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Purification and properties of reductase of the three-component *p*-cymene methyl hydroxylase from *Pseudomonas chlororaphis* subsp. *aureofaciens*

Tapan K. Dutta[a,b,*], Arindam Dutta[a], Joydeep Chakraborty[a], Jayita Sarkar[a], Piyali Pal Chowdhury[a], Irwin C. Gunsalus[b,1]

[a] *Department of Microbiology, Bose Institute, Kolkata 700054, India*
[b] *NHEERL, Gulf Ecology Division, U.S. EPA, Gulf Breeze, FL 32561-5299, USA*

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ABSTRACT

A novel three-component *p*-cymene methyl hydroxylase from *Pseudomonas chlororaphis* subsp. *aureofaciens* was reported earlier on the basis of genetic characterization and their expression catalyzing methyl group hydroxylation. This enzyme system was inductively synthesized when grown on *p*-cymene and had an important role in initiating *p*-cymene metabolism in vivo. In the present study, a NADH-dependent cytochrome c reductase protein has been purified to an electrophoretically homogeneous state and found to be involved in the hydroxylation of methyl group of *p*-cymene. Molecular mass of the reductase appears to be 38 kDa by SDS/PAGE and 39 kDa by gel filtration apart from one molecule of tightly bound FAD and two atoms each of iron and acid-labile sulfur per molecule of the enzyme. An apparent Km value of the enzyme for NADH is 32 ± 1.2 μM. To the best of our knowledge, this is the first report on the purification of reductase component of *p*-cymene methyl hydroxylase.

1. Introduction

A significant amount of alkyl-substituted aromatics are being funneled into the environment by a variety of biological and biogeochemical processes as well as by industrial activities. These alkylated aromatic compounds form an important group of environmental pollutants or their precursors in the ecosystem [1,2]. Fate of these classes of compounds in the environment is largely due to microbial activities leading to their assimilation, primarily initiated via oxidation of side-chain [3–7]. The best known aromatic methyl-substituent oxidation pathway is that encoded by TOL plasmid, pWW0, in *Pseudomonas putida* mt-2 [6,8,9], involving oxidation of toluene, *m*-xylene, and *p*-xylene to benzoate, *m*-toluate, and *p*-toluate, respectively. The pathway is initiated by a monooxygenase, which catalyzes the oxidation of toluene (or *m*- or *p*-xylene) to benzyl alcohol. This monooxygenase is a two-component enzyme consisting of a XylA reductase subunit, which transfers electrons from NADH through FAD and a [2Fe2S] center to the membrane-associated XylM hydroxylase subunit. There, one atom of activated molecular oxygen is inserted into the methyl group while the other oxygen atom is reduced to water. XylM shares significant amino acid identity with the integral-membrane non-heme diiron AlkB alkane hydroxylases and steroyl-CoA desaturase terminal components [10–12].

The terpene *p*-cymene (also referred to as *p*-isopropyltoluene), is an aromatic-terpenoid of biosynthetic origin [13,14] that contains an isopropyl group attached to a benzene ring with a *para*-oriented methyl group. Oxidative catabolism of *p*-cymene had been studied and the assimilating pathway was already established [3,15–17]. Oxidation of methyl side-chain is the committed step in the degradation of *p*-cymene and is converted to *p*-cumic alcohol, *p*-cumic aldehyde and *p*-cumate, which is further metabolized to the intermediates isobutyrate, acetyl CoA and pyruvate. Previous studies found that *P. putida* F1 genes involved in the degradation of *p*-cymene are located on the *cym* operon and cymene monooxygenase is a two-component enzyme system [16], although not much information is known about structural aspect and the mechanistic details of enzyme involved in *p*-cymene methyl group hydroxylation.

The genetic characterization and expression of the three-component *p*-cymene monooxygenase involved in *p*-cymene methyl hydroxylation in *Pseudomonas aureofaciens* reclassified as *Pseudomonas chlororaphis* subsp. *aureofaciens* [18] was reported in our previous communications [19,20]. The present study reports the purification and properties of the NADH-dependent cytochrome c reductase enzyme involved in *p*-cymene methyl group hydroxylation.

