations were determined by the Bio-Rad protein assay in accordance with manufacturer directions. Bovine serum albumin was used as the standard.

Preparation of crude cell extract. Unless indicated otherwise, all purification procedures were conducted at 4°C. Toluene-grown cells which had been stored at −20°C were thawed and diluted with an equal amount (wt/vol) of KH_2PO_4-KOH buffer, pH 7.45, containing 10% ethanol and 10% glycerol (PEG buffer). DTT (1.0 mM) and DNase I (0.002%) were added to the suspension, which was stirred for 20 min. The cells were lysed by passage through a French pressure cell at 10,000 lb/in^2 and 4°C. The resulting extract was centrifuged at 100,000 × *g* for 1 h. The supernatant solution was used as the source of crude cell extract.

DEAE-cellulose chromatography of crude cell extract. A DEAE-cellulose column (10 by 4.6 cm) was prepared by equilibration with 1.0 M KCl, followed by washing with 20 column volumes of PEG buffer. Crude cell extract (4,700 mg of protein) was applied to the top of the column, and unbound protein was eluted with 20 column volumes of PEG buffer. Bound protein was eluted with a linear gradient of 0 to 350 mM KCl in PEG buffer, and fractions were collected. The A_{280} and activity in the toluene-4-monooxygenase assay of each fraction were measured. Active fractions were pooled (component A, 400 to 620 ml; component B, 710 to 880 ml; component C, 920 to 1,060 ml) and dialyzed against PEG buffer containing 1.0 mM DTT. Dialyzed protein solutions were concentrated by adsorption to small DEAE-cellulose columns (1.0 by 8.0 cm), followed by elution with 400 mM KCl. Each component was dialyzed against PEG buffer containing 1.0 mM DTT. Concentrated solutions of components A, B, and C were used immediately or stored at −20°C.

Partial purification of component A. Component A was dialyzed against 50 mM KH_2PO_4 buffer, pH 7.45, to remove ethanol and glycerol. The dialyzed protein solution (90- to 100-mg samples) was applied to an SI-17 anion-exchange column which had previously been equilibrated with 600 mM KH_2PO_4 buffer, pH 7.45, and then washed with 10 column volumes of 50 mM KH_2PO_4 buffer, pH 7.45. Protein bound to the column was eluted at room temperature with a 30-ml linear gradient of 50 to 400 mM starting buffer at a flow rate of 1.2 ml/min. Fractions were collected at 1.0-min intervals, and those exhibiting activity for component A were pooled, dialyzed against PEG buffer containing 1.0 mM DTT, and either used immediately or stored at −20°C.

Partial purification of component B. Component B obtained by DEAE-cellulose chromatography of 8,000 mg of crude cell extract was dialyzed against PEG buffer and further purified by applying 90- to 100-mg protein samples to a column of SI-17 anion-exchange resin. The conditions were identical to those described above for component A, except for the elution gradient, which was changed to 50 to 500 mM KH_2PO_4 buffer, pH 7.45. Fractions exhibiting component B activity were pooled and applied to a column

(2.6 by 90 cm) of Sephacryl S-200. Proteins were eluted from the column with PEG buffer at a flow rate of 7.0 ml/h. Fractions showing component B activity were pooled and used immediately or stored at −20°C.

Partial purification of component C. Component C was partially purified by DEAE-cellulose chromatography of 8,000 mg of crude cell extract, followed by SI-17 anion-exchange and Sephacryl S-200 chromatography. The procedure used was identical to that described above for component B, except for the elution gradient, which was changed to 50 to 600 mM KH_2PO_4 buffer, pH 7.45. Fractions exhibiting component B activity were pooled and applied to a column (2.6 by 90 cm) of Sephacryl S-200. Proteins were eluted from the column with PEG buffer at a flow rate of 7.0 ml/h. Fractions showing component C activity were pooled and used immediately or stored at −20°C.

Enzymatic transformation of toluene in controlled atmospheres. Each reaction mixture contained 200 nmol of $FeSO_4$, 350 nmol of NADH, 3.3 mU of component A, 0.2 mU of component B, 1.7 mU of component C, 6.8 nmol of [^{14}C]toluene (in 20 μl of DMF), and BTP buffer to give a final volume of 400 μl. The procedure used was as follows. Identical reaction mixtures (minus NADH and [^{14}C]toluene) were prepared in sealed containers and made anaerobic by alternate evacuation and flushing with oxygen-free argon. A separate mixture of NADH and [^{14}C]toluene was prepared and flushed with argon. After purging, radioactivity was measured in a sample of the [^{14}C]toluene-NADH mixture to determine the exact amount of toluene present. To initiate the reaction, a portion of the [^{14}C]toluene-NADH mixture was added to each reaction mixture. The cap was immediately removed from the control (aerobic) reaction container. After 5 min of incubation, the reaction was stopped by injection of 20 μl of 12 N HCl and the amount of metabolite formed was determined as described previously.

