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Metabolism of naphthalene by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1

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Abstract

Pseudomonas paucimobilis Q1 originally isolated as biphenyl degrading organism (Furukawa et al. 1983), was shown to grow with naphthalene. After growth with biphenyl or naphthalene the strain synthesized the same enzyme for the ring cleavage of 2,3-dihydroxybiphenyl or 1,2-dihydroxynaphthalene. The enzyme, although characterized as 2,3-dihydroxybiphenyl dioxygenase (Taira et al. 1988), exhibited considerably higher relative activity with 1,2-dihydroxynaphthalene. These results demonstrate that this enzyme can function both in the naphthalene and biphenyl degradative pathway.

Abbreviations: DHBP – dihydroxybiphenyl, DHBPDO – 2,3-dihydroxybiphenyl dioxygenase, DHDHNDH – 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase, DHN – 1,2-dihydroxynaphthalene, DHNDO – 1,2-dihydroxynaphthalene dioxygenase, HBP – *cis*-2'-hydroxybenzalpyruvate, HOPDA – 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, PCB – polychlorinated biphenyl, 2NS – naphthalene-2-sulfonic acid

Introduction

We have recently reported the purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase (DHNDO) from the naphthalenesulfonic acids degrading bacterium strain BN6 (Kuhm et al. 1991). The size of the enzyme, the subunit structure and the NH_2-terminal amino acid sequence of the DHNDO from strain BN6 showed a high degree of homology with the 2,3-dihydroxybiphenyl dioxygenase (DHBPDO) from the PCB converting strain *Pseudomonas paucimobilis* Q1 (Taira et al. 1988). Immunological and DNA hybridization studies indicate that the DHBPDO from *Pseudomonas paucimobilis* Q1 was (if at all) only distantly related to the isofunctional enzymes from all other well characterized biphenyl degrading organisms (Taira et al. 1988; Furukawa et al. 1989). The possibility that in *Pseudomonas paucimobilis* Q1 the pathway for the degradation of naphthalene and biphenyl were evolutionary closely related or identical is analyzed in the present paper.

Material and methods

Bacterial strains and media

Pseudomonas paucimobilis Q1 and *Escherichia coli* C600 pMFQ1 were kindly provided by K. Furukawa. *Pseudomonas paucimobilis* Q1 was grown in a

mineral medium according to Dorn et al. (1974) with glucose (10 mM), biphenyl (0.05% w/v) or naphthalene (0.05% w/v) as sole source of carbon and energy. *Escherichia coli* C600 pMFQ1 was grown in Nutrient Broth (NB) with streptomycin (150 μg/ml). Induced cells of strain BN6 were obtained as described before (Kuhm et al. 1991).

Enzyme assays

2,3-Dihydroxybiphenyl dioxygenase

The enzyme was determined as described by Taira et al. (1988) with Na/K-phosphate buffer (pH 7.0, 50 mM) and a substrate concentration of 0.1 mM. The increase of absorption at 434 nm was measured photometrically. Calculation of reaction rates were based on an extinction coefficient of 221 · mM · cm^{-1}.

2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase

The reaction mixture contained in 1 ml 100 μmol Na/K-phosphate buffer pH 7.0 and 0.05 μmol HOPDA. The reaction was started by the addition of cell-free extract and the decrease of absorption was followed at 434 nm.

1,2-Dihydroxy-1,2-dihydronaphthalene dehydrogenase, 1,2-dihydroxynaphthalene dioxygenase, and *cis*-2'-hydroxybenzalpyruvate aldolase were determined as described before (Kuhm et al. 1991). Catechol-2,3-dioxygenase was measured as described by Nozaki (1970).

For determination of K_M and K_i-values the ranges of substrate concentration were as follows: HOPDA, 30–200 μM; *cis*-HBP, 3–28 μM for determination of K_M-value and 28–56 μM for determination of K_i-value. Determination of HBP aldolase at 298 nm and HOPDA hydrolase at 434 nm were not disturbed by their respective inhibitors.

Chemicals

For the preparation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) strain BN6 was grown in liquid culture with glucose (10 mM) plus 2NS (1 mM) to induce the 1,2-dihydroxynaphthalene dioxygenase (Kuhm et al. 1991). At the end of the exponential growth phase cells were harvested, washed and (E_{546nm} = 5) incubated with 2,3-dihydroxybiphenyl (1 mM). The increase in absorption at 434 nm was measured and, when the calculated amount of HOPDA was formed, cells were removed by centrifugation, the supernatant appropriately diluted and used for the determination of HOPDA-hydrolase. The product of this reaction was identical (UV-spectra at different pH-values and by HPLC analysis) with the product of the 2,3-dihydroxybiphenyl dioxygenase reaction obtained with cell-free extracts from *Pseudomonas paucimobilis* Q1.

