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Purification and Properties of Component B of 2,4,5-Trichlorophenoxyacetate Oxygenase from *Pseudomonas cepacia* AC1100

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***Pseudomonas cepacia* AC1100 degrades 2,4,5-trichlorophenoxyacetate (2,4,5-T), an herbicide and chlorinated aromatic compound. Although some progress has been made in understanding 2,4,5-T degradation by AC1100 by molecular analysis, little is known about the biochemistry involved. Enzymatic activity converting 2,4,5-T to 2,4,5-trichlorophenol in the presence of NADH and O_2 was detected in cell extracts of AC1100. Phenyl agarose chromatography of the ammonium sulfate-fractionated cell extracts yielded no active single fractions, but the mixing of two fractions, named component A and component B, resulted in the recovery of enzyme activity. Component B was further purified to homogeneity by hydroxyapatite and DEAE chromatographies. Component B had a native molecular weight of 140,000, and it was composed of two 49-kDa α-subunits and two 24-kDa β-subunits. Component B was red, and its spectrum in the visible region had maxima at 430 and 560 nm (shoulder), whereas upon reduction it had maxima at 420 (shoulder) and 530 nm. Each mole of αβ heterodimer contained 2.9 mol of iron and 2.1 mol of labile sulfide. These properties suggest strong similarities between component B and the terminal oxygenase components of the aromatic ring-hydroxylating dioxygenases. Component A was highly purified but not to homogeneity. The reconstituted 2,4,5-T oxygenase, consisting of components A and B, converted 2,4,5-T quantitatively into 2,4,5-trichlorophenol and glyoxylate with the coconsumption of NADH and O_2.**

The herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T) is a major component of Agent Orange, which was used in the Vietnam War for defoliation. Because of its toxicity, the United States and several other countries have banned its usage (4). However, its biodegradation intermediate 2,4,5-trichlorophenol (2,4,5-TCP) and many other polyhalogenated aromatic compounds are still widely used as herbicides, fungicides, and general biocides and are a major group of environmental pollutants (2, 12). In addition, 2,4-dichlorophenoxyacetate and other phenoxyacetate compounds are still widely used as herbicides. *Pseudomonas cepacia* AC1100, isolated from an enrichment culture in a chemostat under strong selective pressures, can completely mineralize 2,4,5-T (9, 10).

Genes for an oxygenase responsible for the conversion of 2,4,5-T to 2,4,5-TCP have recently been cloned and characterized from AC1100 (3, 7, 15). As indicated by sequence analysis, the cloned genes may encode only parts of an enzyme system and other proteins may be needed for the enzyme activity. We report here the identification, purification, and characterization of an enzyme system converting 2,4,5-T to 2,4,5-TCP from AC1100. This report confirms that the cloned genes are only part of the enzyme system and that other proteins are required for enzyme activity.

Identification of 2,4,5-T oxygenase activities in cell extracts. Since the enzyme(s) responsible for the conversion of 2,4,5-T to 2,4,5-TCP is constitutively produced (8), AC1100 was cultured with sodium succinate (2 g/liter) and sodium glutamate (1 g/liter) in a mineral medium (10). The washed cells converted 2,4,5-T to 2,4,5-TCP at a very low rate of 9.2 μM/h by a cell suspension in 20 mM Tris buffer (pH 7.5) with an optical density of 2.5 at 600 nm. The cell extract also showed a very low rate of conversion of 2,4,5-T to 2,4,5-TCP in the presence of NADH and O_2. Cell extracts (55 mg of protein ml^{-1}) produced 20 μM 2,4,5-TCP in a mixture containing 200 μM 2,4,5-T in 40 mM potassium phosphate (KPi) buffer (pH 7.8) after a 120-min incubation. When the extracts were dialyzed against 1 liter of 20 mM KPi (pH 6.9) containing 1 mM dithiothreitol (DTT) for 2 h, 77 μM 2,4,5-TCP was produced after a 20-min incubation with the same amount of protein. The activity increased about 23-fold after dialysis. Since we used a large volume of the cell extracts for the activity assay, chemicals present in the cell extracts may have interfered with the enzyme activity. Dialysis against 20 mM KPi (pH 6.9) was necessary for activity assays even when the cells were suspended in 20 mM KPi (pH 6.9) before cell disruption.