For transformations in the presence of isotopic oxygen, each reaction mixture contained 13 μmol of $FeSO_4$, 136 mU of component A, 9.0 mU of component B, 84 mU of component C, 14 μmol of NADH, 1.1 μmol of toluene in 30 μl of DMF, and BTP buffer to give a final volume of 10 ml. All components except toluene were added together in a 50-ml Erlenmeyer flask fitted with a gas-tight serum stopper. Flasks were alternately purged with oxygen-free argon and evacuated. $^{18}O_2$ and $^{16}O_2$ were added with a gas-tight syringe, and the atmosphere was sampled and analyzed by electron impact mass spectrometry. The toluene-DMF solution was purged with argon for 2 min, and the reaction was initiated by addition of 30 μl of the solution. After 20 min at room temperature, the atmosphere above the mixture was sampled and analyzed by electron impact mass spectrometry. The reaction was stopped by addition of 20 μl of concentrated H_2SO_4 and extracted three times with diethyl ether, and the solvent was dried over anhydrous Na_2SO_4. The solvent was removed under a stream of N_2, and the residue was analyzed by thin-layer chromatography. The solvent used was chloroform-acetone (80:20). *p*-Cresol was located by reference to an authentic sample. The sample and standard were analyzed by electron impact mass spectrometry.

RESULTS

Oxidation of toluene by cell extracts. Cell extracts prepared from toluene-grown cells of *P. mendocina* KR1 oxidized [^{14}C]toluene to a radioactive polar metabolite which cochromatographed with *m*-cresol and *p*-cresol when analyzed by reverse-phase HPLC. The rate of product formation was nonlinear with respect to the protein concentrations in cell extracts. Similar results were reported previously for the multicomponent toluene dioxygenase from *P. putida* F1 (37).

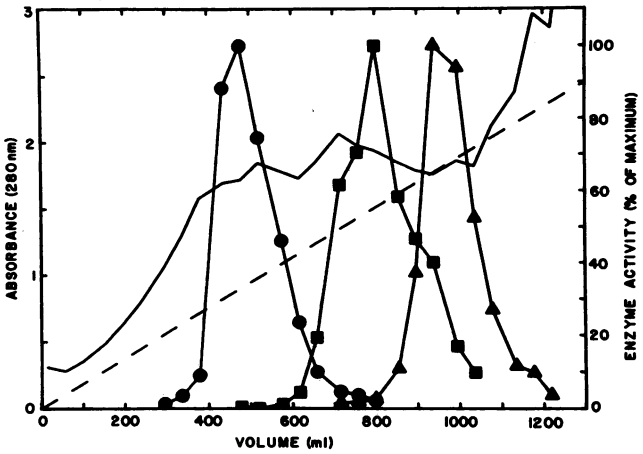

FIG. 1. DEAE-cellulose chromatography of crude cell extract. Details are described in Materials and Methods. Symbols: ●, component A; ■, component B; ▲, component C; ——, A_{280}; – – –, 0.0 to 350 mM KCl gradient.

DEAE-cellulose chromatography of cell extracts led to isolation of three protein fractions that were required for toluene oxidation (Fig. 1). The individual components were designated A, B, and C on the basis of the order of their elution from the DEAE-cellulose column.

Partial purification of component A. Solutions of component A, obtained by DEAE-cellulose chromatography, were yellow. Subsequent HPLC anion-exchange chromatography removed 50% of the protein present in the DEAE-cellulose fraction. Although some inactivation of component A was observed, this procedure removed all traces of component B. Frequently, preparations of component A obtained from the SI-17 column were colorless and required addition of FAD to restore enzyme activity. Further attempts to purify component A resulted in total loss of enzyme activity. A summary of the partial purification of component A is shown in Table 1.

Partial purification of component B. Solutions of component B were further purified by SI-17 HPLC (Table 1). The protein was not completely homogeneous at this stage of the purification procedure. However, all subsequent attempts to purify component B resulted in substantial loss of enzyme activity. SDS-polyacrylamide gel electrophoresis of component B obtained from the SI-17 column revealed two major bands with M_rs of 50,000 and 32,000. Preliminary experiments with molecular exclusion chromatography indicated that the native M_r of component B was approximately 550,000 (data not shown).

