Characterization of a novel *cis*-benzene dihydrodiol dehydrogenase from *Pseudomonas putida* ML2

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Abstract A second and novel *cis*-benzene dihydrodiol dehydrogenase which is able to dehydrogenate a range of *cis*-dihydrodiols and other vicinal alcohols has been purified from *Pseudomonas putida* ML2. The enzyme is a tetramer of a polypeptide of 39 kDa in molecular mass and has a pH optimum of 9.0. Despite having a primary structure that has significant similarity to glycerol dehydrogenases, the k_{cat}/K_m value of the enzyme for *cis*-benzene dihydrodiol is 4300-fold higher compared to glycerol. The apparent K_m values of the enzyme for *cis*-benzene dihydrodiol and glycerol are 0.01 mM and 46 mM, respectively, and 0.22 mM for NAD^+.

Key words: Benzene catabolism; *Pseudomonas putida* ML2; *cis*-Benzene dihydrodiol dehydrogenase; Type III alcohol dehydrogenase

1. Introduction

Despite the wide variety of natural and man-made aromatic compounds present in the biosphere, soil bacteria utilise, by comparison, only a limited number of catabolic pathways to mineralise these hydrocarbons. A critical and fundamental feature of aerobic degradation of aromatic hydrocarbons by microorganisms is the formation of catechols as central intermediates from diverse aromatic substrates [1]. The initial dihydroxylation reaction is carried out by multicomponent dioxygenase enzymes that incorporate both atoms of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols. The *cis*-dihydrodiols then undergo a dehydrogenation reaction to form catechols [2]. Dehydrogenases that catalyse the oxidation of *cis*-diols from benzoic acid [3], naphthalene [4], toluene [5], chloridazon [6] and phenanthrene [7] have been purified from *Pseudomonas* species. A comparison of these enzymes shows that in most cases they exhibit a high substrate specificity towards *cis*-dihydrodiols and NAD^+, and have pH optima between 7.9 and 9.8. In addition, these dehydrogenases are homotetramers of subunit molecular mass of 28 kDa, and are members of the type II short-chain alcohol dehydrogenase family [8].

In *Pseudomonas putida* ML2 (NCIB 12190), the catabolism of benzene to catechol is catalysed by benzene dioxygenase followed by *cis*-benzene dihydrodiol dehydrogenase. The structural genes encoding benzene dioxygenase, *bedC1C2BA* and *cis*-benzene dihydrodiol dehydrogenase, *bedD*, have been sequenced and are presented elsewhere [9,10]. A *cis*-benzene dihydrodiol dehydrogenase catalysing the conversion of *cis*-benzene dihydrodiol to catechol has previously been purified and characterised from *P. putida* ML2 [11]. The dehydrogenase is unusually large, comprising four subunits each of molecular mass 110 kDa, requires ferrous ions for maximum activity and is specific for the *cis*-isomer of benzene dihydrodiol.

In contrast, molecular work on the catabolic plasmid on which the *bed* genes reside reveals the presence of a dehydrogenase with sequence similarity to glycerol dehydrogenases and capable of complementing a *Pseudomonas cis*-benzene dihydrodiol dehydrogenase mutant [10]. These preliminary findings suggest the involvement of another dehydrogenase enzyme in benzene catabolism. In order to clarify this, we sought to purify the second dehydrogenase enzyme from *P. putida* ML2. Characterisation of the purified enzyme shows that it has different properties compared to those of the earlier enzyme reported and of *cis*-diol dehydrogenases in general. Kinetic data further reveal that the dehydrogenase reported here is more efficient at catalysing the dehydrogenation of *cis*-dihydrodiol to catechol.

2. Materials and methods

2.1. Growth conditions

P. putida ML2 was incubated in minimal medium [12] supplemented with 0.1% (v/v) benzene at 30°C with shaking for 8–10 h. The cells were harvested by centrifugation at $6000 \times g$ for 10 min at 4°C and washed with ice-cold 10 mM Tris-HCl buffer, pH 7.5. The wet weight of the cell pellet was determined and the cell pellet was either used immediately for the preparation of cell-free extract or stored at −20°C until needed.

2.2. Purification of BedD

The *cis*-benzene dihydrodiol dehydrogenase, BedD, was purified from *P. putida* ML2 using a four-step purification procedure involving the preparation of the cell-free extract, followed by ammonium sulphate precipitation, dye-affinity chromatography and NAD^+-agarose chromatography [10].

