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# Purification and some properties of (1*R*,2*S*)-1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate dehydrogenase from *Comamonas testosteroni* T-2

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**Abstract**

Inducible (1*R*,2*S*)-1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate (diene-diol) dehydrogenase was found in extracts of *Comamonas testosteroni* T-2 grown in *p*-toluate-or terephthalate-salts medium and it was purified using anion exchange, hydrophobic interaction and gel filtration chromatography. The enzyme is a homodimer with subunit $M_r$ 39000. It had a specific activity of 500 mkat/kg of protein and was activated by the addition of $Fe^{2+}$. The dehydrogenase converted 1 mol diene-diol and 1 mol $NAD^+$ to 1 mol protocatechuic acid, 1 mol NADH and 1 mol $CO_2$. Apparent $K_m$-values of 43 $\mu$M ($NAD^+$) and about 90 $\mu$M (diene-diol) were determined. The hydride ion was transferred to the *si* face of $NAD^+$.

*Keywords:* Terephthalate; Diene-diol dehydrogenase; Enzyme purification; *Comamonas testosteroni* T-2; Biodegradation

## 1. Introduction

Phthalate, *iso*-phthalate and terephthalate (1,2-, 1,3-and 1,4-dicarboxybenzene, respectively) are unreactive aromatic compounds that can enter the environment from industrial or natural sources [1,2]. Bacterial degradation of all three isomers has been reported [1,3,4] but the metabolism of phthalate itself is best understood [5]. In contrast to the three steps from phthalate to protocatechuate, there are only two steps from terephthalate to this intemediate (Fig. 1). Whereas the multi-component terephthalate dioxygenase (Fig. 1) has been partially characterized [2], the (1*R*,2*S*)-1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate (diene-diol) dehydrogenase has not been described. We now report the purification and some properties of this dehydrogenase, the last step specific to the degradation of *p*-toluate to protocatechuate in *Comamonas testosteroni* T-2 [6].

## 2. Materials and methods

### 2.1. Materials

The diammonium salt of the diene-diol was kindly provided by Celgene (Warren, NJ). We identified the

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diene-diol indirectly, using the characteristic of these compounds that water is lost on acidification [7]; the product was derivatized with diazomethane and identified by GC-MS [2,8] as a dimethylated derivative of 2-hydroxyterephthalate. The preparation of the diene-diol was initially chromatographically pure but it deteriorated over time. Complete enzymic transformation of this diene-diol with excess of $NAD^+$ yielded 0.5 mol protocatechuate as the sole product; the impurity was not altered. We concluded that the preparation used contained 50% (wt/wt) diene-diol. [4-$^3$H]-$NAD^+$ was prepared from D-[1-$^3$H]-glucose [9,10]. The Carbosieve SII column (100/120 mesh; 3 m × 2 mm; stainless steel) was from Supelco (Bellefonte, PA). All other chemicals and materials used were from sources indicated elsewhere [2,8,11].

### *2.2. Organism, growth conditions and preparation of cell-free extracts*

*Comamonas testosteroni* T-2 (DSM 6577 [12]) was grown in toluate-or terephthalate-salts medium as described previously [2,8]. Cells harvested in the mid-log growth phase were disrupted in a French pressure cell to obtain crude extract [2].

Diene-diol dehydrogenase activity was measured photometrically at 340 nm as diene-diol-dependent generation of NADH in an assay containing (in 1 ml) 40 μmol Tris sulfate, pH 7.5, 600 nmol $NAD^+$ and 10 to 100 μg of protein. The reaction was started by the addition of 750 nmol diene-diol [2]. If required, the chelator EDTA (up to 125 μM) was added to the reaction, sometimes with preincubation. The standard reaction, in 8-ml septum vials, was used to follow release of $CO_2$ from diene-diol; samples (0.3 ml) of the gas phase were taken by gas-tight syringe and examined by GC with thermal conductivity detection. The organic product from diene-diol was tentatively identified and quantified by reversed-phase HPLC at room temperature [2,13]. The $M_r$ of the native enzyme was estimated by gel filtration through a Superose 6 column [14]. SDS-PAGE was used for the determination of $M_r$ under denaturing conditions and to monitor the protein purification. Proteins in gels were visualized by staining with Coomassie brilliant blue (the method of Laemmli) or silver [15]. Protein concentration was measured by the method of Bradford with bovine serum albumin as standard. The stereospecificity of the diene-diol dehydrogenase was determined in the standard assay (see above and [2]), containing 0.4 nmol [4-$^3$H]$NAD^+$ (170000 dpm). After completion of the reaction, 10 U of yeast alcohol dehydrogenase (Sigma) and 2 μl acetaldehyde were added to the reaction to reoxidize the generated NADH in a defined stereospecific (pro-*R*) reaction [9]. The reaction products were examined by reversed-phase HPLC and in a liquid scintillation counter as described elsewhere [9].

