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NOTES

Separation and Partial Characterization of the Enzymes of the Toluene-4-Monooxygenase Catabolic Pathway in *Pseudomonas mendocina* KR1

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The route of toluene degradation by *Pseudomonas mendocina* KR1 was studied by separating or purifying from toluene-grown cells the catabolic enzymes responsible for oxidation of *p*-cresol through the ring cleavage step. Enzymatic transformations corresponding to each of the metabolic steps in the proposed degradative pathway were conducted with cell-free preparations. *p*-Cresol was metabolized by the enzyme *p*-cresol methylhydroxylase to *p*-hydroxybenzaldehyde. *p*-Hydroxybenzaldehyde was further oxidized by partially purified enzyme preparations to *p*-hydroxybenzoate and subsequently hydroxylated to form protocatechuate. Protocatechuate was then oxidized by *ortho* ring cleavage.

Three catabolic pathways for the degradation of toluene by bacteria have been elucidated. For one pathway, TOL, toluene is degraded via oxidation of the methyl group to benzoic acid. Further oxidation is through a *cis*-carboxylic acid diol and catechol. Catechol is degraded by enzymes of a *meta* cleavage pathway to acetaldehyde and pyruvate (8, 17). A second pathway, TOD, established by studies with *Pseudomonas putida* F1, is characterized by initial attack of toluene by a dioxygenase enzyme system to form (+)-*cis*-1(*S*),2(*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (3, 7, 18). This compound is oxidized to 3-methylcatechol, which is further degraded by enzymes of a *meta* cleavage pathway (3, 4). A third pathway for toluene degradation has been demonstrated with bacterium G4 (14). This organism also metabolizes toluene through 3-methylcatechol and *meta* cleavage, as in the TOD pathway. However, in contrast, G4 forms 3-methylcatechol from toluene by two successive monooxygenations, forming first *o*-cresol and then 3-methylcatechol.

Recently, a new bacterium, identified as *Pseudomonas mendocina* KR1, was isolated by K. L. Richardson in our laboratory. Preliminary evidence suggested that *P. mendocina* KR1 catabolizes toluene by a pathway different from either of the pathways described above (13, 16). These studies led to the prediction of a novel catabolic pathway for the growth of KR1 on toluene. It was suggested that metabolism proceeds by initial hydroxylation of toluene to form *p*-cresol, further metabolism via oxidation of the methyl group to form *p*-hydroxybenzoate, hydroxylation to form protocatechuate, and then complete dissimilation by an *ortho* cleavage pathway. New evidence is now reported in support of the proposed pathway. This includes the separation and partial characterization of enzymes from toluene-grown cells of strain KR1 which catalyze the oxidation of *p*-cresol to β-carboxy-*cis*,*cis*-muconic acid.

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Bacteria, culture conditions, and reagents. *P. mendocina* KR1 was isolated from an algal-bacterial mat obtained from the Colorado River in Austin, Tex. (13). Unless otherwise noted, bacterial growth and storage conditions and reagents were as previously described (16). All chemicals were of the highest purity commercially available.

Analytical methods. A Du Pont Zorbax 5μ ODS column (6.2 by 250 mm) was used to separate *p*-cresol, *p*-hydroxybenzyl alcohol, and *p*-hydroxybenzaldehyde by high-performance liquid chromatography. The solvent was water-acetonitrile (4:6) containing 1.0% acetic acid. The flow rate was 1.5 ml/min. Capillary gas chromatography-mass spectral (GC-MS) analyses were conducted on a Finnigan-MAT 4023 quadrapole mass spectrometer fitted to a Finnigan 9610 gas chromatograph equipped with an INCOS data system. Separations were performed with a Scientific Glass Engineering (Austin, Tex.) BP5 column (12-m long; 0.25 μm-thick film) at an initial temperature of 125°C with a linear increase of 5°C/min to 250°C. Standards and enzyme reaction products were prepared for GC-MS analysis by derivatization with *N*-trimethylsilyldiethylamine (Pierce Chemical Co.) to form their trimethylsilyl (TMS) derivatives. Absorption spectra were recorded on an Aminco DW-2 recording spectrophotometer. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted as described by Weber and Osborn (15). Protein was denatured by dilution with a solution of 0.1% SDS and 1.0% 2-mercaptoethanol and incubation at 100°C for 5 min prior to electrophoresis. For nondenaturing conditions, addition of 2-mercaptoethanol and SDS and heating were omitted. Protein was estimated in extracts by the Bio-Rad protein assay in accordance with manufacturer directions and in whole cells by the procedure of Lowry et al. (9). Bovine serum albumin was used as the standard.