Purification of component C. The purification procedure for component C was the same as that described in Table 1 for components A and B, except for an additional purification stage which involved chromatography on Sephacryl S-200. The protein solution from this column was brown, and SDS-polyacrylamide gel electrophoresis revealed the presence of a single protein with an M_r of 23,000. The same molecular weight was obtained by gel filtration chromatography. The absorption spectrum of component C showed peaks at 280 nm (ε = 42,500 M^{-1} cm^{-1}), 323 nm (ε = 16,400

TABLE 1. Separation and partial purification of components A, B, and C required for enzymatic oxidation of toluene[a]

Protein fraction	Volume (ml)	Protein concn (mg/ml)	Total protein content (mg)	Total mU[b]	Sp act[c]	Recovery (%)	Purification (fold)
Component A							
Crude cell extract	97	49	4,700	11,000	2.4	100	1.0
DEAE-cellulose	32	12	370	4,600	12	40	5.1
SI-17 HPLC	5	39	200	1,700	8.4	14	3.5
Component B							
Crude cell extract	170	48	8,000	7,200	0.89	100	1.0
DEAE-cellulose	33	28	920	1,000	1.1	14	1.2
SI-17 HPLC	8	18	130	810	5.8	11	6.5
Component C							
Crude cell extract	170	48	8,000	8,000	1.0	100	1.0
DEAE-cellulose	17	18	300	1,200	3.8	14	3.8
SI-17 HPLC	7	3.9	27	570	21	7	21
Sephacryl S-200	18	0.2	3.6	580	160	7	160

[a] Details of the purification procedures are described in Materials and Methods.
[b] One milliunit is the amount of enzyme required to form 1.0 nmol of nonvolatile metabolite per minute.
[c] Milliunits per milligram of protein.

M^{-1} cm^{-1}), 435 nm (ε = 8,700 M^{-1} cm^{-1}), 456 nm (ε = 9,000 M^{-1} cm^{-1}), and 559 nm (ε = 4,500 M^{-1} cm^{-1}).

Requirements for enzymatic toluene oxidation. Table 2 shows that components A, B, and C were required for enzymatic activity. The catalytic activity of each component was destroyed by heating at 100°C for 5 min. NADH and NADPH were equally effective as electron donors, and the reconstituted enzyme system was stimulated by addition of ferrous iron. Essentially no toluene oxidation was observed when the reaction was conducted in an argon atmosphere. The pH optimum of the reaction was 6.8, and maximal activity was observed with 50 mM BTP buffer, pH 6.8.

The effects of metals other than ferrous iron on toluene oxidation were tested by addition of metal salts (500 μM) to the reconstituted reaction mixture. A small degree of stimulation was observed with ferric iron ($FeCl_3$) or cobalt [$Co(NO_3)_2$]. Addition of magnesium ($MgSO_4$), calcium ($CaCl_2$), nickel ($NiCl_2$), or molybdenum (Na_2MoO_4) had no effect on activity. However, addition of either zinc ($ZnSO_4$) or copper ($CuCl_2$) to the reaction mixture resulted in 84 or 89% inhibition, respectively.

Two reduced pterin compounds, DL-6-methyl-5,6,7,8-tetrahydropterine dihydrochloride (1 mM) and 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine-HCl (1 mM), which are known cofactors for phenylalanine hydroxylase (16, 28), were tested for their effects on toluene-oxidizing activity. Neither of the pterins served as an electron donor for the reconstituted enzyme system. The presence of the pterins strongly inhibited activity with NADH (1 mM) alone. However, the presence of FAD (7.5 μM) partially protected the activity from pterin inhibition.

TABLE 2. Requirements for toluene oxidation[a]

Reaction mixture	Enzymatic activity	
	%	dpm
Complete	100	7,400
Minus component A	1	48
Minus component B	2	120
Minus component C	2	110
Minus NADH	1	50
Minus Fe^{2+}	19	1,400
Minus FAD	75	5,500

[a] The complete reaction mixture contained, in a final volume of 0.4 ml, 3.3 mU of component A, 0.2 mU of component B, 1.7 mU of component C, 400 nmol of NADH, 1.1 nmol of FAD, 100 nmol of $FeSO_4$, and 50 mM BTP buffer (pH 6.8). Reactions were initiated by addition of 7.7 nmol of [^{14}C]toluene (specific activity, 28.5 μCi/μmol) in 20 μl of DMF. Enzyme activity was determined as described in Materials and Methods.

