Biodegradation of phenanthrene by *Pseudomonas* sp. strain PPD: purification and characterization of 1-hydroxy-2-naphthoic acid dioxygenase

Jaigeeth Deveryshetty and Prashant S. Phale

Correspondence

Prashant S. Phale
pphale@iitb.ac.in

Biotechnology Group, Department of Biosciences and Bioengineering, Indian Institute of Technology – Bombay, Powai, Mumbai 400 076, India

Pseudomonas sp. strain PPD can metabolize phenanthrene as the sole source of carbon and energy via the 'phthalic acid' route. The key enzyme, 1-hydroxy-2-naphthoic acid dioxygenase (1-HNDO, EC 1.13.11.38), was purified to homogeneity using a 3-hydroxy-2-naphthoic acid (3-H2NA)-affinity matrix. The enzyme was a homotetramer with a native molecular mass of 160 kDa and subunit molecular mass of ~39 kDa. It required Fe(II) as the cofactor and was specific for 1-hydroxy-2-naphthoic acid (1-H2NA), with K_m 13.5 μ M and V_{max} 114 μ mol min⁻¹ mg⁻¹. 1-HNDO failed to show activity with gentisic acid, salicylic acid and other hydroxynaphthoic acids tested. Interestingly, the enzyme showed substrate inhibition with a K_i of 116 μ M. 1-HNDO was found to be competitively inhibited by 3-H2NA with a K_i of 24 μ M. Based on the pH-dependent spectral changes, the enzyme reaction product was identified as 2-carboxybenzalpyruvic acid. Under anaerobic conditions, the enzyme failed to convert 1-H2NA to 2-carboxybenzalpyruvic acid. Stoichiometric studies showed the incorporation of 1 mol O₂ into the substrate to yield 1 mol product. These results suggest that 1-HNDO from *Pseudomonas* sp. strain PPD is an extradiol-type ring-cleaving dioxygenase.

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INTRODUCTION

Phenanthrene, a major pollutant, is the smallest polycyclic aromatic hydrocarbon to have a 'bay-region' and a 'K-region'. The epoxides formed at the bay- and K-region are highly reactive as compared to the parent compound and are suspected carcinogens (Bucker *et al.*, 1979; Mastrangelo *et al.*, 1996). Therefore phenanthrene is often used as a model substrate to study the metabolism of polycyclic aromatic hydrocarbons.

Several bacterial species capable of degrading phenanthrene have been reported (Phale *et al.*, 2007). The metabolic pathway is initiated by a ring-hydroxylating dioxygenase to yield *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, which is subsequently metabolized to 1-hydroxy-2-naphthoic acid (1-H2NA). The 1-H2NA generated is metabolized by one of two distinct routes, the 'phthalic acid' or the 'naphthalene' route (Fig. 1). In the 'phthalic acid' route, 1-H2NA is cleaved by 1-hydroxy-2-naphthoic acid dioxygenase (1-HNDO; EC 1.13.11.38) to yield 2-carboxyben-

zalpyruvic acid, which is further metabolized via phthalic acid to tricarboxylic acid cycle intermediates (Kiyohara *et al.*, 1976). In the 'naphthalene' route, 1-H2NA is metabolized via 1,2-dihydroxynaphthalene and salicylic acid (Evans *et al.*, 1965). Further, alternative routes exist which metabolize 1-H2NA via 1-naphthol (Prabhu & Phale, 2003; Samanta *et al.*, 1999).

Ring-cleaving dioxygenases play an important role in the degradation of aromatic compounds; they catalyse the incorporation of two atoms of molecular oxygen into substrates. Based on the mode of ring-cleavage, they are grouped as (a) extradiol dioxygenases, which require non-haem Fe(II) and cleave the aromatic ring proximal to one of the two hydroxylated carbon atoms, yielding a semialdehyde (Kojima *et al.*, 1961); and (b) intradiol dioxygenases, which require non-haem Fe(III) and cleave the aromatic ring between the two hydroxylated carbon atoms, yielding a muconic acid (Hayaishi & Hoshimoto, 1950). Both groups of enzymes have been studied extensively (Arciero & Lipscomb, 1986; Davis *et al.*, 2002; Kita *et al.*, 1999; Shu *et al.*, 1995; Vetting *et al.*, 2000; Vetting & Ohlendorf, 2000). The ring-cleavage of gentisic acid, homogentisic acid, salicylic acid and 1-H2NA is distinct from that catalysed by intradiol and extradiol ring-cleaving dioxygenases (Adachi *et al.*, 1999; Adams *et al.*, 2006; Barnsley, 1983; Crawford *et al.*, 1975; Feng *et al.*, 1999; Harpel & Lipscomb, 1990; Hintner *et al.*, 2004;

