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TOLUENE DIOXYGENASE: PURIFICATION OF AN IRON-SULFUR PROTEIN BY AFFINITY CHROMATOGRAPHY

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SUMMARY

Toluene dioxygenase, from Pseudomonas putida, oxidizes toluene to (+)-cis-1(S),2(R)-dihydroxy-3-methylcyclohexa-3,5-diene. The oxygenase-component of this multienzyme system was purified to homogeneity by a two-step procedure that utilized affinity and ion exchange chromatography. The purified enzyme would oxidize toluene only in the presence of NADH, ferrous iron and partially purified preparations of NADH cytochrome c reductase and an iron-sulfur protein ($ferredoxin_{TOL}$). Spinach NADPH cytochrome c reductase and NADPH could substitute for the Pseudomonas reductase and NADH. The molecular weight of the oxygenase-component was determined to be 151,000 and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the enzyme is composed of two subunits with molecular weights of 52,500 and 20,800. The absorption spectrum showed maxima at 550 (Shoulder), 450, 326 and 278 nm and preliminary experiments have indicated the presence of 2 gram atoms of iron and 2 gram atoms of acid-labile sulfur per mole of protein. The results indicate that the oxygenase-component of the toluene dioxygenase enzyme system is an iron-sulfur protein that has been designated ISP_{TOL}.

Pseudomonas putida oxidizes toluene to (+)-cis-1(S),2(R)-dihydroxy-3-methylcyclohexa-3,5-diene (cis-toluene dihydrodiol, 1-3). The enzyme catalyzing this reaction incorporates both atoms of molecular oxygen into the aromatic nucleus (4) and has been designated toluene dioxygenase (5). Preliminary investigations resolved toluene dioxygenase into three protein components, A1, A2 and B, that are essential for enzymatic activity (5). Protein A1 was tentatively identified as a flavoprotein that catalyzes a protein B-dependent transfer of electrons from NADH to cytochrome c. Recently we

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have shown that protein B is a [2Fe-2S] ferredoxin that has been designated ferredoxin$_{TOL}$ (6). Protein A2 is presumed to be the terminal oxygenase responsible for the oxidation of toluene to cis-toluene dihydrodiol. However, studies on the mechanism of toluene oxidation have been hindered by the instability of protein A2 during conventional purification procedures. We now describe a rapid method that utilizes affinity chromatography for the purification of substantial amounts of protein A2. Preliminary characterization studies show that this component is an iron-sulfur protein that we have designated ISP$_{TOL}$.

MATERIALS AND METHODS

Materials. ω-Amino hexyl-Sepharose-4B (AH-Sepharose 4B) was obtained from Pharmacia Fine Chemicals, Inc. The following materials were from the sources indicated: cytochrome c, NADPH:ferredoxin oxidoreductase; E.C. No. 1.6.7.1 (spinach reductase), NADH, NADPH and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Sigma Chemical Company; DEAE-cellulose (DE-52), Whatman Ltd., Springfield Mill, Maidstone, Kent, England; [$^{14}CH_3$]-toluene (specific activity 26.4 mCi/mmole), Amersham-Searle Corporation; p-toluic acid, Aldrich Chemical Co.

Enzyme Assays. Toluene dioxygenase activity in crude cell extracts was determined by measuring the formation of radioactive cis-toluene dihydrodiol (5). During purification of the terminal component of the toluene dioxygenase system (ISP$_{TOL}$) a similar procedure was used with the exception that NADPH, spinach-ferredoxin reductase and partially-purified ferredoxin$_{TOL}$ (5,6) were added to the assay mixture. Reaction mixtures (0.4 ml) contained Tris-HCl buffer, pH 7.5, 15 μmoles; spinach reductase, 4.5 units; ferredoxin$_{TOL}$, 110 μg; NADPH, 0.12 μmole; $FeSO_4 \cdot 7H_2O$, 0.15 μmole and appropriate amounts of ISP$_{TOL}$. The reaction was started by the addition of $^{14}CH_3$ -toluene (6.42 nmoles, 373 x 10^3 dpm) in 0.030 ml of dimethylformamide. One unit of enzyme activity is defined as the amount of enzyme required to produce 1.0 nmole of cis-toluene dihydrodiol per minute. Specific activity is defined as units

per mg of protein. NADH-cytochrome c reductase activity was measured as described previously (5). The protein content of enzyme preparations was determined by the method of Lowry et al. (7).