Identification of the reaction product formed from toluene by the reconstituted enzyme system. A reaction mixture containing 33.1 mU of component A, 1.8 mU of component B, 16.7 mU of component C, 4.0 μmol of NADH, and 5.3 μmol of $FeSO_4$ was brought to 3.9 ml with BTP buffer and incubated at 30°C with stirring for 5.0 min. The reaction was initiated by addition of 191 nmol of [^{14}C]toluene (in 100 μl of DMF). The reaction was stopped at 32 min by addition of an equal volume of diethyl ether. NaCl was added to the aqueous phase to enhance the extraction efficiency. The organic phase was removed, and the extraction was repeated for three cycles. The organic phase was dried over anhydrous Na_2SO_4, the solvent was removed under a stream of N_2, and the residue was dissolved in hexane. This procedure resulted in extraction of 84% of the radioactivity detected by counting nonvolatile ^{14}C-labeled metabolites. A sample of the hexane extract was mixed with authentic samples of *o*-cresol, *m*-cresol, and *p*-cresol. The individual cresols were separated by normal-phase HPLC. More than 96% of the recovered radioactivity coeluted with *p*-cresol (Fig. 2). The experiment was repeated with nonradioactive toluene. The only detectable reaction product gave absorption and electron impact mass spectra identical to those given by authentic *p*-cresol.

Incorporation of ^{18}O into *p*-cresol. Toluene was incubated with components A, B, and C; ferrous iron; and NADH in the presence of an ^{18}O-^{16}O atmosphere as described in Materials and Methods. *p*-Cresol isolated at the end of the reaction was analyzed by mass spectrometry and shown to contain ^{18}O. In two separate experiments, the ratios of ^{18}O to ^{16}O in the atmospheres above the reaction mixtures were 56:44 and 96:4 and the ratios in the *p*-cresol reaction product were 62:38 and 96:4, respectively. These results show unequivocally that the oxygen in *p*-cresol is derived from molecular oxygen.

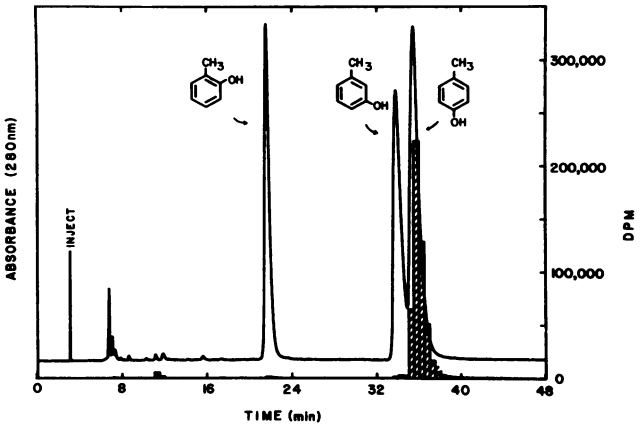

FIG. 2. Normal-phase HPLC of the product formed from [^{14}C]toluene by the reconstituted toluene-oxidizing enzyme system. A complete reaction mixture was extracted as described in the text and mixed with authentic *o*-cresol, *m*-cresol, and *p*-cresol. This mixture was separated on a 5μ Spherisorb CN column (4.6 by 250 mm). The solvent was hexane-CH_2Cl_2 (85:15), and the flow rate was 1.0 ml/min. The eluate was collected at 30-s intervals, and radioactivity was quantitated by scintillation counting. The absorbance is shown as a solid curve, and the disintegrations per minute (DPM) are represented as bars. A total of 569,000 DPM was injected, and 520,000 DPM was recovered, of which 500,000 DPM coeluted with *p*-cresol.

Oxidation of *p*-deuterotoluene by toluene-4-monooxygenase. The mechanism by which the enzyme incorporated molecular oxygen into toluene to form *p*-cresol was investigated by transformation of the toluene analogue *p*-deuterotoluene. A reaction was conducted with partially purified toluene-4-monooxygenase components by using *p*-deuterotoluene as the substrate as described in Materials and Methods. The product was purified by HPLC and analyzed by electron impact mass spectrometry. The spectra of the product and authentic *p*-cresol revealed that 54% of the enzymatically formed *p*-cresol contained deuterium. After correction for the ratio of labeled to unlabeled substrate (*p*-deuterotoluene, 79%) the migration and retention of the deuterium were calculated to be 68%.