2.3. Enzyme assay and protein determination

The activity of NAD^+-dependent dehydrogenase was measured spectrophotometrically at 30°C by the linear increase in absorbance at 340 nm produced by the addition of enzyme preparation to 1 ml reaction mixture containing 100 mM substrate, 0.6 mM NAD^+ and 100 mM potassium carbonate buffer (pH 9.0) [13]. The substrates glycerol, 1,2-propanediol, 1,3-propanediol, *cis*-cyclohexane-1,2-diol and *trans*-cyclohexane-1,2-diol were added to the reaction mixtures at a final concentration of 100 mM. The substrates *cis*-benzene dihydrodiol, *cis*-benzoate dihydrodiol, and *cis*-toluene dihydrodiol, *cis*-naphthalene dihydrodiol, *cis*-chlorobenzene dihydrodiol, *cis*-fluorobenzene dihydrodiol and *cis*-trifluoromethylbenzene dihydrodiol were used at a final concentration of 10 mM. Ethanol, methanol and propanol were used at a final concentration of 10% (v/v) of the reaction mixture. One unit of enzyme activity was defined as the amount of protein required to catalyse the conversion of 1 μmol of NAD^+ to NADH per minute ($\varepsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1}).

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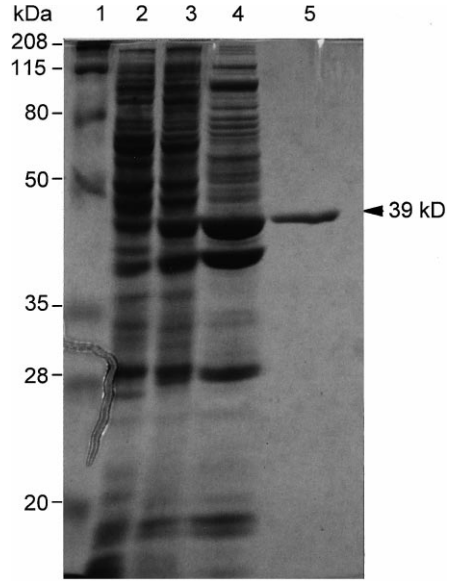

Fig. 1. A 12% SDS-PAGE analysis of samples taken at different stages of BedD purification from *P. putida* ML2. Lane 1, Prestained molecular weight broad range markers (Bio-Rad) used as the standard (top to bottom: myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme); lane 2, crude cell extract; lane 3, 30–45% ammonium sulphate fraction; lane 4, Reactive Red-120 agarose pool; lane 5, NAD^+-agarose pool. The arrowhead marks the position of the 39 kDa protein.

Protein concentrations were determined by the method of Bradford [14]. Bovine serum albumin was used as the standard.

2.4. Kinetic assays

K_m and V_{max} values for the substrates glycerol and *cis*-benzene dihydrodiol and for the coenzyme NAD^+ were determined from Lineweaver-Burk plots derived from the results of experiments in which a fixed concentration of the substrate or the coenzyme and an appropriate range of the other reactant was used. The assays were performed in 100 mM K_2CO_3 buffer, pH 9.0 as described above. The concentrations of the substrates were varied from 25 to 150 mM in the case of glycerol and 0.01 to 0.2 mM in the case of *cis*-benzene dihydrodiol. The concentration of NAD^+ was varied from 0.1 to 0.6 mM.

2.5. Electrophoresis

Proteins were mixed with an equal volume of treatment buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled for 3 min and electrophoresed on a 12% SDS polyacrylamide gel using the discontinuous system of Laemmli [15]. Gels were stained with 0.1% w/v Coomassie blue for protein analysis. The molecular weights of proteins were determined by comparing their electrophoretic mobilities with broad range molecular weight standards (Gibco BRL).

2.6. Analysis of the native BedD protein and activity staining

The purified native BedD protein was analysed on a 6% polyacrylamide gel without SDS. Activity staining was performed by soaking the gel in ice-cold 100 mM K_2CO_3 buffer (pH 9.0) for 5 min at 4°C

