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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

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Abstract

1-Hydroxy-2-naphthoate is formed as an intermediate in the bacterial degradation of phenanthrene. A monooxygenase which catalyzed the oxidation of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene was purified from the phenanthrene- and naphthalene-degrading *Pseudomonas putida* strain BS202-P1. The purified protein had a molecular weight of 45 kDa and required NAD(P)H and FAD as cofactors. The purified enzyme also catalysed the oxidation of salicylate and various substituted salicylates. The comparison of the K_m and V_{max} values for 1-hydroxy-2-naphthoate and salicylate demonstrated a higher catalytic efficiency of the enzyme for salicylate as a substrate. A significant substrate-inhibition was detected with higher concentrations of 1-hydroxy-2-naphthoate. The aminoterminal amino acid sequence of the purified enzyme showed significant homologies to salicylate 1-monooxygenases from other Gram negative bacteria. It was therefore concluded that during the degradation of phenanthrene the conversion of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene is catalysed by a salicylate 1-monooxygenase. Together with previous studies, this suggested that the enzymes of the naphthalene pathway are sufficient to catalyse also the mineralization of phenanthrene.

Introduction

Certain bacteria utilize polycyclic aromatic hydrocarbons (PAHs), including phenanthrene, as a sole source of carbon and energy. The initial steps of phenanthrene degradation lead to the formation of 1-hydroxy-2-naphthoate (Kiyohara & Nagao 1978; Stringfellow & Aitken 1994; Evans et al. 1965; Kiyohara et al. 1976) and might be catalysed by the same enzymes which also convert naphthalene to salicylate (Menn et al. 1993; Kiyohara et al. 1994). The 1-hydroxy-2-naphthoate which is formed from phenanthrene is further metabolized by two different pathways, either through o-phthalate and protocatechuate (Kiyohara et al. 1976) or via salicylate and catechol (Kiyohara & Nagao 1978; Evans et al. 1965) (Figure 1).

The initial enzyme involved in the conversion of 1-hydroxy-2-naphthoate to protocatechuate is a 1-hydroxy-2-naphthoate 1,2-dioxygenase which cleaves 1-hydroxy-2-naphthoate to 2-carboxybenzopyruvate. This enzyme has been previously described and characterized in *Aeromonas* sp. s45pl and *Nocardioides* sp. (Kiyohara et al. 1976; Iwabuchi & Harayama 1998). The amino acid sequence of this enzyme differs from those of other ring-cleaving dioxygenases that oxidize double hydroxylated aromatic rings (Iwabuchi & Harayama 1998).