* Corresponding author at: Department of Microbiology, Bose Institute, P-1/12 CIT Scheme VIIM, Kolkata 700054, India. Tel.: +91 33 25693241; fax: +91 33 2355 3886.
E-mail address: tapan@bic.boseinst.ernet.in (T.K. Dutta).
[1] Professor Irwin C. Gunsalus passed away on October 25, 2008.

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2. Material and methods

2.1. Chemicals

NADH, NADPH, horse heart cytochrome c, and Sephadex G-200 were purchased from Sigma Chemical Co., St. Louis, Mo while DEAE-cellulose (DE 52) anion exchange resin was from Whatman Ltd., Kent, England. Phenyl-Sepharose CL-4B for hydrophobic interaction chromatography and Mono Q HR5/5 anion exchange column (FPLC) were obtained from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. Protein standards for gel filtration were obtained from BioRad, USA while ultrafiltration membranes (PM 10) were from Amicon, Levington, USA. *p*-Cymene, *p*-cumic alcohol, *p*-cumic aldehyde and *p*-cumic acid were purchased from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals were obtained from commercially available sources and were of analytical reagent grade.

2.2. Organism and culture conditions

Pseudomonas chlororaphis subsp. *aureofaciens* strain PJC was cultured in PAS medium [21] with *p*-cymene (1 g l^{-1}) as the sole source of carbon and energy as described earlier [19,20]. Large quantities of cells were grown overnight at 30 °C under constant shaking (OD_{660} 1.2) in 2.8-liter Fernbach flasks containing 800 ml of the minimal medium (pH 7.0). Cells were harvested by centrifugation and washed twice with 50 mM potassium phosphate buffer (pH 7.0) and the cell pellets were frozen in liquid nitrogen and stored at −70 °C until used. Frozen cells (40 g, wet weight) were suspended in 100 ml of 50 mM potassium phosphate buffer (pH 7.0) and cell suspension was loaded into pre-cooled French press, Constant Cell Disruption System, One Shot Model (Constant System Ltd. United Kingdom) fitted with a 8.0-ml cell and lysed at 30,000 psi for two cycles. Particulate material was removed by centrifugation at 48,000 × *g* for 30 min at 4 °C. Bacto agar (1.5%), Difco Laboratories, Detroit, MI was added for solid media.

2.3. Enzyme assay

The reductase activity was assayed spectrophotometrically by measuring the reduction of cytochrome c at 550 nm in the presence of NADH. Reaction was carried out at 25 °C in 1 ml of 50 mM Tris–HCl buffer (pH 8.4) containing 50 μM horse heart cytochrome c and 1 mM NADH. To determine the optimal conditions, different pH values in the range of 7.6–8.6 of Tris–HCl buffer and different incubation temperatures in the range of 15–50 °C were tested for reductase activity. A molar extinction coefficient of 21,000 M^{-1} cm^{-1} [22] for reduced cytochrome c was used to calculate the enzyme activity. Specific activity was defined as units/mg of enzyme where one unit of the enzyme was the amount catalyzing the reduction of 1 μmol cytochrome c min^{-1} under the standard assay conditions.

2.4. Enzyme purification

All purification steps were performed at 4 °C.

2.4.1. Anion-exchange chromatography on DEAE-cellulose

A 100 ml volume of crude extract obtained from 40 g of *P. chlororaphis* subsp. *aureofaciens* cells was applied to a DEAE-cellulose DE-52 anion-exchange column (bed volume 100 ml) previously equilibrated with 50 mM potassium phosphate buffer (pH 7.0). After the column was washed with 500 ml of same buffer, the enzymes were eluted by a linear gradient of 0 – 0.5 M of NaCl in 500 ml of 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 4 ml min^{-1}. The elutant was collected in 5 ml fractions.

2.4.2. Ammonium sulfate precipitation

Active fractions from DEAE-cellulose column were pooled and brought to 30% ammonium sulfate saturation by slow addition of solid ammonium sulfate under gentle stirring at 4 °C. During addition of ammonium sulfate, the pH of the protein solution was adjusted to 7.0 using 1 N NaOH. After 3 h of stirring, the precipitated proteins were removed by centrifugation (20,000 × *g*, 30 min). The supernatant was then brought to 60% ammonium sulfate saturation and the precipitated proteins were centrifuged as described above.

