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ORIGINAL PAPER

1-Naphthol 2-hydroxylase from *Pseudomonas* sp. strain C6: purification, characterization and chemical modification studies

Shivjee Sah · Prashant S. Phale

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Abstract 1-Naphthol 2-hydroxylase (1-NH) which catalyzes the conversion of 1-naphthol to 1,2-dihydroxynaphthalene was purified to homogeneity from carbaryl-degrading *Pseudomonas* sp. strain C6. The enzyme was found to be a homodimer with subunit molecular weight of 66 kDa. UV, visible and fluorescence spectral properties, identification of flavin moiety by HPLC as FAD, and reconstitution of apoenzyme by FAD suggest that enzyme is FAD-dependent. 1-NH accepts electron from NADH as well as NADPH. Besides 1-naphthol (K_m, 9.1 μM), the enzyme also accepts 5-amino 1-naphthol (K_m, 6.4 μM) and 4-chloro 1-naphthol (K_m, 2.3 μM) as substrates. Enzyme showed substrate inhibition phenomenon at high concentration of 1-naphthol (K_i, 283 μM). Stoichiometric consumption of oxygen and NADH, and biochemical properties suggest that 1-NH belongs to FAD containing external flavomonooxygenase group of oxido-reductase class of enzymes. Based on biochemical and kinetic properties, 1-NH from *Pseudomonas* sp. strain C6 appears to be different than that reported earlier from *Pseudomonas* sp. strain C4. Chemical modification and protection by 1-naphthol and NADH suggest that His, Arg, Cys, Tyr and Trp are at or near the active site of 1-NH.

S. Sah · P. S. Phale (✉)
Department of Biosciences and Bioengineering, Indian Institute of Technology-Bombay, Powai, Mumbai 400 076, India
e-mail: pphale@iitb.ac.in

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Introduction

Carbaryl (1-naphthyl-*N*-methylcarbamate), a carbamate group of insecticide, is used in agriculture to control variety of pests (Kaloyanova and Simeonova 1994). Widespread and repeated applications lead to pollution of soil and ground water. The toxicity of carbaryl is due to the ester bond between *N*-methylcarbamic acid and 1-naphthol. The degradation products, *N*-nitrosocarbamates and 1-naphthol are potent mutagens, more toxic and recalcitrant than carbaryl itself (Elespuru et al. 1974; Obulakondaiah et al. 1993; Rickard and Dorough 1984; Shea and Berry 1983; Wilson et al. 1985). Several microbial strains have been reported to degrade carbaryl as the sole source of carbon and energy (Chapalamadugu and Chaudhry 1991; Doddamani and Ninnekar 2001; Hayatsu et al. 1999; Larkin and Day 1986; Swetha and Phale 2005). *Pseudomonas* sp. strain C6, a soil bacterium isolated by enrichment culture technique, metabolizes carbaryl to tri-carboxylic acid cycle intermediate via 1-naphthol, 1,2-dihydroxynaphthalene (1,2-DHN), salicylate and gentisate (Swetha and Phale 2005). 1-Naphthol 2-hydroxylase (1-NH), the key enzyme of the carbaryl degradation pathway,

converts 1-naphthol to 1,2-DHN and was found to be inducible in nature. 1-NH from carbaryl-degrading *Pseudomonas* sp. strain C4 was purified and found to be a single-component flavin monooxygenase and required 1-naphthol, NAD(P)H and molecular oxygen for its activity (Swetha et al. 2007). Flavin hydroxylases involved in the degradation of aromatics perform initial reaction of ring-hydroxylation and reported to be either single- or two-component enzymes (Swetha et al. 2007; van Berkel et al. 2006). Using chemical modification, site-directed mutagenesis and structural studies, the role of His, Arg, Cys, Lys, Tyr, Pro, Ser, and Trp at the active site of various aromatic hydroxylases was proposed (Moran et al. 1996; Neujahr and Kjellen 1980; Palfey et al. 2002; Shoun et al. 1980; Shoun and Beppu 1982; Shoun 1990; Sumathi and Dasgupta 2000; Vanberkel et al. 1988). *p*-Hydroxybenzoate hydroxylase is the most extensively studied enzyme with respect to structure–function and reaction mechanism (Ballou et al. 2005; Gatti et al. 1994).

In the present study, we report purification, molecular characterization, and chemical modification of 1-NH from *Pseudomonas* sp. strain C6. The molecular and kinetic properties of the enzyme from strain C6 were compared with that of 1-NH from strain C4. Based on chemical modification and protection studies, we propose involvement of His, Cys, Arg, Tyr, and Trp at or near the active site of 1-NH.

Materials and methods

Bacterial strain and growth condition

Pseudomonas sp. strain C6 was grown on minimal salt medium (150 ml in 500 ml baffled Erlenmeyer flask) supplemented with carbaryl (0.1%) at 30°C on a rotary shaker at 200 rpm (Swetha and Phale 2005).

1-Naphthol 2-hydroxylase assay

The activity of 1-NH was monitored spectrophotometrically (Lambda 35; Perkin–Elmer) by measuring the decrease in the absorbance of NAD(P)H at 340 nm (ε_{340nm} 6220 M^{-1} cm^{-1}) (Swetha et al. 2007). Assay mixture (1 ml) contained phosphate buffer (KPi 50 mM, pH 8.5), NAD(P)H (250 μM), FAD (6.5 μM), substrate (50 μM) and appropriate amount of enzyme. Activity was expressed as μmole of NAD(P)H oxidized per min. Alternatively, the activity was also monitored polarographically by measuring substrate-dependent oxygen consumption at 30°C using Oxygraph (Hansatech, United Kingdom) fitted with a Clark's O_2 electrode (Swetha et al. 2007). The enzyme activity was expressed as nmole of O_2 consumed per min. The specific activity was reported as μmole min^{-1} mg^{-1} protein. Estimation of protein was performed using BSA as the standard (Bradford 1976).