### *2.3. Purification of the diene-diol dehydrogenase*

The diene-diol dehydrogenase was routinely purified from toluate-grown cells because the enzyme

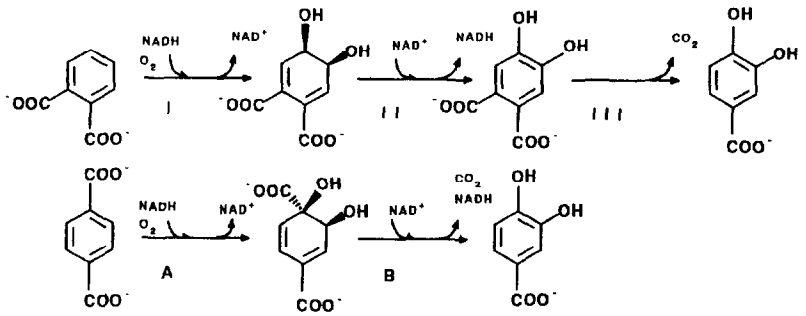


Fig. 1. Initial steps in the degradative pathways of phthalate and terephthalate. The phthalate dioxygenase system (reaction I) is one of the best-characterized dioxygenase systems [26]. The oxidation of the diene-diol to 4,5-dihydroxyphthalate (reaction II) has not been studied in detail, whereas the enzyme catalyzing decarboxylation to protocatechuate (reaction III) has been purified from two organisms [27,28]. The terephthalate dioxygenase system (reaction A) has been partially characterized [2] and the diene-diol dehydrogenase (reaction B) was detected in our preliminary experiments (cited in [6]); the reactions have also been detected in *Pseudomonas putida* (cited in [6]).

cochromatographed on two columns (see below) with component A (oxygenase) of 4-sulfobenzoate 1,2-dioxygenase system [16], which is expressed in terephthalate-but not in toluate-grown cells (Schläfli Oppenberg, 1995, unpublished data). 20 mM Tris sulfate, pH 7.5, containing 1 mM dithiothreitol (buffer A), was used as basis for all eluents. The purification procedure is, in most practical aspects, similar to that of the terephthalate dioxygenase system [2].

Step 1: the preparation of crude extracts free of nucleic acids [2,16], which were removed by protamine sulfate precipitation.

Step 2: anion-exchange chromatography on a Mono Q HR 10/10 (Pharmacia, 10 × 100 mm) with a gradient of $Na_2SO_4$ as described for the terephthalate dioxygenase system [2]. The diene-diol dehydrogenase activity eluted at the beginning of the gradient, after fraction R (containing the reductase function of the terephthalate dioxygenase system) and overlapping with any component A of the 4-sulfobenzoate dioxygenase system. Fractions containing significant diene-diol dehydrogenase activity were pooled and concentrated by ultrafiltration (Centriprep, 10 kDa exclusion limit).

Step 3: hydrophobic interaction chromatography was done with a Phenyl-Superose HR 5/5 column (5 × 50 mm, Pharmacia) [cf. 2]. The diene-diol dehydrogenase from step 2 was loaded on to the column and a gradient of $(NH_4)_2SO_4$ in buffer A was applied which decreased over the course of 30 min from 0.5 M to 0 M. A single diene-diol dehydrogenase activity eluted at the end of the gradient which was essentially pure when the purification was accomplished from toluate-extract. The diene-diol dehydrogenase from terephthalate-extract was contaminated after this step with oxygenase A from the 4-sulfobenzoate dioxygenase system. Crude diene-diol dehydrogenase was pooled and concentrated (Centricon, see above).

Step 4: a Superose 6 column (10 × 300 mm, Pharmacia) was equilibrated (flow of 0.5 ml $min^{-1}$) on buffer A, containing 0.2 M $Na_2SO_4$. The diene-diol dehydrogenase (200 $\mu$l) from step 3 was loaded onto the column and diene-diol dehydrogenase activity eluted after 30 min in a symmetrical peak, containing essentially one protein as monitored by SDS-PAGE.

## 3. Results and discussion

The diene-diol dehydrogenase was detected in extracts of cells of *C. testosteroni* T-2 which could utilize terephthalate (e.g. toluate-or terephthalate-grown) and was not observed under other conditions (e.g. succinate-grown cells). We consider the dehydrogenase to be an inducible enzyme. We obtained 90 $\mu$g of pure enzyme from about 54 mg protein in the extract (Table 1). Given a purification factor of 55, the amount of diene-diol dehydrogenase in the cell is calculated to be about 2% of total soluble protein. The amount of the terephthalate dioxygenase system is in the same range [cf. 2]. The diene-diol dehydrogenase activity observed in the extract (10 mkat/kg of protein; Table 1) is five times the activity needed to support the growth rate observed in terephthalate-salts medium [2].