Enzyme assay. A standard assay mixture contained 40 mM KPi buffer (pH 7.8), 200 μM 2,4,5-T, 10 μM flavin adenine dinucleotide, 10 μM $FeSO_4$, 5 mM NADH, 0.4% Tween 20, and various amounts of protein in a 40-μl volume at 35°C. The reaction was initiated by adding NADH to the reaction mixture and stopped by adding an equal volume of acetonitrile. Incubation times varied from 20 to 40 min to allow about 20 μM 2,4,5-T to be consumed. The 2,4,5-T oxygenase activity was assayed by measuring the production of 2,4,5-TCP by high-performance liquid chromatography (HPLC) analysis (20). The retention times for 2,4,5-T and 2,4,5-TCP were 8.9 and 9.4 min, respectively. Both 2,4,5-T and 2,4,5-TCP were quantified by comparing their peak areas with those of authentic standards. One unit of the enzyme activity was defined as the production of 1 pmol of 2,4,5-TCP per min. Tween 20 was

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included in the reaction mixture because it was found to facilitate reproducible quantitations of chlorinated phenols by HPLC analysis (20). A stock solution (100 mM) of 2,4,5-T was prepared in absolute ethanol, and a stock solution (100 mM) of NADH was prepared in 10 mM Tris base (pH > 13). NADPH could replace NADH, but the enzyme activity was reduced by half. The optimal temperature for 2,4,5-T production was 39°C. Maximum activities of 46, 80, and 41% were retained at 24, 35, and 49°C, respectively. The optimal pH was pH 7.8 in 50 mM KPi buffer; at pH 7.0 the activity was 38% of that at pH 7.8. The best activity was obtained in 40 mM KPi buffer (pH 7.8) at 39°C. Since the activity was more reproducible at 35°C, we routinely assayed enzyme activity at 35°C.

Purification of 2,4,5-T oxygenase. All operations were performed at 6°C. Ammonium sulfate saturation levels reflect a temperature of 25°C. Approximately 15 g (wet weight) of cell paste was suspended in 30 ml of 20 mM Tris buffer (pH 8.0) containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The slurry was passed through a French pressure cell (FA-030; Aminco, Urbana, Ill.) three times at 260 MPa. The product was centrifuged at 17,000 × *g* for 12 min to obtain cell extracts, and nucleic acids in the extracts were precipitated by the addition of protamine sulfate to a final concentration of 0.5 mg/ml. Proteins were fractionated by adding solid ammonium sulfate to 33% saturation with constant stirring. After 10 min of stirring, the mixture was centrifuged at 17,000 × *g* for 10 min. The precipitate containing no activity was discarded. Additional solid ammonium sulfate was added to the supernatant to 65% saturation. The solution was stirred and centrifuged as described above. The precipitate containing the activity was saved and suspended in an equal volume of 20 mM Tris buffer (pH 8.0) containing 1 mM DTT and 1 mM EDTA. The suspension was centrifuged at 17,000 × *g* for 10 min, and the supernatant was loaded onto a phenyl agarose (Sigma) column (1.5 by 20 cm) previously equilibrated with 20 mM Tris buffer containing 1 mM DTT, 1 mM EDTA, and 20% saturation of ammonium sulfate. The enzyme was eluted with 300 ml of a linear gradient of 20 to 0% saturation of ammonium sulfate. This step separated the enzyme activity into two components. The first component, which eluted off the column around 17% saturation of ammonium sulfate, was named component A (cA), and the second component, which eluted around 10% saturation, was named component B (cB). Neither of the components degraded 2,4,5-T alone, but the combination of the two components converted 2,4,5-T to 2,4,5-TCP. The fractions containing cA and cB were pooled separately and precipitated by adding ammonium sulfate to 70% saturation. The precipitates were collected by centrifugation at 17,000 × *g* for 10 min. The precipitated cB was dissolved in a minimal volume of 10 mM KPi buffer (pH 6.9) containing 1 mM DTT and was desalted by passing it through a PD-10 gel-filtration column (Pharmacia, Alameda, Calif.). The desalted sample was loaded onto a Macro-Prep ceramic hydroxyapatite (Bio-Rad) column (1.5 by 20 cm) previously equilibrated with 10 mM KPi buffer (pH 6.9) containing 1 mM DTT. Proteins were eluted with 300 ml of a linear gradient of 10 to 200 mM KPi buffer (pH 6.9) containing 1 mM DTT. cB was eluted off the column around 64 mM KPi. The fractions containing enzyme activity were concentrated by Centriprep-30 (Amicon) to about 1 ml. The sample containing about 64 mM KPi was loaded directly onto a Macro-Prep DEAE (Bio-Rad) column (1.5 by 20 cm) previously equilibrated with 50 mM KPi (pH 6.9) containing 1 mM DTT. The column was washed with 10 ml of the equilibrating buffer, and proteins were eluted with 250 ml of a linear gradient of 0 to 125 mM NaCl in the equilibrating buffer. cB was eluted around 100 mM NaCl. The fractions containing cB were pooled and concentrated to 1 ml by Centriprep-30, and the buffer was changed to 25 mM KPi (pH 6.9) containing 1 mM DTT by Centriprep-30. The protein preparations were stored at −80°C.