Enzyme purification

1-NH was purified from carbaryl-grown *Pseudomonas* sp. strain C6 to homogeneity using following steps. All steps of purification were performed either at 4°C or on ice.

(i) *Preparation of cell-free extract* Cells grown on carbaryl (late log phase culture, 13–14 h) were harvested by centrifugation at 10,000×*g* for 10 min, washed twice with KPi buffer (50 mM, pH 7.5) and resuspended in ice-cold Buffer A (KPi 50 mM, pH 7.5; EDTA, 0.5 mM; and glycerol, 5%). The cells were disrupted using the Ultrasonic Processor (model GE130, USA) on ice, with 10 cycles of 20 pulses each (1 s pulse, 1 s interval, cycle duration 40 s, output of 20 W, 2–3 min interval between two cycles). Cell homogenate was centrifuged at 40,000×*g* for 45 min. The supernatant obtained was referred to as cell-free extract and processed further.

(ii) *Q-Sepharose anion-exchange chromatography* The cell-free extract was loaded onto a Q-Sepharose column (150 × 15 mm, bed vol. 24 ml) pre-equilibrated with Buffer A containing $(NH_4)_2SO_4$ (50 mM). The column was washed (250 ml) with Buffer A containing $(NH_4)_2SO_4$ (50 mM) and the bound enzyme was eluted with an increasing linear gradient of $(NH_4)_2SO_4$ (50–400 mM) in Buffer A (200 ml). Fractions (2 ml each, flow rate 30 ml h^{-1}) were collected by using the fraction collector (Redifrac 920; GE Healthcare). The fractions containing 1-NH activity (≥1 U) were pooled and processed further.

(iii) *Phenyl-Sepharose hydrophobic interaction column chromatography* The enzyme from step (ii) was brought to 30% $(NH_4)_2SO_4$ saturation and centrifuged at 15,000 × *g* for 15 min. The supernatant containing the activity was loaded onto a Phenyl-Sepharose column

(100 × 10 mm, bed vol. 7 ml) pre-equilibrated with Buffer A containing $(NH_4)_2SO_4$ (30% saturation). The column was washed (100 ml) with the same buffer and the bound enzyme was eluted using an increasing linear gradient of isopropanol (0–30%) in Buffer A (120 ml) containing $(NH_4)_2SO_4$ (30% saturation). Fractions (1.2 ml each, flow rate 21 ml h^{-1}) containing 1-NH activity were pooled and concentrated using ultra-filtration (membrane cut-off, 30 kDa; Millipore Amicon, USA).

(iv) *Sephacryl S-200 gel filtration chromatography* The enzyme from step (iii) was loaded onto a Sephacryl S-200-HR gel filtration column (950 × 10 mm, bed vol. 76 ml) pre-equilibrated with Buffer A containing NaCl (0.1 M). Fractions (1.2 ml each, flow rate 3 ml h^{-1}) containing 1-NH activity were pooled and used for characterization.

Spectroscopy

The UV–visible absorption spectra of purified 1-NH (210 μg/ml) was recorded in Buffer A containing NaCl (0.1 M). The emission spectrum of the enzyme was recorded using the fluorescence spectrometer (Jasco V-750).

Molecular weight determination and NH_2-terminal sequencing

The native and subunit molecular weight of 1-NH was determined by using Sephacryl S-200 gel filtration chromatography and SDS-PAGE, respectively. To determine NH_2-terminal sequence, 1-NH from SDS-PAGE (12%) was electro-blotted on to a PVDF membrane (0.45 μ, Pall, USA) in CAPS buffer (10 mM, pH 11) at 100 V for 8 h, stained with Coomassie Brilliant Blue R250 and subjected to automated Edman sequencing (Applied Biosystems 470, USA).

Apoenzyme preparation

The apoenzyme (flavin-free protein) was prepared by acid-ammonium sulfate method as described (Elmorsi and Hopper 1977) with minor modifications. The enzyme (300 μg/ml) was dialyzed sequentially against Buffer A with pH 7.5, 7.0, … to 4 containing $(NH_4)_2SO_4$ (30% saturation) for 12 h at 4°C against 200 ml of buffer and finally the enzyme was dialyzed against Buffer A.

Identification of cofactor by HPLC

1-NH (860 μg/ml) was incubated with trichloroacetic acid (5%) for 5 min on ice followed by centrifugation at 35,000×*g* at 4°C for 15 min. The supernatant was collected and subjected to HPLC analysis using solvent (methanol, 40% and 10 mM *ortho*-phosphoric acid, 60%) as described (Swetha et al. 2007). The eluent was identified by comparing the retention time to authentic FAD (retention time, 3.0 min) and FMN (retention time, 4.1 min).

Kinetic characterization

The kinetic constants of 1-NH were determined using enzyme (2 μg), NAD(P)H (250 μM), FAD (6.5 μM) and varying concentration of substrate (1–300 μM). The K_m, V_{max} and K_i with substrates were determined by using the equation, $V = V_{max}/[1 + (K_m/S) + (S/K_i)]$. The kinetic constants for NAD(P)H were determined at fixed concentration of 1-naphthol (50 μM) and varying concentration of NAD(P)H (5–330 μM). The K_m and V_{max} were determined by using the equation, $V = V_{max.}[S]/K_m + [S]$.

