JOURNAL OF BACTERIOLOGY, May 1996, p. 2645–2649 Vol. 178, No. 9
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Purification and Characterization of Chlorophenol 4-Monooxygenase from *Burkholderia cepacia* AC1100

LUYING XUN[1,2]*

Department of Microbiology, Washington State University Tri-Cities[1] and Environmental Microbiology Group, Pacific Northwest National Laboratory,[2] Richland, Washington 99352

Received 12 December 1995/Accepted 23 February 1996

***Burkholderia* (formerly *Pseudomonas*) *cepacia* AC1100 mineralizes the herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T), and the first intermediate of 2,4,5-T degradation is 2,4,5-trichlorophenol. Chlorophenol 4-monooxygenase activity responsible for 2,4,5-trichlorophenol degradation was detected in the cell extract. The enzyme consisted of two components separated during purification, and both were purified to more than 95% homogeneity. The reconstituted enzyme catalyzed the hydroxylation of several tested chlorophenols with the coconsumption of NADH and oxygen. In addition to chlorophenols, the enzyme also hydroxylated some chloro-*p*-hydroquinones with the coconsumption of NADH and oxygen. Apparently, the single enzyme was responsible for converting 2,4,5-trichlorophenol to 2,5-dichloro-*p*-hydroquinone and then to 5-chlorohydroxyquinol (5-chloro-1,2,4-trihydroxybenzene). Component A had a molecular weight of 22,000 and contained flavin adenine dinucleotide. Component A alone catalyzed NADH-dependent cytochrome *c* reduction, indicating that it had reductase activity. Component B had a molecular weight of 58,000, and no catalytic activity has yet been shown by itself.**

Chlorinated phenols and their derivatives have been widely used as pesticides and herbicides, and they are a major group of environmental pollutants (3, 7, 13). Several microorganisms have been isolated to mineralize these compounds. *Burkholderia* (formerly *Pseudomonas*) *cepacia* AC1100 is the only pure culture that can degrade 2,4,5-trichlorophenoxyacetate (2,4,5-T). It was isolated from an enrichment culture which had been selected in a chemostat under a strong selective pressure with 2,4,5-T as the sole carbon source (10, 11). An oxygenase converting 2,4,5-T to 2,4,5-trichlorophenol (2,4,5-TCP) has been identified; the genes encoding the oxygenase component have been cloned and sequenced (4), and the oxygenase component has been purified and characterized (24). A 2,4,5-T-negative mutant PT88 accumulates 5-chlorohydroxyquinol (5-CHQ) when growing with 2,4,5-T and glucose (17), suggesting that 2,4,5-TCP is converted to 5-CHQ through two hydroxylations. The genes responsible for 5-CHQ breakdown have recently been cloned and sequenced, and they can complement PT88 for 2,4,5-T degradation (5). Although whole-cell study with an enzyme inhibitor suggests that a flavin-containing enzyme is responsible for the hydroxylation of 2,4,5-TCP (20), little is known about either the genes or the enzyme(s) responsible for the conversion of 2,4,5-TCP to 3-CHQ. Since the conversion involves two hydroxylations, it has been assumed that two enzymes are required. I report here the identification, purification, and characterization of chlorophenol 4-monooxygenase that converted 2,4,5-TCP to 2,5-dichloro-*p*-hydroquinone (2,5-DiCH) and then to 5-CHQ from *B. cepacia* AC1100.

MATERIALS AND METHODS

Materials. Pentachlorophenol (PCP), 2,3,5,6-tetrachlorophenol (TeCP), 2,4,6-TCP, 2,4,5-TCP, tetrachloro-*p*-hydroquinone (TeCH), 2,6-DiCH, and 2,5-DiCH were all purchased from Aldrich (Milwaukee, Wis.). 6-CHQ (6-chloro-1,2,4-trihydroxybenzene) was a gift from Ronald L. Crawford, University of Idaho.

Bacterium and culture conditions. *B. cepacia* AC1100 was kindly provided by A. M. Chakrabarty, University of Illinois at Chicago. AC1100 was cultured with sodium glutamate (2 g/liter) and 2,4,5-T (0.15 g/liter) in a mineral medium (11). Large quantities of cells were obtained by culturing AC1100 in a 50-liter carboy containing 30 liters of the medium bubbled with sterile air for 1 day at 24°C. When 2,4,5-T was almost completely consumed (<0.001 g/liter), cells were harvested by concentrating the culture to 2 to 3 liters in a hollow-fiber filtration unit (model DC10L; Amicon, Beverly, Mass.) and then by centrifuging it at 17,000 × *g* for 12 min at 4°C. The cell pellets were stored at −20°C.