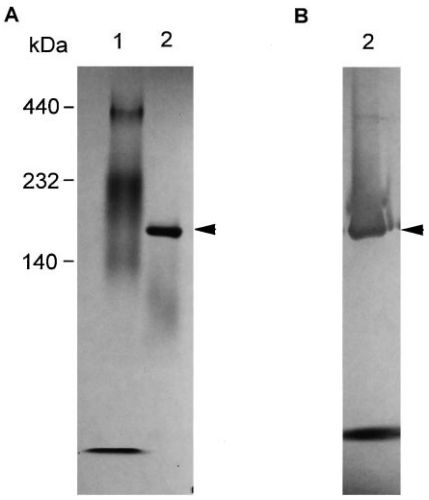

Fig. 2. Samples of the purified BedD enzyme from *P. putida* ML2 analysed on a 6% native polyacrylamide gel. A: Polyacrylamide gel of the native BedD enzyme stained with Coomassie blue. Lane 1, Pharmacia high molecular weight calibration proteins used as markers; lane 2, native BedD enzyme. B: Polyacrylamide activity gel of the native BedD enzyme. Lane 2, native BedD enzyme stained for activity using *cis*-benzene dihydrodiol as the substrate at a final concentration of 10 mM. The arrowhead marks the position of the native protein.

followed by activity staining solution (30 mM ammonium sulphate, 10 mg/ml NAD^+ and 1 mg/ml each of *p*-iodonitrotetrazolium violet and phenazine ethosulphate (Sigma) in 100 mM K_2CO_3 buffer, pH 9.0) containing *cis*-benzene dihydrodiol as substrate. The gel was incubated in activity staining solution at 4°C in the dark for 15–20 min to develop the active band.

2.7. N-terminal amino acid sequence determination

The NAD^+-dependent dehydrogenase, obtained from the last purification step and electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, was blotted onto a polyvinylidene difluoride membrane. The protein was stained with 0.1% Coomassie blue R-250 in 50% methanol for 1–2 min and destained with 30% methanol and 10% acetic acid. The band of interest was excised and subjected to N-terminal sequencing at the Bioprocessing Technology Centre, National University of Singapore.

3. Results

3.1. Purification of BedD

In order to obtain information on the biochemical characteristics of the dehydrogenase, BedD, from *P. putida* ML2, the cell extract was subjected to the four-step purification procedure described. A purification fold of 41 was attained with a 5% recovery of the enzyme activity originally present in the crude extract (Table 1). The purified BedD preparation from *P. putida* ML2 was judged to be essentially pure and homogeneous on the basis of SDS-PAGE analysis (Fig. 1). The

Table 1
Steps in the purification of the BedD protein from *P. putida* ML2

Purification procedure	Total protein (mg)	Total activity (U)	Specific activity[a] (U/mg)	Purification (fold)	Recovery (%)
Cell extract	136.16	635.54	4.6	1	100
Ammonium sulphate (45%)	35.15	277.69	7.9	2	44
Reactive Red-120 agarose	12.96	273.46	21.1	5	43
NAD^+-agarose	0.16	29.92	187.0	41	5

[a]Activity was measured as described in Section 2 with *cis*-benzene dihydrodiol as substrate at a final concentration of 10mM. NAD^+ was added at a final concentration of 0.6 mM.

Table 2
Substrate specificity of the dehydrogenase, BedD, from *P. putida* ML2

Substrate	Specific activity[a] (U/mg)
Glycerol	68 ± 16
1,2-Propanediol	513 ± 46
1,3-Propanediol	0
cis-Benzene dihydrodiol	153 ± 12
cis-Benzoate dihydrodiol	0
cis-1,2-Cyclohexanediol	313 ± 23
trans-1,2-Cyclohexanediol	42 ± 10
cis-Toluene dihydrodiol	147 ± 42
cis-Naphthalene dihydrodiol	53 ± 3
cis-Chlorobenzene dihydrodiol	87 ± 12
cis-Fluorobenzene dihydrodiol	140 ± 24
cis-Trifluoromethylbenzene dihydrodiol	0
Methanol	0
Ethanol	0
Propanol	0
$NADP^+$	0[b]

[a]Activity was measured as described by Tang et al. [13]. Glycerol, 1,2-propanediol, 1,3-propanediol, *cis*- and *trans*-1,2-cyclohexanediol were used at a final concentration of 100 mM while *cis*-benzene dihydrodiol, *cis*-benzoate dihydrodiol, *cis*-toluene dihydrodiol, *cis*-naphthalene dihydrodiol, *cis*-chlorobenzene dihydrodiol, *cis*-fluorobenzene dihydrodiol and *cis*-trifluoromethylbenzene dihydrodiol were used at a final concentration of 10 mM. Methanol, ethanol and propanol were used at a final concentration of 10% v/v. The values represent the mean ± S.D. of three different determinations.
[b]NAD^+ was replaced by $NADP^+$ at a concentration of 0.6 mM. The dehydrogenase activity was determined using 100 mM glycerol as substrate.

purity of the enzyme preparation was also determined by non-denaturing PAGE (Fig. 2) followed by staining for protein and for enzyme activity. In each case, a single band that stained for protein as well as for enzyme activity was observed. The purified BedD protein migrated as a single band corresponding to an estimated molecular mass of ~39 kDa during SDS-PAGE which is in agreement with a calculated molecular mass of 38 448 Da deduced from the amino acid sequence [10]. The molecular mass of the native enzyme under non-denaturing polyacrylamide gel electrophoresis was estimated to be ~166 kDa, suggesting that the dehydrogenase is a tetramer consisting of four apparently identical subunits (Fig. 2).