Chemical modification

1-NH (1–2.8 μM) was treated with diethylpyrocarbonate (DEPC, 15–125 μM), *N*-acetylimidazole (NAI, 5–200 mM) and *N*-ethylmaleimide (NEM, 1–20 mM) in KPi buffer (50 mM, pH 7). The modification of 1-NH with phenylglyoxal (PGO, 0.1–13 mM) was performed in bicarbonate buffer ($NaHCO_3$ 50 mM, pH 7. 5) and KPi buffer (50 mM, pH 8). The modification of the enzyme with 1,2-cyclohexanedione (1,2-CHD, 1–300 mM) was performed in KPi-borate buffer (K_2HPO_4 50 mM, H_3BO_3 50 mM, pH 8). Modification using *p*-chloromercuribenzoate (*p*-CMB, 0.1–20 μM) was carried out in KPi buffer (50 mM, pH 8.5). All the reactions were performed at 30°C in dark. 1-NH was treated with NBS (15–100 μM) in KPi buffer (50 mM, pH 5) containing FAD (6.5 μM) at 4°C in dark. All chemical modifiers were prepared freshly in the corresponding modification buffer. The enzyme in the respective modification buffer was treated with

varying concentrations of modifiers at specific time intervals, aliquots (10–20 µl each) were drawn and residual activity was monitored. Control reactions were performed under identical condition but without modifier. The protection of 1-NH against inactivation by chemical modifier was carried out in the presence of 1-naphthol, its analogs, NAD(P)H, $NAD(P)^+$ or 1,2-DHN.

Results

Purification of 1-naphthol 2-hydroxylase

1-NH was purified to homogeneity up to 22-fold with 15.5% yield and the specific activity of 6.6 µmole min^{-1} mg^{-1} of protein with NADH. The summary of purification is shown in Table 1. Enzyme was eluted as a single activity and protein peak from gel filtration column. The native molecular mass of 1-NH was determined to be 130 kDa (Fig. 1a). Using SDS-PAGE, the subunit molecular weight was determined to be 66 kDa (Fig. 1b). 1-NH exhibited maximum activity at pH 8.5 and found to be stable at 4°C as well as at −20°C for at least a month without any loss of activity. The NH_2-terminal sequence was determined to be Q L A/E N I F L A N E I.

Spectral properties

Purified 1-NH was yellow in color. UV–visible spectrum showed absorption maxima at 274, 375 and 445 nm (Fig. 2a). In presence of sodium dithionite (1–2 mg), the enzyme lost its absorption at 445 nm (Fig. 2a). The fluorescence emission spectrum of 1-NH showed maximum intensity at 525 nm when excited at 450 nm (Fig. 2b).

Cofactor requirement

1-NH failed to oxidize NAD(P)H in the absence of 1-naphthol suggesting that the enzyme was free from nonspecific NAD(P)H oxidase activity. Enzyme showed comparable activity with NADH or NADPH, however, with FAD it showed ~20% more activity as compared to FMN (data not shown). Apoenzyme prepared by acid-ammonium sulfate method retained 5% activity as compared to non dialyzed enzyme. Reconstitution of apoenzyme with FAD leads to increase in the activity up to 50% as compared to FMN (10%). HPLC analysis of flavin moiety extracted from 1-NH gave the retention time of 3 min which was similar to that of authentic FAD (3 min) as compared to FMN (4.1 min).

Stoichiometry and substrate specificity

The ratio of NADH oxidation to oxygen consumption under identical condition was determined to be 0.86 suggesting that the enzyme is an oxygenase and oxidizes one mole of NADH per mole of molecular oxygen consumed. The enzyme showed maximum activity with 1-naphthol (100%) as compared to 5-amino 1-naphthol (49%) and 4-chloro 1-naphthol (35%). The enzyme showed marginal activity (3–9%) with 2-naphthol, 2-naphthaldehyde, 2-naphthoic acid, 1-naphthoic acid, 3-hydroxy 2-naphthoic acid, 1-hydroxy 2-naphthoic acid, 2-hydroxy 1-naphthoic acid, naphthalene, 2-naphthylamine, salicylic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid and protocatechuic acid.

Table 1 Purification of 1-naphthol 2-hydroxylase from *Pseudomonas* sp. strain C6

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)[b]
Cell extract	73	440	155	0.3	1	100
Q-Sepharose	33	22	70	3	10	45
Phenyl-Sepharose	15	5.3	31	5.8	19	20
Sephacryl S-200	12	3.6	24	6.6	22	15.5

[a] One unit (U) is the amount of enzyme that catalyzes the oxidation of 1 µmole of NADH min^{-1}

[b] The best purification profile/data is presented here. The observed variation is in the range of ±5–7%