Enzyme assays. *p*-Cresol methylhydroxylase (PCMH) was assayed as described by Hopper and Taylor (6), except that the buffer was 50 mM Tris-HCl (pH 7.6). One unit of activity is the amount of enzyme required to reduce 1.0 μmol of 2,6-dichlorophenol-indophenol per min (ε, 21,000 M^{-1} cm^{-1}). NAD^+-dependent *p*-hydroxybenzylalcohol dehydrogenase was assayed as previously described (6). *p*-Hydroxy-

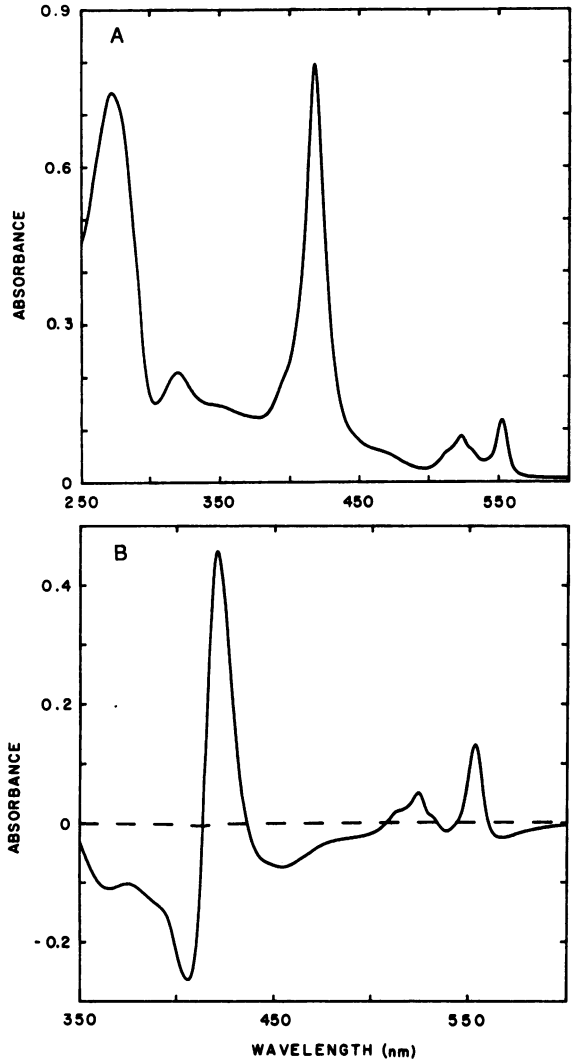

FIG. 1. (A) Absorption spectrum of purified PCMH. The sample cuvette contained 560 μg of PCMH in 1.0 ml of Tris buffer, and the reference cuvette contained only Tris buffer. (B) Difference spectra of purified PCMH. The sample and reference cuvettes each contained 540 μg (4.4 nmol) of PCMH in 1.0 ml of Tris buffer which had been oxidized with 13 μmol of H_2O_2. A baseline difference spectrum of the identical solutions was recorded (– – –). To the sample cuvette was added 2.0 μl of a 4.0 mM solution of *p*-cresol (8.0 nmol) in 10% dimethylformamide, and the spectrum was recorded (——).