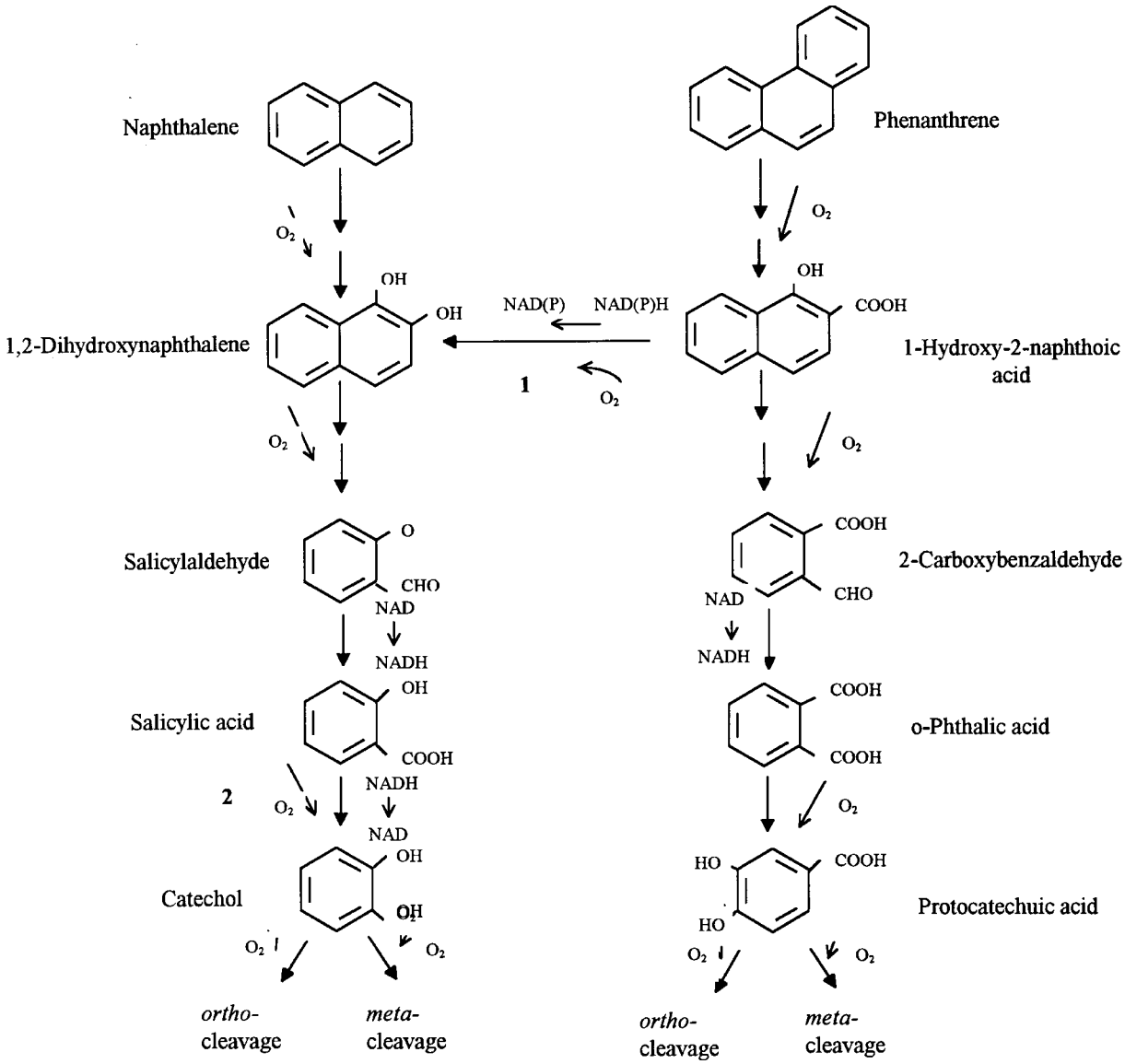


Figure 1. Phenanthrene and naphthalene degradation pathways (Kiyohara & Nagao, 1978). (1) 1-Hydroxy-2-naphthoate hydroxylase, (2) salicylate hydroxylase.

In contrast to the situation for the direct ring-fission of 1-hydroxy-2-naphthoate, the enzyme which catalysis the oxidative decarboxylation of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene has not been characterized previously. To find out the enzyme which is responsible for this reaction, we have chosen to study the metabolism of 1-hydroxy-2-naphthoate by the phenanthrene-degrading strain *Pseudomonas putida* BS202-P1.

Methods

Media and growth conditions

Pseudomonas putida strain BS202-P1 was cultivated at 30 °C on a rotary shaker (150 rpm) in 500-ml Erlenmeyer flasks containing 200 ml of the mineral-salt Evans medium (Evans et al. 1970). Naphthalene and phenanthrene were added to liquid media as crystals to final concentrations of 1 and 0.2 g l^{-1}, respectively. 1-Hydroxy-2-naphthoate and salicylate were added as growth substrates to final concentra-

tions of 0.5 and 1 g l^{-1}, respectively. Growth with 1-hydroxy-2-naphthoate for enzyme purifications was performed at 30 °C in a "1601 Ultroferm" fermenter (7 l working volume, LKB, Sweden). The fermenter was oxygenated by air (agitation at 250 rpm).

Cells used in induction experiments were grown in liquid mineral salt medium containing 1 g l^{-1} succinate. In the early exponential growth phase salicylate and 1-hydroxy-2-naphthoate were added at 0.1 g l^{-1}, respectively, and the bacteria grown for another two hours, before the cells were harvested.

Preparation of cell extracts

Cells were harvested by centrifugation at 4 °C, washed first with 0.5 M NaCl, then with 80 mM NaCl and finally with 20 mM KH_2PO_4 buffer, pH 7.5. Approximately 6 g (wet weight) of cell paste was suspended in 10 ml of 20 mM KH_2PO_4 buffer, pH 7.5. Cells were lysed using a IBPM-press (Russia) at 7 mPa and stored at −40 °C until use. Cell debris was removed by centrifugation at 100,000 × g for 90 min at 4 °C. The supernatant solutions were passed through 0.22 μm-pore-size filters and were used as a crude cell extract for enzyme purification or enzyme assays.

Enzyme assay

All enzymes assays were performed at 30 °C using a UVIKON-spectrophotometer 810P (Kontron Instruments, Germany). One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min. Catechol 1,2- and 2,3-dioxygenase, 2-carboxybenzaldehyde dehydrogenase, 1-hydroxy-2-naphthoate dioxygenase, and protocatechuate 3,4- and 4,5-dioxygenase activities were determined as described previously (Hegeman 1966; Feist & Hegeman 1969; Kiyohara et al. 1976; Fujisawa & Hayaishi 1968; Ono et al. 1970).

Salicylate and 1-hydroxy-2-naphthoate hydroxylase activities were measured at 340 nm by a modification of the salicylate hydroxylase assay described by Yamamoto et al. (Yamamoto et al. 1965). The reaction mixture (1 ml) contained 100 μM NADH, 100 μl cell extract, 20 mM KH_2PO_4 (pH 7.5) and 50 μM salicylate or 1-hydroxy-2-naphthoate, respectively. The extinction coefficient used to determine the reaction of salicylate was 6.22 mM^{-1} cm^{-1}.