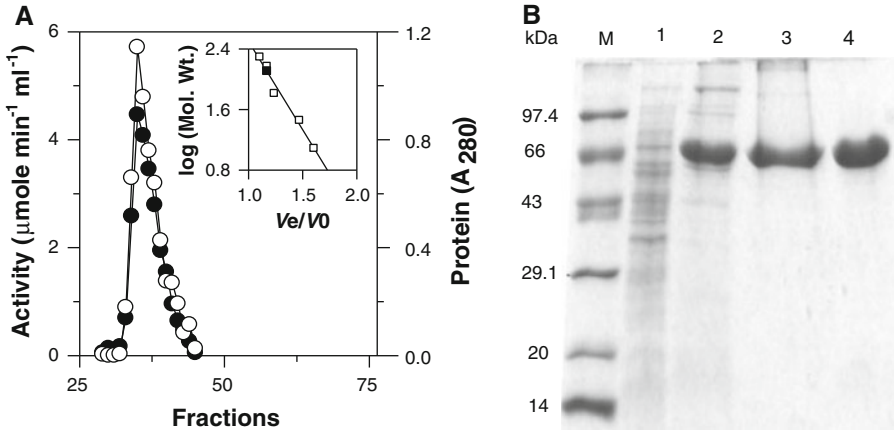

Fig. 1 **a** Protein elution (*open circle*) and activity (*filled circle*) profile of 1-naphthol 2-hydroxylase from Sephacryl S-200-HR gel filtration column. *Inset* plot of log (Mol. Wt.) versus V_e/V_0: Std. Mol. wt. protein markers (kDa, *open square*) are α-amylase (200), alcohol dehydrogenase (150), BSA (66), carbonic anhydrase (29), and cytochrome c (12.4); and 1-NH (*filled square*). **b** SDS-PAGE analysis of 1-naphthol 2-hydroxylase at different steps of purification. Each lane contains 8 μg protein: *Lane M* Protein Mol. wt. markers (kDa): phosphorylase B (97.4), BSA (66), ovalbumin (43), carbonic anhydrase (29), soybean trypsin inhibitor (20.1) and lysozyme (14.3), *lane 1* cell-free extract, *lane 2* Q-Sepharose pool, *lane 3* Phenyl-Sepharose pool; *lane 4* Sephacryl S-200-HR pool

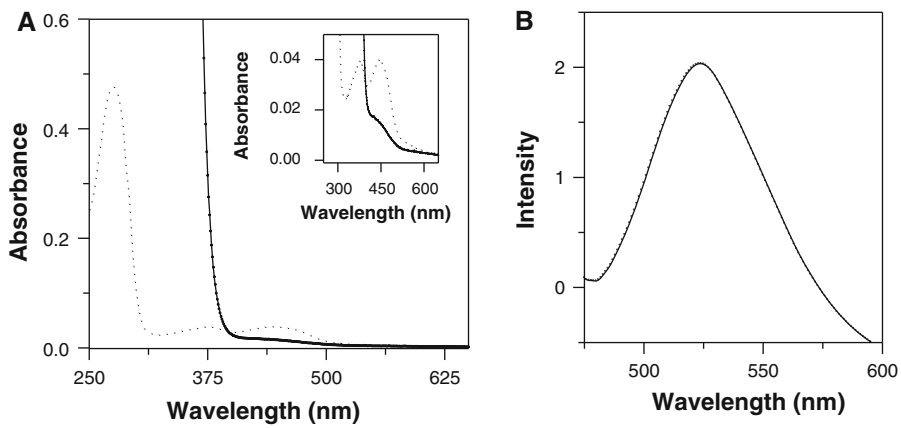

Fig. 2 Spectral properties of 1-naphthol 2-hydroxylase. **a** Absorption spectra of 1-NH (210 μg ml^{-1}) in absence (*dotted line*) and presence (*solid line*) of sodium dithionite (1–2 mg). *Inset* represents the magnified absorption spectra of 1-NH in the visible region. **b** Fluorescence emission spectrum of 1-NH

Kinetic constants

The initial reaction velocities of 1-NH with varying concentrations of 1-naphthol, NADH, or NADPH were determined spectrophotometrically. A representative substrate saturation plot for 1-naphthol using NADH as the electron donor is shown in Fig. 3. Increasing the concentration of 1-naphthol showed linear increase in the activity up to 20 μM. Further increase to 200 and 300 μM resulted in 15 and 30% decrease in the activity, respectively indicating the substrate inhibition (Table 2). Similar pattern of inhibition was observed with 5-amino 1-naphthol and 4-chloro 1-naphthol (data not shown). The kinetic constants derived for 1-NH are summarized in Table 2. Enzyme showed comparatively higher affinity for 1-naphthol with NADH (K_m, 9.1 μM) as compared to NADPH (16.5 μM), and almost similar affinity for NADH (142 μM) and NADPH (198 μM, Table 2).

Chemical modifications of 1-naphthol 2-hydroxylase

1-NH was inactivated with NEM and displayed pseudo-first-order kinetics (Fig. 4a). Under these

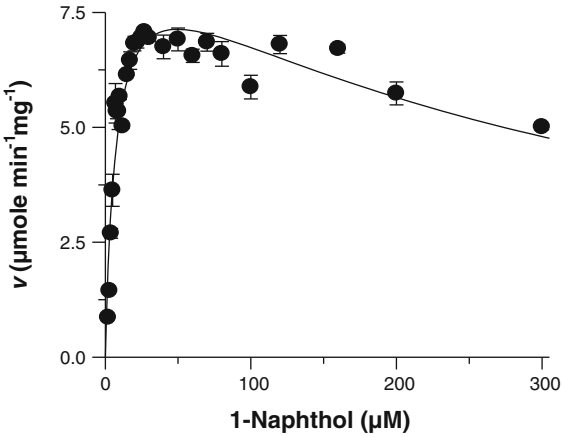

Fig. 3 Reaction velocity (v) versus [S] plot for 1-naphthol 2-hydroxylase. The reactions were performed using varying concentrations of 1-naphthol. The graph was fitted using the model for substrate inhibition (uncompetitive) with the following equation: $v = \{V_{max}/[1 + (K_m/S) + (S/K_i)]\}$