Enzyme assay. The chlorophenol 4-monooxygenase activity was assayed by measuring the production of TeCH. A standard assay mixture contained 40 mM potassium phosphate (KP_i) buffer (pH 7.4), 100 μM TeCP, 10 μM flavin adenine dinucleotide (FAD), 5 mM NADH, 0.4% Tween 20, and various amount of proteins in a 40-μl volume at 35°C. Tween 20 was included in the reaction mixture because it was found to facilitate reproducible quantitation of chlorinated phenols by high-performance liquid chromatography (HPLC) analysis (21). Stock solutions (100 mM) of chlorophenols and chlorohydroquinones were prepared in absolute ethanol, and a stock solution (100 mM) of NADH was prepared in 10 mM Tris base (pH > 13). The reaction was initiated by adding NADH to the reaction mixture. Kinetic analysis of PCP, TeCP, or 2,4,6-TCP was done by measuring TeCH or 2,6-DiCH production. Kinetic analysis for 2,4,5-TCP or 2,5-DiCH was done by measuring the decrease of the substrate.

Activities of cytochrome *c* reduction were measured in a total volume of 500 μl of 40 mM KP_i buffer (pH 7.4) containing 200 μM ferricytochrome *c* (Sigma, St. Louis, Mo.), 10 μM FAD, 1 mM NADH, and various amount of proteins at 24°C. Changes in A_{550} were monitored at 10-s intervals over the first 2 min. The absorption was first recorded without the enzyme to get a baseline, and then 4 μl of enzyme preparations was added to initiate the reaction. The activities of cytochrome *c* reduction were calculated by using $18.5 \times 10^6\ M^{-1}\ cm^{-1}$ as the extinction coefficient of cytochrome *c* (reduced − oxidized) (19). FAD reduction was measured in a total volume of 500 μl of 40 mM KP_i buffer (pH 7.4) containing 40 μM FAD, 1 mM NADH, and various amount of proteins at 24°C under anaerobic conditions. Anaerobic condition was achieved by adding the reaction mixture into a gastight cuvette inside an anaerobic glove box filled with 10% hydrogen and 90% nitrogen.

Purification of chlorophenol 4-monooxygenase. The enzyme was separated into two components, component A (cA) and component B (cB), through a strong anion-exchange column, MonoQ (Pharmacia). The initial purification steps for each component were the same until MonoQ separated cA from cB. After separation on MonoQ, the two components were purified separately.

Purification steps. All operations were performed at 6°C. The levels of ammonium sulfate saturation are those at 25°C.

(i) Extraction of cells. About 10 to 15 g of frozen cells was thawed at room temperature and suspended in 30 ml of 20 mM Tris buffer (pH 8.0) containing 5 mM EDTA. The protease inhibitor phenylmethylsulfonyl fluoride was freshly prepared in absolute ethanol at a concentration of 30 mM and added to the cell suspension to a final concentration of 0.5 mM. The slurry was passed through a French pressure cell (model FA-030; Aminco, Urbana, Ill.) three times at 260 MPa. The product was centrifuged at 17,000 × *g* for 12 min, and the supernatant was saved.

(ii) Protamine sulfate fractionation. A 2% solution of protamine sulfate in 20

* Corresponding author. Phone: (509) 375-9327. Fax: (509) 375-6666. Electronic mail address: L_XUN@pnl.gov.

mM Tris buffer (pH 8.0) was added to the supernatant slowly to 0.05% with constant stirring. After 5 min, the mixture was centrifuged at 17,000 × *g* for 12 min, and the supernatant was saved.

(iii) Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant to 33% saturation with constant stirring. The pH of the solution was not adjusted. After 10 min of stirring, the mixture was centrifuged at 17,000 × *g* for 10 min. The precipitate was discarded. Additional solid ammonium sulfate was added to the supernatant to 65% saturation with constant stirring. After 10 min of stirring, the mixture was centrifuged at 17,000 × *g* for 10 min. The precipitate was saved, and the supernatant was discarded.

(iv) Ultracentrifugation. The precipitate was suspended in an equal volume of 25 mM KP_i buffer (pH 6.9) containing 1 mM dithiothreitol (DTT). The suspension was dialyzed against 1 liter of the same buffer for 2 h. The dialyzed sample was centrifuged at 331,000 × *g* for 30 min, and the supernatant was saved.