Abbreviations: 2-CBADH, 2-carboxybenzaldehyde dehydrogenase; GDO, gentisic acid 1,2-dioxygenase; 2-H1NA, 2-hydroxy-1-naphthoic acid; 1-H2NA, 1-hydroxy-2-naphthoic acid; 3-H2NA, 3-hydroxy-2-naphthoic acid; 6-H2NA, 6-hydroxy-2-naphthoic acid; 1-HNDO, 1-hydroxy-2-naphthoic acid dioxygenase; P3,4DO, protocatechuate 3,4-dioxygenase; P4,5DO, protocatechuic acid 4,5-dioxygenase; SDO, salicylic acid 1,2-dioxygenase.

Fig. 1. Metabolic pathways for the degradation of phenanthrene in bacteria: *Aeromonas*, *Alcaligenes*, *Bacillus* and *Micrococcus* sp. degrade 1-H2NA via the 'phthalic acid' pathway (Doddamani & Ninnekar, 2000; Ghosh & Misra, 1983; Kiyohara *et al.*, 1976) while *Pseudomonas* spp. metabolize 1-H2NA via the 'naphthalene' pathway (Evans *et al.*, 1965). Diversity in the metabolism is generated by degrading phenanthrene via 1-naphthol (Prabhu & Phale, 2003; Samanta *et al.*, 1999).

Iwabuchi & Harayama, 1998; Matera *et al.*, 2008). So far there are only two reports on 1-HNDO, from Gram-negative bacterium strain B156 and *Nocardioideis* sp. strain KP7 (Barnsley, 1983; Iwabuchi & Harayama, 1998). *Pseudomonas* sp. strain PPD can utilize phenanthrene, hydroxybenzoic acids (*o*-, *m*- and *p*-) and phthalic acid isomers (*o*-, *m*- and *p*-) as the sole source of carbon and energy (Deveryshetty *et al.*, 2007; Vamsee-Krishna *et al.*, 2006). Here, we report the metabolic pathway for degradation of phenanthrene in *Pseudomonas* sp. strain PPD, and the affinity purification and characterization of 1-HNDO from this bacterium.

METHODS

Chemicals. Phenanthrene, 1-H2NA, salicylic acid, gentisic acid, 3-hydroxy-2-naphthoic acid (3-H2NA), 2-hydroxy-1-naphthoic acid (2-H1NA), 6-hydroxy-2-naphthoic acid (6-H2NA), 1-naphthol, 2-naphthol, 1-naphthoic acid, 2-naphthoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 1,2-dihydroxynaphthalene, catechol, protocatechuic acid, EGTA, EDTA, 1,10-phenanthroline, 2,2'-dipyridyl, Sepharose CL-4B, DEAE-Sephacel, Sephacryl S-200-HR and benzidine were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade and purchased locally.

Bacterial culture and growth conditions. *Pseudomonas* sp. strain PPD, originally isolated from soil, was grown in 150 ml minimal salt medium (MSM) plus phenanthrene (0.1%) or glucose (0.25%) in 500 ml baffled Erlenmeyer flasks at 30 °C on a rotary shaker at 200 r.p.m. (Vamsee-Krishna *et al.*, 2006).

Whole-cell oxygen uptake. Cells grown on appropriate carbon sources were used to monitor whole-cell O₂ uptake. Rates were measured in the presence of various probable metabolic intermediates at 30 °C using an oxygraph (Hansatech) fitted with a Clark-type electrode as described previously (Deveryshetty *et al.*, 2007).

Preparation of cell-free extract and enzyme assays. Cells were harvested by centrifugation, washed twice with Tris/HCl (50 mM, pH 7.5), and cell-free extract was prepared as described previously (Deveryshetty *et al.*, 2007).