3.2. N-terminal sequence

The first 15 residues of the purified protein were identified to be Met-Asp-Arg-Ala-Ile-Gln-Ser-Pro-Gly-Lys-Tyr-Val-Gln-Gly-Ala, identical to the deduced amino acid sequence of *bedD* [10].

3.3. Enzyme stability

The purified enzyme preparation was less stable at 4°C than at −20°C. Loss of activity occurred at 4°C gradually over a few days and could not be restored by the addition of NAD^+ or metal ions. Because of this instability, the enzyme was prepared fresh for each study.

3.4. Substrate specificity

The substrates oxidised by the dehydrogenase and the respective specific activities are shown in Table 2. *cis*-Benzene dihydrodiol, *cis*-benzoate dihydrodiol, *cis*-toluene dihydrodiol, *cis*-naphthalene dihydrodiol, *cis*-chlorobenzene dihydrodiol, *cis*-fluorobenzene dihydrodiol and *cis*-trifluoromethylbenzene dihydrodiol were used at a final concentration of 10 mM, since higher concentrations of the aromatic compounds interfered with the absorbance readings at 340 nm. The BedD enzyme exhibited a rather broad substrate specificity, being able to recognise *cis*-toluene, *cis*-naphthalene, *cis*-chlorobenzene and *cis*-fluorobenzene dihydrodiols in addition to *cis*-benzene dihydrodiol, *cis*- and *trans*-1,2-cyclohexanediol, 1,2-propanediol and glycerol.

Substrates possessing hydroxyl groups on adjacent carbon atoms or vicinal alcohol groups showed higher specific activities compared to substrates such as *trans*-1,2-cyclohexanediol and glycerol whose hydroxyl groups are not in the *cis* configuration. No activity was detected for 1,3-propanediol or for primary alcohols. Hence, for the reduction of NAD^+, substrates possessing vicinal alcohol groups appear to be a prime requirement. Of the substrates tested 1,2-propanediol had the highest specific activity followed by *cis*-1,2-cyclohexanediol. *cis*-Toluene, *cis*-fluorobenzene and *cis*-benzene dihydrodiol had comparable activities. The rates of dehydrogenation of *cis*-chlorobenzene dihydrodiol and *cis*-naphthalene dihydrodiol were, respectively, 1.8- and 2.9-fold lower than *cis*-benzene dihydrodiol. It is interesting to note that the specific activity of the enzyme decreased with increasing size of the substituent. No activity was observed for *cis*-benzoate and *cis*-trifluoromethylbenzene dihydrodiol. The pure enzyme showed an absolute requirement for NAD^+ as the electron acceptor. No activity was detected when NAD^+ was replaced by $NADP^+$.

3.5. Kinetic properties

The Michaelis-Menten kinetic parameters of the purified NAD^+-dependent dehydrogenase for the substrates glycerol, *cis*-benzene dihydrodiol and NAD^+ were determined from Lineweaver-Burk (double reciprocal) plots (Table 3). The apparent K_m values of the enzyme for *cis*-benzene dihydrodiol and NAD^+ were 0.01 mM and 0.22 mM, respectively. Although the BedD protein bears significant sequence similarity to glycerol dehydrogenases [16–18], the affinity of BedD for glycerol is significantly lower than that for *cis*-benzene dihydrodiol, with the apparent K_m for glycerol being 46 mM.

Table 3
Kinetic constants of the NAD^+-dependent dehydrogenase, BedD, from *P. putida* ML2

Substrate	K_m (mM)	V_{max} (M min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Glycerol	46.0 ± 13.6	$(1.5 \pm 0.2) \times 10^{-5}$	1.3 ± 0.5
cis-Benzene dihydrodiol	$(1.1 \pm 0.1) \times 10^{-2}$	$(5.8 \pm 0.3) \times 10^{-6}$	5597.7 ± 176.3
NAD^+	$(21.6 \pm 1.2) \times 10^{-2}$	$(7.9 \pm 0.9) \times 10^{-6}$	378.6 ± 36.2

The activity of the NAD^+-dependent dehydrogenase was measured in 100 mM K_2CO_3 buffer at pH 9.0 as described in Section 2. NAD^+ was used at a final concentration of 0.6 mM for the determination of K_m and V_{max} for glycerol and *cis*-benzene dihydrodiol while *cis*-benzene dihydrodiol was used at a final concentration of 10 mM for the determination of K_m and V_{max} for NAD^+. The results shown are the mean ± S.D. of three independent experiments.