(v) Dye chromatography. The supernatant was loaded onto a 20-ml Cibacron Blue 3GA agarose (Sigma) column (1.5 by 11.3 cm) previously equilibrated with the 25 mM KP_i buffer with 1 mM DTT. After the proteins were loaded, the column was washed with 80 ml of the equilibrating buffer and then washed with 80 ml of 1 M NaCl in the same KP_i buffer. Most proteins did not bind to the column and washed off with the starting buffer. The enzyme consisting of both cA and cB bound to the column, and it was eluted with the 1 M NaCl solution. The eluant containing the enzyme activity was concentrated to 4 ml by Centriprep-10 (Amicon). The buffer was changed to 25 mM KP_i (pH 6.9) containing 1 mM DTT by Centriprep-10.

(v) MonoQ chromatography. The enzyme from the dye column in 25 mM KP_i (pH 6.9) containing 1 mM DTT was injected onto a MonoQ HR 5/5 column (Pharmacia) equilibrated with the same buffer. Proteins were eluted with a step-and-linear gradient of NaCl (percentages of 1 M NaCl in the same buffer: 0%, 4 ml; 10 to 35%, 20-ml linear gradient; 100%, 5 ml; and 0%, 2 ml) by a fast protein liquid chromatography (FPLC) system (Pharmacia). cA did not bind to the column at all, and cB was eluted as a major peak around 230 to 280 mM NaCl. The fractions containing each component were separately pooled and concentrated to less than 1 ml by Centriprep-10.

(v) Second MonoQ chromatography for cA. The buffer for cA was changed to 20 mM Tris (pH 8.0) containing 1 mM DTT by Centriprep-10. The sample was injected onto the MonoQ column equilibrated with the same buffer. Proteins were eluted with a step-and-linear gradient of NaCl (percentages of 1 M NaCl in the same buffer: 0%, 3 ml; 0 to 25%, 20-ml linear gradient; 100%, 5 ml; and 0%, 2 ml). cA was eluted as a major peak around 100 mM NaCl. The fractions containing enzyme activity were pooled and concentrated.

(vi) Hydroxyapatite chromatography for cA and cB. The buffer for both cA and cB was changed to 8 mM sodium phosphate (pH 6.6) containing 0.3 mM $CaCl_2$ and 1 mM DTT by Centriprep-10. Either cA or cB was injected onto a Bio-Scale CHT2-I hydroxyapatite column (Bio-Rad) equilibrated with the same buffer. Each component was eluted separately by the same linear gradient of sodium phosphate (pH 6.6) (concentrations of sodium phosphate: 8 mM, 3 ml; 8 to 125 mM, 20 ml; 500 mM, 6 ml; and 8 mM, 4 ml). cA was eluted as a single peak around 50 mM sodium phosphate, and cB was eluted as single peak around 45 mM sodium phosphate. cA and cB was individually concentrated to 1 to 2 ml and stored at −80°C.

(vii) Gel filtration chromatography. Gel filtration chromatography was used to estimate the native molecular weights of cA and cB. Purified cA or cB was injected onto a Superose 12 column (Pharmacia) equilibrated with 25 mM KP_i (pH 6.9) containing 1 mM DTT and 150 mM NaCl. The protein was eluted with the same buffer by the FPLC system. The cA was eluted from the column as a single peak with a retention volume of 14.2 ml. The cB was eluted from the column as a single peak with a retention volume of 12.3 ml.

Analytical methods. A Waters HPLC with a Nova-Pak C_{18} column (3.9 by 150 mm) was used to analyze chlorinated phenols and hydroquinones. The compounds were eluted by an 11 mM H_3PO_4-acetonitrile gradient (percentages of acetonitrile; 5 to 60%, 6-ml linear gradient; 60%, 4 ml; 100%, 5 ml; and 100 to 5%, 1-ml linear gradient). Absorption within the range of 250 to 350 nm was monitored by a Waters 996 photodiode array detector operated by Millennium 2010 version 2.1 program (Waters) on a personal computer (NEC). The retention time and absorption spectrum of each compound or reaction product were compared with those of authentic standards for identification, and the peak area was used for quantification.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (12). Gels were stained for proteins with Coomassie brilliant blue R-250. Protein concentrations were determined with a protein dye reagent (2), with bovine serum albumin as the standard.

pH and temperature optima. Chlorophenol 4-monooxygenase activities were measured at various pH values within the range of 6.6 to 7.8 by using 40 mM KP_i buffer in a total volume of 40 μl. The reaction mixture was otherwise the same as described above for the enzyme assay. The temperature optimum for the enzyme activity was determined in a similar way in 40 mM KP_i buffer (pH 7.4). The reaction mixture without NADH was incubated at the corresponding temperature for 4 min, and then 1 μl of 100 mM NADH solution was added to the mixture to start the reaction.