2.4.3. Hydrophobic interaction chromatography

The pellet obtained from 60% ammonium sulfate precipitation was resuspended in a minimum volume of 50 mM potassium phosphate buffer containing 1.7 M ammonium sulfate (pH adjusted to 7.0). Protein was loaded onto a phenyl-Sepharose column (15 ml bed volume) previously equilibrated with 1.7 M ammonium sulfate in 50 mM potassium phosphate buffer as above. The column was washed with 5 bed volumes of the same buffer. Enzymes were eluted with 150 ml of linearly decreasing concentration of ammonium sulfate (1.7–0 M) in 50 mM phosphate buffer (pH 7.0) at a flow rate of 1 ml min^{-1} in 2 ml fractions.

2.4.4. Mono Q anion-exchange chromatography

The active fractions from phenyl-Sepharose column were pooled and concentrated on an Amicon concentrator using PM-10 ultrafiltration membrane under nitrogen atmosphere. The concentrate (2 ml) was dialyzed against 4-liter of ice-cold 50 mM potassium phosphate buffer (pH 7.0) with three changes at 4 °C. Dialyzed protein was loaded onto a Mono Q HR5/5 column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0) designed for fast-performance liquid chromatography (FPLC) system, which consisted of a GP250 controller, two P500 pumps, a UV-1 monitor, a REC-482 recorder and a FRAC200 autosampler (all from GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was washed with 40 ml of phosphate buffer and enzymes were eluted with 40 ml of a pre-programmed gradient of NaCl (0 – 350 mM) in 50 mM potassium phosphate buffer (pH 7.0) at the rate of 0.5 ml min^{-1} in 0.5 ml fractions.

2.5. Analyses

In vitro transformation of *p*-cymene to 4-isopropylbenzyl alcohol (*p*-cumic alcohol) was analyzed by reverse phase high performance liquid chromatography (HPLC) using a Hewlett-Packard 1090 system equipped with a diode array detector. The reaction mixture for determining *p*-cumic alcohol from *p*-cymene consisted of 5 mM *p*-cymene, 2 mM of NADH, 20 μg of purified reductase and saturating amount of membrane fraction (pellet obtained from disrupted cell suspension following centrifugation at 48,000 × *g*) in a total volume of 2 ml in 50 mM potassium phosphate buffer (pH 7.0). Following incubation for 2 h at 25 °C, the reaction mixture was acidified to pH 2.0 using concentrated hydrochloric acid, then centrifuged and the supernatant extracted thrice with equal volume of ethyl acetate. Extract was concentrated and injected onto the HPLC C_{18} column system, then eluted with acetonitrile-water (65:35) for 20 min, and metabolite(s) was detected at 254 nm. Identity of metabolite was confirmed from comparison of retention time and UV spectrum with authentic samples.

Protein concentration in cell extract was measured by the method of Bradford [23], with bovine serum albumin as the standard.

Enzyme purity and relative mobility (M_r) of polypeptides were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [24] with 10% gels and low-molecular weight standard proteins (M_rs 14,400–94,000). Proteins were

stained with Coomassie blue R250. The relative mobility of native protein was estimated by gel filtration [25] from a Sephadex G-200 column (1.0 cm × 90 cm) that had been calibrated with a set of molecular weight standards namely γ-globulin, 158 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa and lysozyme, 14.3 kDa. The void volume of the column was determined by using blue dextran 2000 (Sigma, USA). Protein was eluted by 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 0.5 ml min^{-1}. The elution profile was continuously monitored by measuring absorbance at 280 nm.

