Purification and Characterization of Toluene 2-Monooxygenase from *Burkholderia cepacia* G4[†]

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ABSTRACT: Recent *in vivo* studies indicate that ring monooxygenation is a widespread mechanism by which bacteria metabolize aromatic hydrocarbons and obtain carbon and energy. In this study, toluene 2-monooxygenase from *Burkholderia* (formerly *Pseudomonas*) *cepacia* G4 was purified to homogeneity and found to be a three-component enzyme system. The reconstituted enzyme system oxidized toluene to *o*-cresol and *o*-cresol to 3-methylcatechol, an important intermediate for growth of the bacterium on toluene. Steady-state kinetic parameters measured for the water-soluble substrate *o*-cresol were a K_m of 0.8 μM and a V_{max} of 131 nmol min^{-1} (mg of hydroxylase protein)$^{-1}$. The three protein components were (1) a 40 kDa polypeptide containing one FAD and a [2Fe2S] cluster, (2) a 10.4 kDa polypeptide that contained no identifiable metals or organic cofactors, and (3) a 211 kDa $\alpha_2\beta_2\gamma_2$ component containing five to six iron atoms. The 40 kDa flavo−iron−sulfur protein oxidized NADH and transferred electrons to cytochrome *c*, dyes, and the $\alpha_2\beta_2\gamma_2$ component. It is analogous to other NADH oxidoreductase components found in a wide range of bacterial mono- and dioxygenases. The 10.4 kDa component, added to the other two components and NADH, increased toluene oxidation rates 10-fold. The $\alpha_2\beta_2\gamma_2$ component was indicated to contain the site for toluene binding and hydroxylation by the following observations: (1) tight binding to a toluene affinity column; (2) oxidation of toluene after reduction of the protein with dithionite and adding O_2; (3) H_2O_2-dependent toluene oxidation and catalase activity; and (4) spectroscopic studies of the iron atoms in the component. The $\alpha_2\beta_2\gamma_2$ component had no significant absorbance in the visible region. EPR spectroscopy yielded a signal at $g = 16$ upon addition of >2 equiv of electrons per 2 Fe atoms. Taken with the quantitation of five to six iron atoms, the data suggest that the $\alpha_2\beta_2\gamma_2$ component contains two binuclear iron centers. In total, the structural, spectroscopic, and catalytic features of toluene 2-monooxygenase are reminiscent of soluble methane monooxygenase obtained from methanotrophic bacteria. The two enzyme systems also differ in many subtle ways; for example, they oxidize toluene with completely different regiospecificity.

Aerobic bacteria are known to metabolize aromatic hydrocarbons, such as toluene, as their sole source of carbon and energy. The first reports of the biochemical mechanisms underlying initial attack suggested aromatic ring epoxidation and subsequent epoxide hydrolysis to yield *trans*-dihydrodiols (Walker & Wiltshire, 1953; Marr & Stone, 1961). Further investigations showed that the intermediate was actually a *cis*-dihydrodiol and its formation was catalyzed by a dioxygenase (Gibson et al., 1970). For example, toluene dioxygenase oxidizes toluene to (+)-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (Kobal et al., 1973). Later, another pathway of bacterial toluene metabolism was shown to occur via initial methyl group oxidation yielding benzyl alcohol (Worsey & Williams, 1975). More recently, bacterial monooxygenation of toluene has been demonstrated: (1) *Pseudomonas mendocina* KR1 produces 4-hydroxytoluene or *p*-cresol (Whited & Gibson, 1985); (2) *Pseudomonas picketii* produces *m*-cresol (Olsen et al., 1994); and (3) *Burkholderia cepacia* G4 produces *o*-cresol (Shields et al., 1989). Out of these pathways, only the enzyme system responsible for the formation of *cis*-toluene dihydrodiol, toluene dioxygenase, has been purified to homogeneity (Wackett, 1990). Toluene 4-monooxygenase has been partially purified (Whited & Gibson, 1991), but detailed biochemical characterization has yet to be performed. Sequence alignment protocols indicate that toluene 4-monooxygenase may be related to proteins containing binuclear iron centers such as soluble methane monooxygenase, fatty acid desaturase, and ribonucleotide reductase (Fox et al., 1994).

Early work by Shields and co-workers suggested that toluene degradation by whole cells of *Burkholderia cepacia* G4 represented a novel pathway of toluene catabolism in which toluene was sequentially hydroxylated to form 3-methylcatechol through the intermediate *o*-cresol (Shields et al., 1989). The addition of either toluene or phenol to cultures of *B. cepacia* G4 induced the enzyme(s) responsible for the degradation of toluene (Nelson et al., 1987). The organism was originally isolated for its ability to oxidize trichloroethylene (TCE), a common groundwater pollutant (Nelson et al., 1986). Further *in vivo* studies suggested that the enzyme(s) oxidizing TCE also oxidized toluene and phenol (Shields

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et al., 1991). Previous attempts to obtain enzyme activity in a cell-free form were unsuccessful (Nelson et al., 1987; Shields et al., 1989). The ability of *B. cepacia* G4 to degrade trichloroethylene and other halogenated solvents has increased interest in using this bacterium for bioremediation purposes (Krumme et al., 1993; Winkler et al., 1995). Such applications might be facilitated by a greater knowledge of the enzyme system(s) responsible for solvent oxidation. The present study reports the purification and characterization of the enzyme system, toluene 2-monooxygenase, and provides evidence that this enzyme system catalyzes two consecutive monooxygenation reactons to yield 3-methylcatechol. Evidence is also provided to suggest that this enzyme system belongs to the binuclear-iron protein family (Wilkins, 1992).

MATERIALS AND METHODS

Chemicals and Reagents. DEAE Sepharose Fast Flow, Sephacryl S200 HR and S100, and Reactive Green 19 were obtained from Sigma (St. Louis, MO). Biogel P-60 and P-10 were obtained from Bio-Rad (Richmond, CA). MOPS,[1] Trizma base, and proteins used in molecular weight determinations were obtained from Sigma. Phast-gel System reagents were obtained from Pharmacia (Uppsala, Sweden). [U-^{14}C]Toluene (specific activity, 56.2 μCi/μmol) was obtained from Sigma. Standards used in the determination of toluene hydroxylation (3-methyl- and 4-methylcatechol; *o*-, *m*-, and *p*-cresols) were obtained from Aldrich (Millwaukee, WI). All other chemicals used were reagent grade or better.

Growth of Bacterial Strains. Burkholderia cepacia G4 was provided by Dr. Malcolm Shields (University of West Florida). *Burkholderia* [formerly *Pseudomonas* (J. Int. Syst. Bacteriol., 1993)] *cepacia* G4 was maintained on minimal salts buffer (MSB) (Stanier, 1966) agar plates containing 20 mM lactate. For large-scale growth of *B. cepacia* G4, a 12 L carboy containing 9 L of MSB media was inoculated with a 500 mL overnight culture that had been induced with toluene vapor. The 9 L culture was continuously stirred and sparged with a filter-sterilized air/toluene vapor mixture at 23 °C. The toluene vapor was supplied by diverting half of the air flow through a 1 L flask containing 100 mL of toluene. Cells were harvested at an optical density of 600 nm (OD_{600}) of 2.0 by centrifugation at 5000*g* for 15 min. The cell pellet was washed with 25 mM MOPS (pH 7.5) and stored frozen at −70 °C until use. A typical yield from a 9 L culture was approximately 18−24 g wet weight of cell paste.