1-HNDO activity was monitored spectrophotometrically (Perkin Elmer Lambda 35) by measuring the rate of product formation as the increase in absorbance at 300 nm. The reaction mixture (1 ml) contained 1-H2NA (100 μM), an appropriate amount of enzyme and Tris/HCl buffer (50 mM, pH 7.5). Enzyme activity of 1-HNDO was calculated using an absorption coefficient difference of 11.5 mM⁻¹ cm⁻¹ between 2-carboxybenzalpyruvic acid and 1-H2NA as described by Iwabuchi & Harayama (1998). Phthalic acid dioxygenase-oxygenase and -reductase component (Vamsee-Krishna *et al.*, 2006), catechol 2,3-dioxygenase (Kojima *et al.*, 1961), catechol 1,2-dioxygenase (Hayaishi & Hoshimoto, 1950), gentisic acid dioxygenase (Harpel & Lipscomb, 1990), 2-carboxybenzaldehyde dehydrogenase (Iwabuchi & Harayama, 1997), and protocatechuic acid 3,4- and 4,5-dioxygenase (Ono *et al.*, 1970; Stanier & Ingraham, 1954) were monitored as described in the references cited. Enzyme activities were expressed as units (nanomoles or μmoles of product formed or substrate disappeared, NADH formed or O₂ consumed) min⁻¹ ml⁻¹. Specific activities were expressed as units (mg protein)⁻¹. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Identification of metabolites from biotransformation and bulk enzyme reactions.

Biotransformations were carried out with cells grown on phenanthrene to the late-exponential phase. Cells were harvested, washed twice with Tris/HCl buffer (50 mM, pH 7.5), resuspended in MSM (~200 mg wet weight cells in 80 ml) containing the desired compound (0.1%) and incubated at 30 °C for 4 h on a rotary shaker. After separating cells, spent medium was acidified to pH 2 (with 1 M HCl) and extracted with an equal volume of ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and concentrated. The reaction products were resolved by TLC on silica gel G, using the solvent system hexane/chloroform/acetic acid (7:3:1, by vol.) and identified by comparing R_f and UV-fluorescence properties at 254 nm with those of authentic compounds.

To identify the enzyme reaction products, bulk enzyme reactions (15 ml) were performed for 1-HNDO in mixtures which contained Tris/HCl buffer (50 mM, pH 7.5), NAD⁺ (200 μM), 1-H2NA (200 μM) and an appropriate amount of cell-free extract or purified

1-HNDO. The reaction was carried out at 30 °C for 80 min with addition of NAD^+ every 20 min. The reaction mixture for 2-carboxybenzaldehyde dehydrogenase (2-CBADH) contained glycine/KOH buffer (50 mM, pH 9.6), NAD^+ (200 μM), 2-carboxybenzaldehyde (200 μM) and an appropriate amount of cell-free extract. Control reactions contained all components except substrate or enzyme. The reaction was terminated by adjusting the pH to 2, and the reaction products were extracted and analysed by TLC as described above.

Purification of 1-HNDO from *Pseudomonas* sp. strain PPD

1-HNDO was purified to homogeneity using following steps. All the steps were carried out at 4 °C or on ice.

(i) **Preparation of cell-free extract.** Cells grown on phenanthrene were harvested and washed twice with buffer 1 [MOPS (50 mM, pH 7.5), glycerol (5 %, v/v), $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0.1 mM) and DTT (2 mM)]. The cells were suspended in ice-cold buffer 1 (~1.8 g in 8 ml) and sonicated using an ultrasonic processor (GE130) on ice, with 10 cycles of 20 pulses each (1 s pulse, 1 s interval, cycle duration 40 s, output of 20 W, 3 min interval between two cycles). The supernatant obtained after centrifuging cell homogenate at 50 000 g for 30 min was referred to as the cell-free extract.

(ii) **Heat treatment.** The cell-free extract was incubated at 60 °C in a water bath in the presence of 3-H₂NA (1 mM) with intermittent shaking. After 20 min incubation, the extract was immediately transferred on to ice. Denatured proteins were removed by centrifugation at 35 000 g for 30 min. Supernatant was dialysed against buffer 2 [MOPS buffer (50 mM, pH 7.5) containing $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0.1 mM), DTT (2 mM), glycerol (5 %) and ethanol (10 %)] and processed further.