NADH and 1-hydroxy-2-naphthoate both show significant absorbance at 340 nm. The molar extinction coefficient for 1-hydroxy-2-naphthoate at 340 nm was determined as 5.08 mM^{-1} cm^{-1}. Therefore, a molar reaction coefficient at 340 nm of 11.3 mM^{-1} cm^{-1} was used to calculate the reaction of 1-hydroxy-2-naphthoate and NADH to 1,2-dihydroxynaphthalene and NAD.

For the determination of the pH optimum of the enzyme, the standard spectrophotometric assay was used and the pH values of the phosphate buffers were varied from 6.0 to 8.0.

Protein was determined by the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

Protein purification

All purification procedures were carried out by using a FPLC system (UV-1 control unit, P-500 conductivity monitor, LCC-501 Plus controller, LKB REC 102 recorder, FRAC-100 collector, UV-1 optical unit, Pharmacia). Crude cell extract was loaded onto a Pharmacia Resource Q column (bed volume 6 ml, 16 × 30 mm). Proteins were eluted with a linear KH_2PO_4 (pH 7.5) gradient (20 mM to 200 mM in buffer with 10% (v/v) glycerol). The most active fractions (eluting at 80 mM KH_2PO_4) containing salicylate hydroxylase and 1-hydroxy-2-naphthoate hydroxylase activity were pooled and chromatographed on a Pharmacia Mono Q HR 5/5 column (bed volume 1 ml, 5 × 50 mm) with a linear KH_2PO_4 (pH 7.5) gradient (20 mM to 200 mM phosphate buffer with 10% (v/v) glycerol). The fractions with salicylate hydroxylase and 1-hydroxy-2-naphthoate hydroxylase activities (activity peak at 60 mM KH_2PO_4) were pooled and were subjected to chromatography on a Pharmacia Superdex 75 Prep-Grade gel filtration column XK16/60 (bed volume 120 ml, 1,6 × 60 cm). Proteins were eluted with 50 mM KH_2PO_4, pH 7.5.

The molecular mass of the protein was measured by gel filtration chromatography on a Pharmacia Superdex 75 Prep-Grade gel filtration column XK16/60 (bed volume 120 ml, 1.6 × 60 cm). The molecular masses of the standards used for gel filtration chromatography were 160 kDa (aldolase), 45 kDa (ovalbumin) and 17.8 kDa (myoglobin) (Serva).

SDS-polyacrylamide gel electrophoresis

The purity of protein preparations was evaluated and molecular mass of the subunits was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. It was performed by a modified Laemmli

(Laemmli 1970) procedure on 0.5-mm-thick, 7 cm-long vertical slab gels with 12% acrylamide in the separation gel. The gels were stained with a Bio Rad silver-staining kit (Pharmacia) by a modification of the method of Merril et al. (Merril et al. 1981).

Identification of products

The reaction products were analysed by using an HPLC system which consisted of a L-4200 UV-Vis detector, L-6000 pump, a D-2000 chromato-integrator (Merck Hitachi) and a Grom SIL 100-Octyl-4 FE5mm reverse phase (4.6 mm × 12.5 cm) column (Grom, Herrenberg, Germany). Reaction mixtures (1 ml) contained 20 mM KH_2PO_4 (pH 7.5), 100 μM NADH, and 50 μM salicylate or 50 μM 1-hydroxy-2-naphthoate, respectively. The reactions were started by the addition of 100 μg of purified enzyme. Reactions were stopped by acidification with 100 mM H_3PO_4. Precipitated protein was removed by centrifugation. The solvent system for the analysis of salicylate (retention volume 14.6 ml) and catechol (retention volume 2.7 ml) consisted of 85% water, 15% methanol and 0.2% H_3PO_4. The solvent system for the analysis of 1-hydroxy-2-naphthoate (retention volume 11.1 ml), 1,2-dihydroxynaphthalene (retention volume 13.8 ml) and 1,2-naphthoquinone (retention volume 15.1 ml) consisted of 80% water, 20% methanol and 0.2% H_3PO_4. The flow rate was 1 ml min^{-1} and the absorbance was monitored at 210 nm.

Sequencing

The aminoterminal amino acid sequence was determined by automated Edman degradation using a model 470A gas-phase sequencer (Applied Biosystems) in accordance with the manufacturer's instructions. The BLAST network services at the National Center for Biotechnology Information (Bethesda, Md.) was used to search protein databases for similar sequences.

Determination of kinetic parameters

The kinetic values were either determined at a constant NADH concentration (100 μM) and varying concentrations of salicylate or 1-hydroxy-2-naphthoate (0.1 μM − 1 mM) or with a constant concentration of salicylate or 1-hydroxy-2-naphthoate (100 μM) and different NADH concentrations (10 μM to 150 μM). The K_m, $V_{\max}$ and K_i values were calculated by nonlinear regression analysis using the Enzfitter programm (Vertion 1.05, Elsevier-Biosoft, 1987, Cambridge, UK).

