Biochem. J. (1987) 244, 585–590 (Printed in Great Britain)

Purification and some properties of a novel heat-stable *cis*-toluene dihydrodiol dehydrogenase

Helen D. SIMPSON, Jeffrey GREEN and Howard DALTON
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

cis-Toluene dihydrodiol dehydrogenase was purified 200-fold from cells of a thermotolerant *Bacillus* species grown with toluene as the sole source of carbon and energy. The purified enzyme preparation was remarkably heat-stable and exhibited a half-life of 100 min at 80 °C, the temperature optimum. The activation energy of the reaction was 36 kJ·mol^{-1}. Isoelectric focusing indicated that the pI of the native enzyme was 6.4 and that of the denatured enzyme 6.5. Although the pH optimum was 9.8, the enzyme was most stable at pH 8. The M_r of the enzyme was approx. 172000 as determined by gel filtration and 166000 by polyacrylamide-gel electrophoresis. The enzyme was composed of six apparently identical subunits with M_r values of 29500. Kinetic analysis revealed that the K_m for *cis*-toluene dihydrodiol was 92 μM and for NAD$^+$ was 80 μM. The apparent K_m values for *cis*-benzene dihydrodiol and *cis*-naphthalene dihydrodiol were 330 μM and 51 μM respectively. The enzyme was inhibited by mercurials but was unaffected by metal-ion chelators. Steady-state kinetics and product-inhibition patterns suggested that the enzyme mechanism was ordered Bi Bi.

INTRODUCTION

We have recently isolated a thermotolerant *Bacillus* species from the aeration tank of a waste treatment works which degraded toluene via *cis*-toluene dihydrodiol. *cis*-Toluene dihydrodiol undergoes dehydrogenation, resulting in the formation of 3-methylcatechol, which is then cleaved by catechol 2,3-oxygenase, eventually giving rise to tricarboxylic-acid-cycle intermediates. Bacteria which catabolize toluene via *cis*-toluene dihydrodiol are of specific interest, since *cis*-toluene dihydrodiol can be polymerized to produce high-M_r polyphenylene, an organic polymer which may have uses in the electronics industry (Green & Street, 1984). Furthermore, enzymes from thermotolerant organisms may be ideally suited for industrial processes, since, in general, they exhibit greater heat-stability than do enzymes from mesophiles (Sonnleitner & Fiechter, 1983).

Dehydrogenases catalysing the oxidation of *cis*-benzoic acid 1,2-diol (3,5-cyclohexadiene-1,2-diol-1-carboxylic acid) (Reiner, 1972), *cis*-benzene dihydrodiol (*cis*-1,2-dihydroxycyclohexa-3,5-diene) (Axcell & Geary, 1973), *cis*-naphthalene dihydrodiol (*cis*-1,2-dihydroxy-1,2-dihydronaphthalene) (Patel & Gibson, 1974), *cis*-toluene dihydrodiol (*cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene) (Rogers & Gibson, 1977) and chloridazodihydrodiol [5-amino-4-chloro-2-(2,3-*cis*-dihydroxycyclohexa-4,6-dienyl)-2*H*-pyridazin-3-one] (Eberspächer & Lingens, 1978) have been purified. These enzymes share similar properties in that they are all tetramers, utilize NAD$^+$ as their primary electron acceptor and oxidize a range of *cis*-dihydrodiols. The *cis*-toluene dihydrodiol dehydrogenase purified from *Pseudomonas putida* (Rogers & Gibson, 1977) consisted of four identical subunits of M_r 27000 and was specific for NAD$^+$ as the electron acceptor. The enzyme was inhibited by reducing agents and had a high affinity for *cis*-toluene dihydrodiol and indeed was specific for *cis*-dihydrodiols.

The present paper describes the purification and properties of a heat-stable *cis*-toluene dihydrodiol dehydrogenase. This enzyme is induced in cells of a thermotolerant *Bacillus* species during growth at 50 °C on a minimal medium with toluene as the sole source of carbon and energy.