(iii) **3-H₂NA-affinity chromatography.** The affinity matrix was prepared using 3-H₂NA as a ligand. Sepharose CL-4B was activated by CNBr as described by March *et al.* (1974). CNBr-activated Sepharose CL-4B was converted to benzidyl Sepharose CL-4B and coupled to 3-H₂NA as described by Sugumaran & Vaidyanathan (1978). The purple-coloured matrix obtained was washed with 750 ml HCl (0.1 M), 1 l Tris/maleate (25 mM, pH 6.0) and 1 l distilled water. The matrix was suspended in distilled water and used for affinity purification.

The extract obtained from step (ii) was loaded onto the 3-H₂NA-affinity column (bed vol. 43 ml; 18 × 200 mm) pre-equilibrated with buffer 2 at the rate of 6 ml h⁻¹. The column was washed with buffer 2 (150 ml) and eluted with a linear gradient of 3-H₂NA (0–5 mM, 200 ml) at a flow rate of 30 ml h⁻¹.

(iv) **DEAE-anion-exchange chromatography.** Fractions from 3-H₂NA-affinity chromatography containing 1-HNDO activity (>1 $\mu\text{mol min}^{-1} \text{ml}^{-1}$) were pooled and loaded onto a DEAE-Sephacel column (bed vol. 19 ml; 18 × 100 mm) equilibrated with buffer 2. The column was washed extensively with the same buffer and eluted with a linear gradient of KCl (0–0.4 M, 100 ml) at flow rate of 30 ml h⁻¹. Active fractions from the DEAE column were pooled and dialysed (12 kDa cut-off) against MOPS buffer (50 mM, pH 7.5) containing $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ (0.1 mM), DTT (2 mM) and glycerol (40 %) for 3 h and stored at –20 °C until further use.

Determination of molecular mass. The subunit molecular mass was determined by 12 % SDS-PAGE under denaturing conditions (Laemmli, 1970). The native molecular mass was determined by Sephacryl S-200-HR gel-filtration chromatography. The column (bed 170 ml; void 55 ml; 16 × 860 mm, flow rate 3.5 ml h⁻¹) was equilibrated with Tris/HCl buffer (50 mM, pH 7.5) containing

glycerol (5 %) and ethanol (10 %) and calibrated with standard protein markers.

RESULTS

Metabolism of phenanthrene by *Pseudomonas* sp. strain PPD

Culture grown on phenanthrene showed a bright orange colour in the early-exponential phase, which subsided during the late-exponential and stationary phases. TLC analysis of the early-exponential phase spent medium showed a single spot, which was identified as 1-H₂NA based on its R_f (0.93) and UV-fluorescence properties (Table 1). Other metabolites could not be detected even in the mid-exponential, late-exponential and stationary phases of growth. This could be due to very low concentrations or absence of these metabolites in the spent medium. Phenanthrene-grown cells showed: (i) biotransformation of 1-H₂NA to 2-carboxybenzaldehyde (R_f 0.90) and of 2-carboxybenzaldehyde to phthalic acid (R_f 0.62) (Table 1); (ii) good O₂ uptake on phenanthrene and 1-H₂NA [5.5 and 3.9 nmol O₂ consumed min⁻¹ (mg wet wt of cells)⁻¹, respectively], but no detectable O₂ uptake with phthalic acid or protocatechuic acid (data not shown); (iii) presence of phthalic acid dioxygenase-oxygenase and -reductase components, 1-HNDO, 2-CBADH and protocatechuic acid 4,5-dioxygenase (P4,5DO) in the cell-free extract (Table 2); and (iv) enzymic conversion of 1-H₂NA to phthalic acid (R_f 0.60) and of 2-carboxybenzaldehyde to phthalic acid (R_f 0.60, Table 1). Time-dependent spectral changes in the protocatechuic acid dioxygenase reaction showed a decrease in the absorbance at 250 nm (disappearance of substrate, protocatechuic acid) with a concomitant increase at 410 nm (appearance of yellow-coloured product, 2-hydroxy-5-carboxymuconic semialdehyde), suggesting the *meta* ring-cleavage of protocatechuic acid by P4,5DO (data not shown). An enzyme activity versus growth profile showed maximum activity of 1-HNDO at 9 h and of P4,5DO at 15 h of growth (data not shown). Cells grown on glucose showed neither oxygen uptake nor enzyme activities in the cell-free extract, indicating that the enzymes of the phenanthrene degradative pathways are inducible (Table 2).