2.6. Reaction stoichiometry and kinetic parameters

The stoichiometry of the reductase catalyzing transfer of electrons from NADH to cytochrome c was determined from 1 ml of 50 mM Tris–HCl buffer (pH 8.4) containing 200 μM cytochrome c and 50 μM NADH and 2.0 μg of reductase, incubated at 25 °C, with the formation of reduced cytochrome c monitored spectrophotometrically at 550 nm. The change in A_{550} which could not be modified by further addition of reductase enzyme or oxidized cytochrome c represented the quantity of cytochrome c reduced by 50 μM NADH. Kinetic parameters were determined by using purified enzyme and monitoring the reduction of cytochrome c as described above. The kinetic values for NADH were obtained with a constant cytochrome c concentration of 50 μM and varying concentrations of NADH from 10 to 250 μM in the presence of 2.0 μg of reductase. Each individual reaction was done in triplicate. Apparent K_m and V_{max} values were obtained using a computer-aided direct fit to the Michaelis-Menten equation (Sigmaplot 10, Enzyme module; Systat Software, San Jose, CA, USA).

2.7. Flavin determination

Flavin was extracted by heating at 95 °C for 2 min from the purified reductase at 1 mg/ml in 50 mM potassium phosphate buffer (pH 7.0). Precipitated protein was removed by centrifugation (10,000 × *g*, 5 min) and the supernatant was lyophilized and dissolved in 10 μl of 50 mM potassium phosphate buffer (pH 7.0). The flavin coenzyme was identified by reverse phase chromatography on a C_{18} column as described above, eluted with acetonitrile-KH_2PO_4 (50 mM, pH 3.5), (10:90) and detected at 254 nm. Identity of flavin component was confirmed from comparison of retention time and UV spectrum with that of standard flavins (FAD and FMN).

2.8. Iron and labile sulfide determination

The iron content of the NADH-dependent cytochrome c reductase was determined by using the *o*-phenanthroline method [26] using the purified protein, which was previously deproteinized with trichloroacetic acid (5% final concentration) to release iron component of the iron-sulfur cluster. The acid-labile sulfide content of the reductase was determined by the method of Brumby et al. [27] modified in the manner suggested by Suhara et al. [28], while prior to the addition of N,N-dimethyl-*p*-phenylenediamine and ferric chloride, the purified enzyme was incubated with the alkaline-zinc reagent for 2 h to release acid labile sulphide component.

2.9. EPR spectroscopy

Electron paramagnetic resonance (EPR) spectrum of the purified reductase was recorded using a Varian E-112 X-band spectrometer equipped with an Air Products (Allentown, PA) variable temperature cryostat and a Varian TE102 mode cavity. The conditions used were microwave frequency 9.05 GHz, microwave power 10 mW, modulation amplitude 5 G, and temperature 20 K.

3. Results and discussion

3.1. Purification of NADH-dependent cytochrome c reductase

The reductase component of *p*-cymene methyl hydroxylase was purified from *Pseudomonas chlororaphis* subsp. *aureofaciens* that had been cultivated aerobically in the presence of *p*-cymene as sole source of carbon and energy. By disrupting the cultivated cells with a high pressure homogenizer device, NADH-dependent reductase activity was easily released into the soluble fraction. However, purification of the terminal oxygenase component has so far been unsuccessful due to the membrane bound nature of the protein [12] and its inactivation during solubilization. Therefore, the reductase enzyme is considered to be a membrane-extrinsic protein that possibly combines with the surface of the cytoplasmic membrane by hydrophobic interactions. Moreover, the *in vitro* formation of *p*-cumic alcohol from *p*-cymene by the purified reductase protein in presence of an otherwise inactive crude membrane fraction containing terminal oxygenase component and NADH was observed during HPLC analysis of the organic extract of the reaction mixture, supporting the role of the reductase as an electron transport component in *p*-cymene hydroxylation (data not shown). In the standard assay conditions, the transformation of *p*-cymene was found to be 1.7 nmol min^{-1} μg^{-1} of purified reductase. However, parallel control with membrane fraction from non-induced culture did not exhibit *p*-cymene hydroxylation activity. The results of the purification procedure of the reductase are given in Table 1. Based on the elution profiles of proteins obtained from various column chromatographic processes, it had been observed that the crude cell extract contained only one protein capable of reducing cytochrome c. These processes led to a 92-fold purification of the reductase with 6% recovery of total activity originally present in the crude extract.