All other culture media, strains, chemicals, the preparation of cell-free extracts, protein estimation, enzyme purification procedures, HPLC analysis and apparatus were the same as described (Kuhm et al. 1991).

Results and discussion

Enzyme activities in cell-free extracts

Pseudomonas paucimobilis Q1 was able to grow on agar-plates or in liquid culture not only with biphenyl but also with naphthalene as sole source of carbon and energy. For comparative studies the strain was grown in liquid culture with biphenyl or naphthalene. The activities of the enzymes involved in the break-down of naphthalene or biphenyl after growth with either substrate were compared in cell free extracts. The levels of 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase (DHDHNDH), 2,3-dihydroxybiphenyl dioxygenase (DHBPDO), 1,2-dihydroxynaphthalene dioxygenase (DHNDO), *cis*-2'-hydroxybenzalpyruvate aldolase (HBPA) and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (HOPDAH) were determined after growth with naphthalene, biphenyl or glucose (Table 1). A DHDHNDH was induced after growth of *Pseudomonas paucimobilis* Q1 both with biphenyl or naphthalene. Only low specific activity of this enzyme was found after growth with glucose. Growth with naphthalene led

to a high activity of DHNDO compared to the enzyme level after growth with glucose or biphenyl. The relative activities with 1,2-dihydroxynaphthalene (DHN) or 2,3-dihydroxybiphenyl were about the same after growth with glucose or naphthalene (about 100 : 3) while the activity with 2,3-dihydroxybiphenyl was slightly higher after growth with biphenyl (activity with DHN: 2,3-dihydroxybiphenyl 100 : 7). HOPDAH, which is involved in the break-down of biphenyl, was induced after growth of strain Q1 with this substrate, whereas only very low enzyme levels were found after growth with glucose or naphthalene. HBPA was induced after growth of strain Q1 with naphthalene and surprisingly also with biphenyl (Table 1). The relative activities of DHNDO and HBPA were 100 : 0.4 in naphthalene grown cells but 100 : 6.4 in biphenyl grown cells. These results suggested an independant regulation of DHNDO and HBPA in *Pseudomonas paucimobilis* Q1.

Conversion of 1,2-dihydroxynaphthalene and 2,3- and 3,4-dihydroxybiphenyls by cell-free extracts

Cell-free extracts of cells of strain Q1 that had been grown with biphenyl or naphthalene converted 3,4-dihydroxybiphenyl to products with an absorption maximum at λ_{max} (pH 7.5) 381 nm and λ_{max} (pH 2) 328 nm. This reaction had also been observed with the purified DHNDO from strain BN6 (Kuhm et al. 1991), but is in contrast to the observation of Taira et al. (1988). Their purified DHBPDO from *Pseudomonas paucimobilis* Q1 exhibited no activity with 3,4-dihydroxybiphenyl. Unfortunately the authors did not report their source of 3,4-dihydroxybiphenyl so that we could not reproduce their results. We have recently characterized the range of substrates converted by the DHNDO from the naphthalenesulfonic acids degrading strain BN6 (Kuhm et al. 1991). The relative activities observed for 2,3-dihydroxybiphenyl, 3,4-dihydroxybiphenyl and DHN with cell-free extracts of *Pseudomonas paucimobilis* Q1 were very similiar to the relative activities determined with the purified enzyme from strain BN6 (Table 2).

Induction of a single enzyme after growth with biphenyl or naphthalene for turnover of 1,2-dihydroxynaphthalene and 2,3-dihydroxybiphenyl

The results described above clearly showed that cell-free extracts of *Pseudomonas paucimobilis* Q1 after growth with biphenyl showed much higher activities with DHN than with 2,3-dihydroxybiphenyl. This unexpected result can be explained in two ways. One possibility is that the enzyme Taira et al. (1988) have purified from *Pseudomonas paucimobilis* Q1 and designated as 2,3-dihydroxybiphenyl

Table 1. Enzyme activities in cell-free extracts from *Pseudomonas paucimobilis* Q1 after growth with different substrates

