Purification and properties of a plasmid-encoded 2,4-dichlorophenol hydroxylase

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2,4-Dichlorophenol hydroxylase, an enzyme involved in the bacterial degradation of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) was purified from two bacterial strains that harbored the same 2,4-D plasmid, pJP4. The purified enzymes (M_r 224000) from the two transconjugants were indistinguishable; they contained FAD and were composed of non-identical subunits, M_r 67000 and 45000, respectively. Various substituted phenols were hydroxylated, using either NADH or NADPH. The amino acid composition of the native enzyme was determined.

2,4-Dichlorophenol hydroxylase *2,4-Dichlorophenoxyacetate plasmid*
2,4-Dichlorophenoxyacetate degradation Alcaligenes eutrophus Pseudomonas putida

1. INTRODUCTION

Strains of *Alcaligenes* have been isolated that are capable of degrading the herbicide 2,4-D [1,2]. One of these strains, *A. eutrophus* JMP134, carries a transmissible plasmid, pJP4, which confers the ability to degrade 2,4-D when transferred by conjugation. We have shown that the second enzyme on the degradative pathway, i.e., 2,4-dichlorophenol hydroxylase, is present in cell-free extracts of parental and transconjugant strains. Phenol hydroxylase has been purified from the soil yeast *Trichosporon cutaneum* [3] and another similar enzyme 2,4-dichlorophenol hydroxylase was isolated from a strain of *Acinetobacter* that degraded 2,4-D [4]. We now report the purification of a 2,4-dichlorophenol hydroxylase encoded by the 2,4-D plasmid and compare its properties with the hydroxylase previously described [4]. The two transconjugant strains carrying the plasmid pJP4 served as sources of the enzyme.

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Abbreviation: 2,4-D, 2,4-dichlorophenoxyacetate

2. MATERIALS AND METHODS

2.1. *Bacterial strains and cell extracts*

Pseudomonas putida 3CB5/pJP4 was constructed in this laboratory by transferring the plasmid pJP4 from *A. eutrophus* JMP134 [1,2] to a 3-chlorobenzoate-degrading strain *P. putida* 3CB5. *A. eutrophus* 335/pJP4 was constructed in the same way except that *A. eutrophus* 335 was used as the recipient. Both strains were grown with aeration at 30°C in a basal medium [5] with the addition of 0.1% 2,4-D as carbon source. When large quantities (250 g) were required, cells were grown in a 100-l fermentor and pastes of washed cells were broken in a Hughes bacterial press [6]. Cell extracts were prepared in KH_2PO_4/Na_2HPO_4 buffer (100 mM), pH 7.6, containing 1 mM β-mercaptoethanol, 0.1 mM EDTA and 2 μM FAD as in [3]. This solution, referred to as phosphate buffer, was used throughout the purification unless otherwise stated.

2.2. *Purification procedures*

All operations were at 4°C. After removal of cell debris, the crude cell extracts were fractionated with ammonium sulfate. For extracts of *P. putida*

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3CB5/pJP4, fractions were taken at 0.2–0.4 saturation with solid $(NH_4)_2SO_4$, at pH 7.6, and for *A. eutrophus* 335/pJP4, 0.3–0.5 saturation was used. Precipitates were suspended in 30 ml phosphate buffer and each solution was dialyzed overnight against 2 l buffer. Each dialyzed solution was loaded onto a column (2.4 × 30 cm) of DEAE–cellulose (DE-52) and was eluted with an increasing gradient of KCl (50–250 mM in 20 mM phosphate buffer; total volume 600 ml). Active fractions were pooled and concentrated using an Amicon PM10 membrane. The protein solution was then applied to a column (1.5 × 22 cm) of phenyl Sepharose Cl-4B and eluted with 30–60% ethylene glycol in 50 mM phosphate buffer. The active fractions were pooled, concentrated as before, and chromatographed on a gel-filtration column (1.2 × 110 cm) of Sepharose 4B. Upon elution with 20 mM phosphate buffer, active fractions were collected and checked for homogeneity by disc gel electrophoresis [7]. Two more chromatographic steps using first a column (1.5 × 19 cm) of DEAE–Sephacryl and then a column (1.2 × 110 cm) of Sepharose 4B gavé an enzyme of 99% purity as shown by gel electrophoresis.

2.3. *Enzyme assays*

2,4-Dichlorophenol hydroxylase was assayed by following the decrease in absorbance at 340 nm due to the substrate-dependent oxidation of NAD(P)H [3]. Each assay mixture contained: phosphate buffer, 0.94 ml; NADPH (65 mM), 0.04 ml; enzyme, 0.01 ml; 2,4-dichlorophenol (10 mM), 0.01 ml.

2.4. *Protein determination*

A spectrophotometric assay for protein [8] was used throughout the purification and the assay in [9] was used for the purified enzyme.

2.5. *Gel electrophoresis*

Analytical disc gel electrophoresis [7] and electrophoresis in the presence of SDS [10] were performed as in [11].