Results

Phenanthrene and naphthalene degradation by Pseudomonas putida BS202-P1

The wild-type of *P. putida* BS202 was isolated from oil-contaminated soil samples after enrichment with naphthalene and could grow on naphthalene and salicylate but was unable to grow on phenanthrene and 1-hydroxy-2-naphthoate (Borisoglebskaya & Boronin 1983). However, after incubation of this strain for a period of one month in liquid culture with phenanthrene, the mutant strain BS202-P1 was isolated. It simultaneously acquired the ability to grow on phenanthrene and 1-hydroxy-2-naphthoate as sole source of carbon and energy (Balashova et al. 1999).

To test which metabolic pathway the mutant strain utilized to degrade phenanthrene, several key enzymes for the degradation of aromatic compounds were determined in cell extracts after growth of the strain on different carbon sources. Thus, enzyme activities for the oxidation of catechol and for the oxidation of NADH in the presence of salicylate or 1-hydroxy-2-naphthoate were detected in the cultures grown on phenanthrene or naphthalene (Table 1). In contrast, no o-carboxybenzaldehyde dehydrogenase, 1-hydroxy-2-naphthoate dioxygenase, protocatechuate 3,4- and 4,5-dioxygenase activities were found in these samples (Table 1). This suggested that phenanthrene was degraded via 1-hydroxy-2-naphthoate and 1,2-dihydroxynaphthalene and further by the enzymes of the naphthalene degradative pathway.

Induction experiments

The similarity of the salicylate 1-monooxygenase reaction and the reaction proposed for 1-hydroxy-2-naphthoate conversion (Figure 2) and the joint induction of both activities during growth on naphthalene and phenanthrene suggested that both reactions could be catalysed by the same enzyme. Therefore, the induction of salicylate hydroxylase and 1-hydroxy-2-naphthoate hydroxylase activities was compared after growth on 1-hydroxy-2-naphthoate, salicylate or after the addition of salicylate (0.1 g l^{-1}) to succinate-grown cells. Both salicylate and 1-hydroxy-2-naphthoate hydroxylase activities were detected in crude extract of salicylate and 1-hydroxy-2-

Table 1. Enzyme activities found in cell extracts of *P. putida* BS202-P1 after growth on different carbon sources. SH – salicylate hydroxylase, HH – 1-hydroxy-2-naphthoate hydroxylase, C12O – catechol 1,2-dioxygenase, C23O – catechol 2,3-dioxygenase, 2CBAD – 2-carboxybenzaldehyde dehydrogenase, HO – 1-hydroxy-2-naphthoate dioxygenase, P34O – protocatechuate 3,4-dioxygenase, P45O – protocatechuate 4,5-dioxygenase

Growth substrate	Specific activity (μmol min^{-1} mg^{-1} of protein)							
	SH	HH	C12O	C23O	2CBAD	HO	P45O	P34O
phenanthrene	0.14	0.03	0.04	0.1	–[1]	–	–	–
naphthalene	0.12	0.04	0.02	–	–	–	–	–
1-hydroxy-2-naphthoate	0.11	0.05	0.01	0.06	–	–	–	–
salicylate	0.2	0.07	0.01	0.04	–	–	–	–
succinate	–	–	–	0.04	–	–	–	–
succinate + salicylate (0.1 g l^{-1})	0.1	0.04	ND[2]	ND	ND	ND	ND	ND
succinate + 1-hydroxy-2-naphthoate (0.1 g l^{-1})	–	–	ND	ND	ND	ND	ND	ND

[1] "–" – activity smaller than 0.005.
[2] ND – not determined.

naphthoate grown and salicylate induced cells. Thus, salicylate served as a common inductor of salicylate and 1-hydroxy-2-naphthoate hydroxylase activities (Table 1). No activities for the salicylate or 1-hydroxy-2-naphthoate dependent oxidation of NADH were detected in crude extracts of succinate grown cells.

Cofactor requirements

Many monooxygenases contain flavins in their catalytic center (Yamamoto et al. 1965; Beadle & Smith 1982). It was therefore tested, if the salicylate 1-monooxygenase from strain BS202-P1 also required flavins for activity. Thus, 1 ml of crude extract (4 mg of protein) was dialyzed at 4 °C against 5 l of 20 mM KH_2PO_4 buffer (pH 7.5). The preparation lost about half of its salicylate and 1-hydroxy-2-naphthoate hydroxylase activities after five days of dialysis. Then, FAD, FMN or riboflavin (2 μM each) were added to aliquots of the dialysed cell extracts and the salicylate and 1-hydroxy-2-naphthoate hydroxylase activities were tested after one hour of incubation with the flavins. The addition of FAD resulted in a complete recovery of both enzyme activities. In contrast, no effect was found after the addition of FMN or riboflavin. The effects of different FAD concentrations (0.5–4 μM) were compared and a maximal recovery of the enzyme activity was found after the addition of 1.5 μM FAD.

