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Inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. strain CB406 by 3,4-dihydroxybiphenyl (4-phenylcatechol)

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Summary

3,4-dihydroxybiphenyl is not a substrate for the 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) from biphenyl-degrading *Pseudomonas* sp. strain CB406. It acts as both a reversible inhibitor and a potent inactivator of the enzyme. The inactivation process requires the presence of O_2 and can be reversed by the removal of the 3,4-dihydroxybiphenyl followed by incubation of the enzyme in the presence of dithioerythritol and Fe^{2+} under anaerobic conditions. Two other extradiol dioxygenases behave similarly, the catechol 2,3-dioxygenase (BphE) from strain CB406 and the BphC from *Pseudomonas* sp. strain LB400. The BphC from *P. testosteroni* B-356 also did not cleave 3,4-dihydroxybiphenyl but it was not inactivated.

Abbreviations: C23o – Catechol 2,3-dioxygenase; 34DHBP – 3,4-dihydroxybiphenyl

Introduction

The meta-cleavage dioxygenases are central enzymes in the catabolism of aromatic compounds. They catalyse the extradiol ring cleavage of catechols which are obligatory metabolites in the aerobic catabolism of virtually all aromatic growth substrates. The three main classes with neutral *vic*-arene diol substrates are catechol-2,3-dioxygenase (C23o; typified by XylE in TOL plasmid-mediated catabolism of toluene and xylenes (Assinder & Williams 1990; Nozaki 1970), 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC) in biphenyl catabolism (Furukawa & Miyazaki 1986; Mondello 1989), and 1,2-dihydroxynaphthalene dioxygenase (NahC) in naphthalene catabolism (Harayama & Rekik 1989).

The *meta*-cleavage dioxygenases are non-haem Fe^{2+} enzymes which are readily inactivated. Oxidising agents, including O_2 and H_2O_2 convert the iron to Fe^{3+} but the enzyme could be reactivated by anaerobic incubation in the presence of Fe^{2+} and reducing agents such as thiols or borohydrides (Nozaki et al. 1968). Specific chelators for Fe^{2+} such as α,α'- dipyridyl inhibit catalysis but protect the Fe^{2+} from oxidative inactivation (Nozaki et al. 1968).

Substrate analogues can also inactivate extradiol ring cleavage enzymes. 3- and 4-chlorocatechols were shown to act as mixed-type inhibitors of the C23o from a *P. putida* and incubation with 3-chlorocatechol leads to its inactivation but the inactive enzyme can be reactivated (Klecka & Gibson 1981). However with the XylE protein from *P. putida* mt-2 3-chlorocatechol is a suicide substrate since the product of its *meta*-cleavage is a highly reactive acyl chloride and the inactivated enzyme cannot be regenerated (Bartels et al. 1984). Chlorocatechols, or their *meta*-cleavage products, have also been reported as inhibitors of BphC (Adams et al. 1992; Sondossi et al. 1992). A second class of substrate inactivators are alkyl catechols. 4-Ethylcatechol is a potent inactivator of XylE (Ramos et al. 1987) and this is one of the contributory reasons why the host strain is unable to utilise 4-ethylbenzoate as a growth substrate. This effect must be a function of the enzyme structure since mutant XylE proteins with a single amino acid substitution have been selected which are much more resistant to inactivation by 4-ethylcatechol and permit growth on 4-ethylbenzoate (Ramos et al. 1987). Even catechols which are metabolites of growth substrates, such as 3- and 4-methylcatechols, inactivate C23o at a substantial rate during the course of their catalysis: it has been proposed that in vivo the inactive C23o

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source of reference
Bacteria:		
Pseudomonas sp. CB406	Wild type; Bph$^+$ contains plasmid pWW100	Lloyd-Jones et al. (1994)
Pseudomonas sp. LB400	Wild type; Bph$^+$	Mondello (1989)
P. putida KT2440		Bagdasarian et al. (1981)
E. coli XL1Blue	Tc^r	Stratagene
Plasmids:		
pDA2	Sm^{r}Ap^r *bphABC*$^+$ (*P. testosteroni* B-356)	Ahmed et al. (1991)
pG6	pUC18 carrying 5.5 kbp *Eco*RI fragment of pWW100; Ap^r*bphC*$^+$	Lloyd-Jones et al. (1994)
pG7	pUC18 carrying 2 kbp *Eco*RI/*Pst*I fragment of pWW100:Ap^r*bphE*$^+$	Lloyd-Jones et al. (1994)

molecules are reactivated through the action of a small ferredoxin-like molecule the gene for which is directly upstream of the C23o gene in *meta* pathway operons (Polissi & Harayama 1993).