TABLE 1. Purification scheme for the cA of chlorophenol hydroxylase

Purification step	Vol (ml)	Protein (mg)	Activity (U)[a]		Recovery (%)
			Total	Specific	
Cell extract	46.5	990.0	11.33	0.011	100.0
Protamine sulfate treatment	47.0	868.6	11.53	0.013	101.8
Ammonium sulfate fractionation	29.0	596.0	21.48	0.036	189.6
Ultracentrifuge	28.0	467.6	17.63	0.037	155.6
Dye column	4.0	73.0	6.30	0.086	55.4
MonoQ KP_i buffer	1.6	8.6	2.32	0.283	20.5
MonoQ Tris buffer	2.0	1.45	1.88	1.298	16.6
Hydroxyapatite	2.0	0.21	1.22	5.824	10.8

[a] One unit is defined as the conversion of 1 μmol of TeCP to 1 μmol of TeCH per min.

RESULTS

Identification of chlorophenol 4-monooxygenase activities in cell extracts. Since the enzyme(s) responsible for the degradation of 2,4,5-TCP is induced by 2,4,5-T (9), AC1100 was cultured with sodium glutamate and small amount of 2,4,5-T in a mineral medium. AC1100 cells were actively degrading 2,4,5-TCP when harvested before 2,4,5-T had been completely degraded. The cell extract degraded 2,4,5-TCP in the presence of NADH and O_2; however, the proposed product, 2,5-DiCH (17), was not detected, possibly because of the further degradation of 2,5-DiCH by subsequent enzymes. Since it is more conclusive to monitor the formation of a product than to check the decrease of a substrate for activity assay, other chlorophenols were tested for the accumulation of a product. When TeCP was used as a substrate, TeCH was produced by the cell extract in the presence of NADH and O_2. Therefore, TeCP was used as a substrate to monitor the enzyme activity for TeCH production during purification of the enzyme. FAD but not flavin mononucleotide was found to stimulate the reaction. NADPH could replace NADH, but the enzyme activity was significantly reduced.

The effects of temperature, pH, and ionic strength on chlorophenol 4-monooxygenase activity were determined in cell extracts. The optimal temperature for TeCH production was 35°C, with 64, 89, 83, and 10% of the optimal activity retained at 26, 30, 40, and 45°C, respectively. The enzyme had a broad optimal pH range. Its highest activity was observed at pH 7.4 in 40 mM KP_i buffer; the activities were 93, 94, and 84% at pH

TABLE 2. Purification scheme for the cB of chlorophenol hydroxylase

Purification step	Vol (ml)	Protein (mg)	Activity (U)[a]		Recovery (%)
			Total	Specific	
Cell extract	34.0	524.6	3.825	0.007	100.0
Protamine sulfate treatment	34.0	522.6	2.685	0.005	71.2
Ammonium sulfate fractionation	9.7	300.7	2.233	0.007	58.4
Ultracentrifuge	9.2	276.4	2.231	0.008	58.3
Dye column	3.1	51.2	0.786	0.015	20.5
MonoQ KP_i buffer	1.4	5.5	0.346	0.063	9.0
Hydroxyapatite	1.4	0.9	0.150	0.160	3.9

[a] One unit is defined as the conversion of 1 μmol of TeCP to 1 μmol of TeCH per min.

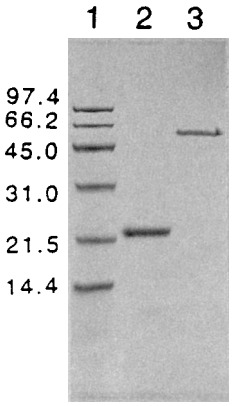

FIG. 1. SDS-PAGE of the purified cA and cB of chlorophenol 4-monooxygenase. Lane 1, molecular weight standards (Bio-Rad) (positions are indicated in kilodaltons); lane 2, 2.1 μg of cA; lane 3, 1.2 μg of cB.