3.2. Properties of NADH dependent cytochrome c reductase

Polyacrylamide gel electrophoresis (PAGE) of the purified reductase enzyme preparation in the presence and absence of sodium dodecyl sulfate (SDS) revealed one Coomassie Brilliant Blue-positive band on the gel. The enzyme was composed of only one kind of polypeptide, which had an estimated relative mobility of ~38,000 from SDS-PAGE determination (Fig. 1A, Lane 2). The molecular mass of the reductase was determined to be 39 kDa on a calibrated Sephadex G-200 gel filtration column, as compared to 38.433 kDa calculated from the amino acid sequence of the protein [19], suggesting that the purified reductase was a monomer of the 38 kDa polypeptide.

Solution of the enzyme was brownish yellow in color and gave absorption maxima at 460 nm, a wavelength slightly longer than the absorbance maximum of free FAD (448 nm, Fig. 1B). The spectrum bore similarity to NADH:acceptor reductase components of methane and xylene monooxygenases [29,30]. The flavin coenzyme isolated from the reductase protein had a spectrum corresponding to that of free FAD (Fig. 1B). The flavin exhibited the same mobility and UV spectrum as FAD as compared with that of FMN on reverse phase chromatography. The concentration of FAD was determined at 1.07 mol/mol of enzyme, indicating that each molecule of NADH-cytochrome c reductase contains one FAD molecule. Furthermore, cloning and sequencing of the reductase gene (*cymA*) and the deduced amino acid sequence showed the typical FAD binding motif as well as the N-terminal cysteine residues finger print for the presence of chloroplast type [2Fe–2S] redox center [19]. These observations suggest the protein is the reductase component of

Table 1
Purification of reductase.

Purification step	Protein (mg)	Activity[a] (U)	Sp act (U/mg)	Recovery (%)	Purification fold
Cell-free extract	3200	1612	0.5	100	1
DEAE-cellulose DE-52	228	616	2.7	38	5
Ammonium sulfate fractionation	42.6	408	9.6	25	19
Phenyl sepharose	6.8	268	39.4	17	79
Mono Q	2.2	101	45.9	6	92

[a] Enzyme activity was determined by the NADH-dependent cytochrome c reductase assay.

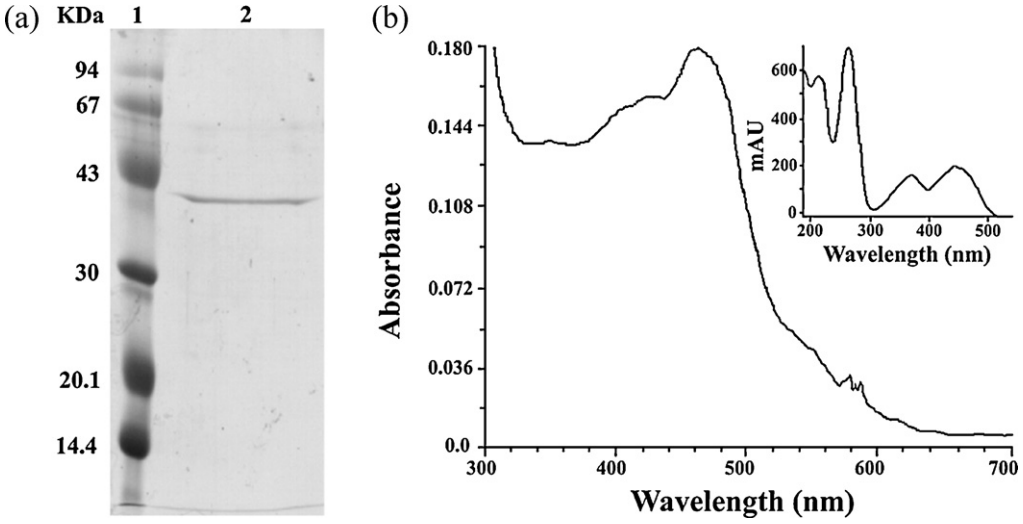

Fig. 1. (a) SDS-PAGE analysis of the NADH dependent cytochrome c reductase. Lane 1, molecular weight markers (kDa) containing phosphorylase *b* (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), and α-lactalbumin (14.4); and lane 2, purified reductase, 0.5 μg. (b) Absorption spectra of NADH dependent cytochrome c reductase in 50 mM potassium phosphate buffer (pH 7.0). Inset, cofactor isolated from the reductase. mAU, milli-absorbance unit.

p-cymene methyl hydroxylase, an iron–sulfur flavoprotein, containing 1 molecule each of FAD and [2Fe–2S] center/molecule of enzyme.