Escherichia coli DE3(pET-A5) containing the cloned toluene 2-monooxygenase 40 kDa component (reductase) gene was provided by Dr. Steve Francesconi (EPA Laboratory, Gulf Breeze, FL). *E. coli* DE3(pET-A5) was maintained on Luria−Bertani (LB)(Sambrook et al., 1989) agar plates containing 100 μg/mL ampicillin. The *E. coli* DE3-(pET-A5) was routinely grown in LB containing 100 μg/mL ampicillin at 23 °C. At an OD_{600} of 2.5, the cells were harvested by centrifugation at 5000*g* for 15 min, washed in 25 mM MOPS (pH 7.5), and stored frozen at −70 °C. A typical yield was approximately 5 g/L of culture.

Enzyme Assays. All assays were performed at 23 °C. Toluene 2-monooxygenase activity was assayed by determining the amount of nonvolatile products formed from the oxidation of [^{14}C]toluene (Wackett, 1990). The assay was performed in 1.8 mL Teflon-lined septum sealed vials. The assay mixture contained, in a final volume of 400 μL, 25 mM MOPS (pH 7.5), all three enzyme components, NADH to a final concentration of 1.25 mM, and 5 μM radiolabeled toluene in *N*,*N*-dimethylformamide (DMF). After incubation for 15 min, a 20 μL aliquot of the reaction mixture was spotted onto a 1 cm^2 silica gel square of plastic-backed thin-layer chromatography sheet (Kodak). Any unreacted [^{14}C]-toluene was removed through volatilization in a fume hood after which the silica gel square was placed in a scintillation vial, 7 mL of scintillation fluid was added, and the radioactivity remaining was quantitated in a scintillation counter. When *o*-cresol was the substrate, a coupled assay was used in which the wild-type catechol 2,3-dioxygenase was included in the reaction mixture to monitor the formation of 2-hydroxy-6-oxohepta-2,4-dienoate at 388 nm (ϵ = 13 800 M^{-1} cm^{-1} (Bayly et al., 1966) from the 3-methylcatechol formed during the hydroxylation reaction. The assay mixture contained, in a final volume of 200 μL, 25 mM MOPS (pH 7.5), all three enzyme components, NADH to a final concentration of 0.5 mM, and saturating amounts of catechol 2,3-dioxygenase. The assay was initiated by the addition of varying concentrations of *o*-cresol, and the absorbance at 388 nm was followed over time. Reductase activity was assayed by following the NADH-dependent reduction of cytochrome *c* spectrophotometrically at 550 nm (Wackett, 1990). The small component was assayed throughout the purification using the radiometric assay described above. Fractions containing the small component were those that reconstituted monooxygenase activity in a mixture containing hydroxylase, reductase, NADH, and radiolabeled toluene. Specific activity values reported for the hydroxylase and small component were determined using the radiolabeled assay in the presence of optimal concentrations of the other components of toluene 2-monooxygenase. Optimal concentrations of the components were determined by varying the concentration of one of the components in relation to fixed concentrations of the other two components.

Preparation of Cell-Free Extract. Approximately 20 g of *Burkholderia cepacia* G4 cell paste was suspended in a 1:4 (w/v) ratio in 25 mM MOPS (pH 7.5), 2 mM cysteine, and 200 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ buffer (buffer A) containing 170 μg/mL phenylmethanesulfonyl fluoride (PMSF). The cell suspension was passed 3 times through an Amicon French Pressure cell at 20 000 psi, at 4 °C. Cell debris was removed from the suspension by centrifugation at 27000*g* for 90 min after the addition of 10 μg/mL DNase I. The supernatant was carefully decanted and immediately fractionated as described below.

Cell-free extract from *E. coli* DE3(pET-A5) was prepared by resuspending approximately 15 g of cell paste, at a 1:4 ratio, in 25 mM Tris-HCl (pH 7.5), 10% glycerol, and 5 mM thioglycolate buffer (buffer B) with the addition of 170 μg/mL PMSF. The cell suspension was passed 3 times through a French Pressure cell at 20 000 psi, at 4 °C. Cell debris was removed from the suspension by centrifugation at 27000*g* for 60 min after the addition of 10 μg/mL DNase I.

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADH, nicotinamide adenine dinucleotide reduced form; PAGE, polyacrylamide gel electrophoresis; FAD, flavin adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride; EPR, electron paramagnetic resonance.

The supernatant was carefully decanted and stored under argon at −70 °C.

Separation of Components. All column chromatography in the purification of the enzyme components was conducted at 4 °C. Cell-free extract from *B. cepacia* G4 in buffer A was immediately adjusted to 0.1 M NaCl and loaded onto a DEAE Sepharose Fast Flow column (2.5 cm × 20 cm) equilibrated in buffer A, 10% glycerol, and 0.1 M NaCl at a flow rate of 3.0 mL/min. The column was further washed with an additional 200 mL of the same buffer. The toluene 2-monooxygenase components were eluted using a 700 mL linear gradient of 0.1−0.35 M NaCl at a flow rate of 1.5 mL/min. Fractions later established to be the small component, hydroxylase, and reductase eluted at 0.15, 0.20, and 0.28 M NaCl, respectively.

Purification of the Hydroxylase. Fractions containing the hydroxylase were pooled and immediately concentrated by ultrafiltration. The concentrated protein was applied to a Sephacryl S-200HR column (2.5 cm × 90 cm) equilibrated in buffer A, 10% glycerol, and 0.1 M NaCl at a flow rate of 0.25 mL/min. Fractions containing hydroxylase were pooled, concentrated by ultrafiltration, and stored at −70 °C.

Purification of the Small Component. Fractions containing the small component were pooled and concentrated by ultrafiltration and applied to a Biogel P-60 column (1.5 cm × 90 cm) equilibrated in 25 mM MOPS (pH 7.0), 0.1 M NaCl at a flow rate of 0.45 mL/min. Fractions containing the small component were pooled, concentrated by ultrafiltration, and stored at −70 °C.

Purification of the Reductase. Cell-free extract from *E. coli* DE3(pET-A5) in buffer B was loaded onto a DEAE Sepharose Fast Flow column (2.5 cm × 20 cm) equilibrated in buffer B, 0.15 M NaCl, at a flow rate of 1.0 mL/min. The column was further washed with an additional 300 mL of buffer B, 0.15 M NaCl at a flow rate of 2.5 mL/min. The reductase was eluted with a 1 L gradient of 0.15−0.45 M NaCl at a flow rate of 2.5 mL/min. Fractions containing the reductase were pooled, concentrated by ultrafiltration, diluted 1:4 in fresh buffer B, and loaded onto a Reactive Green 19 column (2.5 cm × 10 cm) equilibrated in buffer B at a flow rate of 0.5 mL/min. The column was washed with an additional 100 mL of buffer B at a flow rate of 1.0 mL/min. The reductase was eluted with 0.5 M NaCl in buffer B at a flow rate of 1.0 mL/min. Fractions containing the reductase were pooled, concentrated by ultrafiltration, and stored at −70 °C.

Pooled fractions containing wild-type reductase from the separation of all three enzyme components described under Separation of Components were further purified using the Reactive Green 19 column method described above. The reductase eluted from this column is approximately 80−90% pure, whereas the recombinant reductase is >90% pure as determined by silver-stained SDS−PAGE and quantitative amino acid determinations (data not shown).

Electron Paramagnetic Resonance Spectroscopy Methods. X-band EPR spectra were obtained on a Varian E-109 spectrometer equipped with an Oxford Instruments ESR-10 liquid helium cryostat, Hewlett Packard 436A power meter, and Hewlett Packard 5350B microwave frequency counter (Fox et al., 1989). Anaerobic reductions of the hydroxylase with NADH were carried out in an EPR tube made anaerobic with argon. Reductase was added to the hydroxylase sample at a ratio of 0.05 nmol to 1 nmol, respectively, to allow the reduction of the hydroxylase component with NADH. Samples of mixed-valent hydroxylase were prepared by addition of equimolar concentrations of anaerobic standardized NADH ($\epsilon_{340} = 6.3\ mM^{-1}\ cm^{-1}$) based on two binuclear iron clusters per hydroxylase molecule. Fully reduced hydroxylase samples were prepared by addition of excess anaerobic NADH. After the addition of NADH, the samples were incubated 5 min at 23 °C before freezing in liquid nitrogen. Oxidized samples were prepared by allowing air into the EPR tube, mixing, and refreezing in liquid nitrogen following incubation at 23 °C. All additions to samples were made using gas-tight syringes.