MATERIALS AND METHODS

Materials

NAD$^+$, NADH, Trizma base, α,α'-dipyridyl, bathophenanthroline, *p*-hydroxymercuribenzoate, Nitro Blue Tetrazolium, potassium ferricyanide, phenazine methosulphate, cytochrome *c* and standard proteins were purchased from Sigma (Poole, Dorset, U.K.). Calibration kits for protein standards to determine M_r values were obtained from Pharmacia (Hounslow, Middx., U.K.). pI markers for isoelectric focusing were obtained from LKB (Bromma, Sweden). 8-Hydroxyquinoline-5-sulphonic acid monohydrate was supplied by Aldrich (Gillingham, Dorset, U.K.). 3-Methylcatechol was obtained from Phase Separations (Queensferry, Clwyd, Wales, U.K.). *cis*-Toluene dihydrodiol and *cis*-benzene dihydrodiol were gifts from Dr. S. C. Taylor, ICI, Billingham, Cleveland, U.K. *cis*-Naphthalene dihydrodiol was a gift from Dr. R. O. Jenkins, University of Warwick, Coventry, U.K. All other chemicals, except where stated in the text, were obtained from BDH (Poole, Dorset, U.K.).

Growth of organism and preparation of cell extract

The organism was isolated from the aeration tank of a waste treatment works and was designated '*Bacillus* sp. AT50'. The organism was grown at 50 °C and pH 6.8 on a minimal medium containing (g·l^{-1}): KH_2PO_4, 2; NH_4Cl, 3; $MgSO_4,7H_2O$, 0.4; trace-element solution (Vishniac & Santer, 1957), but containing only 0.22% $ZnSO_4,7H_2O$, 2 ml·l^{-1}. Cells were grown in a 1-litre-

working-volume chemostat (LH Engineering, Stoke Poges, Slough, Berks., U.K.) at a dilution rate of 0.1 h^{-1} with toluene vapour as the sole carbon source.

Cells were collected by centrifugation and washed once in 0.05 M-potassium phosphate buffer, pH 7.2. The pellet was routinely resuspended (1 g wet wt./ml of buffer) in 0.05 M-potassium phosphate buffer, pH 7.2, containing 10% (v/v) ethanol and 10% (v/v) glycerol. Cells were stored at −70 °C until required. The cell suspension was disrupted by three passages at 137 MPa through a chilled French pressure cell (Aminco, Silver Spring, MD, U.S.A.), followed by centrifugation at 100000 *g* for 30 min. The supernatant was used as the source of enzyme in subsequent purification procedures.

Protein was determined by a Bio-Rad (Watford, Herts., U.K.) assay, with bovine serum albumin as standard.

Enzyme assay

The enzyme was assayed spectrophotometrically by a modification of the method of Reiner (1972). Assays were routinely carried out at pH 8.0 and 45 °C. The reaction mixture (3 ml) contained 20 mM-Tris/HCl, pH 8.0, *cis*-toluene dihydrodiol (0.5 mM), NAD^+ (0.5 mM) and enzyme. NADH formation was monitored at 340 nm by using a Pye–Unicam SP.8-200 spectrophotometer.

For pH stability and optima studies the following buffers were substituted for the Tris/HCl buffer: 50 mM-citrate buffer (pH range 3–6); 50 mM-Tris/HCl buffer (pH range 6–9); and 50 mM-carbonate/bicarbonate buffer (pH range 9–10.4). The change of buffer had minimal effect on the activity of the enzyme. The stability studies were performed by incubating the enzyme at the selected pH value for 30 min at 4 °C and then titrating back to pH 8.0 and assaying at pH 8.0.

One unit of enzyme activity was defined as the amount of enzyme required to reduce 1.0 μmol of NAD^+/min. Specific activities are expressed as units per mg of protein.

Purification procedure

The enzyme was purified from cell extracts by using a four-step purification procedure described below. Steps 2–4 were carried out at 4 °C.

Step 1: heat treatment. The cell extract was heated for 90 min at 75 °C in a water bath, centrifuged at 38000 *g* for 30 min, and the precipitate discarded.

Step 2: DEAE-Sepharose chromatography. The supernatant from step 1 was applied to a column (2 cm × 28 cm) of DEAE-Sepharose CL-6B (Sigma) previously equilibrated with 20 mM-potassium phosphate buffer, pH 7.2. The column was washed with 20 ml of the same buffer, after which the *cis*-toluene dihydrodiol dehydrogenase activity was eluted with a linear gradient of 0–0.5 M-NaCl in the same buffer, and 5 ml fractions were collected.

Step 3: hydroxyapatite column chromatography. The peak fractions (37–39) from step 2 were diluted 1:1 in 20 mM-potassium phosphate buffer, pH 7.2, to reduce the concentration of NaCl in the solution. The resulting protein solution was applied to a column (2.5 cm × 5 cm) of hydroxyapatite (Bio-Rad). The column was washed with 40 ml of buffer before the enzyme activity was eluted with 400 ml of a linear gradient of 20–500 mM-potassium phosphate buffer, pH 7.2; 5 ml fractions were collected.