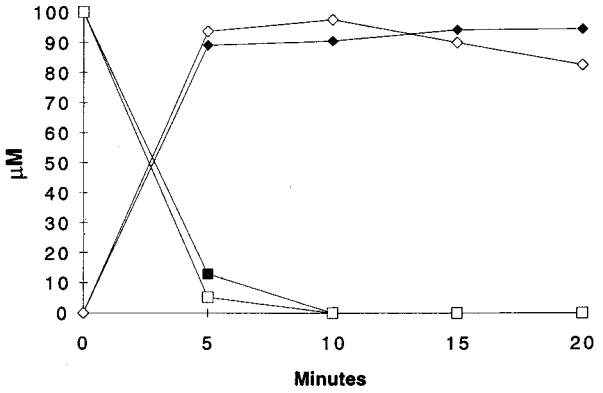

FIG. 3. TeCP and 2,4,6-TCP degradation by reconstituted chlorophenol 4-monooxygenase. Assay conditions were the same as described in the legend to Fig. 2. Only one substrate, either 2,4,6-TCP or TeCH, was added to each assay. Symbols: ■, 2,4,6-TCP; □, TeCP; ◆, 2,6-DiCH; ✧, TeCH.

6.6, 7.0, and 7.8. The best activity was obtained in 40 mM KP_i buffer (pH 7.4) at 35°C.

Enzyme purification. Chlorophenol 4-monooxygenase was purified by monitoring TeCH production in reaction mixtures following each purification step. The enzyme was separated into two components by MonoQ. Neither of the components alone degraded TeCP, but the combination of the two components converted TeCP to TeCH. First, cB was further purified to homogeneity by using cA from the MonoQ column to reconstitute the enzyme activity, and then cA was purified by using purified cB to reconstitute the enzyme activity.

The results of a typical enzyme purification of cA are summarized in Table 1. The specific activity of cA was estimated with excess cB for TeCH production. The increase in total activity for TeCP hydroxylation after ammonium sulfate fractionation was unexpected. Since the activity depended on two protein components, the increase could be due to increased coupling of the two components. Therefore, the total cA activity was underestimated in the cell extract (Table 1). The results of a typical enzyme purification of cB are presented in Table 2. The specific activity of cB was estimated with excess cA. The maximal activity for either cA or cB was reached when the other component was in threefold excess on a molar basis.

Enzyme properties. cA was apparently purified to homogeneity, as indicated by SDS-PAGE analysis, which revealed a single 22-kDa band (Fig. 1). The native cA was estimated to be a monomer by gel filtration. The purified cA was slightly yellowish, and FAD but not flavin mononucleotide stimulated its cytochrome *c* reduction, indicating that cA uses FAD as its prosthetic group. A solution containing 68.2 μM cA (1.5 mg of protein per ml; molecular weight, 22,000) gave an A_{450} of 0.15. The flavin content was calculated to be 13.2 μM by using a molar extinction coefficient of 11,300 M^{-1} cm^{-1} for FAD (18). The molar ratio of flavin to protein was 0.19, indicating that only about 19% of the purified cA contains FAD.

The SDS-PAGE analysis of purified cB revealed a protein of 58 kDa (Fig. 1). The enzyme preparation was colorless. The native cB was estimated to be about 60 kDa by gel filtration, indicating that the purified cB is a monomer.

Enzyme activity. Chlorophenol 4-monooxygenase (a mixture of purified cA and cB) converted TeCP to TeCH. Both NADH and O_2 were required for the conversion. The enzyme was not active when the reaction was carried out with 2 mM sodium dithionite in an anaerobic glove box. The optimal enzyme activity occurred in 40 mM KP_i buffer (pH 7.4) at 35°C in the presence of 10 μM FAD. With one preparation of cA, the reconstituted enzyme was less active without FAD, and 10 μM FAD stimulated the activity fourfold. The purified cA catalyzed the NADH-dependent reduction of cytochrome *c* at 6.38 μmol min^{-1} mg of $protein^{-1}$ in the presence of 10 μM FAD. The reaction rate was significantly reduced without FAD. However, cA did not catalyze NADH-dependent FAD reduction under anaerobic conditions when FAD was added to the reaction mixture, indicating that FAD is likely bound to the enzyme when it is reduced. About 90% of cB activity and 55% of cA activity remained after 40 days of storage at −80°C.