Catalytic Turnover of Hydroxylase Component. Single-turnover experiments were conducted under strictly anaerobic conditions (Fox et al., 1989). Protein components (3 nmol of hydroxylase or 1.5 nmol of reductase or 1.9 nmol of small component) in 100 mM MOPS (pH 7.5) containing 4 μM methyl viologen were made anaerobic in a 1.0 mL conical bottom, septum-sealed vial by repeated evacuation and flushing with oxygen-free argon gas. Anaerobic solutions of sodium dithionite were standardized by titration with potassium ferricyanide ($\epsilon_{420} = 1.03\ mM^{-1}\ cm^{-1}$). Fully reduced samples were prepared by addition of 4 reducing equiv of sodium dithionite (based on four iron atoms per hydroxylase protein) and incubated 5 min at 23 °C. Excess sodium dithionite was not present as determined by the absence of blue color indicating methyl viologen radical. Nonradioactive toluene was mixed with [^{14}C]toluene to yield a final concentration of 1 mM in 100 mM MOPS (pH 7.5) in a 1.8 mL septum-sealed reaction vial previously made anaerobic by repeated flushing with argon. Sodium dithionite (15 μM) was added to remove residual oxygen present in the substrate solution. The reduced protein samples were anaerobically transferred to the reaction vial and incubated 5 min at 23 °C. The final volume of the reaction mixture was 0.4 mL. The reaction was initiated by the addition of 0.36 mL of 100% O_2 at 1 atm. Upon addition of oxygen, the reaction vial was vigorously mixed and incubated an additional 5 min at 23 °C. Aliquots of the reaction mixture were then spotted onto 1 cm^2 TLC squares and allowed to dry, and the radioactivity remaining was determined by counting in a scintillation counter. Reaction products were also determined by extracting the samples with an equal volume of diethyl ether, reducing the volume of the ether extract under a stream of argon, and analyzing the radiolabeled product(s) present by TLC analysis as described under Analytical Methods.

Hydrogen peroxide dependent turnover reactions (Andersson et al., 1991) were performed with the hydroxylase component at a concentration of 23 μM in 25 mM MOPS (pH 7.5), and varying concentrations of [^{14}C]toluene in DMF. The reaction was initiated by the addition of 10 mM H_2O_2, mixed, and incubated for 30 min at 23 °C. Aliquots of the reaction mixture were spotted onto 1 cm^2 TLC squares, allowed to dry in a hood, and counted to determine the amount of nonvolatile radioactivity present. The concentration of H_2O_2 remaining was determined (Hildebrandt et al., 1978).

Toluene Oxidation Product Determination. Purified enzyme components (1.13 nmol of hydroxylase, 0.8 nmol of small component, and 2.35 nmol of reductase) were incubated in 1.8 mL septum-sealed vials in 25 mM MOPS (pH 7.5) containing 10 μM [^{14}C]toluene at a final volume of 0.4

mL. The reaction was initiated upon addition of 1.25 mM NADH. The vials were incubated with shaking at 200 rpm, and 23 °C. At appropriate time points, the reactions were terminated by addition of 8 μL of 1 N H_2SO_4. The samples were immediately extracted 3 times with an equal volume of diethyl ether. The extracts were dried over anhydrous sodium sulfate, and concentrated under a stream of argon. The extracts were then analyzed for radiolabeled oxidation products as described under Analytical Methods.

The purified hydroxylase was found to contain trace amounts of catechol 2,3-dioxygenase activity as evidenced by the formation of the yellow product, 2-hydroxy-6-oxohepta-2,4-dienoate, after the addition of 3-methylcatechol. Due to the high rate of turnover of ring-cleavage dioxygenases, it was necessary to remove the trace amount present in the hydroxylase fraction before performing the toluene oxidation experiment. Further purification of the hydroxylase was attempted to rid the fractions of catechol dioxygenase activity. Additional ion chromatography using a MonoQ 5/5 column (Pharmacia), gel filtration, and hydrophobic interaction chromatography did not remove the catechol dioxygenase activity. The passage of the hydroxylase fractions over these additional columns resulted in a loss of hydroxylase activity. The activity of the catechol 2,3-dioxygenase was inhibited by the addition of 20 mM hydrogen peroxide (Nozaki et al., 1968). The activity of the hydroxylase after treatment with hydrogen peroxide was 90%, indicating little or no inhibition of the hydroxylase component had occurred. Dialyzed fractions of the treated hydroxylase contained undetectable catechol 2,3-dioxygenase activity.

Molecular Weight Determinations. Molecular weights of the reductase and small component were determined by gel filtration on a Superose 12 HR column (1.0 × 30 cm) equilibrated in 25 mM MOPS buffer (pH 7.5) containing 0.1 M NaCl using an FPLC system (Pharmacia). The column was calibrated with sheep γ-globulin, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, cytochrome *c*, and aprotinin. The molecular weight of the hydroxylase component was determined by gel filtration on a Superose 6 HR column (1.0 × 30 cm) equilibrated in 25 mM MOPS buffer (pH 7.5) containing 0.1 M NaCl and calibrated with apoferritin, β-amylase, alcohol dehydrogenase, and bovine serum albumin. Molecular weights were also determined by native polyacrylamide gel electrophoresis (PAGE) using a Phast-gel System (Pharmacia); by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on a 5−30% gradient gel using a Mini-Protean II gel appartus (Bio-Rad); and by calculation of the molecular weight from quantitative amino acid analysis performed at the Microchemical Facility, University of Minnesota, Minneapolis MN.

Other Methods. Protein concentration was determined by the Lowry method (Lowry et al., 1951) after precipitation of protein (Brown et al., 1989). N-Terminal sequencing of the first 10 amino acids was performed at the Microchemical Facility, University of Minnesota, Minneapolis. The subunits of the hydroxylase were separated for N-terminal sequencing on a Model 491 Prep Cell (Bio-Rad). Iron content was determined spectrophotometrically by complexation with Ferene S [3-(2-pyridyl)-5,6-bis[2-(5-furylsulfonic acid)]-1,2,4-triazine, disodium] using a combination of published methods (Zabinski et al., 1972; Gottschall et al., 1982). Varying amounts of the protein were diluted in 0.4 mL of distilled, deionized water, and 0.05 mL of 8 N HCl was added. After 10 min at 23 °C, the protein was precipitated with the addition of 0.05 mL of 50% trichloroacetic acid and removed by centrifugation. The clear supernatants were transferred to clean tubes, and 0.1 mL of 45% sodium acetate was added with mixing. Then 0.9 mL of Ferene S reagent (0.8 mM Ferene S, 10 mM L-ascorbic acid in 45% sodium acetate) was added and mixed, and the absorbance was read at 593 nm. Iron standards were prepared using iron atomic absorption standard solution (Aldrich). Metal analysis was also determined on enzyme solution directly by inductively coupled plasma emission spectroscopy performed by Soil Science Services, University of Minnesota, St. Paul. Inorganic sulfide was determined by methylene blue formation (Chen & Mortensen, 1977). The flavin content of the reductase was determined by reverse phase HPLC using a 10−100% methanol gradient in 5 mM ammonium acetate buffer (pH 7.2) (Hausinger et al., 1986). The flavin content was also quantitated spectrophotometrically (ϵ_{456} = 11.3 mM^{-1} cm^{-1}) after heat denaturation of the reductase. Metal chelators and sulfhydryl alkylating reagents were assayed for the ability to inhibit reductase activity (Subramanian et al., 1981). Isoelectric point determinations were performed using a Phast-gel System and IEF 3−9 media (Pharmacia). Catechol 2,3-dioxygenase activity was detected spectrophotometrically by monitoring the formation of 2-hydroxy-6-oxohepta-2,4-dienoate at 388 nm (ϵ = 13 800 M^{-1} cm^{-1}) (Bayly et al., 1966) when 3-methylcatechol was used as a substrate.