It was tested if NADH could be replaced by NADPH as cofactor for the conversion of salicylate and 1-hydroxy-2-naphthoate. In the concentration range tested (50–100 μM), NADH was the preferred donor of reduction equivalents in respect to both salicylate and 1-hydroxy-2-naphthoate. Only 50% activity for salicylate and 1-hydroxy-2-naphthoate was detected when NADPH replaced NADH as cofactor.

Purification of the enzyme

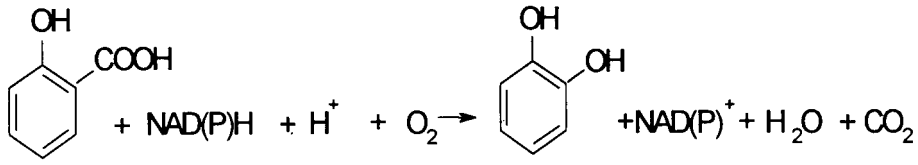
The results shown above suggested that salicylate 1-monooxygenase might be responsible for the conversion of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene in strain BS202-P1 grown on phenanthrene. To prove this hypothesis, the salicylate 1-monooxygenase was further characterized and purified.

Initial studies of salicylate and 1-hydroxy-2-naphthoate hydroxylase activities with crude extracts from cells grown on phenanthrene, salicylate or 1-hydroxy-2-naphthoate showed that both activities were always present with similar relative activities. Because growth on phenanthrene was very slow and only low amounts of biomass could be produced, a crude extract from 1-hydroxy-2-naphthoate grown cells was used for enzyme purification.

Crude extracts from 1-hydroxy-2-naphthoate grown cells were fractionated by anion-exchange chromatography and enzyme activities with 1-hydroxy-2-naphthoate and salicylate were tested in the presence of NADH. It was shown that the enzyme activities for both substrates were found in the same fractions. Therefore, the enzyme was purified further. The complete purification confirmed the presence of a single enzyme responsible for the conversion of both salicylate and 1-hydroxy-2-naphthoate.

A typical purification procedure is shown in Table 2. The enzyme was purified approximately 25-fold in three chromatographic steps. Since the enzyme gradually lost FAD during the purification, it was

A



B

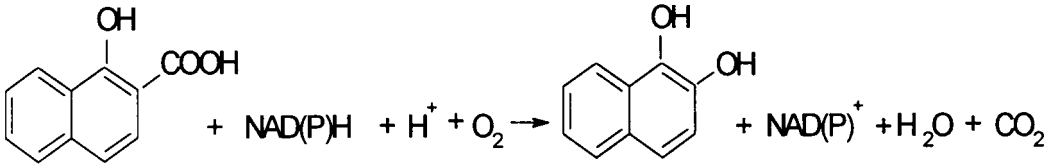


Figure 2. Conversion of salicylate (A) and 1-hydroxy-2-naphthoate (B) by salicylate hydroxylase from *P. putida* BS202P-1.

routinely reactivated by addition of FAD (1.5 μM) after every purification step. This resulted in a preparation that gave a single band upon SDS-polyacrylamide gel electrophoresis. The molecular mass of the holoenzyme was determined by SDS-polyacrylamide gel electrophoresis and gel-filtration was 45 kDa. Thus the enzyme appears to be catalytically active as a monomer.

Catalytic properties

The pH optimum of the purified salicylate and 1-hydroxy-2-naphthoate hydroxylase was 7.0–7.5. Activities at pH 6.0 and 8.0 were 65 and 96%, respectively, of the maximal activity.

The temperature optimum for the hydroxylase activity was 30 °C. The enzyme activities when assayed at 55, 50, 45, 35, 20 °C were 0, 70, 83, 98 and 62%, respectively, of that observed at 30 °C.

The purified enzyme could be stored in 20 mM KH_2PO_4 buffer, pH 7.5 at −20 °C without loosing any activity.