Affinity chromatography. p-Toluic acid (16 mmoles) was dissolved in 140 ml of a 50% (v/v) solution of 1,4-dioxane in distilled water. The final pH of the mixture was adjusted to 4.5 with 0.1M NaOH. To this solution was added 15 g of washed AH-Sepharose 4B. Coupling with EDC (3.75 mmoles) and subsequent treatment of the gel was carried out according to the manufacturer's instructions. Before use in enzyme purification the gel (4-8 µmoles of ligand per ml) was equilibrated with nitrogen-saturated 0.05M PEG buffer.

Other analytical methods. Polyacrylamide gel electrophoresis was performed as described by Davis (8). Molecular weight determinations were carried out with a calibrated Sephadex G-200 column as described by Andrews (9). Inorganic iron analyses were performed with an atomic absorption spectrophotometer (Model 400) from Perkin-Elmer. Acid-labile sulfide was measured by the method of Fogo and Popowsky (10) as described by King and Morris (11).

RESULTS

Preliminary experiments revealed that toluene dioxygenase activity in crude cell extracts was not retained by columns of AH-Sepharose-4B that contained p-toluic acid as an affinity ligand. However, dithionite-treatment of crude cell extracts prior to chromatography resulted in the appearance of a brown band at the top of the p-toluic acid affinity column and toluene dioxygenase activity could not be detected in the column eluate. Attempts to elute the bound material by raising the ionic strength of the buffer or by by eluting with buffer saturated with toluene did not result in the recovery of enzyme activity.

The binding of one of the oxygenase components to the affinity column was also observed when crude cell extracts were pretreated with NADH. In this case enzyme activity could be recovered from the column by elution with a linear gradient of KCl in PEG buffer (Fig. 1). In this experiment crude

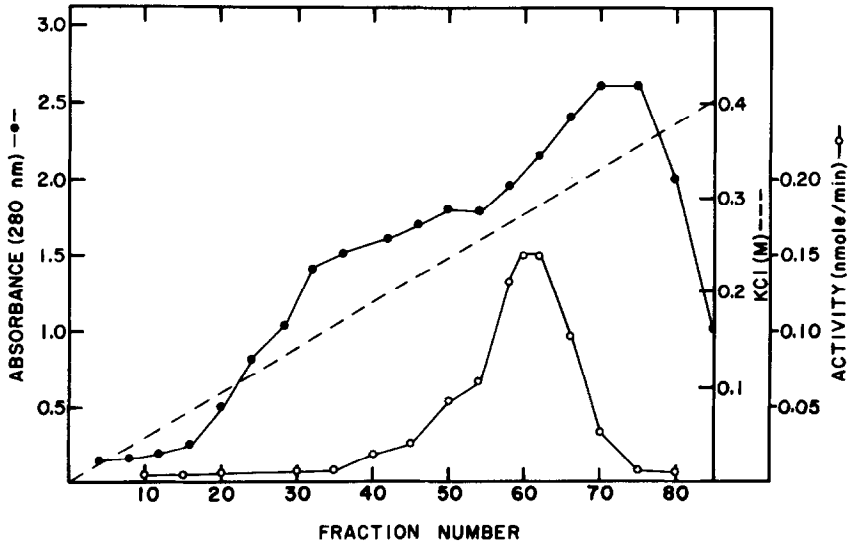

FIGURE 1: Resolution of toluene dioxygenase activity in crude cell extracts by affinity chromatography. Fractions eluting from the column were assayed for toluene dioxygenase activity as described in Materials and Methods.