Analytical Methods. Spectrophotometric measurements were made using a Beckman DU 7400 diode array or Beckman DU 640 spectrophotometer. Scintillation counting was conducted with a Beckman LS3801 scintillation counter. Thin-layer chromatography of the products of the toluene hydroxylation reactions was carried out using 20 × 20 cm silica G plates (250 μm hard surface analytical layer with preadsorbant spotting area and 19 channels of 8 mm width; J. T. Baker, Phillipsburg, NJ) in a benzene/methanol (95:5) solvent system (Stahl, 1969). Radiolabeled products were detected and quantitated by scraping each lane in 1 cm intervals, collecting the silica gel, and counting in 7 mL of scintillation fluid. Cold standards of 4-methylcatechol, 3-methylcatechol, *o*-cresol, *m*-cresol, and *p*-cresol were added to samples and chromatographed. Spots were visualized using an iodine vapor chamber. The R_f values were determined from these standards and compared to the locations of the radiolabeled oxidation products. Oxygen and the rate of oxygen formation from the peroxidase activity of the hydroxylase component were measured using a YSI 5331 oxygen electrode obtained from Fisher (Pittsburgh, PA).

RESULTS

Purification of the Components. Previous attempts to obtain cell-free toluene 2-monooxygenase from *B. cepacia* G4 were unsuccessful (Nelson et al., 1987). In this study, toluene 2-monooxygenase activity was reproducibly obtained in cell extracts prepared using toluene-grown cells that were lysed in a MOPS−iron−cysteine buffer (Fox et al., 1989). Active crude extract was obtained only when Fe^{2+} was present in the buffer. All activity was present in the soluble fraction of the crude extract. The rate of formation of nonvolatile metabolites from the oxidation of [^{14}C]toluene

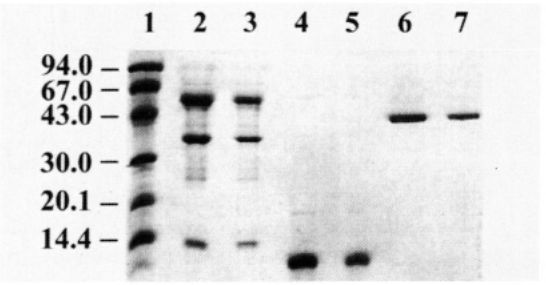

FIGURE 1: Denaturing polyacrylamide electrophoresis of purified toluene 2-monooxygenase components: hydroxylase (4 μg, 2 μg; lanes 2, 3); small component (2 μg, 1 μg; lanes 4, 5); and reductase (2 μg, 1 μg; lanes 6, 7). Molecular weight markers (lane 1) were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

was not linear with respect to protein concentration, suggesting that toluene 2-monooxygenase was a multicomponent enzyme system (Yeh et al., 1977). The purification of the enzyme system was monitored by SDS−PAGE and indicated that three components were necessary for the oxidation of toluene. All three components were obtained at >90% of homogeneity (Figure 1).

Initial attempts to purify the enzyme components centered around the use of affinity chromatography. Resins were chosen for their ability to mimic substrates of the enzyme system, toluene and phenol. Phenyl-Sepharose and *p*-toluic acid columns were unsuccessful in obtaining active hydroxylase fractions. The hydroxylase component bound strongly to each column. However, it could not be eluted from these columns in an active form.

The purification of the components was as described under Materials and Methods. As shown in Table 1, the three components were separated from one another by passage over a DEAE ion exchange column. Further purification of the hydroxylase and small component was carried out through the use of gel filtration chromatography. The reductase active fractions from the DEAE column were subsequently passed over a Reactive Green 19 dye-affinity column. The purification of the wild-type reductase in this manner resulted in an 80% purification as indicated by N-terminal amino acid sequencing data. Further purification of the wild-type reductase could be performed by passage through a Sephacryl S100 column which removed faint contaminating protein bands. However, this additional step resulted in a 2-fold loss of activity. The recombinant reductase could be purified from the *E. coli* DE3(pET-A5) clone containing the wild-type reductase gene in a similar manner. The fractions eluting from the dye-affinity column were 90% pure with 30% recovery of activity.

The reductase and the small protein component are single polypeptides, and the hydroxylase is comprised of three distinct polypeptides. The smallest subunit of the multisubunit protein, approximately 13 500 molecular weight, stained lighter with Coomassie Blue than the other two subunits. However, it consistently purified with the other two subunits. Silver-stained gels showed a uniform intensity of staining of all three subunits, suggesting that they are present in equal amounts. Molecular weight determinations of this large protein component were consistent with the presence of three subunits of the sizes shown (Figure 1). N-Terminal sequences determined for the subunits of the hydroxylase and the reductase and small component matched the N-terminal sequence predicted from the gene sequence (Francesconi et al., 1995).

Characterization of Components. (A) Reductase. The reductase component of the multicomponent enzyme system has a molecular weight of approximately 42 000 as measured by gel filtration chromatography and denaturing gel electrophoresis (Table 2). Quantitative amino acid analysis indicated a molecular weight of 40 000, and this molecular weight was used in all calculations. The flavin cofactor was not covalently bound and was readily lost during purification. FAD was found to be substoichiometrically bound to the isolated protein, and it, compared to FMN, restored activity, on admixture, at a lower concentration (K_m = 16 nM). After reconstitution of the protein with FAD followed by passage over a Biogel P-10 desalting column, the reductase was found to contain 1.2 mol of FAD/mol of protein. Iron and inorganic sulfide analysis revealed the presence of 2.2 and 2.9 mol of iron and inorganic sulfide, respectively, per mole of reductase. This ratio suggests the presence of a [2Fe2S] cluster in the protein. The optical absorbance spectra (Figure 2) reveal absorbance maxima at 270, 341, 423, and 457 nm, which is consistent with other purified bacterial reductase proteins containing a flavin cofactor and [2Fe2S] cluster

Table 1: Purification of the Components of Toluene 2-Monooxygenase

fraction	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purification (*x*-fold)
hydroxylase					
crude	1550.0	373.0[a]	0.2	100	1.0
DEAE	238.2	317.3	1.3	85	5.5
Sephacryl S200	89.7	149.6	1.7	40	6.9
small component					
crude	1886.5	536.0[a]	0.3	100	1.0
DEAE	59.5	112.7	1.9	21	6.8
Biogel P60	2.3	178.0	79.4	27	283.0
reductase					
crude	1886.5	3351.0[b]	1.8	100	1.0
DEAE	122.6	2236.0	18.2	66	10.2
Reactive Green 19	5.0	707.0	141.4	21	79.4
reductase[c]					
crude	742.0	3128.0[b]	4.2	100	1.0
DEAE	22.7	2536.0	11.7	81	26.5
Reactive Green 19	1.8	938.0	512.0	30	122.0

[a] 1 unit of enzyme activity is defined as the conversion of 1 nmol of [^{14}C]toluene/min at 23 °C. [b] 1 unit of enzyme activity is defined as 1 μmol of cytochrome *c* reduced/min at 23 °C. [c] Purified from *E. coli* DE3(pET-A5).