Enzyme	Specific activities after growth with:					
	Glucose		Biphenyl		Naphthalene	
DHDHNDH	37 ± 9	(2)	355 ± 22	(15)	250 ± 13	(1,5)
DHNDO	1600 ± 137	(100)	2310 ± 230	(100)	16030 ± 1310	(100)
DHBPDO	50 ± 2	(3)	160 ± 25	(7)	450 ± 56	(3)
HBPA	14 ± 3	(1)	150 ± 30	(6)	69 ± 3,5	(0.4)
HOPDAH	3 ± 0,2	(0.2)	50 ± 3	(2)	30 ± 3	(<0.1)

DHDHNDH 1,2-Dihydroxy-1,2-dihydronaphthalene dehydrogenase; DHNDO 1,2-dihydroxynaphthalene dioxygenase; DHBPDO 2,3-dihydroxybiphenyl dioxygenase; HBPA *cis*-2'-hydroxybenzalpyruvate aldolase; HOPDAH 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase.

Pseudomonas paucimobilis Q1 was grown with the indicated substrates. Cells were harvested during exponential growth. Enzyme activities were determined as described under Material and methods and are expressed as absolute specific activities (μmol per minute per gram of protein). The assays were repeated 3–4-times, the average values and the mean square deviations are given. The values given in parenthesis are referred to the activities with 1,2-dihydroxynaphthalene as 100%.

1,2-dioxygenase actually shows higher activity with DHN than with 2,3-dihydroxybiphenyl. This hypothesis was strengthened by the fact that the purified DHNDO from the naphthalenesulfonic acid degrading strain BN6 converted not only DHN but also 2,3-dihydroxybiphenyl with high activity. It would also explain the low homology of the DHBPDO from *Pseudomonas paucimobilis* Q1 with the isofunctional enzymes from other biphenyl degrading bacteria (Taira et al. 1988; Furukawa et al. 1989). The other explanation for the high activity of cell-free extracts after growth of the strain with biphenyl for DHN would be the induction of a DHNDO distinct from the DHBPDO. This possibility was supported by the differences in the relative activities for DHN and 2,3-dihydroxybiphenyl of cell-free extracts from naphthalene and biphenyl grown cells.

To distinguish between the two possibilities proteins from a cell-free extract of strain Q1 were separated by column chromatography. Two bands were resolved by anion-exchange chromatography which showed activity with 2,3-dihydroxybiphenyl (Fig. 1). One of the enzymes found (fraction 29) showed a broad substrate specifity and oxidized 2,3-dihydroxybiphenyl, 3,4-dihydroxybiphenyl and DHN. The second protein band (fraction 38) exhibited high specifity for 2,3-dihydroxybiphenyl and did not convert 3,4-dihydroxybiphenyl and DHN. The protein in fraction 29 showed similiar relative activities as the DHNDO from strain BN6 (Table 2). The high homology of the NH_2-terminal amino acid sequence of the enzyme presented by Taira et al. (1988) with the DHNDO from strain BN6 and the similarities in the substrate range indicated that Taira et al. (1988) had cloned the 'wide substrate range enzyme' which they called DHBPDO. According to Taira et al. this enzyme should not have any activity with 3,4-dihydroxybiphenyl. The enzyme in fraction 29, however, possessed such activity.

Since Taira et al. (1988) described some catechol 2,3-dioxygenase activity with catechol as substrate (22% referred to 2,3-dihydroxybiphenyl as 100%) we used this trait to identify this enzyme in our protein fractions. In fraction 29 (Fig. 1) the relative activities with 2,3-dihydroxybiphenyl (c = 0.1 mM) compared to catechol (c = 0.2 mM) were 100 : 44; while in fraction 38 the relative activities were 100 : 2040. It was therefore deduced that fraction 29 contained the '2,3-dihydroxybiphenyl dioxygenase' described by Taira et al. (1988) and that fraction 38 exhibited catechol 2,3-dioxygenase activity with some activity for 2,3-dihydroxybiphenyl as substrate. Obviously this *meta*-cleavage activity is due to the catabolism of benzoic acid, which is a metabolite in the degradative pathway of biphenyl.