conditions, the second-order rate constant was determined to be 16.2 M^{-1} min^{-1} (Fig. 4a, Inset). The inactivation of the enzyme was protected with 1-naphthol depending upon the concentration used (Fig. 4b). The dissociation constant was determined to be 1.6 μM for 1-naphthol-1-NH complex from the slope (Fig. 4b, Inset). The summary of chemical modification of 1-NH with chemical modifiers is given in Table 3. The inactivation of 1-NH with *p*-CMB, DEPC, PGO, 1,2-CHD or NAI also displayed pseudo-first-order kinetics. The inactivation was much faster in *p*-CMB plus 1-naphthol than *p*-CMB plus NADH or *p*-CMB treated enzyme. However, in presence of DTT, *p*-CMB failed to inactivate enzyme. The protection of 1-NH against the inactivation by DEPC was maximum with 1-naphthol than 5-amino 1-naphthol and 4-chloro 1-naphthol. The co-substrates, NAD(P)H and products, $NAD(P)^+$, 1,2-DHN failed to protect the enzyme. The dissociation constant (K_{diss}) was determined to be 1.12 μM for 1-naphthol-1-NH complex. The absorption spectrum of DEPC inactivated 1-NH showed a maximum absorption at 235 nm, a characteristic of *N*-carbethoxyhistidyl derivative of His residue. In addition, very less broad trough appeared in the region of 300 nm (data not shown). Neither 1-naphthol nor NADH protected the enzyme from the inactivation incurred by PGO. The enzyme was protected with 1-naphthol but not with NADH against the inactivation incurred by 1,2-CHD. The enzyme was partially protected with 1-naphthol against the inactivation incurred by NAI. The activity was lost more than 90% within one min of treatment with NBS (100 μM). The inactivation of the enzyme was very rapid and failed to follow pseudo-first-order kinetics. NADH and 1-naphthol offered ~80 and 40% protection, respectively even after 25 min of incubation with NBS (100 μM).

Discussion

1-Naphthol 2-hydroxylase, a key enzyme in the carbaryl degradation pathway, hydroxylates 1-naphthol at *ortho* position to yield 1,2-dihydroxynaphthalene. 1-NH was purified to homogeneity with specific activity of 6.6 μmole min^{-1} mg^{-1} of protein with NADH from carbaryl-grown *Pseudomonas* sp. strain C6. The enzyme was found to be homodimer with subunit mol. wt. of 66 kDa. The NH_2-terminal sequence was found to be different from previously

Table 2 Kinetic properties of 1-naphthol 2-hydroxylase from *Pseudomonas* sp. strain C6

Condition	K_m (μM)	V_{max} (Umg^{-1})	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
1-Naphthol with NADH	9.1 ± 2.5	10.6 ± 1.4	283 ± 75	21.4 ± 2.6	2.4 ± 0.4
1-Naphthol with NADPH	16.5 ± 2.5	13.4 ± 2.9	237 ± 44	29.2 ± 6.4	1.7 ± 0.3
5-Amino 1-naphthol with NADH	6.4 ± 3.1	5.2 ± 0.4	135 ± 30	11.3 ± 1	1.9 ± 0.7
4-Chloro 1-naphthol with NADH	2.3 ± 0.2	2.8 ± 0.2	326 ± 169	6.0 ± 0.6	2.6 ± 0.05
NADH	142 ± 18	11 ± 1.4	–	23.9 ± 3.2	0.17 ± 0.03
NADPH	198 ± 24	13.4 ± 1.6	–	29 ± 3.5	0.14 ± 0.02

One unit (U) is the amount of enzyme that catalyzes the oxidation of 1 μmole of NAD(P)H min^{-1}. – Not detected. K_m, V_{max}, and K_i values for 1-naphthol, 5-amino 1-naphthol and 4-chloro 1-naphthol were derived by using a fixed concentration of NAD(P)H (250 μM). The kinetic constants for NADH and NADPH were determined by keeping the 1-naphthol concentration at 50 μM. The standard error was determined from at least 3 independent experiments