2.6. *Determination of relative molecular mass*

The relative molecular mass of native enzyme was determined as in [12] from sedimentation equilibrium studies [13]. The partial specific volume used for calculation was obtained from the amino acid composition of the native protein [13].

2.7. *Characterization of the flavin cofactor*

The flavin component was extracted from the enzyme [14] and examined by high-performance liquid chromatography (HPLC) using a solvent system of 30% methanol and 70% 5 mM ammonium acetate, pH 6.0 [15].

Table 1

Purification of 2,4-dichlorophenol hydroxylase

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)
(1) Crude cell-free extract	800	12464	926	0.0743	100	–
(2) $(NH_4)_2SO_4$ fractionation, concentration and dialysis	50	2856	482	0.17	52	2.3
(3) DEAE–cellulose chromatography and concentration	82	430	264	0.613	28.5	8.25
(4) Phenyl-Sephacryl chromatography and concentration	3	82	88	1.064	9.5	14.3
(5) DEAE–Sephacryl chromatography and concentration	2.8	70.6	25.6	1.187	2.7	16
(6) Sepharose 4B chromatography and concentration	2.8	9	13.5	1.482	1.5	20

Crude extract was prepared from 250 g wet wt of *Alcaligenes eutrophus* 335/pJP4

3. RESULTS

3.1. *Purification procedure*

The same purification procedure, which is summarized in table 1, was used both for *P. putida* 3CB5/pJP4 and *A. eutrophus* 335/pJP4. The yields of the enzyme from both sources were the same.

3.2. *Homogeneity*

Purified enzyme showed a single band on polyacrylamide gel after staining with Coomassie blue. Unstained gel was sliced and assayed for the enzyme activity. Only the slice of the corresponding protein band had activity.

3.3. *pH optimum*

The maximal enzyme activity was reached at the range pH 7.3–8.0 in 100 mM KH_2PO_4/Na_2HPO_4 buffer and pH 8.1–8.5 in 100 mM Tris–HCl buffer. At pH 7.7, the enzyme activity in Tris–HCl buffer was 60% of that in KH_2PO_4/Na_2HPO_4 buffer.

3.4. *Sedimentation properties and subunit structure*

The enzyme sedimented as a single component of M_r 224000 ± 4000, calculated from data obtained at 3 protein concentrations (0.1, 0.2, 0.4 mg per ml). The partial specific volume calculated from the amino acid composition was 0.7. SDS-polyacrylamide gel electrophoresis gave two bands corresponding to M_r 67000 and 45000, indicating the native enzyme may be a tetramer composed of pairs of the subunits.

3.5. *Absorption spectrum and flavin content*

The spectrum of the native protein showed two maximal points at 375 and 450 nm, characteristics of a flavoprotein. The flavin was extracted and shown to be FAD using HPLC, as described in section 2. From the absorbance at 450 nm, each mol protein was calculated to contain 3.5 mol FAD.

3.6. *Stoichiometry*

The enzyme catalyzed the hydroxylation of 2,4-dichlorophenol with the consumption of equimolar amounts of NAD(P)H and oxygen as shown in table 2.

Table 2

Stoichiometry of the hydroxylase reaction

2,4-Dichlorophenol supplied	NADPH supplied	O_2 consumed	NADPH consumed
0.05	0.2	0.0590	0.051
0.05	0.05	0.0534	0.05
0.05	0.03	0.03	0.03
0.03	0.2	0.03	0.0304

NADPH oxidation and oxygen consumption were measured by spectrophotometric and polarographic assay, respectively. The reactions were initiated by addition of the appropriate amount of 24-dichlorophenol to the assay mixture described in section 2. Values are expressed in μmol

3.7. *Substrate specificity*

Various substituted phenols were substrates for the hydroxylase (table 3). In addition to 2,4-dichlorophenol, 2,4-dibromophenol and 4-chloro-2-methylphenol were also readily converted to the corresponding catechols. Furthermore, 4-chlorophenol, 4-chloro-2-nitrophenol, 2-chloro-4-

Table 3

Substrate specificity of 2,4-dichlorophenol hydroxylase

Aromatic compound	Activity (%) with	
	Purified enzyme	Crude extract
4-Chloro-2-methylphenol	90	83
2,4-Dibromophenol	90	70
4-Chlorophenol	24	30
4-Chloro-2-nitrophenol	18	22
2-Chloro-4-nitrophenol	15	17
2,4,5-Trichlorophenol	13	13
2-Chlorophenol	10	13
4-Nitrophenol	6	8
3-Chlorophenol	3.5	6
4-Fluorophenol	3	4
2-Fluorophenol	2	N.D.