TABLE 1. Purification of cB of 2,4,5-T oxygenase

Step	Vol (ml)	Total protein (mg)	Activity[a]		Recovery (%)
			Specific (U mg^{-1})	Total (U)	
Cell extracts	20.0	933.9	0.30	280.2	100.0
Protamine treatment	19.0	718.4	0.28	201.2	71.8
$(NH_4)_2SO_4$, 65% saturation	16.3	357.4	0.48	171.6	61.2
Phenyl agarose	6.0	37.1	2.45	90.9	32.4
Hydroxyapatite	0.8	7.7	3.64	28.0	10.0
DEAE	0.7	2.2	6.92	15.2	5.4

[a] Activities (2,4,5-TCP production) were determined at 35°C.

The results of a typical enzyme purification of cB are summarized in Table 1. Protein concentrations were determined with a protein dye reagent (1), with bovine serum albumin as the standard. The specific activity of cB was estimated with excess cA, leaving cB as the limiting factor. The purification scheme resulted in a 23-fold increase in cB specific activity and enabled us to purify cB to apparent homogeneity (Fig. 1A) as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (11). The gels were stained for proteins with Coomassie brilliant blue R-250. Approximately 5.4% of the activity of cB in the cell extract was recovered. cB consisted of two polypeptides that were visible in cell extracts by SDS-PAGE analysis (data not shown). On the basis of the activity recovery and the final amount of purified cB (Table 1), we estimated that 40.7 mg of cB was present in the cell extract or 4.4% of the total protein in the cell extract was cB.

We could not purify cA to homogeneity although we tried many purification schemes. One scheme, consisting of DEAE, Cibacron Blue 3GA dye (Sigma), and Mono Q (Pharmacia) column chromatographies, resulted in a highly purified cA with three major bands and several minor bands (Fig. 1B). The highly purified cA showed a specific activity of 79.0 nmol mg^{-1} min^{-1} for 2,4,5-TCP production with excess cB. When we used the highly purified cA to estimate the amount of cA in the cell extract, cA was about 0.28% of the total protein in the cell extract. This indicates that cB was at least 12.3 times more

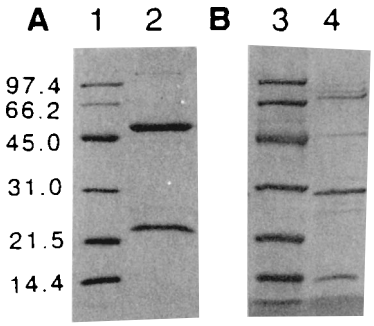

FIG. 1. Results of SDS-PAGE of purified cB (A) and cA (B). Lanes 1 and 3 contained marker proteins (Bio-Rad), lane 2 contained 5 μg of cB, and lane 4 contained 1.2 μg of cA.

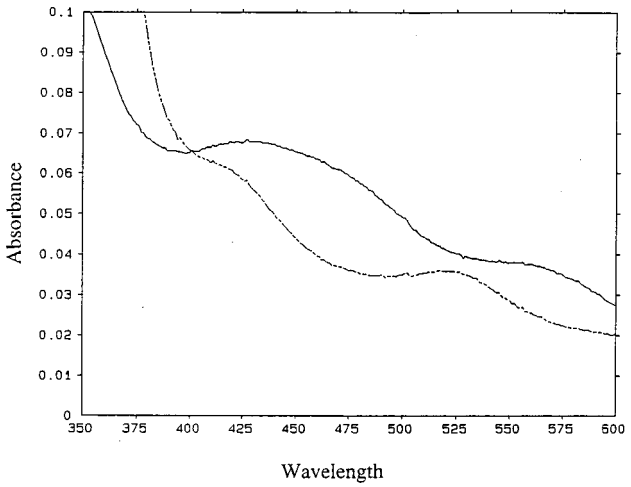

FIG. 2. Spectra of cB at 1.2 mg ml^{-1} in 20 mM KPi, pH 6.9. The same buffer without protein is used as the photometric reference. Solid line, oxidized cB; broken line, reduced cB.

abundant than cA was in the cell extract. Since cA was not abundant, its purification was difficult.