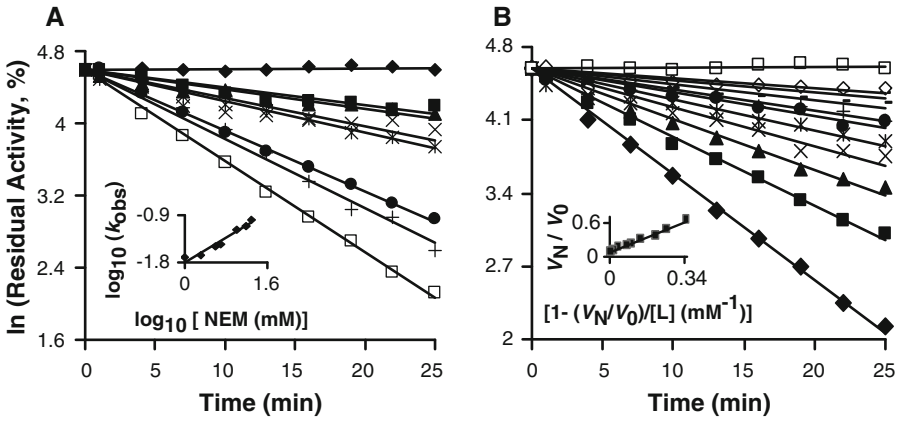

Fig. 4 **a** Semilogarithmic plot of time-dependent inactivation of 1-naphthol 2-hydroxylase as a function of NEM concentration and reaction order plots. 1-NH (1.7 μM) was incubated with NEM: 0 (*filled diamond*), 1 (*filled square*), 2 (*filled triangle*), 4 (*times*), 5 (*asterisk*), 10 (*filled circle*), 15 (*plus*) and 20 mM (*open square*). *Inset* The double logarithmic plot shows the linear relationship between apparent pseudo-first-order rate constant (k_{obs}) and NEM according to the method of Hollenberg et al. (1971). **b** Protection of 1-naphthol 2-hydroxylase with 1-naphthol against inactivation by NEM. 1-NH (1.7 μM) was incubated with NEM—0 (*open square*) and 20 mM (*filled diamond*); and NEM 20 mM plus varying concentration of 1-naphthol 1 (*filled square*), 2 (*filled triangle*), 3 (*times*), 5 (*asterisk*), 8 (*filled circle*), 10 (*plus*), 20 (*minus*), 50 (*n-dash*), 100 (*open diamond*) μM. *Inset* Plot for determination of the K_{diss} for the 1-NH-1-naphthol complex according to method of Scrutton and Utter (1965). V_N and V_0 are the pseudo-first-order-rate constants for inactivation of 1-NH in the presence and absence of 1-naphthol, respectively. [L] is the concentration of 1-naphthol

Table 3 Summary of chemical modification for 1-naphthol 2-hydroxylase

Amino Acid	Cys		His	Arg		Tyr	Trp
Modifiers	NEM	*p*-CMB	DEPC	PGO	1,2-CHD	NAI	NBS
Pseudo-first-order rate observed	Yes	Yes	Yes	Yes	Yes	Yes	No
Second-order rate constant (k_1, M^{-1} min^{-1})	16.2	3000	700	8[a] 19.5[b]	4.2	3.7	–
Protection by NADH/1-Naphthol	1-Naphthol	No	1-Naphthol	No	1-Naphthol	Partial with 1-Naphthol	1-Naphthol and NADH
No. of essential amino acid modified	0.6	0.7	1.1	0.8[a] 1[b]	0.5	0.7	–

Modification of 1-NH was performed in: [a] phosphate buffer (50 mM KPi, pH 8); [b] bicarbonate buffer (50 mM $NaHCO_3$, pH 7.5).
– Data not determined

reported 1-NH from *Pseudomonas* sp. strain C4 (Swetha et al. 2007), suggesting that both the enzymes are different. 1-NH from strain C6 as a FAD-dependent flavoenzyme was supported by: (i) UV–visible and fluorescence properties; (ii) preparation of apoenzyme followed by its reconstitution with FAD; (iii) extraction of flavin moiety and its identification as FAD by HPLC. The successful preparation of apoenzyme and its reconstitution with FAD for 1-NH from strain C6 suggest that apoenzyme is conformationally stable even in the absence of FAD. The apoenzyme preparation from strain C4 using various methods was successful, however the apoenzyme could not be activated by reconstituting FAD or FMN, suggesting the loss of enzyme conformation (Swetha et al. 2007). Flavin monooxygenases require external electron donor and accept electron from NADH, NADPH, or both (Balashova et al. 2001; Husain and Massey 1979; Suske et al. 1997; Swetha et al. 2007; Vanberkel and Vandentweel 1991). 1-NH from strain C6 showed comparable activity with NADH or NADPH, suggesting that it accepts electron

from both. In this respect, 1-NH from strain C6 is different than that from strain C4 which showed maximum activity with NADPH (Swetha et al. 2007). Purified enzyme showed stoichiometric (equimolar) consumption of oxygen and NADH. These molecular and biochemical properties suggest that 1-NH from strain C6 is a single-component flavin hydroxylase similar to others e.g. salicylate hydroxylase, *p*-hydroxybenzoate hydroxylase and 2-hydoxybiphenyl 3-monooxygenase (Balashova et al. 2001; Jadan et al. 2001; Suske et al. 1997). 1-NH showed comparable affinity for NADH and NADPH (Table 2). Enzyme showed higher affinity for 1-naphthol in presence of NADH (K_m, 9.1 μM) as compared to that in presence of NADPH (K_m, 16.5 μM); however, k_{cat} and k_{cat}/K_m values were comparable (Table 2). Enzyme also showed limited substrate specificity, accepting also 5-amino 1-naphthol and 4-chloro 1-naphthol as substrates. Inability to accept mono aromatic compounds like benzoate, hydroxybenzoate; or diaromatic compounds like 2-naphthol and other derivatives suggests that, to act as the substrate, compound should be diaromatic with hydroxyl group at 1 position. 1-Naphthol with substitution at *para* position can be accepted as substrate by the enzyme but with significantly lower activity. Similar to other reported monooxygenases, 1-NH from strain C6 showed substrate inhibition phenomenon. The kinetic properties (Table 2) are different than those reported for 1-NH from strain C4 (Swetha et al. 2007).