Substrate specificity and inhibition

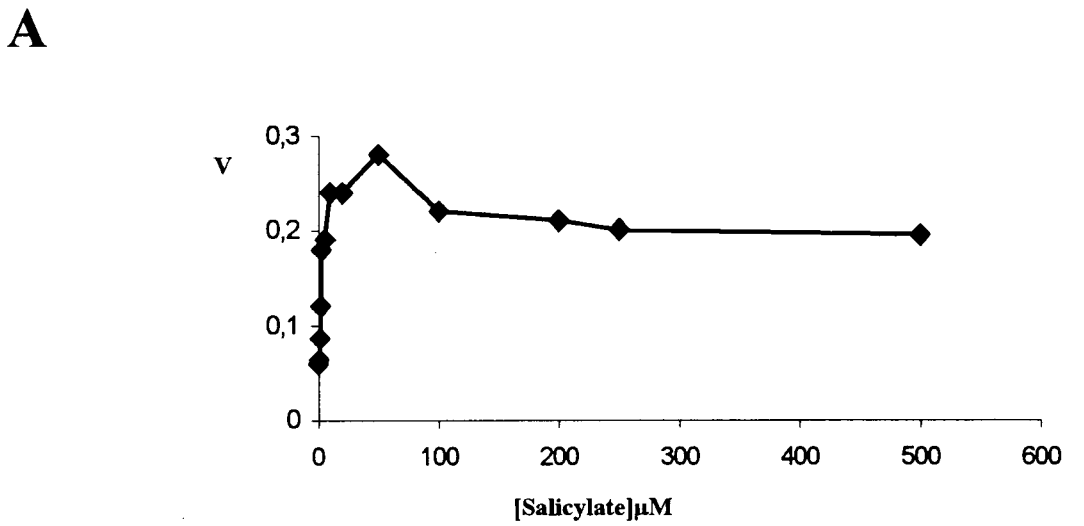
To compare the catalytic efficiencies of the enzyme with both substrates, different concentrations of salicylate and 1-hydroxy-2-naphthoate were converted by the purified enzyme. 100 μM NADH concentration was used to provide excess of cofactor in reaction mixture. NADH concentrations over 100 μM were inhibitory (10–150 μM concentrations were tested). A significant substrate inhibition was observed with 1-hydroxy-2-naphthoate concentrations >100 μM and almost no reaction was observed with 1-hydroxy-2-naphthoate concentrations >500 μM. In contrast, almost no substrate inhibition was found with the same concentrations of salicylate as a substrate (Figure 3). The kinetic data are presented in Table 3.

A number of other compounds were tested as substrates for the enzyme. A substrate-dependent oxidation of NADH was observed with many substituted salicylates, but 1-hydroxy-2-naphthoate was the only naphthoic acid, which was converted by the enzyme (Table 4).

Products of hydroxylation

The conversion of salicylate and NADH to catechol and NAD by the purified enzyme could be analysed by overlay-spectra, because NADH, catechol and salicylate were distinguishable by their absorbance maxima at 340 nm, 260 nm and 296 nm, respectively.

However, the spectrophotometric analysis of the conversion of NADH and 1-hydroxy-2-naphthoate was impeded by the very similar absorption maxima of NADH and 1-hydroxy-2-naphthoate. Furthermore, the expected product of the reaction, 1,2-dihydroxynaphthalene, and its autoxidation product,



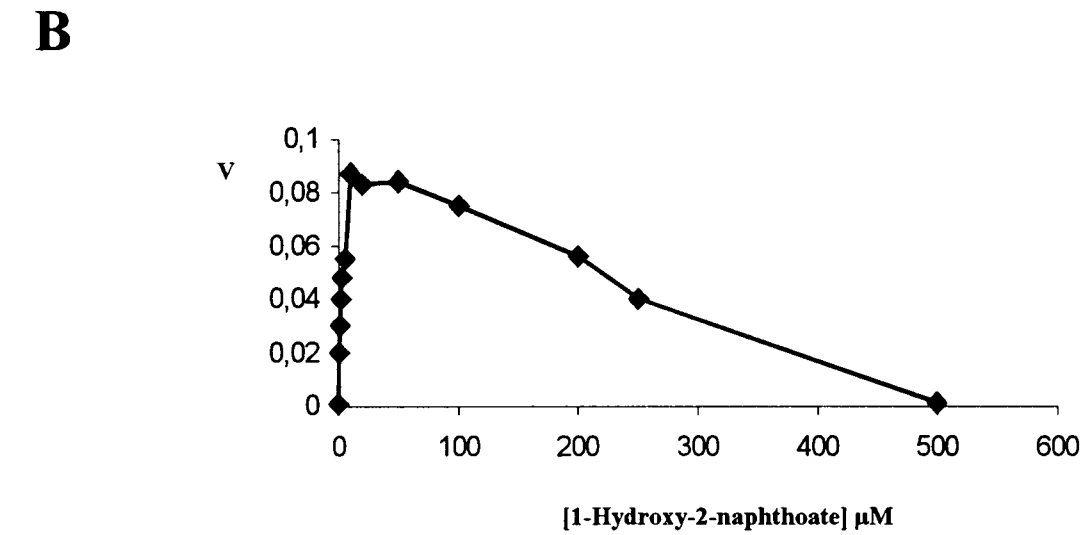


Figure 3. Michaelis-Menten plots of salicylate hydroxylase (A) and 1-hydroxy-2-naphtoate (B) activities. V is presented in μmol min^{-1} mg^{-1}.