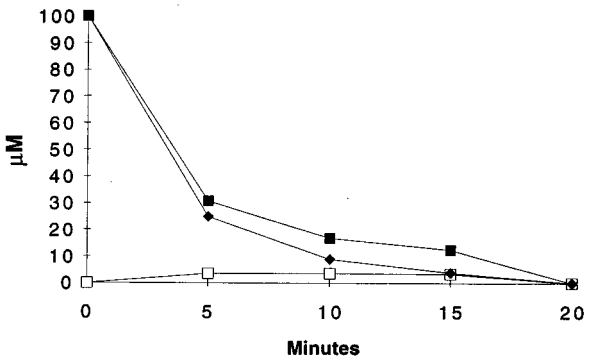

FIG. 2. 2,4,5-TCP and 2,5-DiCH degradation by reconstituted chlorophenol 4-monooxygenase (the concentration of cA was 34.3 μg/ml, and that of cB was 224.5 μg/ml). Only one substrate, either 2,4,5-TCP or 2,5-DiCH, was added to each assay. Symbols: ■, 2,4,5-TCP; □, 2,5-DiCH produced from 2,4,5-TCP degradation; ◆, 2,5-DiCH-added as the substrate.

TABLE 3. Substrate specificity of chlorophenol 4-monooxygenase[a]

Substrate	K_m (mM) (10^{-3})	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
PCP	36.5	0.008	0.2
2,3,5,6-TeCP	10.5	0.193	18.4
2,4,5-TCP	37.9	0.327	8.6
2,4,6-TCP	41.3	0.306	7.4
2,5-DiCH	2.2	0.219	100.8

[a] Kinetic experiments were performed in 40 mM KP_i buffer (pH 7.4) at 25°C. Since the activity was analyzed with excess of cA, leaving cB as the limiting factor, k_{cat} values were calculated with the molecular weight of cB.

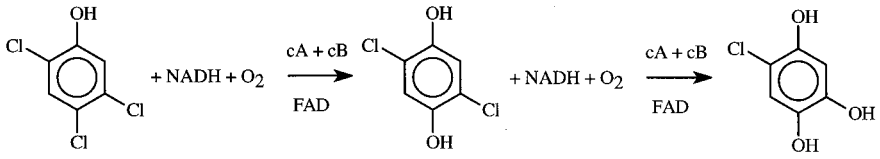

FIG. 4. Proposed conversion of 2,4,5-TCP to 2,5-DiCH and then to 5-CHQ by chlorophenol 4-monooxygenase.

Even when the purified enzyme was used to degrade 2,4,5-TCP, 2,5-DiCH was only slightly accumulated during 2,4,5-TCP degradation (Fig. 2). Subsequently, the enzyme was found to use 2,5-DiCH as a substrate (Fig. 2). Both 2,4,5-TCP hydroxylation and 2,5-DiCH consumption required cA and cB as well as NADH and oxygen. FAD stimulated both reactions. The final product from either 2,4,5-TCP or 2,5-DiCH was identified as a CHQ (chloro-1,2,4-trihydroxyquinol) by HPLC analysis. The final product and authentic 6-CHQ had the same retention time of 6.2 min and the same absorption maximum of 288 nm. When TeCP was used as the substrate, TeCH was produced and accumulated (Fig. 3). The enzyme also quantitatively converted 2,4,6-TCP to 2,6-DiCH, which was not further degraded (Fig. 3). Although TeCH was slightly decreased (Fig. 3), it was not conclusive whether TeCH was converted to 3,5,6-trichlorohydroxyquinol by the enzyme.

Kinetic analysis. The rate of enzymatic reaction at low substrate concentrations is dependent on the specificity constant, k_{cat}/K_m (6): The greater the constant, the higher the reaction rate. The kinetic parameters were determined for PCP, TeCP, 2,4,6-TCP, 2,4,5-TCP, and 2,5-DiCH (Table 3). The k_{cat}/K_m value for 2,5-DiCH was 10 times higher than that for 2,4,5-TCP, which explains why 2,5-DiCH was only slightly accumulated during 2,4,5-TCP degradation (Fig. 2). The relatively high k_{cat}/K_m value for TeCP was primarily due to its low K_m value. When used at a high concentration, 2,4,6-TCP could also be a good substrate for monitoring the enzyme activity because of its high k_{cat} value and because its reaction product, 2,6-DiCH, was accumulated.