Table 2: Molecular Properties of Purified Components

property	hydroxylase	small component	reductase
subunit structure	$(\alpha\beta\gamma)_2$	monomer	monomer
subunit mol masses (kDa)	54.4, 37.7, 13.5	10.4	40.0
mol mass (kDa)			
gel filtration	216	19.3	45.8
native PAGE	190	*e*	*e*
SDS−PAGE	*e*	10.5	41.8
calculated (aa quantitation)	*e*	10.4	40.0
metal content			
Fe content (mol/mol)	5.3	nd[d]	2.3
inorganic S^- content (mol/mol)	nd	nd	2.9
FAD (mol/mol)	nd	nd	1.2
p*I*	4.5	4.3	5.8
absorption max (nm)	282	277	270, 341, 423, 457
sp act. (units/mg)	1.7, 87[a]	79.4[b]	512.0[c]
% recovery	40	27	30

[a] 1 unit is defined as 1 nmol of [^{14}C]toluene/min at 23 °C and 1 nmol of *o*-cresol/min at 23 °C, respectively. [b] 1 unit is defined as 1 nmol of [^{14}C]toluene/min at 23 °C. [c] 1 unit is defined as 1 μmol of cytochrome *c* reduced/min at 23 °C. [d] nd, not detected. [e] Not determined.

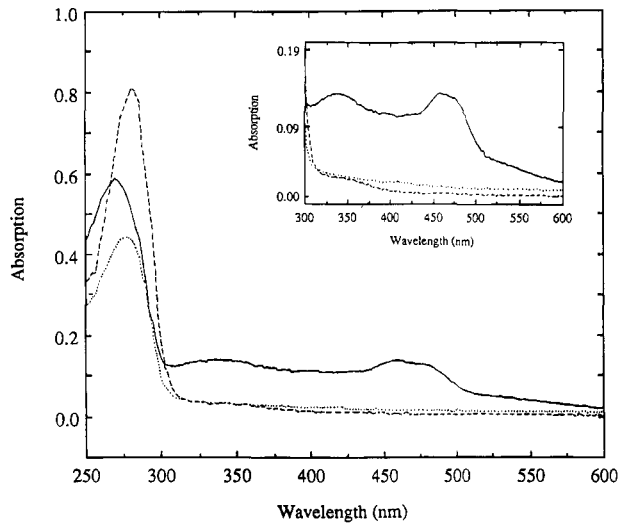

FIGURE 2: Electronic absorption spectra of purified toluene 2-monooxygenase components. Hydroxylase [(- - -) 0.4 mg/mL protein, 1.9 μM]; small component [(•••) 0.38 mg/mL protein, 37 μM]; reductase [(—) 0.28 mg/mL protein, 7 μM]. The inset shows the spectra of the three components from 300 to 600 nm enlarged 5-fold.

(Batie et al., 1987; Colby & Dalton, 1979; Fox et al., 1989; Haigler & Gibson, 1990; Powlowski & Shingler, 1990; Yamaguchi & Fujisawa, 1978). Reductase activity was inhibited by sulfhydryl alkylating reagents and metal chelating reagents, which is consistent with the presence of an iron−sulfur cluster in the protein. The reduction of cytochrome *c* was dependent upon the addition of NADH to the reaction mixture. The addition of NADPH resulted in a 4.4-fold lower rate of reduction than that observed upon addition of NADH. The reductase could be reduced by sodium dithionite and reoxidized with potassium ferricyanide. The reductase was capable of reducing nitroblue tetrazolium, 2,6-dichlorophenolindophenol, and potassium ferricyanide. The specific activities of the purified wild-type and recombinant reductase were 141 and 512 μmol of cytochrome *c* min^{-1} (mg of protein)$^{-1}$, respectively. The yield and fold purification obtained for the wild-type reductase suggest that this component comprises approximately 1.3% of the soluble cell protein. On a molar basis, the wild-type reductase is present at half the amount of the hydroxylase.

(B) Small Component. The small component was determined to consist of a small protein of approximately 10.5 kDa as determined from denaturing PAGE. Gel filtration of purified small component suggested a molecular weight of 19 300. This is nearly twice the molecular weight determined by denaturing gel electrophoresis and suggested that the small component may be eluting from the column as a dimer. Calculation of the molecular weight from quantitative amino acid analysis gave a molecular weight of 10 480. This molecular weight was used in all calculations. The optical absorbance spectrum revealed an absorbance maximum at 277 nm and no appreciable absorbance above 300 nm (Figure 2). Metal analysis revealed no significant amounts of metals associated with the protein. The addition of the small component to mixtures of hydroxylase and reductase enhanced toluene oxidation activity. Optimal concentrations of small component resulted in a 10-fold increase in activity. The specific activity of the purified small component was 80 nmol of toluene min^{-1} (mg of protein)$^{-1}$. The yield and fold purification obtained for the small component suggest that it accounts for 0.35−0.55% of the soluble cellular protein. On a molar basis, the small component is present at approximately 75% that of the hydroxylase.

(C) Hydroxylase. Denaturing gel electrophoresis of active hydroxylase fractions during the purification consistently showed the presence of three protein bands of molecular weights 54 500, 37 700, and 13 500. Holoenzyme molecular weight determinations of purified hydroxylase yielded a value of 210 000. Gel filtration and nondenaturing PAGE gave similar molecular weights that ranged from 190 000 to 216 000. The molecular weight used for all calculations was the average value, 211 000. This molecular weight and the molecular weights of the three subunits suggest an $(\alpha\beta\gamma)_2$ quaternary structure for the holoenzyme. The amino acid compositions of the translated genetic sequences of the three subunits were compared to the amino acid quantitation of the holoenzyme and found to be consistent with an $(\alpha\beta\gamma)_2$ quaternary structure for the hydroxylase component. The UV/visible absorbance spectrum of the hydroxylase showed an absorbance maxima at 282 nm with a relatively low absorbance >300 nm that recedes to the base line without prominent features (Figure 2). There was no optical absorbance spectrum in the visible region that suggested the presence of a heme or iron−sulfur center. Concentrated solutions of hydroxylase were light brown in color. Colo-

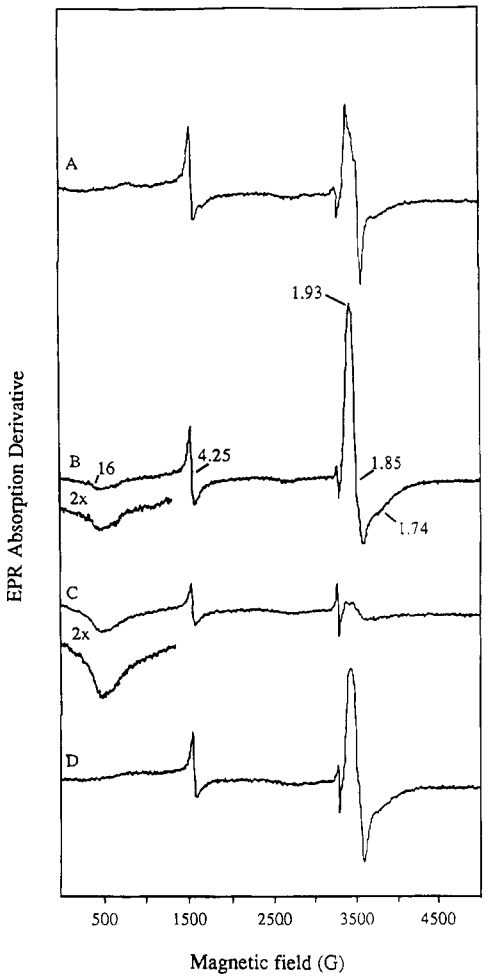

FIGURE 3: X-band EPR spectra of the hydroxylase component. (A) Hydroxylase sample as purified; (B) anaerobic sample of 52 nmol of hydroxylase in 200 μL of 25 mM MOPS (pH 7.5), with 2.6 nmol of reductase and 42 nmol of NADH added (0.75 mol of NADH/mol of hydroxylase); (C) sample from (B) after addition of excess NADH; (D) sample from (C) after the readmission of 100% oxygen to the anaerobic sample. Instrumental conditions were the following: scan range, 5000 G; field set, 2500 G; time constant, 0.128 s; modulation amplitude, 10 G; modulation frequency, 100 kHz; scan time, 4 min; 0.2 mW; 8.0 K; 9.231 GHz.