Purification of 1-HNDO from *Pseudomonas* sp. strain PPD

The enzyme was very unstable in the cell-free extract and required Fe(II) (0.1 mM), DTT (2 mM) and ethanol (10 %) for stability. Interestingly, the enzyme was stable at 60 °C for 5 min and showed prolonged stability for 20 min in the presence of 3-H₂NA (1 mM). Kinetic analysis of partially purified enzyme revealed competitive inhibition with 3-H₂NA (data not shown). 1-HNDO was purified by using heat treatment, 3-H₂NA-affinity matrix and DEAE ion-exchange column chromatography to 12.5-fold, yield 4.2 %, with a specific activity of 61.5 μmol

Table 1. Detection of metabolites from the spent culture medium, whole-cell biotransformation and bulk enzyme reactions

Sample		R_f	Fluorescence properties (254 nm)	Compound identified
1-H2NA		0.93	Fluorescent blue with quench in centre	Standard
2-Carboxybenzaldehyde		0.90	Black quench	Standard
Phthalic acid		0.62	Black quench	Standard
Protocatechuic acid		0.77	Dark blue quench	Standard
Spent medium of culture grown on phenanthrene				
	Spot 1	0.93	Fluorescent blue with quench in centre	1-H2NA
Whole-cell biotransformation* with:				
1-H2NA	Spot 1	0.93	Fluorescent blue with quench in centre	1-H2NA
	Spot 2	0.90	Black quench	2-Carboxybenzaldehyde
2-Carboxybenzaldehyde	Spot 1	0.90	Black quench	2-Carboxybenzaldehyde
	Spot 2	0.62	Black quench	Phthalic acid
Bulk enzyme reaction† with:				
1-H2NA	Spot 1	0.60	Black quench	Phthalic acid
2-Carboxybenzaldehyde	Spot 1	0.60	Black quench	Phthalic acid

*Cells were grown on phenanthrene, harvested, and the biotransformation reaction was performed using 1-H2NA and 2-carboxybenzaldehyde as described in Methods.

†Cell-free extract was prepared from phenanthrene-grown cells; bulk enzyme reactions were performed with 1-H2NA and 2-carboxybenzaldehyde as described in Methods.

$\text{min}^{-1} \text{mg}^{-1}$ from PPD cells grown on phenanthrene (Table 3). SDS-PAGE showed a single band at a molecular mass of 39 kDa (Fig. 2a). The enzyme was eluted as a single activity peak from Sephacryl S-200-HR gel filtration chromatography, with a native molecular mass of 160 kDa (Fig. 2b). The enzyme showed activity in the pH range 6–9 with a maximum at pH 7.5 (data not shown).

Table 2. Activities of enzymes involved in phenanthrene degradation by *Pseudomonas* strain PPD

Enzyme	Specific activity [nmol min^{-1} (mg protein^{-1}) from cells grown on	
	Phenanthrene	Glucose
1-HNDO	4640	–
2-CBADH	95	–
Phthalic acid dioxygenase, oxygenase	20	–
Phthalic acid dioxygenase, reductase	499	–
P4,5DO	1095	62
Catechol 2,3-dioxygenase	14	–
Catechol 1,2-dioxygenase	14	17
GDO	8.6	–

–, Activity could not be detected.

Effect of metal chelators and metal ions

The metal chelators EGTA and EDTA (1 mM) inactivated 1-HNDO by 30–40 %, while phenanthroline and 2,2'-dipyridyl (1 mM) inactivated it completely. Phenanthroline-inactivated enzyme regained 100 % activity following addition of Fe(II) (1 mM). Other divalent metal ions (Zn^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} and Cu^{2+}) failed to reactivate the enzyme (data not shown).