DISCUSSION

This is the first report on chlorophenol 4-monooxygenase. Since the enzyme oxidized TeCP to TeCH, 2,4,6-TCP to 2,6-DiCH, and 2,4,5-TCP to 2,5-DiCH and since the hydroxylating sites for these chlorophenols are all at the 4 position, the enzyme should be named chlorophenol 4-monooxygenase. In addition to chlorophenols, the enzyme oxidized 2,5-DiCH to 5-CHQ, which also required NADH and O_2. The HPLC analysis could not distinguish chlorine substitution isomers of 2,5-DiCH versus 2,6-DiCH or 5-CHQ versus 6-CHQ. I suggest that 5-CHQ is the end product because it is produced from 2,5-DiCH (Fig. 4) and because it was accumulated by mutant PT88 (17). In addition. 6-CHQ is produced from 2,6-DiCH in 2,4,6-TCP degradation by *Azotobacter* sp. strain GP1 (25). In both cases, a chlorine is replaced by a hydroxyl group. The enzyme did not use 2,6-DiCH as a substrate, but it might use TeCH as a substrate at a very slow rate. Apparently the single enzyme catalyzed the sequential hydroxylation of 2,4,5-TCP to 2,5-DiCH and then to 5-CHQ (Fig. 4). Most monooxygenases introduce a single hydroxyl group to their substrates, and sequential hydroxylation is not common. However, there is another bacterial monooxygenase that oxidizes toluene to *o*-cresol and then to 3-methylcatechol (15). The enzyme, toluene 2-monooxygenase, consists of five polypeptides (15).

Chlorophenol 4-monooxygenase has both similarities and differences from pentachlorophenol 4-monooxygenase (14, 21–23). Both enzymes can use many chlorinated phenols as substrates, and both use FAD as their prosthetic group. On the other hand, chlorophenol 4-monooxygenase was not effective in catalyzing PCP oxidation, and the same is true when PCP 4-monooxygenase is used to hydroxylate 2,4,5-TCP (22). There is no evidence that PCP 4-monooxygenase can use any hydroquinones as substrate, either. In addition, PCP 4-monooxygenase is a single flavin-containing protein with a molecular weight of 60,000 (14, 21). Unlike PCP 4-monooxygenase, chlorophenol 4-monooxygenase consists of two components and is more similar to the newly reported 4-hydroxyphenylacetate 3-hydroxylase, a two-component flavomonooxygenase (16).

Several lines of evidence indicate that cA requires FAD: FAD stimulated the enzyme activity and also stimulated the cA reductase activity toward cytochrome *c*, and a small fraction of purified cA still contained FAD. cB probably does not require FAD because the purified cB did not contain FAD and some chlorophenol hydroxylating activity could be reconstituted by combining cA and cB without addition of FAD.

So far, all of the enzymes involved in the initial steps of 2,4,5-T degradation until ring cleavage have been identified in AC1100. 2,4,5-T oxygenase converts 2,4,5-T to 2,4,5-TCP (4, 24), and then chlorophenol 4-monooxygenase oxidizes 2,4,5-TCP to 5-CHQ. Chlorohydroxyquinol-1,2-dioxygenase catalyzes the ring cleavage of 5-CHQ (5). Nonetheless, some detailed work regarding 2,4,5-T degradation remains to be accomplished.

The breakdown of mono- or dichlorinated phenols is usually through a catechol intermediate before ring cleavage (8). On the other hand, all polychlorinated phenols have been shown to be degraded through a CHQ before ring cleavage (1, 5, 25). Chlorophenol 4-monooxygenase is the first reported enzyme that produces a CHQ. The enzyme is probably not involved in PCP degradation because PCP was a poor substrate and 2,6-DiCH, a PCP degradation intermediate, was not a substrate for chlorophenol 4-monooxygenase. The enzyme can effectively oxidize 2,4,6-TCP to 2,6-DiCH but not 2,6-DiCH to a CHQ. Therefore, a different enzyme must be responsible for converting 2,6-DiCH to 6-CHQ for PCP and 2,4,6-TCP degradation.

ACKNOWLEDGMENTS

This research was supported by NSF grant MCB-9218783.

I thank Kenneth Wagnon and Caroline Auker for technical help, Jang-Young Lee for reviewing the manuscript, and R. L. Crawford for his gift of CHQ.

REFERENCES

1. **Apajalahti, J. H. A., and M. S. Salkinoja-Salonen.** 1987. Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced *Rhodococcus chlorophenolicus*. J. Bacteriol. **169:**5125–5130.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Chaudhry, G. O., and S. Chapalamadugu.** 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. **55:**59–79.
4. **Danganan, C. E., R. W. Ye, D. L. Daubaras, L. Xun, and A. M. Chakrabarty.**

1994. Nucleotide sequence and functional analysis of the genes encoding 2,4,5-trichlorophenoxyacetic acid oxygenase in *Pseudomonas cepacia* AC1100. Appl. Environ. Microbiol. **60:**4100–4106.