Activity was determined at 25°C by spectrophotometric assay. Each assay mixture contained 0.1 μmol aromatic substrates. The results are given as a percentage of the activity towards 2,4-dichlorophenol. N.D., not determined

nitrophenol, 2-chlorophenol and 2,4,5-trichlorophenol were hydroxylated by the enzyme, although with lower rate. Phenol, pentachlorophenol, 4-nitrothiophenol, 4-cyanophenol, *o*-, *m*- and *p*-cresols, resorcinol, 3,5-dichlorocatechol and catechol were not substrates for the hydroxylase. Either NADH or NADPH was used as cosubstrate, the specific activity with NADPH being twice that for NADH. No pseudo-substrates were found which reduced O_2 to H_2O_2 as an alternative to hydroxylation. The hydroxylation products of 2,4-dichlorophenol, 4-chloro-2-methylphenol, 4-chlorophenol and 2,4,5-trichlorophenol have been identified by gas-chromatography mass-spectrometry to be the corresponding catechols.

3.8. *Kinetics*

The purified enzymes from both transconjugants showed the same substrate affinities. Apparent K_m values were obtained from double-reciprocal plots of reaction velocity *vs* substrate concentration. The apparent K_m for NADPH (2 μM) was much lower than for NADH (300 μM), but V_{max} for NADPH (6 μmol/min per mg protein) was only twice that for NADH (3 μmol/min per mg protein) as shown in table 4.

3.9. *Effect of heavy metals, thiols and chelating agents*

Heavy metals, $CuSO_4$ and $HgCl_2$, severely inhibited the enzyme activity (93 and 100% inhibition, respectively). $AgNO_3$ had only 30% inhibitory effect. High concentration of $FeSO_4$ (0.1 mM) also showed 50% inhibition. Dithiothreitol and dithionitrobenzoate had little effects on the enzyme activity. Ion chelators, *o*-phenanthroline and diethyldithiocarbamate, inhibited the enzyme activity by 40 and 50%. The heme inhibitor, KCN, had no effect. Incubating the enzyme with $CuSO_4$ also decreased the enzyme activity.

Table 4

Kinetic parameters of 2,4-dichlorophenol hydroxylase

K_m determined for	Saturating substrate	Apparent K_m (μM)
2,4-Dichlorophenol	NADPH	2
2,4-Dichlorophenol	NADH	4
NADPH	2,4-dichlorophenol	48
NADH	2,4-dichlorophenol	300

Details of the assay conditions are given in section 2

Table 5

Amino acid composition of 2,4-dichlorophenol hydroxylase

Amino acid	mol/mol enzyme (M_r 224000)
Aspartic acid	182
Threonine	74
Serine	219
Glutamic acid	242
Cystine	19
Glycine	509
Alanine	139
Valine	73
Methionine	7
Isoleucine	56
Leucine	82
Tyrosine	44
Phenylalanine	46
Lysine	63
Histidine	0
Arginine	81

Cystine was determined as cysteic acid as in [16]. Tryptophan was not determined

3.10. *Inhibition by salt and aromatic substrates*

High salt concentrations, 0.05, 0.1 and 0.2 M KCl, inhibited 40, 50 and 70% of the enzyme activity. The maximal enzyme activity was reached at substrate concentration of 0.01 mM 2,4-dichlorophenol. Higher concentration (>0.1 mM) of 2,4-dichlorophenol had inhibitory effect. 25% inhibition was found at 0.5 mM 2,4-dichlorophenol.

3.11. *Amino acid composition*

The amino acid composition of the native protein is shown in table 5.

4. DISCUSSION

To study the 2,4-D plasmid pJP4-encoded 2,4-dichlorophenol hydroxylase, the enzyme was purified from two transconjugants that harbored

the plasmid by conjugation. The construction of these transconjugant strains will be discussed in detail elsewhere. Purified enzyme from both transconjugants possessed the same relative molecular mass and range of substrate specificities as those reported, strongly indicating that the enzymes examined were plasmid-borne. Our sedimentation equilibrium studies gave M_r 224000, a value very close to that obtained for the *Acinetobacter* enzyme by authors in [4] who also found 4 subunits that were identical; however, we found two non-identical subunits with M_r 67000 and 45000. The substrate specificity of our plasmid-encoded enzyme differed from that from *Acinetobacter*; thus, monochlorophenols and cresols were not good substrates (table 3) while some phenols were attacked that are not substrates for the *Acinetobacter* enzyme. The plasmid-encoded enzyme utilized both NADH and NADPH whereas the *Acinetobacter* enzyme uses only NADPH. The plasmid-encoded hydroxylase contained 3.5 mol FAD/mol enzyme, suggesting that each subunit carries one FAD. This enzyme is similar in several respects to that from *Acinetobacter*. The minor differences between them can be explained by the host modification of the enzyme. The 2,4-D plasmid, pJP4, was first isolated in Australia from an *Alcaligenes* strain. It is likely that a similar plasmid may also be present in the *Acinetobacter* strain isolated in England [4]. It would be interesting to compare the origins of these degradative plasmids, their transfer and evolutions.

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