Possible inhibitors of 2,3-dihydroxybiphenyl-1,2-dioxygenases are analogues of its natural substrate 2,3-dihydroxybiphenyl (4-phenylcatechol). 3,4-dihydroxybiphenyl (34DHBP) is not a substrate for 2,3-dihydroxybiphenyl 1,2-dioxygenases from *P. pseudoalcaligenes* KF707 (Furukawa & Miyazaki 1986) or *Pseudomonas* sp. strain LB400 (Eltis et al. 1992). Although first reports indicated that the atypical BphC from *P. paucimobilis* Q1 (Taira et al. 1988) was unable to attack 34DHBP, subsequent studies have shown some limited activity (Kuhm et al. 1991a). However, strain Q1 is also able to degrade naphthalene, apparently using the same set of enzymes which are involved in biphenyl catabolism (Kuhm et al. 1991a). The 1,2-dihydroxynaphthalene dioxygenase (NahC) from the naphthalene sulphonic acid-degrading strain BN6 also showed activity with 34DHBP at 7% of the activity towards 1,2-dihydroxynaphthalene (Kuhm et al. 1991b). Thus it would seem that BphC dioxygenases are specific for 2,3-dihydroxybiphenyl and have no activity towards 34DHBP whereas NahC dioxygenases do possess some limited activity.

This paper details the reversible inactivation of the *meta*-cleavage dioxygenase BphC cloned from *Pseudomonas* sp. strain CB406, a 4-chlorobiphenyl-degrading strain, by 34DHBP which does not serve as a substrate for the enzyme. In addition the BphC in biphenyl-grown *Pseudomonas* sp. strain LB400 is also reversibly inactivated by 34DHBP, as is the catechol-2,3-dioxygenase cloned from *Pseudomonas* sp. strain CB406. We refer to this enzyme as BphE by analogy with the designation XylE for the isofunctional enzyme from the TOL plasmid (Carrington et al. 1994; Lloyd-Jones et al. 1994).

Materials and methods

Bacterial strains and plasmids used during the course of this work are listed in Table 1.

Batch growth

Batch growth was carried out at 30° C (*Pseudomonas*) and 37° C (E. coli) in 50 ml of LB medium in 250 ml Erlenmeyer flasks shaken at 200 rpm.

Preparation of cell-free extracts

Cells were harvested by centrifugation in a Beckman centrifuge, washed in 50 mM phosphate buffer pH 7.5, and the cell pellet resuspended in 50 mM phosphate

buffer pH 7.5 containing 10% v/v acetone at 0.5 g wet weight/ml. The cells were disrupted by sonication (three 30 s bursts interspersed by 1 min cooling on ice), and the cell debris cleared by ultracentrifugation at 50000 rpm in a TY65 rotor (Beckman). The clear supernatant was carefully decanted and referred to as the cell-free extract.

Subcloning and analysis of dioxygenase genes

The two dioxygenase clones, 2,3-dihydroxybiphenyl dioxygenase (BphC) and catechol 2,3-dioxygenase (BphE) were obtained as separate clones in pUC18 from digests of the Bph plasmid pWW100 (Lloyd-Jones et al. 1994) (Table 1). Cloned dioxygenase genes were characterised by determining the substrate specificities towards various catechols.

Sublimation and sources of substrates

2,3-dihydroxybiphenyl was obtained from Wako Chemicals (W-4040, Neuss, Germany), 3,4-dihydroxybiphenyl from Promochem Ltd. (Welyn Garden City, AL1 4TB, England), and catechol from BDH Ltd. (Poole, England). Any catechols which became discoloured through partial oxidation were repurified by sublimation in vacuo at 60–70° C to yield pure white crystals.

Enzyme assays

Catechol 2,3-dioxygenase was assayed using an adaptation of the assay detailed by Nozaki (1970) using 50 mM phosphate buffer pH 7.5 containing 10% (v/v) acetone with an initial substrate concentration of 10 mM catechol. The increase of absorption at 375 nm was measured and reaction rates calculated using a molar extinction coefficient of 36000 $M^{-1}cm^{-1}$. 2,3-dihydroxybiphenyl 1,2-dioxygenase was assayed according to Taira et al. (1988) in 50 mM phosphate buffer pH 7.5 containing 10% v/v acetone with an initial substrate concentration of 0.1 mM 2,3-dihydroxybiphenyl. The increase of absorption at 434 nm was measured, and reaction rates were calculated using a molar extinction coefficient of 22000 $M^{-1}cm^{-1}$. When using 3,4-dihydroxybiphenyl as a substrate scans over the absorbance range 250–450 nm were used to follow any appearance of meta-cleavage product. The anticipated *meta*-cleavage product from 3,4-dihydroxybiphenyl has an absorbance maximum at 380 nm with a shoulder at 324 nm (pH 7) (Kuhm et al. 1991b). 1,2-dihydroxynaphthalene dioxygenase was assayed as detailed by Kuhm et al. (1991b). Protein was estimated by the method of Lowry et al. (1951).