DISCUSSION

P. mendocina KR1 was found to elaborate a multicomponent enzyme system which catalyzed a monooxygenase reaction. The activity of the enzyme was dependent on a source of reducing equivalents (NADH or NADPH) and O_2. It catalyzed the incorporation of one atom of O_2 into the substrate toluene to form *p*-cresol as the first detectable metabolite. This enzyme system was designated toluene-4-monooxygenase.

The multicomponent nature of toluene-4-monooxygenase is typical of other bacterial oxygenases that attack unactivated aromatic rings. A nonlinear relationship of protein concentration to enzyme activity was observed in crude cell extract prepared from toluene-grown cells of *P. mendocina* KR1. This relationship has also been reported for multicomponent aromatic hydrocarbon oxygenases isolated from other bacteria (2, 7, 37). Toluene-4-monooxygenase has been shown to be a multicomponent enzyme system which is composed of three proteins designated components A, B, and C. Component A was located in a yellow fraction which eluted from a column of DEAE-cellulose. Further purification of this enzyme by HPLC anion-exchange chromatography resolved the yellow fractions from fractions containing activity. This purification resulted in protein which required addition of FAD to restore full activity. Whether the additional FAD was required for the function of component A or one of the other toluene-4-monooxygenase proteins was not investigated.

Toluene-4-monooxygenase component C is a red-brown protein with spectrophotometric properties similar to those of the ferredoxin proteins purified from benzene dioxygenase (2) and toluene dioxygenase (35). The extinction values reported for ferredoxin$_{TOL}$ at 327 and 460 nm are in close agreement with the values determined for component C at 323 and 456 nm, respectively. The molecular weight of component C, 23,000, was larger than those reported for ferredoxin$_{TOL}$ (M_r 15,300 [35], ferredoxin from the benzene dioxygenase system (M_r, 12,000 [2]), ferredoxin$_{NAP}$ (M_r, 13,600 [17]), and the ferredoxin from the pyrazon dioxygenase system (M_r, 12,000 [32]). By analogy to the above enzyme systems, component C is proposed to function as a ferredoxin in the toluene-4-monooxygenase system.

Toluene-4-monooxygenase component B was probably composed of at least two subunits with M_rs of 50,000 and 32,000. The native molecular weight appeared to be very large, i.e., >500,000. The most purified preparation obtained was colorless. The absorption spectrum showed a peak at 280 nm, due to protein, and a shoulder at about 390 nm (data not shown). No further physical data were obtained for this protein.

All three toluene-4-monooxygenase components and NAD(P)H were required for catalytic activity. In addition, more purified preparations also required exogenous Fe^{2+} and FAD. These data suggest a hypothetical electron flow from NAH(P)H to a flavin-containing protein, to an iron-sulfur protein, to a colorless iron-containing protein. Methane monooxygenase from *Methylococcus capsulatus* (29) and *Methylosinus trichosporium* OB3b (8) is catalytically organized in this manner. The oxygenase of this enzyme system is a large, colorless, iron-containing protein. However, the flavin and the iron-sulfur center are contained in the same protein (4, 5, 8).

The reaction mechanism for *p*-cresol formation by toluene-4-monooxygenase was investigated by using the substrate analog *p*-deuterotoluene. Three possible routes to *p*-cresol can be envisioned. One route is direct hydroxylation of the S_N2-type mechanism, in which oxygen is inserted on one face of the ring and deuterium is lost from the other. Hydroxylation by this route would result in complete loss of deuterium. The second route is the result of an oxenoid type of reaction which yields an arene oxide from oxygen transfer to an aromatic ring (18). Isomerization of the unstable arene oxide proceeds with a 1, 2, deuteride shift in an NIH type of reaction (6). The product of an NIH shift rearrangement will retain a percentage of the *para* substituent which has migrated. The extent of migration and retention is dependent on the substituent and the electron-withdrawing nature of the other ring substituents. Finally, a similar type of NIH shift rearrangement would be expected from a third possible reaction mechanism, dehydration of a dihydrodiol compound. Loss of a hydroxyl at the 3 position from a 3,4-dihydrodiol would yield an intermediate similar to that formed during the rearrangement of an arene oxide. Again, migration and retention of the deuterium would be expected (21).