To identify various amino acids at the active site, classical chemical modification and protection approach was followed. Results obtained suggest the involvement of Cys, His, Arg, Tyr and Trp at or near the active site of the enzyme (Table 3). Involvement of these residues in the catalysis or substrate binding site has been reported from various aromatic flavin hydroxylases (Ballou et al. 2005; Sumathi and Dasgupta 2000; Vanderbolt et al. 1994). Inactivation of 1-NH by Cys, His, Arg and Tyr modifiers and its protection in the presence of 1-naphthol suggests possible interaction of these amino acids with the substrate, 1-naphthol at the active site. The probable interaction could be proton transfer network which makes overall negative charge at C2 carbon of the substrate. As a result, one oxygen atom of the flavin C4a-hydroperoxide intermediate is introduced into the C2 carbon of 1-naphthol or its analogs by electrophilic aromatic substitution. Such interaction has been reported from aromatic hydroxylases such as phenol hydroxylase, 3-hydroxybenzoate hydroxylase and *p*-hydroxybenzoate hydroxylase (Ballou et al. 2005; Enroth et al. 1998; Hiromoto et al. 2006). In *p*-hydroxybenzoate hydroxylase, structure–function relationship studies suggest that Tyr222 and Arg220 are responsible for 'in' and 'out' conformation of flavin during the catalytic process (Moran et al. 1996; Palfey et al. 1997). Besides that, Tyr385 and His72 are implicated in the proton transfer network (Palfey et al. 1997; Schreuder et al. 1994). Rapid inactivation of 1-NH with *p*-CMB in the presence of 1-naphthol as compared to NEM could be due ability of the aromatic modifier to compete with aromatic substrate, 1-naphthol or increased susceptibility of Cys due to change in the conformation of the enzyme when bound to 1-naphthol. However, in presence of 1-naphthol, enzyme is protected from NEM modification suggests that Cys is probably at the substrate binding site. Similar role was postulated for Cys in *p*-hydroxybenzoate hydroxylase (Vanderbolt et al. 1994). The inactivation of 1-NH with NBS did not display pseudo-first-order kinetics. Similarly, the inactivation of 3-HBA-6-hydroxylase with NBS did not follow the pseudo-first-order kinetics (Sumathi and Dasgupta 2000). The inactivation of enzyme was protected by NADH and 1-naphthol, suggesting Trp residues at or near the active site, interacting (hydrophobic or stacking interaction) with NADH and 1-naphthol.

Conclusion

1-NH was purified to homogeneity from carbaryl-degrading *Pseudomonas* sp. strain C6. Biochemical properties suggest that enzyme is a FAD-dependent aromatic hydroxylase. As compared to 1-NH from strain C4, the NH_2-terminal sequence of 1-NH from strain C6 was different, the enzyme showed higher K_i for 1-naphthol, higher K_m for NAD(P)H, significantly lower activity on 4-chloro 1-naphthol and the apoenzyme could be reconstituted to active enzyme with FAD. These results suggest that both enzymes are different. Amino acid modification and protection studies suggest the participation of His, Arg, Cys, Tyr and Trp at or near the catalytic site of 1-NH of strain C6.

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References

Balashova NV, Stolz A, Knackmuss HJ, Kosheleva IA, Naumov AV, Boronin AM (2001) Purification and characterization of a salicylate hydroxylase involved in 1-hydroxy-2-naphthoic acid hydroxylation from the naphthalene and phenanthrene-degrading bacterial strain *Pseudomonas putida* BS202–P1. Biodegradation 12:179–188

Ballou DP, Entsch B, Cole LJ (2005) Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases. Biochem Biophys Res Commun 338:590–598

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254

Chapalamadugu S, Chaudhry GR (1991) Hydrolysis of carbaryl by a *Pseudomonas* sp and construction of a microbial consortium that completely metabolizes carbaryl. Appl Environ Microbiol 57:744–750

Doddamani HP, Ninnekar HZ (2001) Biodegradation of carbaryl by a *Micrococcus* species. Curr Microbiol 43:69–73

Elespuru R, Lijinsky W, Setlow JK (1974) Nitrosocarbaryl as a potent mutagen of environmental significance. Nature 247:386–387

Elmorsi EA, Hopper DJ (1977) Purification and properties of 4-hydroxyisophthalate hydroxylase from *Pseudomonas putida* NCIB-9866. Eur J Biochem 76:197–208

Enroth C, Neujahr H, Schneider G, Lindqvist Y (1998) The crystal structure of phenol hydroxylase in complex with FAD and phenol provides evidence for a concerted conformational change in the enzyme and its cofactor during catalysis. Structure 6:605–617

Gatti DL, Palfey BA, Lah MS, Entsch B, Massey V, Ballou DP, Ludwig ML (1994) The mobile flavin of 4–OH benzoate hydroxylase. Science 266:110–114

Hayatsu M, Hirano M, Nagata T (1999) Involvement of two plasmids in the degradation of carbaryl by *Arthrobacter* sp. strain RC100. Appl Environ Microbiol 65:1015–1019

Hiromoto T, Fujiwara S, Hosokawa K, Yamaguchi H (2006) Crystal structure of 3-hydroxybenzoate hydroxylase from *Comamonas testosteroni* has a large tunnel for substrate and oxygen access to the active site. J Mol Biol 364: 878–896

Hollenberg PF, Flashner M, Coon MJ (1971) Role of lysyl epsilon-amino groups in adenosine diphosphate binding and catalytic activity of pyruvate kinase. J Biol Chem 246:946–953

Husain M, Massey V (1979) Kinetic studies on the reaction of *p*-hydroxybenzoate hydroxylase—agreement of steady-state and rapid reaction data. J Biol Chem 254:6657–6666

Jadan AP, van Berkel WJH, Golovleva LA, Golovlev EL (2001) Purification and properties of *p*-hydroxybenzoate hydroxylases from *Rhodococcus* strains. Biochemistry-Moscow 66:898–903

Kaloyanova F, Simeonova PP (1994) International programme on chemical safety, environmental health criteria, vol. 153. United Nations Environment Programme, International Labour Organisation and World Health Organization, Geneva