Inactivation kinetics

At time zero cell-free extract was added to a 0.1 mM solution of 3,4-dihydroxybiphenyl in phosphate buffer (50 mM) pH 7.5, containing 10% v/v acetone, and incubated at 20° C. Samples were withdrawn at timed intervals and the residual *meta*-cleavage dioxygenase activity assayed.

Reactivation of dioxygenase activities

The procedure was adapted from Bartels et al. (1984). Samples were dialysed overnight at 4° C, against two 2000-fold volumes of 50 mM phosphate buffer pH 7.5 containing 10% (v/v) acetone. N_2 was then bubbled through the samples for 1 min/ml after which aqueous dithioerythritol and $FeSO_4$ were added to give final concentrations of 1 mM respectively. N_2 bubbling was continued for a further 2 min when the tubes were capped and incubated at room temperature for 1 hour.

Results and discussion

Determination of the substrate specificities of both BphC and BphE from the *Bph* plasmid pWW100 carried by *Pseudomonas* sp. strain CB406 revealed that 34DHBP was not a substrate for either enzyme: incubation of 34DHBP with either dioxygenase enzyme for 30 min did not result in any change in the absorption spectrum over the range 250–450 nm. The *meta*-cleavage product from 34DHBP has been produced by the action of the 1,2-dihydroxynaphthalene dioxygenase (NahC) from strain BN6 and has an absorbance maximum at 380 nm with a shoulder at 324 nm (pH 7) (Kuhm et al. 1991b).

Incubation of the dioxygenase expressed by the pWW100 BphC in the presence of 34DHBP resulted in a time-dependent loss of activity (Fig. 1). This inactivation depends on the presence of oxygen since incubation of the enzyme with 34DHBP under anaerobic conditions did not produce such a rapid loss of activity. The inactivation observed was not reversed solely by removal of 34DHBP by dialysis. Furthermore no activity, as the production of yellow ring-

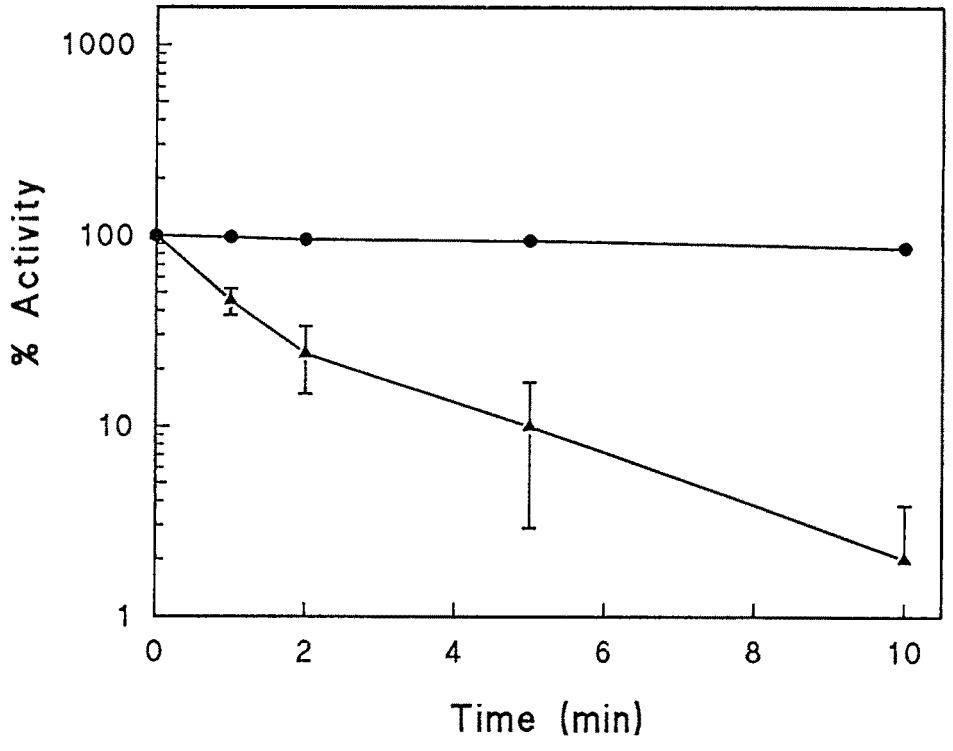

Fig. 1. Inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) by 0.1 mM 3,4-dihydroxybiphenyl (34DHBP) (▲) compared with a control in the absence of 34DHBP (●).