The stoichiometry of the NADH-cytochrome c reductase activity was determined and the oxidation of 50 nmol NADH produced 104 nmol of reduced cytochrome c. The activity of the enzyme varied with pH with maximum activity between pH 7.6 and 8.6 (optimum pH 8.4) while the optimum temperature was found to be 25 °C. The reductase was inactivated when exposed to higher temperatures, incubation at 30 °C, 37 °C and 50 °C for 10 min reduced its activity by 42%, 66% and 98% respectively. The reductase could be stored at −70 °C in 50 mM potassium phosphate buffer (pH 7.0) containing 60% glycerol (v/v) for a month without major loss of activity.

The activity of the purified enzyme could not be stimulated by the addition of FAD indicating tight association with the enzyme molecule which was not lost during the purification process. The content of iron and acid-labile sulfide was determined to be in ratios of 1.92 ± 0.14 and 1.86 ± 0.11 atoms molecule^{-1} of the NADH-dependent cytochrome c reductase enzyme, respectively, confirming the presence of one [2Fe–2S] center per enzyme molecule. Moreover, the EPR spectrum of the dithionite-reduced purified reductase revealed *g* values at 2.04, 1.95, and 1.89 (Fig. 2), which agrees very well with those of typical chloroplast type ferredoxins [31]. The NADH oxidation catalyzed by the reductase displayed Michaelis–Menten kinetics, with apparent K_m and V_{max} values for NADH of 32 ± 1.2 μM and 1412 ± 38 μmol min^{-1} mg^{-1} of protein respectively. With the purified enzyme, NADPH could not replace the cofactor requirement for cytochrome c reductase activity.

The *p*-cymene methyl hydroxylase (monooxygenase) system encoded in *P. chlororaphis* subsp. *aureofaciens* catalyses the hydroxylation of a methyl side-chain of *p*-cymene similar to that reported in *P. putida* F1. However, genetic studies have suggested that this monooxygenase consists of a novel three-component system, where products of the *cymA*, *cymB* and *cymM* genes function as an electron-transfer protein, enhancer/activator protein and a

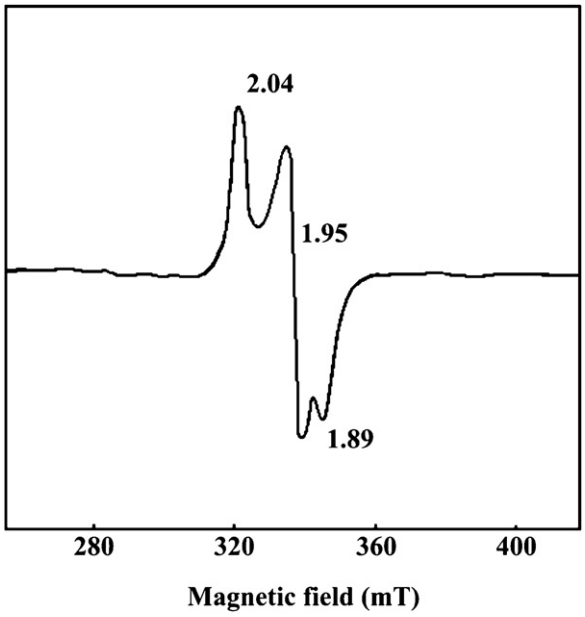

Fig. 2. EPR spectrum of the NADH dependent cytochrome c reductase. A 200-μl volume of the reductase (40 μM) was anaerobically reduced with a slight excess of sodium dithionite.

terminal hydroxylase, respectively. The reductase component CymA from *P. chlororaphis* subsp. *aureofaciens* showed 87% identity in 349 amino acid residues with the corresponding reductase component CymAb from *P. putida* F1. In this study, the electron-transfer component of *p*-cymene monooxygenase, the product of *cymA*, was purified to homogeneity followed by the characterization of the properties of this reductase enzyme. Understanding the mechanistic details of this complex protein including that of the role of the possible enhancer/activator protein will be the subject of further investigations.

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