cell extract (2.38 g of protein) was incubated with 0.12 mmole of NADH for 15 minutes. The extract was then dialyzed against 4 liters of nitrogen-saturated PEG buffer for two hours before being applied to the top of a p-toluic acid-Sepharose column (20 x 1.6 cm). Prior to the addition of crude cell extract the column was equilibrated with nitrogen-saturated PEG buffer. The same buffer was used to elute all protein that did not bind to the affinity matrix. This protein fraction, which contains NADH cytochrome c reductase and ferredoxin$_{TOL}$ was stored at -20 C as a future source of these two proteins. Subsequent elution was carried out with a 400 ml linear gradient (0.0 - 0.4M) of KCl in PEG buffer. Fractions (4.0 ml) were collected and 20 µl aliquots assayed for toluene dioxygenase activity (Fig. 1). Fractions 50-66 were pooled to give a brown colored protein solution (protein A2). This procedure gave a 16-fold purification with a 77 percent recovery of enzyme activity. The eluate (114 mg of protein) was diluted to 120 ml with PEG; dialyzed for 3 hours against 2 liters of PEG buffer and then applied to the top of a DEAE cellulose column (24 x 2.4 cm). The column was washed with PEG

Table 1. Purification of ISP_{TOL} from toluene grown cells of *Pseudomonas putida*

Step	Volume ml	Protein mg	Oxygenase (ISP_{TOL}) activity		
			Total units	Specific units/mg protein	Recovery %
1. Crude extract[1]	175	2,380	760	0.32	100
2. Affinity chromatography[2]	120	114	588	5.14	77
3. DEAE cellulose[2]	75	62	469	7.50	62

[1]Activity in crude extracts was measured in the absence of spinach reductase and $ferredoxin_{TOL}$. NADH, 0.12 µmole was used to replace NADPH.

[2]Assayed as described in Materials and Methods.

buffer containing 0.1M KCl until protein could not be detected in the eluate. A linear gradient of KCl (0.1 - 0.45M) in PEG buffer was used to elute protein from the column and 7.0 ml fractions were collected. Enzyme activity was detected in fractions 62-70 and the contents of these tubes were pooled. A summary of the purification procedure is given in Table 1. A 24-fold purification was achieved with a 62 percent recovery of enzyme activity.

The purified protein gave a single major band that stained for protein when analyzed by polyacrylamide gel electrophoresis. Several minor bands of lower mobility were also observed. These components were greatly reduced when electrophoresis was conducted in the presence of 2-mercaptoethanol (Fig. 2). Similar results were reported by Yu and Gunsalus for crystalline cytochrome c $P\text{-}450_{CAM}$ which is the terminal oxygenase component of the camphor 5-exo hydroxylase system (12).

The molecular weight of the purified oxygenase was determined by gel filtration on a standardized column of Sephadex G-200. From the results obtained the molecular weight was determined to be 151,000. The subunit composition was determined by disc-gel electrophoresis of the enzyme in the pres-

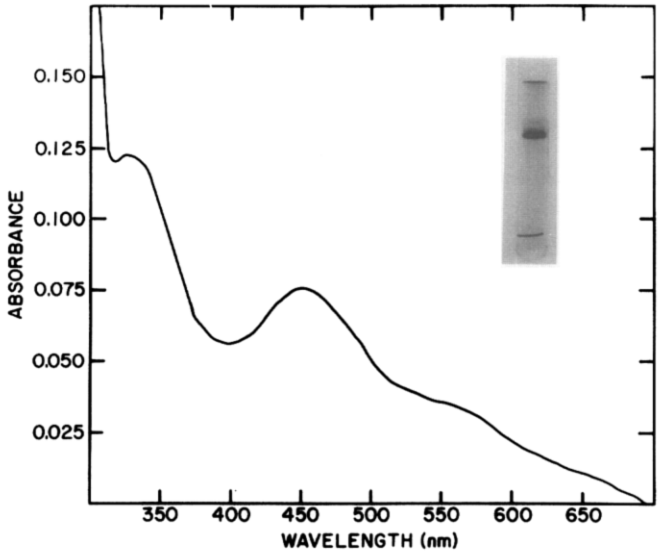

FIGURE 2: Absorption spectrum of purified ISP_{TOL}. The protein concentration was 0.92 mg/ml in PEG buffer. The spectrum was recorded on a Cary model 14 recording spectrophotometer. The inset shows the result of polyacrylamide gel (7.5%) electrophoresis at pH 8.3 in the presence of 14 mM 2-mercaptoethanol.

ence of 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol. Two subunits were identified with molecular weights of 52,500 and 20,800.