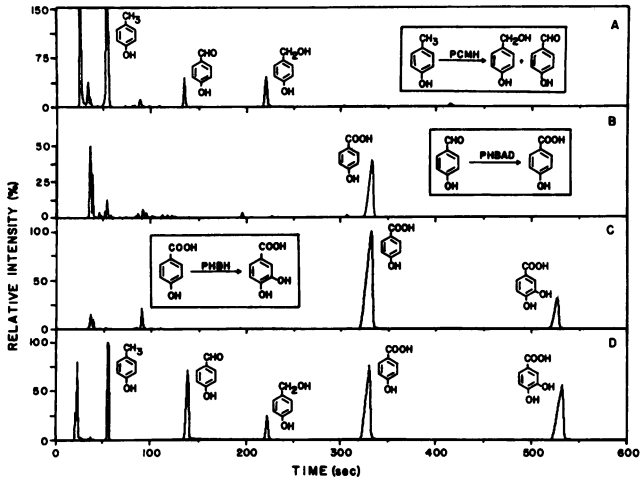

FIG. 2. GC-MS analysis of enzymatic transformations. The enzyme reactions, TMS derivatizations, and chromatography conditions are described in the text. The relevant peaks are identified in each panel for transformations by PCMH (A), *p*-hydroxybenzaldehyde dehydrogenase (B), and *p*-hydroxybenzoate hydroxylase (C). A mixture of authentic standards is shown for comparison (D).

benzaldehyde dehydrogenase was measured spectrophotometrically by monitoring the decrease in A_{330} in reaction mixtures which was due to conversion of *p*-hydroxybenzaldehyde to *p*-hydroxybenzoate. The reaction mixture contained 600 nmol of $NADP^+$, 40 nmol of *p*-hydroxybenzaldehyde, appropriate amounts of enzyme, and glycine buffer to 1.0 ml. One unit of activity is the amount of enzyme required to oxidize 1.0 μmol of *p*-hydroxybenzaldehyde per min (ε, 28,800 M^{-1} cm^{-1}). *p*-Hydroxybenzoate hydroxylase was assayed spectrophotometrically by monitoring NADPH oxidation (2). One unit of activity is the amount of enzyme required to oxidize 1.0 μmol of NADPH per min. Protocatechuate-3,4-dioxygenase was measured polarographically with a Clark-type electrode at 30°C. The reaction mixture contained 400 nmol of protocatechuate, appropriate amounts of enzyme, and 50 mM KH_2PO_4 buffer (pH 7.2) to give a final reaction volume of 1.4 ml. One unit of activity is the amount of enzyme required to catalyze the consumption of 1.0 μmol of O_2 per min.

Separation of enzyme components. All procedures were performed at 4°C unless otherwise stated. Toluene-grown cells which had been stored at −20°C were thawed and diluted with an equal amount (wt/vol) of 50 mM KH_2PO_4 buffer (pH 7.45) containing 10% ethanol and 10% glycerol (PEG buffer). After addition of dithiothreitol (1.0 mM) and DNase I (0.002%), the cell suspension was lysed by a single 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, and the supernatant solution was used as a source of crude cell extract. The crude cell extract contained PCMH, *p*-hydroxybenzaldehyde dehydrogenase, *p*-hydroxybenzoate hydroxylase, and protocatechuate-3,4-dioxygenase with specific activities of 0.06, 0.55, 0.25, and 0.62 U mg of $protein^{-1}$, respectively. No NAD^+-dependent *p*-hydroxybenzyl-alcohol dehydrogenase activity was detected. The crude cell extract was applied to the top of a column of DEAE-cellulose which had been previously equilibrated with PEG buffer. The column was washed with 10 column volumes of PEG buffer, and bound protein was eluted with a gradient of 0 to 350 mM KCl in PEG buffer. Fractions were collected and assayed for enzyme activity. PCMH, *p*-hydroxybenzaldehyde dehydrogenase, and protocatechuate-3,4-dioxygenase were not bound by DEAE-cellulose under these conditions and were recovered in the unbound protein fractions. *p*-Hydroxybenzoate hydroxylase was retained and eluted with the KCl gradient. Fractions containing activity were pooled, dialyzed against PEG buffer, and applied to a small DEAE-cellulose column. The protein was eluted batchwise by application of 0.5 M KCl, and the concentrated eluate was collected, dialyzed against PEG buffer, and stored at −20°C until used for transformations. The yield was 14% of that in the crude extract, and the specific activity was 0.41 U mg of $protein^{-1}$.