Step 4: 5′-cyclic AMP–Sepharose column chromatography. The peak fractions (24–26) from step 3 were pooled and further purified by affinity chromatography on a 5′-cyclic AMP–Sepharose (Sigma) column (2 cm × 11 cm). The column was washed with 20 mM-potassium phosphate buffer, pH 7.2, until protein could no longer be detected in the eluate. The *cis*-toluene dihydrodiol dehydrogenase was eluted by passing 100 ml of 10 mM-NADH through the column, and 5 ml fractions were collected. The peak fractions (24 and 25) were pooled and concentrated by ultrafiltration through an Amicon (Lexington, MA, U.S.A.) PM10 membrane to remove NADH.

M_r determination by gel filtration

The M_r of the purified *cis*-toluene dihydrodiol dehydrogenase was determined by chromatography on a calibrated column of Ultrogel AcA34 (LKB, Croydon, Surrey, U.K.).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970). Acrylamide gradient (10–30%, w/v) gels were used. For non-dissociating gels, electrophoresis was carried out in Tris/glycine buffer, pH 9.5, for 2000 V·h. For SDS-containing dissociating gels, a constant current of 7 mA was applied until the Bromophenol Blue marker reached the bottom of the gel. Protein was stained by immersing the gel in a 0.1% solution of Coomassie Blue in 'destain' (methanol/acetic acid/water, 4:1:5, by vol.).

Isoelectric focusing

Isoelectric focusing was performed as described by O'Farrell (1975). Gels were made in glass tubes (130 mm × 2.5 mm) and were run under dissociating conditions (in the presence of urea). The pI value was determined by using standards of pH range 3–10 obtained from Pharmacia, by measuring the pH in the gradient at the position of the major band. The pI value for the native protein was determined using LKB Ampholine PAG plates with pI markers in the pH range 5.65–8.3.

Stoichiometry of reaction

To investigate the stoichiometry of the reaction the assay was carried out at 45 °C and pH 7.0 with 0.5 mM-*cis*-toluene dihydrodiol, 0.5 mM-NAD^+ and 40 μg of purified enzyme. The amount of NAD^+ converted into NADH was monitored at 340 nm in a Pye–Unicam spectrophotometer. After 30 min a sample of the assay mixture was removed and the amount of 3-methyl catechol produced and *cis*-toluene dihydrodiol consumed was determined using h.p.l.c. (Jenkins & Dalton, 1985).

RESULTS

Purification

A summary of the purification procedure is illustrated in Table 1. A 210-fold purified preparation of *cis*-toluene

Table 1. Summary of purification

Step	Volume (ml)	Protein (mg/ml)	Enzyme (units)	Specific activity	Purification factor	Recovery (%)
Cell extract	30	54	636	0.39	0	100
1. Heat treatment	20	32.5	578.4	0.89	2	91
2. DEAE-Sepharose CL-6B	20	8.95	265.2	1.48	4	42
3. Hydroxyapatite	10	0.81	130.2	16.1	41	20
4. 5'-Cyclic AMP–Sepharose	2	0.5	80.5	81.0	210	13

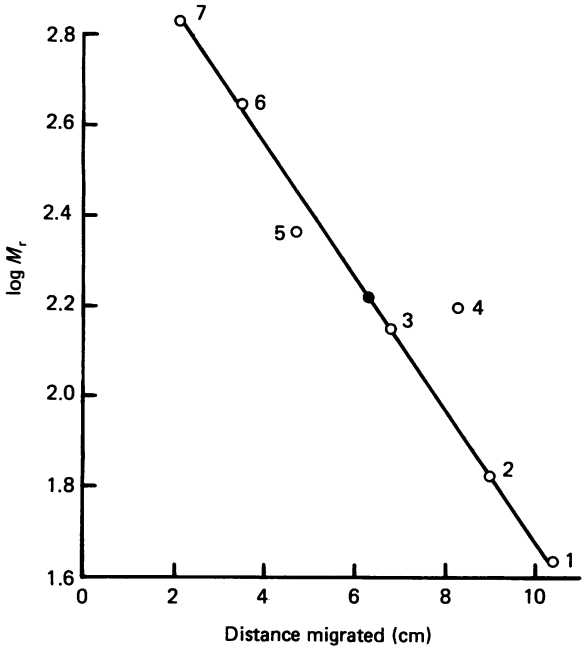

Fig. 1. M_r determination of purified *cis*-toluene dihydrodiol dehydrogenase using a non-denaturing polyacrylamide gel