Larkin MJ, Day MJ (1986) The metabolism of carbaryl by three bacterial Isolates, *Pseudomonas* sp. (NCIB-12042 and NCIB-12043) and *Rhodococcus* sp. (NCIB-12038) from garden soil. J Appl Bacteriol 60:233–242

Moran GR, Entsch B, Palfey BA, Ballou DP (1996) Evidence for flavin movement in the function of *p*-hydroxybenzoate hydroxylase from studies of the mutant Arg220Lys. Biochemistry 35:9278–9285

Neujahr HY, Kjellen KG (1980) Phenol hydroxylase from Yeast—A lysyl residue essential for binding of reduced nicotinamide adenine-dinucleotide phosphate. Biochemistry 19:4967–4972

Obulakondaiah M, Sreenivasulu C, Venkateswarlu K (1993) Nontarget effects of carbaryl and its hydrolysis product, 1-naphthol, towards *Anabaena torulosa*. Biochem Mol Biol Int 29:703–710

Palfey BA, Ballou DP, Massey V (1997) Flavin conformational changes in the catalytic cycle of *p*-hydroxybenzoate hydroxylase substituted with 6-azido- and 6-aminoflavin adenine dinucleotide. Biochemistry 36:15713–15723

Palfey BA, Basu R, Frederick KK, Entsch B, Ballou DP (2002) Role of protein flexibility in the catalytic cycle of *p*-hydroxybenzoate hydroxylase elucidated by the Pro293Ser mutant. Biochemistry 41:8438–8446

Rickard RW, Dorough HW (1984) Invivo formation of nitrosocarbamates in the stomach of rats and guinea-pigs. J Toxicol Environ Health 14:279–290

Schreuder HA, Mattevi A, Obmolova G, Kalk KH, Hol WGJ, Vanderbolt FJT, Vanberkel WJH (1994) Crystal-structures of wild-type p-hydroxybenzoate hydroxylase complexed with 4-aminobenzoate, 2, 4-dihydroxybenzoate, and 2-hydroxy-4-aminobenzoate and of the Tyr222Ala mutant complexed with 2-hydroxy-4-aminobenzoate - evidence for a proton channel and a new binding mode of the flavin ring. Biochemistry 33:10161–10170

Scrutton MC, Utter MF (1965) Pyruvate carboxylase: V. Interaction of enzyme with adenosine triphosphate. J Biol Chem 240:3714–3723

Shea TB, Berry ES (1983) Toxicity and intracellular-localization of carbaryl and 1-naphthol in cell-cultures derived from goldfish. B Environ Contam Toxicol 30:99–104

Shoun H (1990) *p*-Hydroxybenzoate hydroxylase from *Pseudomonas desmolytica*—relative molecular mass, amino-acid-composition, and reactivity of sulfhydryl-groups. Agr Biol Chem 54:329–336

Shoun H, Beppu T (1982) A histidine residue in *p*-hydroxybenzoate hydroxylase essential for binding of reduced nicotinamide adenine-dinucleotide phosphate. J Biol Chem 257:3422–3428

Shoun H, Beppu T, Arima K (1980) An essential arginine residue at the substrate-binding site of *p*-hydroxybenzoate hydroxylase. J Biol Chem 255:9319–9324

Sumathi S, Dasgupta D (2000) Reactivity of 3-HBA-6-hydroxylase with diethylpyrocarbonate and *N*-bromosuccinimide: effect of chemical modifications on kinetic and spectral properties of the enzyme. Biotechnol Prog 16: 577–582

Suske WA, Held M, Schmid A, Fleischmann T, Wubbolts MG, Kohler HPE (1997) Purification and characterization of 2-hydroxybiphenyl 3-monooxygenase, a novel NADH-dependent, FAD-containing aromatic hydroxylase from *Pseudomonas azelaica* HBP1. J Biol Chem 272: 24257–24265

Swetha VP, Phale PS (2005) Metabolism of carbaryl via 1, 2-dihydroxynaphthalene by soil isolates *Pseudomonas* sp. strains C4, C5, and C6. Appl Environ Microbiol 71: 5951–5956

Swetha VP, Basu A, Phale PS (2007) Purification and characterization of 1-naphthol-2-hydroxylase from carbaryl-degrading *Pseudomonas* strain C4. J Bacteriol 189: 2660–2666

van Berkel WJH, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J Bacteriol 124:670–689

Vanberkel WJH, Vandentweel WJJ (1991) Purification and characterization of 3-hydroxyphenylacetate 6-hydroxylase—a novel FAD-dependent monooxygenase from a *Flavobacterium* species. Eur J Biochem 201:585–592

Vanberkel WJH, Muller F, Jekel PA, Weijer WJ, Schreuder HA, Wierenga RK (1988) Chemical modification of tyrosine-38 in p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* by 5'-p-fluorosulfonylbenzoyladenosine—A probe for the elucidation of the NADPH binding-site—Involvement in catalysis, assignment in sequence and fitting to the tertiary structurer. Eur J Biochem 176:449–459

Vanderbolt FJT, Drijfhout MC, Eppink MHM, Hagen WR, Vanberkel WJH (1994) Selective cysteine-serine replacements in *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* allow the unambiguous assignment of Cys211 as the site of modification by spin-labeled *p*-chloromercuribenzoate. Protein Eng 7:801–804

Wilson GD, Doherty MD, Cohen GM (1985) Selective toxicity of 1-naphthol to human colorectal tumor-tissue. Brit J Cancer 51:853–863