The unbound protein from the previous column was dialyzed against 20 mM Tris-HCl buffer, pH 7.6 (Tris buffer),

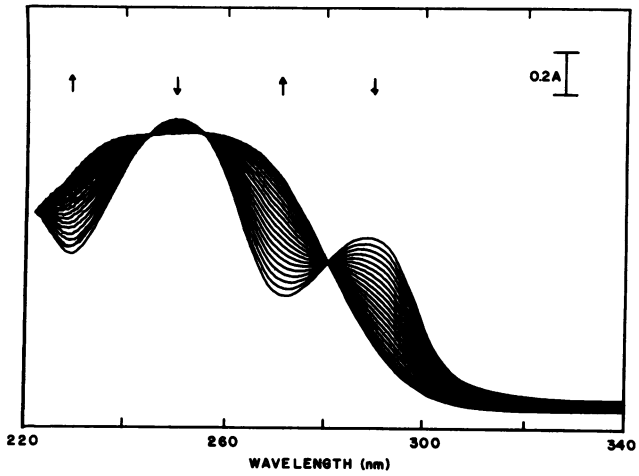

FIG. 3. Spectral changes observed during oxidation of protocatechuate by protocatechuate-3,4-dioxygenase from *P. mendocina* KR1. The sample cuvette contained 190 nmol of protocatechuate and 96 μg of protocatechuate-3,4-dioxygenase (described in the text) in 1.0 ml of KH_2PO_4 buffer, pH 7.2. The reference cuvette contained only buffer. Scans were recorded at 238-s intervals. The arrows indicate the absorption spectrum of the reaction mixture at the initiation of the reaction.

and applied to the top of a column of DEAE-cellulose equilibrated with Tris buffer. Unbound protein was washed from the column with 10 column volumes of Tris buffer, and bound protein was eluted with a gradient of 0 to 300 mM KCl in Tris buffer. Fractions were collected and measured for enzyme activity. Protocatechuate-3,4-dioxygenase was not bound by DEAE-cellulose, and all of the activity was recovered in the unbound protein fractions. The pooled protein was precipitated with NH_4SO_4 between 20 and 70% saturation, dialyzed, and stored at −20°C until used. The extract had a specific activity of 0.21 U mg of protein^{-1} and represented a total yield of 6% of the activity in the crude extract.

PCMH and *p*-hydroxybenzaldehyde dehydrogenase, which were bound by the DEAE-cellulose column, were eluted with the gradient and dialyzed and concentrated on small DEAE-cellulose columns as described above. The resulting *p*-hydroxybenzaldehyde dehydrogenase was stored at −20°C until used. The yield was 1.2% of that in the crude extract, and the specific activity was 0.17 U mg of protein^{-1}.

PCMH was applied to the top of a Sephacryl S-200 column which was equilibrated in PEG buffer containing 100 mM NaCl. The column was eluted with PEG buffer, and fractions containing PCMH were pooled and stored at −20°C until used. The yield of PCMH activity was 67% of that in the crude extract, and the specific activity was 14.0 U mg of protein^{-1}. SDS-polyacrylamide gel electrophoresis of the purified protein showed the presence of a single band with an M_r of 56,000. Polyacrylamide gel electrophoresis of this sample under nondenaturing conditions resulted in a single band which migrated with a larger M_r. The native size of PCMH (M_r, 120,000) was estimated by Sephacryl S-200 chromatography. This indicates that the protein is composed of two subunits.