Further evidence for the 'wide substrate range DHBPDO' cloned by Taira et al. (1988) was obtained by analyzing the cell-free extract from *Escherichia coli* C600 pMFQ1, which contained the cloned DHBPDO from strain Q1 (Taira et al. 1988). Although the expression of DHBPDO was only weak (0.003 U/mg of protein) relative activities for 2,3DHBP: 3,4DHBP: 1,2-DHN: catechol could be determined to be 3 : 2 : 100 : 1. When the crude extract from *Escherichia coli* C600 pMFQ1 was analyzed by FPLC the activity for 2,3-dihydroxybiphenyl eluted at the same NaCl-concentration as the 'broad substrate range' enzyme from the cell-free extract of *Pseudomonas paucimobilis* Q1. These results clearly demonstrate that the enzyme

Table 2. Relative activities of a cell-free extract from *Pseudomonas paucimobilis* Q1 after growth of cells with biphenyl and separated fractions derived thereof by column chromatography with different aromatic 1,2-diols in comparison to the purified DHNDO from strain BN6

	Substrate		
	2,3-DHBP	3,4-DHBP	DHN
Cell-free extract	100 ± 7 (477)	47 ± 3	3400 ± 630
Fraction 29	100 ± 4 (3456)	86 ± 2	7060 ± 620
Fraction 38	100 ± 7 (3697)	< 1	< 1
DHNDO from BN6	100	53	3570

The relative activities of a cell-free extract from *Pseudomonas paucimobilis* Q1 and two fractions from the column chromatography shown in Fig. 1 with different ortho-hydroxylated aromatic compounds were determined as described before (Kuhm et al. 1991). The assays were repeated 3–4-times, the average values and the mean square deviations are given. Absolute specific activities (units per gram of protein) are given in parentheses for the relative rates reported as 100%. The values for the purified DHNDO from strain BN6 were taken from Kuhm et al. (1991).