The diene-diol dehydrogenase could be purified to homogeneity in three steps (Fig. 2, Table 1) and an $M_r$-value of 39000 was observed under denaturing conditions (Fig. 2). The $M_r$ obtained under native conditions was 60000 ± 10000. These values suggest a homodimeric structure. The known diol dehydrogenases are described as dimeric [14], tetrameric

Table 1
Purification of the diene-diol dehydrogenase from *C. testosteroni* T-2

| Protein fraction | Volume (ml) | Total protein ($\mu$g) | Total activity (nkat) | Specific activity (mkat/kg of protein) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 0.91 | 53700 | 518 | 9.6 | 100 | 1 |
| Anion exchange chromatography | 3.63 | 4200 | 109 | 25.8 | 21 | 2.7 |
| Hydrophobic interaction chromatography | 0.93 | 128 | 51 | 400 | 9.8 | 41.5 |
| Gel filtration chromatography | 2.0 | 90 | 48 | 533 | 9.3 | 55.3 |

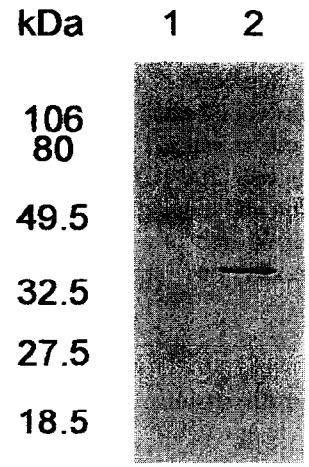


Fig. 2. SDS-PAGE of purified diene-diol dehydrogenase. Proteins in the gel were visualized by silver-staining. 1, prestained low-range marker proteins (Bio-Rad); 2, 1 $\mu$g of the diene-diol dehydrogenase purified by gel filtration chromatography.

[17–22] or hexameric [23], with $M_r$s of about 25 000 to 30 000 [14,18–20,22,23], 50 000 [21] or 110 000 [17]. The diol dehydrogenase from *C. testosteroni* T-2 therefore appears to be unusual both in subunit-$M_r$ and subunit composition.

Enzyme catalysis gave a 98% yield of NADH from $NAD^+$ and a 93% yield of protocatechuate from $NAD^+$. The anticipated gaseous product of the reaction, $CO_2$, was detected in the gas phase in 57–83% of the theoretical yield by GC. This product, which was absent from control experiments, had the same retention time as authentic material. The data indicate that 1 mol diene-diol and 1 mol $NAD^+$ yield 1 mol protocatechuate, 1 mol NADH, and 1 mol $CO_2$.

The enzyme was specific for $NAD^+$, $NADP^+$ was not a cofactor. The apparent $K_m$ for $NAD^+$ (43 $\mu$M) was low, similar to that for benzene dihydrodiol dehydrogenase [17], whereas other diene-diol dehydrogenases have values ranging up to about 900 $\mu$M. The apparent $K_m$ for the diene-diol, about 90 $\mu$M, is at the higher end of the range observed, values $< 2$ $\mu$M being known [18]. Addition of FAD or FMN had no effect on the enzyme activity.

We examined the stereospecificity of hydride transfer by the diene-diol dehydrogenase to [4-$^3$H]$NAD^+$. In experiments with different enzyme preparations we found that the secondary enzyme, yeast alcohol dehydrogenase, which is specific for the pro-*R* hydride of NADH, removed more than 85% of the tritium label from the [4-$^3$H]NADH generated by the diene-diol dehydrogenase. We therefore conclude that the diene-diol dehydrogenase specifically transfers hydride ions to the (4*S*)-position (*si*-face) of $NAD^+$.

We observed the highest activity of the diene-diol dehydrogenase at pH 8.2 in 20 mM Tris-sulfate. Two described diol dehydrogenases have a similar optimal pH [17,22], and most other enzymes a significantly higher optimal pH (pH 9.0 and more) [18–21,23].

The UV-visible spectrum of the enzyme, with no absorbance in the visible or near UV (a $\lambda_{max}$ at 280 nm) gave no evidence for prosthetic groups with chromophores. The reaction was activated by a factor of about two on the addition of iron (saturation at 60 $\mu$M $Fe^{2+}$). The reaction could be totally inhibited by the addition of EDTA, the amount of which decreased with increasing preincubation. Addition of iron could overcome the inhibition.

In a recent review, Reid and Fewson [24] classified the microbial alcohol dehydrogenases into three major categories, the first category comprizing NAD(P)-dependent enzymes in three sub-groups. The known properties of the diene-diol dehydrogenase do not fit the characteristics of any of these sub-groups. Group I enzymes are pro-*R* specific, zinc-dependent enzymes consisting of about 350 amino acid residues. Some aromatic diene-diol dehydrogenases can be attributed to group II, which consists mainly of smaller (25–30 kDa), metal-independent homomultimeric enzymes with predominant pro-*S* specificity and distinct sequence similarities [24,25]. The diene-diol dehydrogenase would be an unusual member of group II for its subunit size and iron requirement. However, determination of an apparent $M_r$ by SDS-PAGE does not yield absolute values and the native $M_r$ could indicate a smaller subunit size. Group III of NAD(P)H requiring alcohol dehydrogenases contains long-chain enzymes (40–100 kDa), that can be activated by iron. No aromatic diol dehydrogenase has been attributed to this group of alcohol dehydrogenases so far, but the diene-diol dehydrogenase and also *cis*-benzene dihydrodiol dehydrogenase could be members of this group. With respect to the known enzymes of this reaction type,

the diene-diol dehydrogenase shows most similarity to benzene dihydrodiol dehydrogenase (pH optimum, $K_m$ ($NAD^+$), activation by $Fe^{2+}$) [17].

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