Identification of the reaction product of 1-HNDO

To identify the product, large-scale enzyme reactions were performed under aerobic and anaerobic conditions (for 4 h) using Thunberg tubes. Under aerobic conditions, a major spot with R_f 0.28 (blue quench) was detected on TLC. After removing the enzyme by Centricon filtration (10 kDa, Pall Life Sciences), the filtrate gave an absorption maximum at 300 nm at pH 7, and at 276 and 282 nm at pH 2 (Fig. 3), similar to that observed for 2-carboxybenzalpyruvic acid (Adachi *et al.*, 1999; Barnsley, 1983). Under anaerobic conditions, a single spot with R_f 0.95 corresponding to the substrate, 1-H2NA, was observed.

Stoichiometry of the reaction

Purified 1-HNDO showed a specific activity of $36.8 \mu\text{mol product formed min}^{-1} \text{mg}^{-1}$ by spectrophotometry and $34.4 \mu\text{mol O}_2 \text{ consumed min}^{-1} \text{mg}^{-1}$ by oxygraph. The ratio of O_2 consumed to product formed was 0.93, suggesting that $\sim 1 \text{ mol O}_2$ is consumed to yield 1 mol 2-

Table 3. Purification of 1-HNDO from *Pseudomonas* sp. strain PPD

Step	Total vol. (ml)	Total protein (mg)	Total activity*	Sp. act.†	Purification (fold)	Yield (%)
Cell-free extract	8.5	134	660	4.9	1	100
Heat treatment (60 °C)	6.6	53.3	571	11.2	2.3	86
Dialysis I	5.8	40.7	619	15.2	3.1	94
3-H2NA-affinity chromatography	60.5	4.11	218	53	11	33
Anion-exchange chromatography	13	0.52	15.2	29	6	2.3
Dialysis II	11.2	0.45	28	61.5	12.5	4.2

* $\mu\text{mol min}^{-1}$.† $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

carboxybenzalpyruvic acid. This observation, along with the product identification data, suggests that the enzyme requires oxygen.

Substrate specificity

The enzyme showed activity with 1-H2NA but failed to show activity (spectral changes or O_2 uptake) with gentisic acid, salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 1-naphthol, 2-naphthol, 1-naphthoic acid, 2-naphthoic acid, 2-H1NA, 3-H2NA or 6-H2NA (data not shown).

Kinetic constants

Initial reaction velocities were measured spectrophotometrically by varying the concentrations of 1-H2NA (1–200 μM). A representative substrate saturation plot for 1-H2NA is depicted in Fig. 4. Increasing the concentration of 1-H2NA led to linear increases in the activity up to 25 μM . With a further increase to 100 and 200 μM there was a 15 and 45 % decrease in activity, respectively, indicating substrate inhibition. A double reciprocal plot gave a K_i of $116 \pm 28 \mu\text{M}$, a K_m of $13.5 \pm 3.2 \mu\text{M}$ and a $V_{\max}$ of $114 \pm 20 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for the substrate 1-H2NA. The k_{cat} and k_{cat}/K_m values were determined to be 70.4 s^{-1} and $520 \times 10^4 \text{ s}^{-1} \text{M}^{-1}$. Of the compounds tested, 3-H2NA showed competitive inhibition with a K_i of $24 \pm 3.4 \mu\text{M}$, and 2-H1NA showed a mixed-partial inhibition pattern with a K_i of $26 \pm 5 \mu\text{M}$. Salicylic acid, gentisic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 1-naphthol, 2-naphthol, 1-naphthoic acid and 2-naphthoic acid did not inhibit the activity of the enzyme (data not shown).