Table 2. Inactivation of extradiol dioxygenases with 3,4-dihydroxybiphenyl and its reversibility.

Source of dioxygenase	Relative specific activities				
	t (0)	t (30) - 34DHBP	t (30) + 34DHBP	Reactivated - 34DHBP	Reactivated + 34DHBP
Wild-type CB406	100 (816)	94	0	56	59
pG6 (BphC)	100 (83500)	61	22	86	82
pG7 (BphE)	100 (32000)	94	31	122	63
Wild-type LB400	100 (656)	85	0	102	107
pDA2	100 (8.6)	60	64	73	64

The enzyme activities were measured by the standard assays using 2,3-dihydroxybiphenyl as the substrate for all except pG7 where catechol was used. The activities were measured initially at t(0) and the specific activities (μmoles substrate converted/ min/mg protein) are presented in parentheses. The cell extract was then incubated for 30 min in the presence and absence of 100 μM 34DHBP. For reactivation, extracts were dialysed overnight against two changes of buffer (× 1000 vol) to remove the 34DHBP and reactivated as described in Methods and then assayed. The activities are presented as the %age of the initial activity.

cleavage product, was detected by addition of excess substrate (2,3-dihydroxybiphenyl) to the inactivated enzyme showing that the loss of activity was not due to a reversible binding by the 34DHBP. The inactivated enzyme was reactivated after removal of 34DHBP by dialysis, followed by incubation in the presence of Fe^{2+} and a reducing agent (Table 2). As a comparison the enzyme was also completely inactivated by incubation with 1 mM H_2O_2 and reactivated under similar conditions.

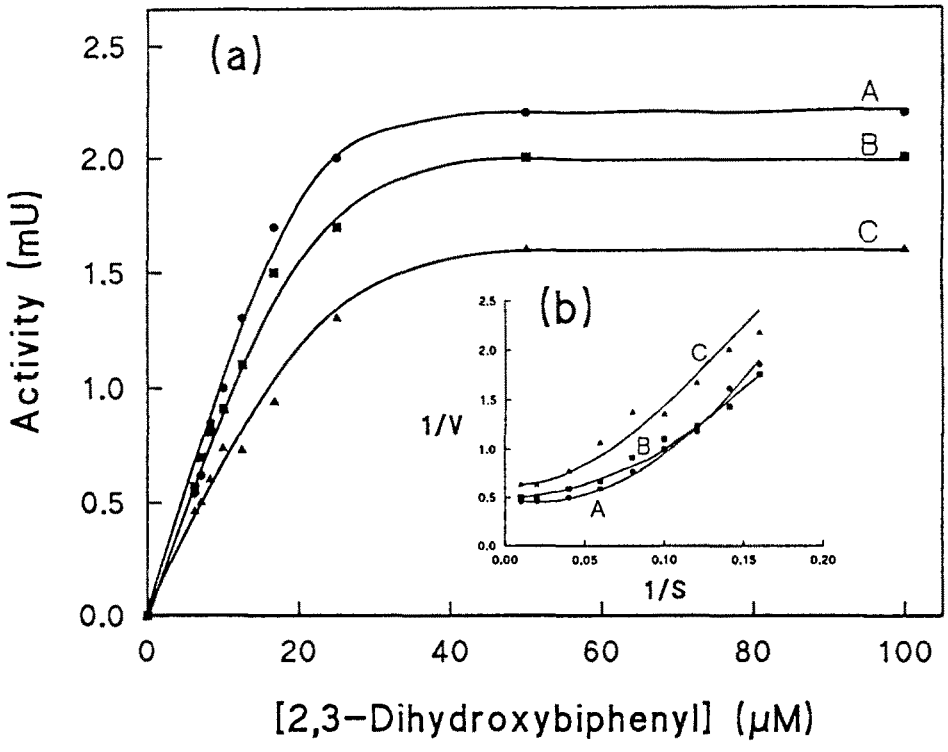

Fig. 2. (a) Inhibition of 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) by 3,4-dihydroxybiphenyl (34DHBP). A. [34DHBP] = 0 (•); B. [34DHBP] = 0.1 mM (■); C. [34DHBP] = 1 mM (▲). (b) Double reciprocal (Lineweaver-Burk) plot of same results.