Table 2. Copurification of salicylate and 1-hydroxy-2-naphthoate hydroxylase activities from *P. putida* BS202-P1. SH – salicylate hydroxylase, HH – 1-hydroxy-2-naphthoate hydroxylase

Purification step	Protein (mg)	Vol. (ml)	Total activity		Specific activity (U/mg)		Recovered activity (%)		Purification factor	
			SH	HH	SH	HH	SH	HH	SH	HH
Cell extract	22.8	6	5.3	1.6	0.2	0.07	100	100	1	1
Resource Q	3.6	4	2.8	0.8	0.7	0.17	52	51	3.5	2.4
MonoQ	0.2	2	2.4	0.9	12	4.5	45	58	60	64
Superdex75	0.08	4	0.4	0.12	5	1.5	7.5	7.7	25	21

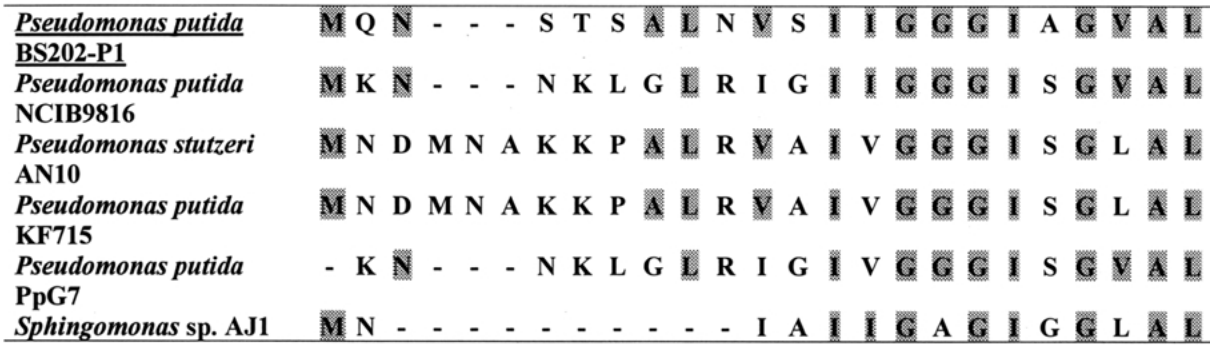


Figure 4. Alignment of the N-terminal amino acid sequence of salicylate and 1-hydroxy-2-naphthoate hydroxylase from *P. putida* BS202-P1 with sequences for salicylate 1-monooxygenases from *P. stutzeri* AN10 (NahG AN10), *P. putida* KF715, *P. putida* PpG7, *P. putida* NCIB9816 (NCBI accession number X83926, deduced from nucleotide sequence data), *Sphingomonas* sp. AJ1 (NCBI accession number AB000564, deduced from nucleotide sequence data). Positions identical with those in the *P. putida* BS202-P1 are shown on a grey background.

Table 3. Kinetic data of the purified salicylate 1-monooxygenase

Substrate	K_M (μM)	V_{max} (μmol min^{-1} mg^{-1}	V_{max}/K_M	K_i (μM)
Salicylate	1.72	0.27	0.16	935
1-Hydroxy-2-naphthoate	4.01	0.12	0.03	127

1,2-naphthoquinone, show some absorbance at 340 nm (Kuhm et al. 1991). Therefore, the products from the conversion of salicylate and 1-hydroxy-2-naphthoate with NADH were identified by using HPLC. This assay indicated that the purified enzyme converted salicylate to a substance with a retention volume corresponding to catechol and 1-hydroxy-2-naphthalene to a substance with a retention volume corresponding to 1,2-dihydroxynaphthalene. 1,2-Naphthoquinone was also detected in the reaction mixture. This is presumably due to the spontaneous autoxidation of 1,2-dihydroxynaphthalene to 1,2-naphthoquinone.

N-terminal sequence

The amino-terminal amino acid sequence of the salicylate and 1-hydroxy-2-naphthoate converting enzyme was determined and compared with those of other salicylate hydroxylases. This analysis indicated that the enzyme from BS202-P1 differed from a previously studied salicylate 1-monooxygenase, but was clearly related to other salicylate hydroxylases (Figure 4). The amino acid sequence of the purified protein showed the highest degree of sequence conservation with the salicylate-1-monooxygenase from *Pseudomonas putida* NCIB9816 (13 aa from 22 were identical).

Discussion

The enzymes of the upper part of the naphthalene pathway have been shown earlier to possess a broad substrate specificity. Thus the enzymes from *Pseudomonas* sp. NCIB 9816 were able to convert anthracene to 2-hydroxy-3-naphthoate and phenanthrene to 1-hydroxy-2-naphthoate (Menn et al. 1993; Kiyohara et al. 1994). It has been repeatedly proposed that 1-hydroxy-2-naphthoate could be converted to 1,2-dihydroxynaphthalene and then further metabolized by the same enzymes which also convert 3,4-dihydroxyphenanthrene to 1-hydroxy-2-naphthoate. Nevertheless, before our study there was no information available which enzyme was responsible for the conversion of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene in those phenanthrene-degrading strains which metabolize phenanthrene via 1,2-dihydroxynaphthalene. This might be one of the limiting steps which prevent growth of many naphthalene degrading bacteria on phenanthrene (Kiyohara et al. 1994, Balashova et al. 1999).