DISCUSSION

Pseudomonas sp. strain PPD can utilize phenanthrene, hydroxybenzoic acids (*o*-, *m*- and *p*-) and phthalic acid isomers (*o*-, *m*- and *p*-) as the sole source of carbon and energy (Deveryshetty *et al.*, 2007; Vamsee-Krishna *et al.*, 2006). Detection of 1-H2NA in the spent medium, whole-cell respiration on phenanthrene and 1-H2NA, and activity

of 1-HNDO, 2-CBADH, phthalic acid dioxygenase and P4,5DO in the cell-free extract suggests that strain PPD degrades phenanthrene via the 'phthalic acid' route (Fig. 1; Tables 1 and 2). Low activity of phenanthrene-degrading enzymes in glucose-grown cells indicates that the enzymes are inducible (Table 2). Interestingly, strain PPD showed a carbon-source-dependent induction of two different ring-cleaving protocatechuate dioxygenases. Phenanthrene-grown cells showed P4,5DO activity, whereas phthalic acid-grown cells showed protocatechuate 3,4-dioxygenase (P3,4DO) activity (Vamsee-Krishna *et al.*, 2006). Phthalic acid-grown cells showed whole-cell O_2 uptake on phthalic acid and protocatechuic acid (Vamsee-Krishna *et al.*, 2006), indicating the induction of phthalic acid permease when grown on phthalic acid only. Phenanthrene-grown cells failed to show O_2 uptake with phthalic acid, although it is one of the proposed intermediate metabolites. This could be due to the inability of phenanthrene or intracellular phthalic acid to induce phthalic acid permease, which is responsible for phthalic acid uptake (Chang & Zylstra, 1999; Vamsee-Krishna & Phale, 2008).

1-HNDO (EC 1.13.11.38) catalyses the conversion of 1-H2NA to 2-carboxybenzalpyruvic acid. 1-HNDO was highly unstable, requiring Fe(II), DTT, ethanol and 3-H2NA as stabilizers; it showed heat stability for 20 min at 60 °C (Table 4). Using heat treatment, 3-H2NA-affinity chromatography and ion-exchange chromatography, 1-HNDO was purified to homogeneity. The subunit molecular mass of the enzyme was determined to be 39 kDa and the native molecular mass to be 160 kDa, indicating that the enzyme is a homotetramer (Fig. 3). 1-HNDO from *Nocardioide* sp. strain KP7 was found to be a homohexamer with native molecular mass 270 kDa and subunit molecular mass 45 kDa (Iwabuchi & Harayama, 1998; Table 4). pH-dependent changes in the spectral properties of the product of 1-HNDO were similar to those reported earlier, confirming the product to be 2-carboxybenzalpyruvic acid (Adachi *et al.*, 1999; Barnsley, 1983). Identification of 2-carboxybenzalpyruvic acid under aerobic conditions, consumption of 1 mol O_2 per 1 mol of product formed and the requirement for Fe(II) suggest that 1-HNDO is an extradiol-type ring-cleaving dioxygenase.

Fig. 2. Purification of 1-HNDO: (a) SDS-PAGE analysis during the different stages of purification. Lanes (each contains 5 μ g protein): 1, cell-free extract; 2, heat-treated fraction; 3, 3-H₂NA affinity-purified pool; 4, DEAE eluted pool. M, molecular mass markers (kDa): phosphorylase *b* (97.4), BSA (66), ovalbumin (43), carbonic anhydrase (29), soy-bean trypsin inhibitor (20) and lysozyme (14.3). (b) Elution profile of 1-HNDO from a Sephacryl S-200-HR gel-filtration column. The dioxygenase activity is represented by crosses and protein elution (A_{280}) by open triangles. Inset: plot of log(molecular mass) versus V_e/V_0 for molecular mass protein markers [β -amylase (200 kDa), alcohol dehydrogenase (150), BSA (66), and carbonic anhydrase (29)], represented by filled circles; the open circle represents 1-HNDO.

1-HNDO from strain PPD showed activity with 1-H₂NA and failed to act on substrate analogues, indicating that the enzyme is specific and similar to that reported from *Nocardioides* sp. strain KP7 (Iwabuchi & Harayama, 1998). Gentisic acid 1,2-dioxygenase (GDO), an extradiol-type dioxygenase, has been shown to act on halogenated and alkylated derivatives of gentisic acid but not on salicylic acid or 1-H₂NA (Crawford *et al.*, 1975; Feng *et al.*, 1999; Harpel & Lipscomb, 1990; Werwath *et al.*, 1998). Salicylic acid 1,2-dioxygenase (SDO) from *Pseudoaminobacter*

Fig. 3. UV-visible absorption spectra for the enzyme reaction product (2-carboxybenzalpyruvate) at pH 7.0 (solid line) and at pH 2.0 (dotted line), and for the substrate (1-H₂NA) at pH 7 (dashed line) and at pH 2.0 (dash-dot-dash line) in phosphate buffer (50 mM).