Table 4
Comparison of the two dehydrogenase isoenzymes of *P. putida* ML2

Substrate	Organism	Number of subunits	Subunit M_r (kDa)	K_m (mM)	pH optimum	Metal requirement	Reference
cis-Benzene dihydrodiol	*P. putida* ML2	4	39	0.011	9.0	none	This study
cis-Benzene dihydrodiol	*P. putida* ML2	4	110	0.286	7.9	Fe^{2+}	[11]

The higher k_{cat}/K_m value of BedD for *cis*-benzene dihydrodiol compared to glycerol also indicates that the enzyme is more specific and efficient at utilising *cis*-benzene dihydrodiol as substrate.

4. Discussion

A second NAD^+-dependent *cis*-benzene dihydrodiol dehydrogenase, BedD, has been purified from *P. putida* ML2. The BedD enzyme is unusual in having a subunit molecular mass of 39 kDa, a broad substrate specificity and sequence similarity to glycerol dehydrogenases. These properties make *cis*-benzene dihydrodiol dehydrogenase the first reported bacterial enzyme to be involved in aromatic hydrocarbon degradation belonging to the type III alcohol dehydrogenase family [19–22]. In comparison, dehydrogenases that catalyse the oxidation of *cis*-diols from benzoic acid, toluene and naphthalene are members of the type II (*Drosophila*-type) short-chain alcohol dehydrogenase family, where the subunit molecular mass is approximately 28 kDa and there is no requirement for metal ions for activity [8].

Besides being able to utilise a variety of aromatic *cis*-dihydrodiols as substrates, the BedD enzyme is also able to dehydrogenate other vicinal alcohols. Among the *cis*-dihydrodiols tested, there appears to be a relationship between the specific activity and the size of the substituent on the benzene nucleus. No activity was observed with *cis*-benzoate and *cis*-trifluoromethylbenzene dihydrodiols suggesting that steric hindrance by the substituent groups may prevent the binding of the substrate to the active site [23,24].

The biochemical properties of this *cis*-benzene dihydrodiol dehydrogenase differ significantly from those of the 110 kDa dehydrogenase reported previously from the same organism [11] in terms of subunit molecular mass, pH optimum and lack of a strict requirement for ferrous ions for activity (Table 4). Interestingly, a comparison of the apparent K_m values of both enzymes for the substrate shows that BedD is 26-fold more efficient at converting *cis*-benzene dihydrodiol to catechol (Table 4). No sequence information is available for the 110 kDa dehydrogenase, but based on the difference in subunit molecular mass, it can be expected that the two enzymes would differ at the nucleotide and amino acid levels, and probably bear little or no sequence similarity to one another. Despite the differences that exist between BedD and the other dehydrogenase, both enzymes are similar with respect to their specificity towards *cis*-benzene dihydrodiol and their absolute requirement for NAD^+ as the electron acceptor.

Isoenzymes of several members of the type III alcohol dehydrogenase family have been isolated from other microorganisms [25–27]. However, it is evident that the 39 kDa BedD enzyme is the one utilised by *P. putida* ML2 in the catabolic pathway for the degradation of benzene. Firstly, the contiguous genetic organisation of the *bedDC1C2BA* genes on a catabolic transposon, Tn*5542*, in *P. putida* ML2 [28] is consistent with other similar aromatic catabolic operons [29,30]. Secondly, biochemical characterisation of the BedD enzyme indicates that it functions more like a *cis*-dihydrodiol dehydrogenase rather than a glycerol dehydrogenase to which it bears greater sequence similarity [10]. Both the affinity and catalytic efficiency of the enzyme for *cis*-benzene dihydrodiol relative to glycerol as substrate are more than 4000 times greater (Table 3). Finally, the apparent K_m value of the BedD enzyme for *cis*-benzene dihydrodiol is lower than that of the 110 kDa dehydrogenase. It is conceivable that the gene encoding the BedD enzyme may have been selectively acquired through transposition and, through genetic rearrangements and point mutations over the course of time, has evolved to encode a more effective *cis*-dihydrodiol dehydrogenase compared to the 110 kDa enzyme identified previously.

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