The absorption spectrum of the purified enzyme showed maxima at 550 (shoulder), 450 and 326 nm which are typical of iron-sulfur proteins (Fig. 2) and a preliminary analysis revealed the presence of 2.2 gram atoms of iron and 2.3 gram atoms of sulfur per 151,000 g of protein. Thus the oxygenase-component of the toluene dioxygenase system appears to be a simple iron-sulfur protein (13) that we have designated *P. putida* ISP_{TOL}.

Enzyme activity was only observed in the presence of ISP_{TOL}, $Ferredoxin_{TOL}$, *P. putida* cytochrome c reductase, NADH and ferrous iron. Spinach cytochrome c reductase and NADPH could substitute for NADH and the bacterial reductase. However, ferredoxin from *Clostridium pasteurianum* or spinach would not substitute for $ferredoxin_{TOL}$ (Table 2).

Table 2. Reconstitution studies on the toluene dioxygenase enzyme system

	Proteins Present[1]	Electron donor	Toluene dioxygenase activity[2] (mU)
1.	ISP_{TOL}		
	P. putida reductase	NADH	109
	$Ferredoxin_{TOL}$	NADPH	4.4
2.	ISP_{TOL}		
	Spinach reductase	NADPH	112
	$Ferredoxin_{TOL}$	NADH	5.8
3.	ISP_{TOL}		
	P. putida reductase	NADPH	0.0
	Ferredoxin (*C. pasteurianum* or spinach)	NADH	0.0
4.	ISP_{TOL}		
	Spinach reductase	NADPH	0.0
	Ferredoxin (*C. pasteurianum* or spinach)	NADH	0.0

[1] In experiments 1 and 2 no enzymatic activity was observed when any one of the three protein components was omitted from the reaction mixture.

[2] Enzyme assays were conducted as described in Materials and Methods. Amounts of individual components in 0.4 ml reaction volume were: ISP_{TOL}, 15 µg; *P. putida* reductase, 9.8 µg; $ferredoxin_{TOL}$, 110 µg; spinach reductase, 34 µg; *C. pasteurianum* ferredoxin, 20 µg; spinach ferredoxin, 20 µg; NAD(P)H, 0.12 µmoles.

DISCUSSION

Previous attempts to purify the oxygenase-component of the toluene dioxygenase system were hindered by the instability of the protein and the low recoveries of enzyme activity during conventional purification procedures. These problems have now been resolved by use of an affinity chromatographic procedure that utilizes a substrate analogue (p-toluic acid) as the ligand. It is of interest to note that the enzyme will only bind to the column after the addition of NADH to the crude cell extract. This may indicate that electrons are transferred to the terminal oxygenase by the flavoprotein reductase

and ferredoxin$_{TOL}$ before substrate binding can occur. The purified enzyme is quite stable at 4 C over a five day period and no loss in activity was observed over a seven week period when samples were stored at -20 C.

The properties of the purified enzyme are similar in several respects to the analogous proteins that function in cis-dihydrodiol formation from benzene (14) and pyrazon (5-amino-4-chloro-2-phenyl-2H-pyridazin-3-one, 15). All three enzymes show similar absorption spectra with absorption maxima in the visible region near 550 and 450 nm. However, the benzene oxygenase has a molecular weight of 215,300 and contains two (2Fe-2S) clusters. In contrast ISP$_{TOL}$ and the terminal component of the pyrazon dioxygenase have molecular weights of 151,000 and 180,000 respectively. Both enzymes are iron-sulfur proteins that contain two atoms each of iron and acid-labile sulfur.

The availability of significant amounts of ISP$_{TOL}$ should facilitate future studies on the mechanism of oxygen activation by the toluene dioxygenase enzyme system.

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