rimetric iron determinations of active purified hydroxylase revealed the presence of 4.9 nmol of iron/nmol of protein. Plasma emission spectroscopy revealed 5.9 nmol of iron/nmol of protein associated with the hydroxylase component. Zinc was also present at 2 nmol/nmol of protein. The specific activity of the purified hydroxylase was 1.7 nmol of toluene min^{-1}, (mg of protein)$^{-1}$ as assayed by the conditions used during purification. With saturating amounts of toluene, the specific activity was 27 nmol min^{-1} (mg of protein)$^{-1}$. The water-soluble substrate *o*-cresol was a better substrate based on the steady-state kinetic parameters determined to be 0.8 μM and 131 nmol/min^{-1} (mg of protein)$^{-1}$ for the K_m and V_{max}, respectively. The yield and fold purification obtained for the hydroxylase component suggest that it accounts for 14.5% of the soluble cellular protein.

EPR Spectroscopy of the Hydroxylase and Reductase Components. EPR spectra were determined for the hydroxylase component treated with 1 reducing equiv, excess reductant, and oxygen to bring about reoxidation (Figure 3). Upon addition of approximately 1 (0.75) reducing equiv per 2 Fe to the anaerobic hydroxylase sample, a signal appeared that displayed rhombic symmetry with $g_z = 1.93$, $g_y = 1.85$, and $g_x = 1.74$ ($g_{av} = 1.84$). This signal is reminiscent of the mixed-valent state of sMMO (Lipscomb, 1994). A signal at $g = 4.25$ may result from ferric iron that is adventitiously associated with the protein. This signal varied in intensity depending on the preparation of the sample and whether the sample was extensively dialyzed to remove the purification buffer. Integration of the $g < 2.0$ signal indicated that 11% of the iron cluster was present in the mixed-valent state. The $g < 2.0$ signal disappeared upon addition of excess NADH. Concomitantly, the signal at $g = 16$ intensified and may be indicative of a binuclear Fe(II)·Fe(II) center (Fox et al., 1988). This $g = 16$ signal was also seen in the partially reduced state, indicating some diferrous form exists under these conditions. Upon exposure of the reduced enzyme sample to air, the $g = 16$ signal disappeared. However, the spectrum revealed a partial restoration, approximately 7%, to the mixed-valent state of the diiron cluster. Repeated incubation of the sample in air and also 100% oxygen did not result in a disappearance of this signal to an EPR-silent Fe(III)·Fe(III) state. This suggested that the mixed-valent form of the enzyme was relatively stable. A sample of purified hydroxylase, as isolated, contained 5% of the iron cluster in the mixed-valent state, giving further evidence for the stability of the Fe(II)·Fe(III) configuration.

The reductase component was also subjected to EPR analysis (Figure 4). Purified wild-type reductase in the resulting state was EPR-silent. Upon partial reduction of the protein, the spectrum revealed a large $g = 2.0$ signal indicative of a flavin semiquinone. Upon further reduction, this $g = 2.0$ signal decreased in intensity, and a signal indicating reduction of a [2Fe2S] cluster appeared. This signal increased in intensity upon addition of excess NADH, but the $g = 2.0$ signal did not completely disappear. Only when excess sodium dithionite was present did the signal at $g = 2.0$ completely disappear and give rise to a $g_z = 2.03$, $g_y = 1.93$, $g_x = 1.91$, ($g_{av} = 1.95$) spectrum indicative of a plant-type ferredoxin [2Fe2S] center (Bertrand et al., 1991; Orme-Johnson, 1973).

Identification of the $\alpha_2\beta_2\gamma_2$ Component as the Hydroxylase. Single-turnover experiments were performed to definitively ascertain which component binds toluene and activates molecular oxygen. The results of the single-turnover experiments in Table 3 indicate that this is played by the $\alpha_2\beta_2\gamma_2$ component. All reactions containing the $\alpha_2\beta_2\gamma_2$ component yielded an oxidation product of toluene whereas reactions lacking this component had no detectable product. The yields were low (4.8−13.9%) in these reactions containing 2 electron equiv of sodium dithionite. Interestingly, even with excess dithionite added to a mixture of the hydroxylase and the small component, the yield of the product did not increase significantly based on the amount of the hydroxylase present. These data suggest that dithionite serves poorly to support turnover of the hydroxylase. As a control, 1.25 mM NADH was added to the fully reconstituted enzyme system after the single-turnover reaction was conducted, and 70% of the original activity was retained. This would suggest that the enzyme components were not inactivated by the experimental procedure. The product of the single-turnover reaction was identified as *o*-cresol by thin-layer chromatography (see section on reaction products below).

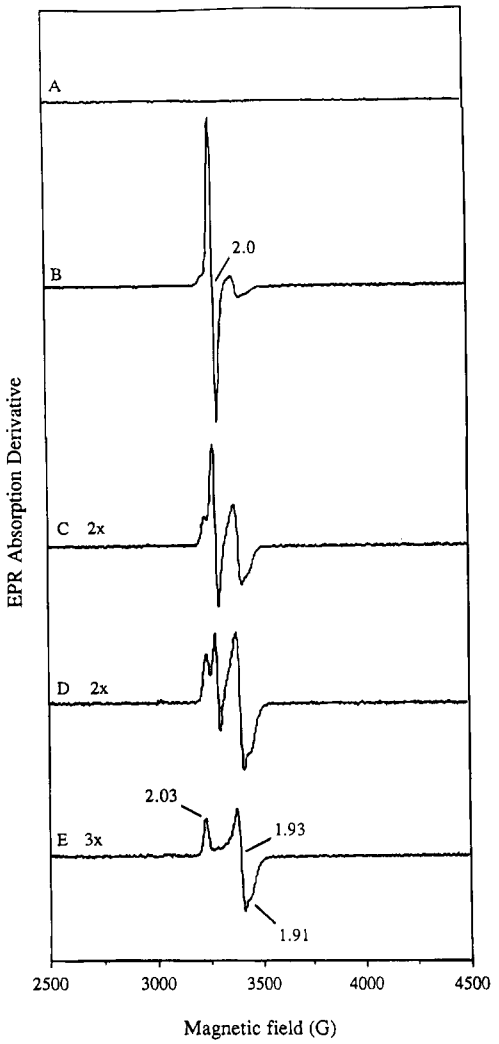

FIGURE 4: X-band EPR spectra of the reductase component. (A) Reductase sample as purified; (B) anaerobic sample of 47 nmol of reductase in 200 μL of 25 mM MOPS (pH 7.5), plus 23.5 nmol of NADH (1-electron reduction); (C) sample from (B) plus an additional 23.5 nmol of NADH (2-electron reduction); (D) sample from (C) plus the addition of excess NADH (94 nmol); (E) sample from (D) plus the addition of excess sodium dithionite (100 μmol). Instrumental conditions were as follows: scan range, 2000 G; field set, 3500 G; time constant, 0.128 s; modulation amplitude, 10 G; modulation frequency, 100 kHz; scan time, 4 min; 0.0125 mW; 8.0 K; 9.233 GHz.