Table 4. Substrate specificity of the purified salicylate 1-monooxygenase

Substrate	Relative activity (%)
Salicylate	100
3-Chlorosalicylate	30
4-Chlorosalicylate	63
5-Chlorosalicylate	39
3-Aminosalicylate	40
4-Aminosalicylate	110
5-Aminosalicylate	110
5-Methylsalicylate	83
3-Methylsalicylate	67
5-Sulfosalicylate	<5
2,3-Dihydroxybenzoate	85
2,5-Dihydroxybenzoate	114
2,6-Dihydroxybenzoate	78
1-Naphthoate	<5
2-Naphthoate	<5
1-Hydroxy-2-naphthoate	30
2-Hydroxy-3-naphthoate	<5
2-Hydroxy-1-naphthoate	<5

The enzyme activity was determined in a reaction mixture (1 ml) which contained 20mM KH_2PO_4 (pH 7.5), 100 μM NADH, 50 μM of the respective substrate and 20 μg of purified enzyme. The oxidation of NADH was determined at 340 nm. The enzyme activity with salicylate (0.9 U/mg) was taken as 100%.

The results of the present manuscript demonstrate that after growth on 1-hydroxy-2-naphthoate or salicylate strain *P. putida* BS202-P1 synthesized a single salicylate 1-monooxygenase, which converted 1-hydroxy-2-naphthoate under the standard reaction conditions with 30% of the activity found with salicylate. The previously studied salicylate 1-monooxygenases from two different bacteria which had been enriched with salicylate also showed some NADH-oxidation after the addition of 1-hydroxy-2-naphthoate but with considerably lower relative rates compared to salicylate (v_{rel} salicylate: 1-hydroxy-2-naphthoate 100: 1.9 and 100: 15, respectively) (Yamamoto et al. 1965; White-Stevens & Kamin 1972). Salicylate 1-monooxygenases are known to perform with certain substrate-analogues (so called pseudo-substrates) NADH-oxidation reactions which do not result in the hydroxylation of the substrates (White-Stevens & Kamin 1972). Thus, the detection of the formation of 1,2-dihydroxynaphthalene from 1-hydroxy-2-naphthoate by the purified salicylate 1-monooxygenase gave the first conclusive evidence that this enzyme could indeed catalyse the productive metabolism of 1-hydroxy-2-naphthoate and salicylate.

The determination of the aminoterminal amino acid sequence and the comparison with previously determined sequences demonstrated that the salicylate 1-monooxygenase from strain BS202-P1 showed high degree of homology to other salicylate 1-monooxygenaes (59–49%). The holoenzyme from *P. putida* BS202-P1 showed a similar size (45 kDa) as the salicylate 1-monooxygenases from *P. putida* S-1 and *P. putida* PpG7 (Yamamoto et al. 1965; You et al. 1990), but differed from the dimeric salicylate 1-monooxygenase described for *Burkholderia cepacia* ATCC 29351 and ATCC 29352 (White-Stevens & Kamin 1972; Wang et al. 1984).

The purified salicylate 1-monooxygenase converted a broad spectrum of substituted salicylates. This was also previously observed for other salicylate hydroxylases from *P. putida* S-1, *Pseudomonas stutzeri* AN10 and *Burkholderia cepacia* ATCC 29352 strains (Yamamoto et al. 1965; Bosch et al. 1999; White-Stevens & Kamin 1972). The purified enzyme oxidized from naphthoic acids tested only 1-hydroxy-2-naphthoate with significant rates (Table 4). This might be the reason why the strain BS202-P1 could not utilize other PAHs, in particular anthracene, which is metabolized via 2-hydroxy-3-naphthoate.

The results of the present study suggested that salicylate 1-monooxygenases are responsible for the oxidation of 1-hydroxy-2-naphthoate in phenanthrene-degrading fluorescent pseudomonads. The attempts to adapt the naphthalene-degrading fluorescent pseudomonads from our collection to growth on phenanthrene showed that most of these strains could be adapted to grow on phenanthrene after a certain period of incubation. In addition to the ability to grow on phenanthrene these bacteria became capable of growing on 1-hydroxy-2-naphthoate. Nevertheless, it was shown that the relative activities of cell extracts from the naphthalene degrading isolates and their phenanthrene-degrading derivatives for the conversion of salicylate and 1-hydroxy-2-naphthoate were almost identical (data not presented).

This suggested that the induction of the upper and lower naphthalene degradative pathway may limit the degradation of phenanthrene by fluorescent pseudomonads. For *Pseudomonas* sp. NCIB 9816 and *Pseudomonas putida* G7 it was previously shown that salicylate (substituted salicylates) could serve as inductors for the relevant enzymes (Barnsley 1975; Shamsuzzaman & Barnsley 1974a; Shamsuzzaman & Barnsley 1974b; Schel 1985). This indicates that during the slow turn-over of phenanthrene by naphthalene

degrading fluorescent pseudomonads only very low amounts of salicylate are accumulating which do not result in a sufficient induction of the enzymes involved in the degradation of naphthalene and phenanthrene. To elucidate what genetic alterations are necessary to acquire the ability to grow on phenanthrene in naphthalene-degrading strains will be the subject of further investigations.

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