salicylatoxidans showed activity on derivatives of salicylic acid, gentisic acid and 1-H₂NA (Hintner *et al.*, 2001, 2004). Based on the structure of GDO and SDO, it was proposed that the N-terminal residues, which contribute to the active site, may be responsible for the wide substrate specificity of SDO (Matera *et al.*, 2008). 1-HNDO from strain PPD showed competitive inhibition with 3-H₂NA. This may be due to the presence of a carboxyl group at the 2 position on 1-H₂NA and 3-H₂NA. With respect to substrate specificity, affinity constants and competitive inhibition, the 1-HNDOS from *Pseudomonas* strain PPD and *Nocardioides* sp. strain KP7 are similar (Table 4). However, interestingly, the enzyme from strain PPD showed substrate inhibition at a higher concentration, with a K_i of 116 μ M. This feature does not appear to have

Fig. 4. Reaction velocity (v) versus substrate concentration $[S]$ plot for 1-HNDO. The reactions were performed with 0.5 μ g enzyme, and with various concentrations of 1-H₂NA from 1 to 200 μ M. The graph was fitted using the model for substrate inhibition (noncompetitive) with the following equation: $v = \{V_{\max} / [1 + (K_m/S) + (S/K_i)]\}$.

Table 4. Comparison of properties of extradiol-type ring-cleaving dioxygenases from various bacteria

Derived from the available data in the respective references.

Enzyme	Organism	Subunit mol. mass (kDa)	Native mol. mass (kDa)	Heat stability (°C)	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)*	Active sites per monomer	AA at Fe(II)- binding site	Structure	Reference
GDO	<i>Comamonas testosteroni</i>	41	158	60	85	4.3×10^6	ND	ND	ND	Harpel & Lipscomb (1990)
	<i>Comamonas acidovorans</i>	37–40	164	60	74	4.3×10^6	ND	ND	ND	Harpel & Lipscomb (1990)
	<i>Pseudomonas alcaligenes</i> P25X	39	154	60	92	0.44×10^6	ND	ND	ND	Feng <i>et al.</i> (1999)
	<i>Pseudomonas putida</i> P35X	41	82	60	143	0.4×10^6	ND	ND	ND	Feng <i>et al.</i> (1999)
	<i>Moraxella osloensis</i> OA3	40	154	60	7.1	ND	ND	ND	ND	Crawford <i>et al.</i> (1975)
	<i>Sphingomonas</i> sp. strain RW5	38.5	177	ND	15	5.1×10^6	ND	ND	ND	Werwath <i>et al.</i> (1998)
	<i>Escherichia coli</i> O157:H7	38.9	154	ND	11	384×10^6	1	H104, H106 and H145	2.4	Adams <i>et al.</i> (2006)
HGO†	<i>Pseudomonas fluorescens</i>	38	ND	ND	600	ND	ND	ND	ND	Adachi <i>et al.</i> (1966)
SDO	<i>Pseudoaminobacter salicylatoxidans</i>	45	180	ND	12	0.2×10^6	1	H119, H121 and H160	2.9	Hintner <i>et al.</i> (2001, 2004); Matera <i>et al.</i> (2008)
1-HNDO	<i>Nocardioides</i> sp. strain KP7	45	270	ND	10	11.4×10^6	ND	ND	ND	Iwabuchi & Harayama (1998)
	<i>Pseudomonas</i> sp. strain PPD	39	148	60	13.5	5.2×10^6	ND	ND	ND	Present study

ND, Not determined.

*Some of the k_{cat}/K_m values were derived from the available data in the respective references.

†Homogentisate oxygenase.

been reported so far for any of the ring-cleaving dioxygenases belonging to this group (Table 4).

The substrate specificity, involvement of Fe(II), position of ring-cleavage and product formed indicate that the catalytic mechanism of 1-HNDO is probably similar to that of GDO. Further characterization, such as determination of the active-site amino acids, reaction mechanism and comparative analysis, may help in engineering these enzymes for wide substrate acceptance and improved catalytic constants for efficient bioremediation.

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