The native M_r of *cis*-toluene dihydrodiol dehydrogenase was determined by measuring the electrophoretic mobility of the protein on a non-denaturing polyacrylamide gradient gel as described in the Materials and methods section. Protein standards represented by open circles (○) were: (1) ovalbumin, (2) bovine serum albumin, (3) lactate dehydrogenase, (4) aldolase, (5) catalase, (6) ferritin and (7) thyroglobulin. The closed circle (●) represents *cis*-toluene dihydrodiol dehydrogenase.

dihydrodiol dehydrogenase was obtained with 13% recovery of activity. Analysis of 25 μg of protein from the purified dehydrogenase preparation by 10–30%-(w/v)-polyacrylamide-gradient-gel electrophoresis resulted in the detection of one protein band when stained with either Coomassie Blue or glycol dehydrogenase gel stain (Rogers & Gibson, 1977).

M_r and subunit structure

The M_r of *cis*-toluene dihydrodiol dehydrogenase was approx. 172000, as determined by gel filtration at both pH 7.2 and pH 9.0. The M_r on a non-denaturing polyacrylamide gel was approx. 166000 (Fig. 1). A polyacrylamide denaturing gel run in the presence of SDS indicated that the subunit M_r was 29500. These results suggest that the *cis*-toluene dihydrodiol dehydrogenase consisted of six apparently identical subunits.

Isoelectric focusing

One major band and three very minor bands were observed. The presence of only one major band indicated that only one type of subunit was present. The pI was determined to be 6.5 for the denatured protein and to be 6.4 for the native protein.

Temperature optimum and stability

Activity of the purified *cis*-toluene dihydrodiol dehydrogenase was assayed over the temperature range 30–88 °C, and the optimum was found to be 80 °C. From an Arrhenius plot, the activation energy of the reaction was calculated to be 36 $kJ \cdot mol^{-1}$.

When stored at −20 °C the purified enzyme appeared to be stable for at least 2 months. Furthermore, a filter-sterilized solution of enzyme was stable at both 4 °C and room temperature for at least 10 days with no loss of enzyme activity. The thermostability of the enzyme was investigated at both 75 °C and 80 °C and the half-lives were found to be 140 min and 110 min respectively. When the enzyme was incubated at 80 °C in the presence of either NAD^+ or *cis*-toluene dihydrodiol the enzyme was less stable, with the half-life falling to 30 min.

pH optimum and pH stability

The pH optimum of the purified enzyme was 9.8 with both *cis*-toluene dihydrodiol and *cis*-benzene dihydrodiol as substrate. When purified enzyme was incubated at 4 °C for 30 min at pH 6.0, 7.0, 8.0 and 9.0, and then assayed for activity at pH 8.0, little effect on enzyme activity was observed. However, beyond this range activity was rapidly lost until, at pH 3.0, the enzyme lost 87% of activity in 30 min. The enzyme was most stable at pH 8.0.

Absorption spectrum of *cis*-toluene dihydrodiol dehydrogenase

The absorption spectrum of the enzyme exhibited absorption maxima at 280 nm and 220 nm, but there was no significant absorbance in the visible part of the spectrum.

Stoichiometry

The stoichiometry of the enzyme reaction was determined as described in the Materials and methods section. Purified enzyme reduced one equivalent of NAD^+ for each equivalent of *cis*-toluene dihydrodiol converted into 3-methylcatechol.