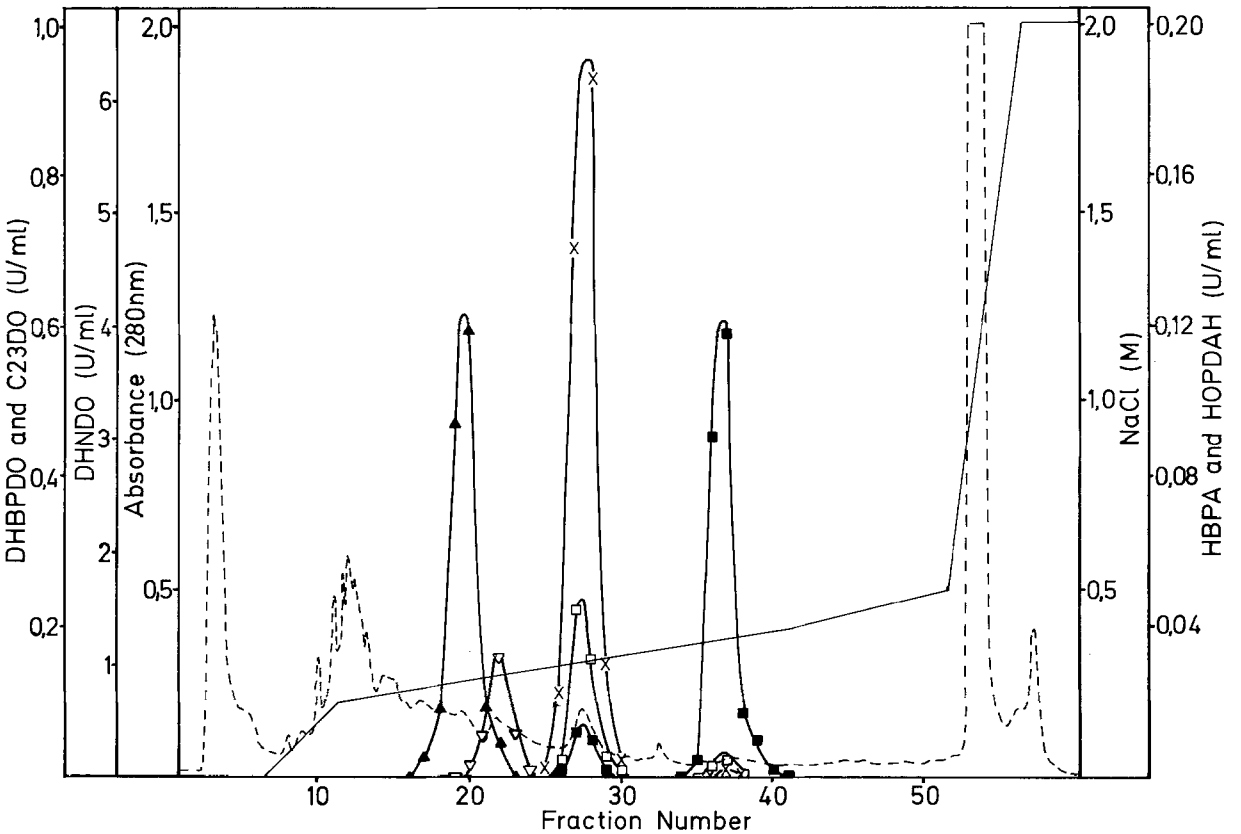

Fig. 1. Elution profile from a Mono-Q-column of a cell-free extract prepared from biphenyl grown cells of *Pseudomonas paucimobilis* Q1. The cell-free extract (2.7 mg protein) was applied to a Mono-Q-column (HR5/5, Pharmacia, Uppsala, Sweden). The sample was eluted with a gradient of Tris/HCl (50 mM, pH 7.5) into Tris/HCl (50 mM, pH 7.5) plus 500 mM NaCl at a flow rate of 1 ml/min. Fractions (0.7 ml each) were collected and the enzyme activities determined. —— NaCl gradient; --- absorbance 280 nm; -□—□- 2,3-dihydroxybiphenyl dioxygenase; -×—×- 1,2-dihydroxynaphthalene dioxygenase; -■—■- catechol 2,3-dioxygenase; -▲—▲- HBP-aldolase; -▽—▽- HOPDA-hydrolase.

cloned by Taira et al. (1988) could be termed 1,2-dihydroxynaphthalene dioxygenase as well as 2,3-dihydroxybiphenyl dioxygenase.

When *Pseudomonas paucimobilis* Q1 was grown with naphthalene the same 'broad substrate range' enzyme (as judged by FPLC chromatography and the relative activities for various ortho-diols) was induced for the conversion of DHN as in the biphenyl grown cells for the conversion of 2,3-dihydroxybiphenyl. It was therefore concluded that the strain actually uses the same ring cleavage enzyme for growth with naphthalene or biphenyl.

Separation of cis-*2'-hydroxybenzalpyruvate aldolase from 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase*

The results presented here and in a previous communication (Kuhm et al. 1991) demonstrate that bacteria can cleave 2,3-dihydroxybiphenyl through enzymes that originally function in naphthalene catabolism. To answer the question whether other enzymes of the naphthalene pathway could also act on metabolites of the biphenyl pathway *cis*-2'-hydroxybenzalpyruvate aldolase and HOPDA hydrolase were compared. The K_M value for HOPDA-hydrolase was determined to be 140 μM with HOPDA and the K_M for HBP-aldolase found to be 22 μM with *cis*-HBP. The HOPDA hydrolase ac-

tivity was competetively inhibited by HBP and a K_i value of 50 μM was calculated. The HBP aldolase reaction was also competetively inhibited by the addition of HOPDA and a K_i of 95 μM was found. Thus the results from the inhibition studies proposed that the HOPDA hydrolase can bind HBP and the HBP aldolase accepted HOPDA at the catalytic site. We tested therefore whether the two activities were really distinct enzymes or if a single enzyme catalyzed both reactions in *Pseudomonas paucimobilis* Q1. A cell-free extract of the strain after growth with biphenyl was separated by column chromatography under the conditions described in Fig. 1. Actually HBP aldolase and HOPDA hydrolase eluted as two well separated protein bands at salt concentrations of 0.25 M and 0.27 M, respectively.

It has been described earlier that naphthalene dioxygenase from *Pseudomonas putida* 119 attacked besides naphthalene a variety of aromatic hydrocarbons including biphenyl (Jeffrey et al. 1975). Also the second enzyme in the degradative pathway of naphthalene *cis*-1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase converted the corresponding *cis*-2,3-dihydroxy-2,3-dihydrobiphenyl with high relative activity (Patel & Gibson 1974). This together with the ability of 1,2-dihydroxynaphthalene dioxygenase from strain BN6 to convert 2,3-dihydroxybiphenyl and '2,3-dihydroxybiphenyl dioxygenase' from *Pseudomonas paucimobilis* Q1 to metabolize 1,2-dihydroxynaphthalene suggests that naphthalene and biphenyl (and presumably their substituted derivatives) can be metabolized to a great extent by a single set of enzymes. Only *cis*-2'-hydroxybenzalpyruvate aldolase (in the naphthalene pathway) and HOPDA hydrolase (in biphenyl degradation), are specific for their respective substrate. It will be of great interest to compare substrate specifities in greater detail and to compare sequence data of these two groups of enzymes.

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