Enzymatic transformation of *p*-deuterotoluene by mam-

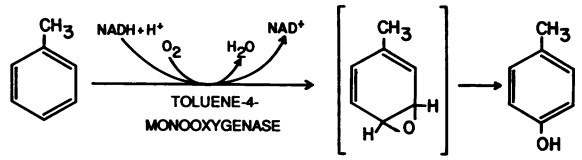

FIG. 3. Proposed reaction of toluene-4-monooxygenase.

malian P-450 enzymes leads to 54% migration and retention of the deuterium (6). Aqueous nonenzymatic isomerization of synthetic 4-deutero-3,4-toluene oxide at pH 8.0 leads to 70% migration and retention of deuterium (22). Nonenzymatic dehydration of 3,4-dihydroxy-4-deuterochlorobenzene, a compound similar to a possible dihydrodiol intermediate (toluene-3,4-dihydrodiol), leads to only about 20% migration and retention of deuterium (21). In this investigation, transformation of *p*-deuterotoluene by toluene-4-monooxygenase led to 68% retention and migration of deuterium. This demonstrated that a reaction intermediate was formed during conversion of *p*-deuterotoluene to *p*-cresol and suggests that an arene oxide is the more likely intermediate (Fig. 3). An epoxide of toluene was not detected in reactions with toluene-4-monooxygenase. However, if an epoxide is formed and is released from the enzyme, it might be detected by catalysis of toluene by the reconstituted oxygenase in the presence of the enzyme epoxide hydroxylase (23). Other bacterial transformations which appear to involve formation of arene oxide intermediates have been recently described. Cerniglia et al. have shown an NIH shift during the cooxidation of naphthalene to α-naphthol by *Bacillus cereus* (3). Zurrer et al. have isolated a number of bacteria which can desulfonate substituted benzenesulfonic and naphthalenesulfonic acids when forced to utilize the sulfur in these compounds as a sole sulfur source (39). The products of these desulfonation reactions are consistent with an arene oxide mechanism. A *Mycobacterium* sp. which degrades pyrene has also been isolated (19). The initial detectable products from this degradation are products from both a dioxygenase and a monooxygenase enzyme. In all of the above cases, the products have been shown to contain oxygen derived from $^{18}O_2$.

An alternative mechanism involving an enzyme-bound *cis*-dihydrodiol can be envisaged. The dihydrodiol could be dehydrated by an enzyme-assisted mechanism to yield *p*-cresol. During characterization of the toluene-4-monooxygenase reaction product, care was taken to treat reaction mixtures in such a manner as not to destroy a dihydrodiol chemically if it was present; i.e., acidic conditions and high temperatures were avoided. A dihydrodiol was not detected. Toluene-grown cells of *P. mendocina* KR1 contain enzymes for metabolism of *p*-cresol through ring fission via protocatechuic acid (35b). An enzyme that catalyzes the formation of a cresol from a dihydrodiol has not been described. The expected catabolic metabolism of a dihydrodiol is formation of a catechol (13). In the case of toluene oxidation by *P. mendocina* KR1, the expected product would be toluene-3,4-diol. Dehydrogenation of this compound would lead to formation of 4-methylcatechol. Toluene-grown *P. mendocina* KR1 cell extract oxidizes 4-methylcatechol at a very slow rate. The same extract rapidly oxidizes protocatechuic acid, the proposed metabolite of further toluene oxidation. Formation of a dihydrodiol by toluene-4-monooxygenase is not expected on the basis of (i) failure to isolate a dihydrodiol compound as an oxidation product of toluene; (ii) the inability to detect enzymes in toluene-grown cells that metabolize the expected oxidation product of toluene-3,4-diol, 4-methylcatechol; and (iii) the high retention and migration of deuterium from *p*-deuterotoluene oxidation.

The pterin enzyme phenylalanine hydroxylase catalyzes the *p*-hydroxylation of phenylalanine to tyrosine (14, 15, 24). This hydroxylation proceeds with a concomitant NIH shift (14). A pterin enzyme purified from a pseudomonad catalyzes the *p*-hydroxylation of benzoate (31). These reactions are not unlike the reaction catalyzed by toluene-4-monooxygenase. However, reduced pterin cofactors, which can serve as cofactors for phenylalanine hydroxylases and benzoate hydroxylase, were not able to function as cofactors for toluene-4-monooxygenase. Rather, the presence of the reduced pterins strongly inhibited the observed activity. This inhibition was partially relieved by the presence of added FAD. Further experiments are necessary to determine the nature of this inhibition. Possibly, pterin uncouples the electron transport of the enzyme. Structural similarity to flavin might allow a pterin molecule to bind to a site where flavin is required for proper electron transfer. Further work is required to determine the reaction mechanism.

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