5. **Daubaras, D. L., C. D. Hershberger, K. Kitano, and A. M. Chakrabarty.** 1995. Sequence analysis of a gene cluster involved in metabolism of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* AC1100. Appl. Environ. Microbiol. **61:**1279–1289.
6. **Fersht, A.** 1985. Enzyme structure and mechanism. W. H. Freeman, New York.
7. **Firestone, D.** 1978. The 2,3,7,8-tetrachlorodibenzo-para-dioxin problem: a review, p. 39–52. *In* C. Ramel (ed.), Chlorinated phenoxyacetic acids and their dioxins, vol. 27. Ecological Bulletin. Stockholm.
8. **Haggblom, M. M.** 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. **103:**29–72.
9. **Karns, J. S., S. Duttagupta, and A. M. Chakrabarty.** 1983. Regulation of 2,4,5-trichlorophenoxyacetic acid and chlorophenol metabolism in *Pseudomonas cepacia* AC1100. Appl. Environ. Microbiol. **46:**1182–1186.
10. **Kellogg, S. T., D. K. Chatterjee, and A. M. Chakrabarty.** 1981. Plasmid assisted molecular breeding—new technique for enhanced biodegradation of persistent toxic chemicals. Science **214:**1133–1135.
11. **Kilbane, J. J., D. K. Chatterjee, J. S. Karns, S. T. Kellogg, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. **44:**72–78.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lindstrom, K., J. Nordin, and F. Osterberg.** 1981. Chlorinated organics of low and high relative molecular mass in pulp mill bleachery effluents, p. 1039–1058. *In* L. H. Keith (ed.), Advances in the identification and analysis of organic pollutants in water, vol. 2. Ann Arbor Science Publishers Inc., Ann Arbor, Mich.
14. **Orser, C. S., C. C. Lange, L. Xun, T. C. Zahrt, and B. J. Schneider.** 1993. Cloning, sequence analysis, and expression of the *Flavobacterium* pentachlorophenol 4-monooxygenase gene in *Escherichia coli*. J. Bacteriol. **175:**411–416.
15. **Newman, L. M., and L. P. Wackett.** 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry **34:** 14066–14076.
16. **Prieto, M. A., and J. L. Garcia.** 1994. Molecular characterization of 4-hydroxyphenylacetate 3-hydroxylase of *Escherichia coli*. J. Biol. Chem. **269:** 22823–22829.
17. **Sangodkar, U. M. X., P. J. Chapman, and A. M. Chakrabarty.** 1988. Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T by *Pseudomonas cepacia* AC1100. Gene **71:**267–277.
18. **Strickland, S., and V. Massey.** 1973. The purification and properties of the flavoprotein melilotate hydroxylase. J. Biol. Chem. **248:**2944–2952.
19. **Tisdale, H. D.** 1967. Preparation and properties of succinic-cytochrome c reductase (complex II-III). Methods Enzymol. **10:**213–215.
20. **Tomasi, I., I. Artaud, Y. Bertheau, and D. Mansuy.** 1995. Metabolism of polychlorinated phenols by *Pseudomonas cepacia* AC1100: determination of the first two steps and specific inhibitory effect of methimazole. J. Bacteriol. **177:**307–311.
21. **Xun, L., and C. S. Orser.** 1991. Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. **173:**4447–4453.
22. **Xun, L., E. Topp, and C. S. Orser.** 1992. Diverse substrate range of a *Flavobacterium* pentachlorophenol hydroxylase and the reaction stoichiometries. J. Bacteriol. **174:**2898–2902.
23. **Xun, L., E. Topp, and C. S. Orser.** 1992. Confirmation of oxidative dehalogenation of pentachlorophenol by a *Flavobacterium* pentachlorophenol hydroxylase. J. Bacteriol. **174:**5745–5747.
24. **Xun, L., and K. B. Wagnon.** 1995. Purification and properties of component B of 2,4,5-trichlorophenoxyacetate oxygenase from *Pseudomonas cepacia* AC1100. Appl. Environ. Microbiol. **61:**3499–3502.
25. **Zaborina, O., M. Latus, J. Eberspacher, L. A. Golovleva, and F. Lingens.** 1995. Purification and characterization of 6-chlorohydoxyquinol 1,2-dioxygenase from *Streptomyces rochei* 303: comparison with an analogous enzyme from *Azotobacter* sp. strain GP1. J. Bacteriol. **177:**229–234.