The absorption spectrum of PCMH is shown in Fig. 1A. The visible spectrum was not changed by addition of 13 μmol of H_2O_2 to 4.4 nmol of PCMH. Chemical reduction of the oxidized PCMH was performed by addition of a few crystals of $Na_2S_2O_4$. The difference spectrum revealed α, β, and γ peaks characteristic of a heme chromaphore at 552.5, 523, and 421 nm, respectively. The same reduced spectra were observed after addition of either *p*-cresol (8.0 nmol; Fig. 1B) or *p*-hydroxybenzyl alcohol to oxidized (H_2O_2) PCMH (4.4 nmol). These results indicate enzymatic reduction of the chromophore by direct interaction with the substrate.

The ferrohemochromagen of PCMH was prepared as previously described (1). A molecular mass of 120,000 Da was used to estimate the heme-to-protein ratio as 1.14 to 1.00. PCMH was found to contain covalently bound flavin by acetone precipitation of the enzyme. The resuspended yellow pellet had spectrophotometric properties at pH 11.6 typical of flavin-containing solutions, with absorbance maxima at 454 and 344 nm (11). The peak at 454 nm was bleached by reduction with $Na_2S_2O_4$. The flavin-to-protein ratios were 0.91 to 1.00 for flavin adenine dinucleotide and 0.80 to 1.00 for flavin mononucleotide. These data for PCMH from strain KR1 are consistent with reports on other PCMH enzymes (12).

Enzymatic transformations. A reaction mixture was prepared to determine the products formed from oxidation of *p*-cresol by purified PCMH containing 8.0 μmol of *p*-cresol, 1.0 μmol of phenozine methosulfanate (PMS) and 95 μg of PCMH in 2.0 ml of glycine buffer. The reactions were initiated by addition of PCMH. Samples were withdrawn at intervals, added to 10 μl of glacial acetic acid, and centrifuged at 6,500 × *g* for 5 min. A sample was then analyzed by high-performance liquid chromatography as described above. The product, *p*-hydroxybenzaldehyde, was formed in approximately equal molar concentrations with respect to the *p*-cresol oxidized. After 1 h, the remaining reaction mixture was extracted with diethyl ether and the extracted material was concentrated and separated by high-performance liquid chromatography. Both *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde were identified by their high-performance liquid chromatography retention times (3.25 and 4.12 min, respectively) and their absorption spectra with respect to authentic standards. Only a small amount of *p*-hydroxybenzyl alcohol was detected in the reaction mixture. No products were formed in a control reaction which contained all components except protein. A separate reaction mixture containing 40 μmol of *p*-cresol, 5.0 μmol of PMS, and 475 μg of PCMH in 10 ml of glycine buffer was prepared for analysis by GC-MS. The reaction was initiated with enzyme and stopped at 25 min by addition of HCl. The

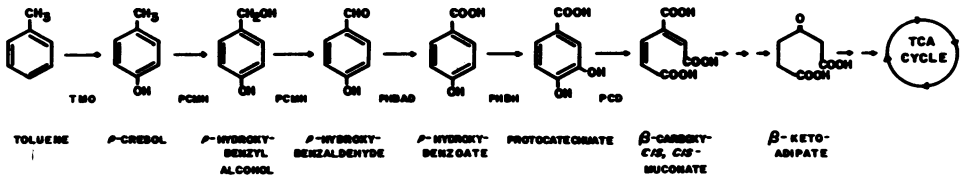

FIG. 4. Pathway proposed for complete dissimilation of toluene by *P. mendocina* KR1. Enzyme designations: TMO, toluene-4-monooxygenase; PHBAD, *p*-hydroxybenzaldehyde dehydrogenase; PCD, protocatechuate-3,4-dioxygenase. Metabolic oxidations for which an enzyme is indicated have been characterized by partial or complete purification of the enzyme(s) involved. TCA, tricarboxylic acid.