Hydrogen peroxide (H_2O_2) dependent turnover of the hydroxylase would be possible if addition of H_2O_2 resulted in formation of a reactive oxygenating species as has been demonstrated for the soluble methane monooxygenase from *M. trichosporium* OB3b (Andersson et al., 1991). The results in Table 4 show that the $\alpha_2\beta_2\gamma_2$ component is needed for product formation and it is driven by H_2O_2 in place of NADH and O_2. No product was observed in incubations of H_2O_2 with the reductase, the small component, or heat-treated hydroxylase. The low yields are due to the rapid depletion of H_2O_2 (Table 4) and not enzyme inactivation. The addition of NADH to the sample containing all three protein components after completion of the H_2O_2 experiment resulted in the conversion of 52% of the radiolabeled substrate to product (data not shown). This was comparable to a control sample incubated with NADH and O_2, indicating that no appreciable loss of enzyme activity occurred due to the presence of H_2O_2. Incubation of hydroxylase with 10 mM *tert*-butyl hydroperoxide resulted in no detectable product formation.

Table 3: Single-Turnover Reactions Catalyzed by the Hydroxylase Component[a]

component	oxidation of toluene: nmol reacted	oxidation of toluene: % yield[c]	product formed[b]
hydroxylase	0.14	4.8	*o*-cresol
and small component	0.30	10.3	*o*-cresol[d]
and reductase	0.25	8.6	*o*-cresol[d]
small component	0.00	0.0	*e*
reductase	0.00	0.0	*e*
all components	0.41	13.9	*o*-cresol[d]
hydroxylase and NADH	0.01	0.4	*o*-cresol
hydroxylase, no treatment	0.00	0.0	*e*
NADH	0.00	0.0	*e*
methyl viologen	0.00	0.0	*e*

[a] Single-turnover reactions were performed as described under Materials and Methods. [b] Determined using undiluted [^{14}C]toluene at a final concentration of 10 μM. [c] Based on nanomoles of hydroxylase present, 3 nmol of toluene would equal 100% yield. [d] Trace amounts of 3-methylcatechol were detected. [e] Not determined.

Table 4: Hydrogen Peroxide Dependent Turnover of the Hydroxylase Component[a]

sample	% [^{14}C]toluene reacted	H_2O_2 remaining (mM)
hydroxylase	1.2	0.1
and small component		
1 nmol	1.9	0.3
3 nmol	2.2	0.3
6 nmol	2.1	0.2
12 nmol	3.1	0.2
and reductase		
2 nmol	1.3	0.1
5 nmol	1.3	0.2
10 nmol	1.4	0.2
20 nmol	1.3	0.2
hydroxylase and reductase (20 nmol), small component (6 nmol)	2.5	0.2
small component (6 nmol)	0.0	6.8
reductase (20 nmol)	0.0	9.1
hydroxylase (heat-killed)	0.0	6.7

[a] Hydrogen peroxide dependent turnover reactions were performed as described under Materials and Methods. The concentration of hydroxylase used in each reaction was 23.7 μM (9.5 nmol). All reactions were performed at 23 °C.

In all of the experimental samples containing active hydroxylase, the amount of H_2O_2 consumed far exceeded the amount of organic product formation. Bubbles were observed to form in the reaction vials after the addition of H_2O_2, and this suggested oxygen was being evolved. This was confirmed with the use of an oxygen electrode. The addition of 356 μM H_2O_2 resulted in the formation of 151 μM O_2, or a product yield of 85% based on the utilization of 2 mol of H_2O_2 per 1 mol of O_2 formed. Increasing the concentration of H_2O_2 resulted in an increase in the rate of oxygen formation. A doubling of the enzyme concentration resulted in a doubling of the rate during the initial time of the reaction. A Lineweaver–Burke plot of the data revealed a K_m of 21 mM and a V_{max} of 2200 nmol min^{-1} (mg of protein)$^{-1}$.

Products of Toluene Oxidation. Experiments were conducted to determine products from the oxidation of toluene by the reconstituted enzyme system. NADH and the complete enzyme system, consisting of hydroxylase, small component, and reductase, supported the oxidation of [^{14}C]-toluene to a nonvolatile product(s). Controls which included

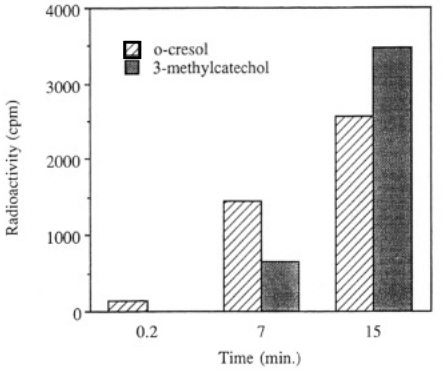

FIGURE 5: Products of the oxidation of toluene by reconstituted toluene 2-monooxygenase. Results of the incubation of radiolabeled toluene with reconstituted enzyme system. Enzyme components were present in the following concentrations: 1.13 nmol of hydroxylase; 0.8 nmol of small component; and 2.35 nmol of reductase. Reaction vials contained 250 μM nonradioactive *o*-cresol. Reactions were performed as described under Materials and Methods.

the reductase and small component alone, or the hydroxylase and small component alone, were incapable of hydroxylating substrate in the presence of NADH (data not shown). In the absence of the small component, the reductase and the hydroxylase components were capable of oxidizing toluene at 10% the level of the fully reconstituted system.

Thin-layer chromatographic analysis of the toluene reaction products revealed a radiolabeled compound that comigrated with authentic 3-methylcatechol. No detection of radiolabeled product migrating with authentic *o*-cresol was detected. *o*-Cresol was not detected in the reaction mixture unless nonradioactive *o*-cresol (250 μM) was included to act as a cold trap (Figure 5). Under these reaction conditions, *o*-cresol could be detected as the first reaction product and was seen to increase over time. The secondary product, 3-methylcatechol, was also detected under these reaction conditions and was seen only after the accumulation of *o*-cresol in the reaction mixture. This demonstrated a dihydroxylation of toluene via two sequential hydroxylations, the first at the *ortho*-position and the second at the *meta*-position to produce 3-methylcatechol via *o*-cresol as an intermediate.

DISCUSSION

Toluene 2-monooxygenase has been purified for the first time from *B. cepacia* G4 and shown to be a soluble, three-component enzyme system. Previous attempts to obtain active cell-free extract were unsuccessful (Shields & Reagin, 1992). It was found in our studies that the addition of Fe^{2+} to the buffer was necessary to obtain active crude extract. In addition, it was necessary to include Fe^{2+} in all buffers during the purification of the hydroxylase component, suggesting the importance of iron in maintaining the activity of this component.

The enzyme complex is made up of a large 211 kDa iron-containing hydroxylase component consisting of three protein subunits in an $(\alpha\beta\gamma)_2$ quaternary structure, a 40 kDa iron–sulfur reductase requiring FAD as a cofactor, and a 10.5 kDa small protein component which contains no metals and no identifiable organic cofactor for activity. These physical characteristics are strikingly different from other bacterial multicomponent oxygenases responsible for the oxidation of aromatic compounds that typically consist of a hydroxylase component with an iron–sulfur cluster, a small ferredoxin component, and a reductase component containing flavin (van der Meer et al., 1992). Low molecular weight ferredoxins in these latter systems function to shuttle electrons from the flavoprotein to the hydroxylase, but there is no hint of a similar role for the low molecular weight component of toluene 2-monooxygenase. In fact, the small component is not obligately required for toluene oxidation but stimulates the reaction and, in this way, resembles the small component of the soluble methane monooxygenase enzyme system. The hydroxylase component of toluene 2-monooxygenase differs substantially from the corresponding component of toluene dioxygenase (Subramanian et al., 1979) or *p*-methoxybenzoate monooxygenase (Bernhardt et al., 1975). The latter oxygenase components are iron–sulfur proteins, but the toluene 2-monooxygenase hydroxylase does not show any appreciable visible absorbance nor does it contain inorganic sulfur.