Enzyme properties. SDS-PAGE analysis of the purified cB revealed one 49-kDa (α-subunit) band and one 24-kDa (β-subunit) band (Fig. 1A). Gradient (4 to 20%) native PAGE analysis (16) revealed that the native molecular weight of cB was about 140,000; gel filtration on a Superose 6 column (Pharmacia) with 20 mM Tris buffer containing 150 mM NaCl as the elution buffer showed a retention volume of 15.3 ml, indicating that the native molecular weight of cB was about 150,000. Both methods suggested that the native cB was a heterotetramer with an $\alpha_2\beta_2$ structure. The reddish color of cB suggested that it had either a heme or an iron-sulfur center. Thus the visible (350 to 600 nm) spectrum was studied with 25 mM KPi buffer (pH 6.9) as the reference. The (oxidized) cB in the KPi buffer had absorption maxima at 430 and 560 nm (shoulder), whereas upon reduction with sodium dithionite it had adsorption maxima at 420 (shoulder) and 530 nm (Fig. 2). These results are characteristic of a Reiske-type [2Fe-2S] center. Labile sulfide (17) and iron (14) were both determined by colorimetric assays. On the basis of a molecular weight of 70,000 per αβ heterodimer, approximately 2.9 mol of iron and 2.1 mol of labile sulfide were found per ml of αβ heterodimer. These data together with the spectral analysis indicate the probability of one [2Fe-2S] center and one free iron per αβ heterodimer. The pI of cB was determined to be approximately 5.4 by analytical isoelectric focusing gels with 5% acrylamide with 2% ampholytes (pH 3 to 10) (Bio-Rad). The isoelectric focusing gels were run on an isoelectric focusing mini electrophoresis system as directed by the manufacturer (Bio-Rad). cB was very similar to the terminal oxygenase components of aromatic-ring-hydroxylating dioxygenases (13). Shared features include (i) an $\alpha_2\beta_2$ structure, (ii) similarities in sizes of α- and β-subunits, (iii) absorption spectra between 350 and 500 nm, and (iv) one [2Fe-2S] center and one free iron per αβ heterodimer.

The highly purified cA preparation was very dilute and colorless. In the presence of flavin adenine dinucleotide, the highly purified cA preparation exhibited NADH-dependent cytochrome *c* reduction (18) at 11.2 μmol min^{-1} mg of $protein^{-1}$, indicating that cA is likely a reductase. Since we did not purify cA to homogeneity and did not have enough of it, we were unable to characterize these proteins.

Enzyme activity. The 2,4,5-T oxygenase (a mixture of highly purified cA and purified cB) converted 2,4,5-T to 2,4,5-TCP. Both NADH and O_2 were required for the reaction. The optimal enzyme activity occurred in 40 mM KPi buffer (pH 7.8) at 39°C in the presence of 10 μM flavin adenine dinucleotide and 10 μM Fe^{2+}. Less than 10% of the activity remained without flavin adenine dinucleotide, and about 70% of the activity remained in the absence of Fe^{2+}. The enzyme (cA and cB) was stored at −80°C, and no detectable activity loss was detected after several weeks. The enzyme also converted 2,4-dichlorophenoxyacetic acid (2,4-D) to 2,4-dichlorophenol at a rate similar to the rate it oxidized 2,4,5-T in the presence of NADH and O_2. When 2-chlorophenoxyacetate was tested, our HPLC program did not separate 2-chlorophenoxyacetate from 2-chlorophenol, but glyoxylate was detected. The glyoxylate production indicates that the enzyme also used 2-chlorophenoxyacetate as a substrate.

The stoichiometry of the reaction has quantitatively been studied for 2,4,5-T; 2,4,5-TCP; NADH; and glyoxylate. In one of the experiments with the reconstituted 2,4,5-T oxygenase, we found that 50 μM 2,4,5-T was quantitatively converted to 50 μM 2,4,5-TCP and 49 μM glyoxylate. Glyoxylate was detected by the phenylhydrazine-$K_3Fe(CN)_6$ method (19). When NADH was used as a limiting factor, only 15 μM 2,4,5-TCP was produced when 50 μM NADH was added to the reaction mixture and the reaction was allowed to go to completion (3 h). Although the consumption ratio of NADH to 2,4,5-T was higher than 1, it is not uncommon that the electron coupling from NADH to the substrate is not very efficient. In most cases, H_2O_2 is produced from NADH and O_2. The ratio of oxygen consumption to 2,4,5-T degradation was not determined. However, the enzyme was not active when the reaction was carried out with 2 mM sodium dithionite in an anaerobic glove box. Since 2,4,5-T was quantitatively converted to 2,4,5-TCP and glyoxylate and both NADH and O_2 were required, the reaction is probably as follows:

$$\text{2,4,5-T} + O_2 + \text{NADH} + H^+ \rightarrow \text{2,4,5-TCP} + \text{glyoxylate} + \text{NAD}^+ + H_2O$$

Although 2,4-D α-ketoglutarate dioxygenase of *Alcaligenes eutrophus* catalyzes an α-ketoglutarate-dependent conversion of 2,4-D to 2,4-dichlorophenol and glyoxylate (5), similar to the reactions catalyzed by 2,4,5-T oxygenase, the two enzymes are different types of oxygenases. The α-ketoglutarate dioxygenase uses α-ketoglutarate as its reducing agent, while 2,4,5-T oxygenase required NADH. The partially purified 2,4-D α-ketoglutarate dioxygenase has a specific activity for 2,4-D oxidation at 6.2 μmol min^{-1} mg of $protein^{-1}$ (5), while the purified 2,4,5-T oxygenase (cB) showed a specific activity for 2,4,5-T at 6.92 nmol min^{-1} mg of $protein^{-1}$ (Table 1). Therefore, 2,4-D α-ketoglutarate dioxygenase oxidizes 2,4-D about 1,000 times faster than 2,4,5-T oxygenase utilized 2,4,5-T. We have found that 2,4,5-T oxygenase oxidized 2,4-D and 2,4,5-T at relatively similar rates. The slow reaction of the enzyme was also suggested by the whole-cell assay of AC1100. The selection of a slow enzyme over a fast enzyme may be essential for the survival of AC1100 during its enrichment in a chemostat because 2,4,5-TCP is more hydrophobic and toxic than 2,4,5-T. The selection of an enzyme that slowly converts 2,4,5-T to 2,4,5-TCP can ensure that 2,4,5-TCP is not accumulated and is used up as soon as it is produced. The slow activity of 2,4,5-T oxygenase made its detection difficult.

The N-terminal sequences of α- and β-subunits. The two polypeptides of cB were separated by HPLC on a Delta Pak C_{18} column (3.9 by 150 mm) (Waters) with a water acetonitrile

gradient containing 0.1% trifluoroacetic acid. The N-terminal amino acid sequences of the HPLC-separated polypeptides were determined on an automated protein sequencer (Applied Biosystems, Foster City, Calif.) at Washington State University. The N-terminal sequence of the α-subunit was determined to be Met-Leu-Asp-Gln-Asn-Ala-Val-Ala-Ile-Ala-Thr-Asn-Val-Leu, and the N-terminal sequence of the β-subunit was determined to be Met-Asn-Thr-Thr-Met-Asn-Thr-Pro-Val-Pro. The genes (*tftA1* and *tftA2*) encoding the two polypeptides of cB have been cloned and sequenced (3). The N-terminal sequences of α- and β-subunits matched exactly those predicted from the nucleotide sequences of *tftA1* and *tftA2*. The predicted amino acid sequences have strong sequence similarities with α- and β-subunits of the terminal dioxygenase of ring-hydroxylating dioxygenases (3). It is interesting that only the genes encoding cB are cloned and expressed in *Pseudomonas aeruginosa* PAO1 and that transformed PAO1 cells can convert 2,4,5-T to 2,4,5-TCP. PAO1 must provide cB with a reductase system because cB alone cannot convert 2,4,5-T to 2,4,5-TCP in vitro. Since *Pseudomonas cepacia* AC1100 was isolated from an enrichment culture in a chemostat under strong selective pressure (9, 10) and since transposable elements have been implicated in the recruitment of the genes encoding 2,4,5-T oxygenase (6), one may wonder whether AC1100 recruited just the genes encoding cB or all the genes encoding both cB and cA. If PAO1 can provide cB with a reductase, AC1100 should be able to do the same. The high ratios of cB to cA in cell extracts of AC1100 suggest that the genes encoding cA and cB may be under different expression controls. If it is true that cA and cB are under different expression controls and are not physically linked together, cA may have other functions for AC1100. To answer these questions, the genes encoding cA must be cloned and characterized.

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