As well as acting as an inactivator, the instantaneous effect of 34DHBP on the enzyme reaction was determined by assaying without preincubation with 34DHBP. The results show that 34DHBP also acts as an enzyme inhibitor (Fig. 2). The data were plotted as a double reciprocal plot but, in place of the expected straight lines, curves were produced. This can be characteristic of inhibition by excess substrate as found by Eltis et al. (1992) but over the substrate range used in this study there is no diminution of reaction rate at high 34DHBP. The type of inhibition by 34DHBP is on this data impossible to categorise as competitive, noncompetitive, etc.

The effect of 34DHBP on four other extradiol oxygenases was investigated. The cloned 2,3-dihydroxybiphenyl dioxygenases (BphC) from both *Pseudomonas* sp. strain CB406 and *Pseudomonas* sp. strain LB400, were also found to be inactivated by 34DHBP, as was the cloned catechol-2,3-dioxygenase (BphE) from strain CB406. All three could be readily reactivated in the presence of ferrous iron and a reducing agent (Table 2). Interestingly the BphC from *Pseudomonas testosteroni* B-356 (Ahmed et al. 1991) which we had available cloned on plasmid pAD2 (Table 1) was also found to lack any activity against 34DHBP but was not susceptible to inactivation (Table 2).

Pseudomonas sp. strain LB400 is capable of hydroxylating PCB congeners in the 3,4 positions, when the 2,5,2′,5′ sites are occupied. This can occur in whole cells (Bedard & Haberl 1990) and with cell-free extracts of the biphenyl 2,3-dioxygenase enzyme BphA (Haddock et al. 1993; Gibson et al. 1993). No specific biphenyl 3,4-dioxygenase has been detected which is able to introduce molecular oxygen into the 3,4 position (Haddock et al. 1993).

In light of the observation that 3,4-dihydroxybiphenyl is toxic to *meta* cleavage dioxygenases, PCB congeners hydroxylated in the 3,4 positions may prove to be unusual metabolites for a PCB-degrading strain to produce. Such PCB intermediates with two hydroxyl groups in the 3,4 positions may be inhibitory to or even inactivate the *meta*-fission dioxygenases in the host strain.

In order to increase in the number of PCB congeners degraded by a single strain, a strategy of altering the substrate specificity of the BphA gene product, biphenyl 2,3-dioxygenase, to catalyse the introduction of molecular oxygen at other positions on the biphenyl

molecule will not operate effectively if the metabolites produced either inactivate critical enzymes or are not further metabolised by subsequent enzymes of the *bph* pathway. The production of 3,4-dihydroxy derivatives of PCBs may not be advantageous unless the *meta*-fission dioxygenases of the strain are capable of both tolerating and metabolisng these compounds. Enzymes which can tolerate 34DHBP, such as the BphC from *P. testosteroni* B-356, do exist: though unable to catalyse the *meta*-cleavage of 34DHBP, it is not inactivated by it. Other *meta*-cleavage dioxygenases which are active against 34DHBP have also been reported (Kuhm et al. 1991a, b).

Thus the biodegradative effectiveness of non-specific biphenyl dioxygenases, such as in LB400, which are capable of dihydroxylating PCB congeners in unexpected positions may be severely limited by the susceptibility to inactivation and inhibition by subsequent enzymes of the pathway. Certainly it would appear from this work that the BphC from *Pseudomonas* strains CB406 and LB400 would not catalyse the further degradation of any 3,4-dihydroxybiphenyl analogues produced endogeneously.

It is not clear from this evidence the mechanism by which 34DHBP inactivates the enzymes. It resembles the inactivation of XylE by 4-ethylcatechol (Ramos et al. 1987) and this similarity may be more than superficial if 34DHBP is considered as 4-phenylcatechol. The simplest hypothesis is that it binds at or around the vicinity of the active site but, perhaps because of the large 4-substituent, does so in an orientation which is unproductive as far as enzyme catalysis is concerned but which renders the catalytic Fe^{2+} far more susceptible to either aerial oxidation or loss from the active site in the presence of O_2. The mechanism must differ from that described for nitrogen bases such as α,α'-dipyridyl and m-phenanthroline which bind to catechol 2,3-dioxygenase and inhibit catalysis of catechol yet protect the Fe^{2+} from inactivation by O_2 (Nozaki et al. 1968).

In the case of 4-ethylcatechol inactivation of XylE, it has proved possible to obtain a mutant XylE protein which was resistant to inactivation (Ramos et al. 1987). It would be interesting to see whether the same could be done with susceptible BphC proteins. Extension of the substrate range of biphenyl and PCB degraders may require this as a strategy.

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