metabolites were extracted with diethyl ether, and the TMS derivatives were prepared as described above. The products, *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde, were identified by their retention times (222 and 139 s, respectively) and their mass spectra with respect to authentic standards (Fig. 2A). Formation of *p*-hydroxybenzaldehyde from *p*-cresol is the expected reaction from this purified enzyme and is consistent with those obtained with PCMH isolated from other sources (12).

Reaction mixtures for the oxidation of *p*-hydroxybenzaldehyde by *p*-hydroxybenzaldehyde dehydrogenase contained 1.6 μmol of *p*-hydroxybenzaldehyde, 6.6 μmol of $NADP^+$, 2.9 U of enzyme, and glycine buffer to a final volume of 4.0 ml. A control mixture contained the same reagents without enzyme. After 30 min of incubation, the reaction was stopped by addition of HCl and the mixture was extracted with diethyl ether. The TMS derivatives were prepared as described above and analyzed by GC-MS. *p*-Hydroxybenzoate was identified by its retention time (331 s) and its mass spectrum with respect to an authentic standard (Fig. 2D). *p*-Hydroxybenzoate was the only metabolite detected, and there was no substrate present in the reaction mixture after 30 min (Fig. 2B). The control reaction mixture, which did not contain the enzyme, showed no conversion of the substrate to a product.

Reaction mixtures for oxidation of *p*-hydroxybenzoate by *p*-hydroxybenzoate hydroxylase contained 35 μmol of *p*-hydroxybenzoate, 25 μmol of NADPH, 15.1 U of enzyme, and 50 mM Tris-HCl buffer (pH 8.0) to a final volume of 22 ml. The reaction was stopped by addition of HCl, and the mixture was extracted with diethyl ether. The TMS derivatives were prepared as described above and analyzed by GC-MS. *p*-Hydroxybenzoate and the product, protocatechuate, were both present in significant amounts when the reaction was stopped at 2 min (Fig. 2C). The compounds were identified by their retention times (331 and 553 s, respectively) and their mass spectra with respect to authentic standards. The control reaction without enzyme contained only *p*-hydroxybenzoate. To determine the product of protocatechuate cleavage by protocatechuate-3,4-dioxygenase, the spectral changes of a reaction mixture with time were observed. The partially purified enzyme converted protocatechuate to a single compound (Fig. 3). The absorption spectrum of the reaction mixture was recorded throughout the transformation and exhibited isosbestic points at 246, 255, and 281 nm. These data suggest that the product of the reaction is β-carboxy-*cis*-*cis*-muconate (10).

The pathway proposed for oxidation of toluene to tricarboxylic acid cycle intermediates is shown in Fig. 4. This pathway has been designated the toluene-4-monooxygenase catabolic pathway to emphasize the unusual initial oxidation of toluene (16). Support for this pathway has now been obtained by separation from toluene-grown cells of enzymes which catalyzed the proposed catabolic reactions. Complete dissimilation of toluene by enzymes of the TOL and TOD pathways and by G4 involves *meta* cleavage of the aromatic ring. In contrast, enzymes of the toluene-4-monooxygenase pathway degrade toluene by a sequence involving *ortho* ring cleavage reactions not previously reported for toluene catabolism. However, metabolism of *p*-cresol via protocatechuate and *ortho* ring cleavage has been reported for *P. putida* PP7-1 (NCIB 9866) (5). The unusual ability of *P. mendocina* KR1 to grow on toluene by the pathway shown in Fig. 4 may be due to acquisition of the ability to hydroxylate toluene by an organism with metabolic properties similar to those of *P. putida* PP7-1.

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