To further investigate the nature of the iron present in the hydroxylase component, EPR spectra of the partially reduced, fully reduced, and oxidized states of the hydroxylase component were obtained. Upon the addition of one electron per two Fe atoms, the EPR spectrum obtained showed rhombic symmetry, a characteristic of a spin-coupled system. Compatible with observations that the hydroxylase contained no inorganic sulfur and no prominent optical absorbance band over 300 nm, we suggest that this EPR signal arises from a binuclear iron cluster, similar to that present in the soluble methane monooxygenase hydroxylase component from *M. trichosporium* OB3b (Lipscomb, 1994; Wilkins, 1992). The partially reduced or mixed-valent state, $[Fe^{II}\cdot Fe^{III}]$, of soluble methane monooxygenase (sMMO) from *M. capsulatus* (Bath) and *Methylococcus* CRL-25 and *Methylobacterium* CRL-26 give similar EPR spectra (Woodland & Dalton, 1984; Woodland et al., 1986; Prince et., 1988). Upon addition of excess reducing equivalents to the hydroxylase sample, the EPR signal ($g_{av} < 2.0$) disappeared, and a signal in the low magnetic field of $g = 16$ increased in intensity. This signal is a characteristic of sMMO in the fully reduced state $\{[Fe^{III}\cdot Fe^{III}]\}$ and disappears when the enzyme is exposed to oxygen (Fox et al., 1989). The $g = 16$ signal seen in the EPR spectrum of the reduced hydroxylase disappeared in a similar manner upon exposure to oxygen. This suggests that the toluene-2-monooxygenase hydroxylase contains oxo-bridged diiron clusters, possibly two per holoenzyme based on iron determinations. Also, to obtain the apparent mixed-valent state as evidenced by EPR, approximately 2 reducing equiv were required, suggesting the presence of two $[Fe^{II}\cdot Fe^{III}]$ clusters per holoenzyme.

The $\alpha_2\beta_2\gamma_2$ component was strongly implicated as the protein that binds toluene and activates molecular oxygen based on several independent observations. The first is the resemblance to the methane monooxygenase hydroxylase component described above. Second, the $\alpha_2\beta_2\gamma_2$ component bound tightly to a toluene affinity column. In fact, the conditions required to elute the hydroxylase component were sufficiently stringent to inactivate the protein, and this purification step had to be replaced by gentler procedures. Third, only the hydroxylase component was singly active in catalyzing toluene monooxygenation following chemical reduction. Fourth, the hydroxylase reacted with H_2O_2, showing substantial peroxidase activity. Soluble methane

monooxygenase also acts as a peroxidase, albeit at a lower rate (Andersson et al., 1991), and it is proposed to bind a 2-electron-reduced form of O_2 during the catalytic cycle (Lipscomb, 1994).

The hydroxylase component, as isolated, contains spectroscopically-homogeneous iron centers except for a minor high-field iron EPR signal, and the centers undergo reduction uniformly. The specific activity of the protein increases during purification, commensurate with the degree of enrichment. These data suggest that the purified hydroxylase is fully active. Moreover, while the measured specific activity with toluene is low, this may be partly due to the sequential hydroxylation of toluene forming the competing substrate *o*-cresol, and this underestimates the specific activity.

Although the toluene 2-monooxygenase enzyme system is quite similar to the sMMO enzyme system in physical characteristics, there are differences in catalytic properties of the enzymes. First, the products of toluene oxidation are different between the two enzyme systems. The sMMO enzyme system oxidizes toluene to the products benzyl alcohol and *p*-cresol (Wilkins et al., 1994), or *m*-cresol and *p*-cresol, with only a minor amount of *o*-cresol detected (Liu, 1995). This is in contrast to the toluene 2-monooxygenase system described here. We detected only the formation of *o*-cresol from the initial oxidation of toluene (see discussion below). Second, the ability to reduce the diiron centers chemically appears to be quite different based on the single-turnover results. Third, the experiments with the hydrogen peroxide coupled oxygenation reactions also suggest differences. Andersson and co-workers report low peroxidase activity and a 2:1 ratio of peroxide utilization to product formed (Andersson et al., 1991). In our studies, a ratio of 100 000:1 was obtained, suggesting that the rate of reaction with peroxide was much greater than that of the toluene reaction.

Previous whole cell studies with *B. cepacia* G4 indicated that toluene was metabolized via sequential hydroxylation reactions yielding *o*-cresol and 3-methylcatechol successively (Shields et al., 1989). It was not definitively known if one enzyme system or multiple enzyme systems operating in the cell were responsible for the sequential hydroxylation reactions. Mutants of *B. cepacia* G4, belonging to a single TOM A^- class, were unable to catabolize toluene, phenol, *o*-cresol, and *m*-cresol, and degrade trichloroethylene, suggesting a single structural gene or regulatory region was responsible for these reactions. However, it could not be ruled out that the mutations in the TOM A^- class were not polar mutations resulting in the absence of expression of two or more genes (Shields et al., 1991). The products detected as a result of the oxidation of radiolabeled toluene by the purified enzyme system demonstrate that the sequential hydroxylation of toluene is catalyzed by a single enzyme complex. The results of the experiment show the first detectable intermediate of the reaction was a radiolabeled compound which comigrated with *o*-cresol. This intermediate was detectable only if the reaction mixture was spiked with *o*-cresol, suggesting that the enzyme affinity for this substrate is higher than for toluene. Upon further incubation, a second product, which comigrated with authentic 3-methylcatechol, accumulated in the reaction mixture. The addition of *o*-cresol strongly inhibited the oxidation of [^{14}C]toluene (data not shown), further suggesting that the same enzyme active site catalyzes the hydroxylation of both substrates.

The genes for the toluene 2-monooxygenase enzyme complex have recently been cloned and sequenced (Francesconi et al., 1995; Johnson & Olsen, 1995). Francesconi and co-workers have shown six open reading frames, *tomA0*–*tomA5*, from a single operon to be involved in the expression of this multicomponent enzyme complex. The N-terminal sequences of the purified toluene 2-monooxygenase match the translated amino acid sequence of these genes. The three hydroxylase subunits of sizes 54.4, 37.7, and 13.5 kDa correspond to the open reading frames designated *tomA3*, *tomA1*, and *tomA4*, respectively. The small component is encoded by the gene designated *tomA2*. The reductase component is encoded by the gene *tomA5*. These gene sequences were reported to be homologous to other oxygenases such as dimethylphenol monooxygenase, soluble methane monooxygenase, toluene 4-monooxygenase, and phenol 2-monooxygenase.

A number of other bacterial oxygenase systems have recently been compared to sMMO and other binuclear iron proteins by sequence alignments. These include phenol hydroxylase from *Pseudomonas* strain CF600 (Shingler et al., 1990), toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 (Fox et al., 1994), and the membrane-associated xylene monooxygenase from *Pseudomonas putida* and alkane hydroxylase of *Pseudomonas oleovorans* (Shanklin et al., 1994). Of these oxygenase systems, alkane hydroxylase (McKenna & Coon, 1970), phenol hydroxylase (Shingler et al., 1990), and toluene 4-monooxygenase (Whited & Gibson, 1991) have been partially purified.

In summary, we present the first report of the purification of the enzyme system toluene 2-monooxygenase from the toluene-degrading bacterium *B. cepacia* G4, and its inclusion in the rapidly growing class of binuclear iron proteins. Further studies on the catalytic and spectroscopic properties of this enzyme system, and further comparisons to other similar enzyme systems, are in progress.

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