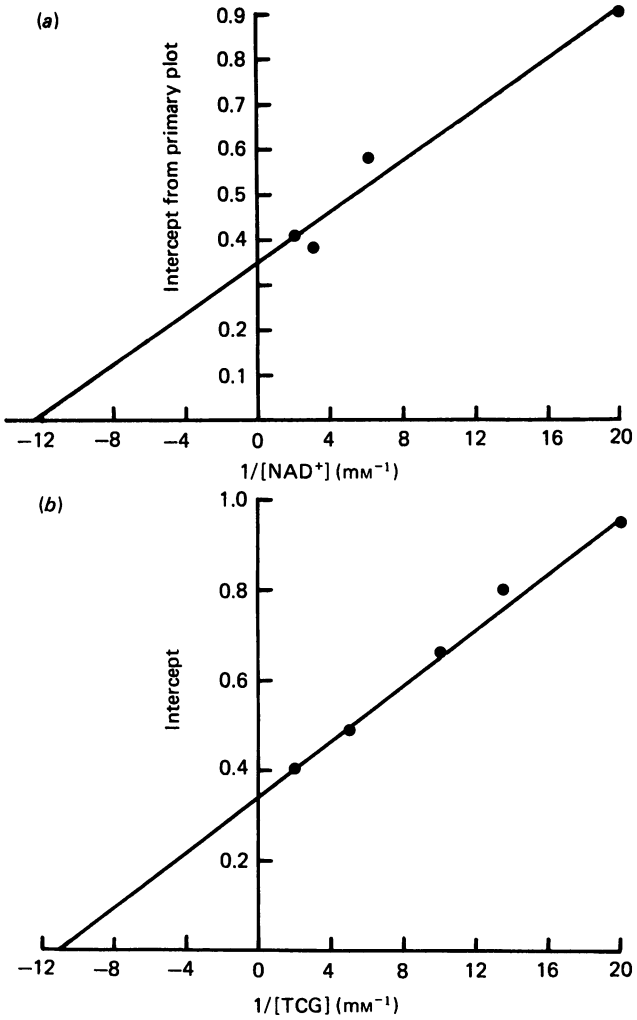

Fig. 2. Secondary plots for K_m determination of *cis*-toluene dihydrodiol dehydrogenase

(*a*) Calculation of K_m for NAD^+; (*b*) calculation of K_m for *cis*-toluene dihydrodiol (TCG). Data was obtained from primary Lineweaver–Burk plots in which *cis*-toluene dihydrodiol was varied at four NAD^+ concentrations.

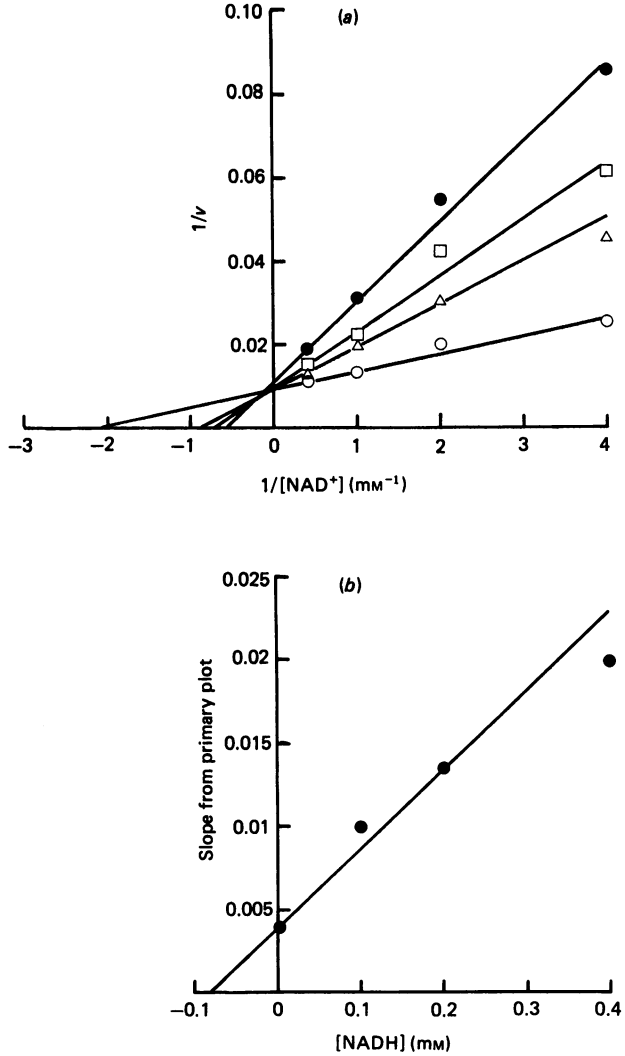

Fig. 3. Determination of the K_i of *cis*-toluene dihydrodiol dehydrogenase for NADH

(*a*) Primary plot of the effect of adding NADH at concentrations of 0.1 mM (△), 0.2 mM (□), 0.4 mM (●) and no inhibitor (○). *cis*-Toluene dihydrodiol was present at a concentration of 0.5 mM. (*b*) Secondary plot of the same data.

Inhibitors

A range of possible inhibitors of *cis*-toluene dihydrodiol dehydrogenase was investigated. Of the compounds tested, mercuric chloride was found to be the best inhibitor. Its effect on enzyme activity was investigated at concentrations of 3 mM and 0.1 mM, which gave 100% and 50% inhibition respectively. Another mercuric compound, *p*-hydroxymercuribenzoate, also inhibited the enzyme, albeit at much higher concentrations (> 10 mM). Metal-ion chelators such as EDTA, KCN, α,α′-dipyridyl and bathophenanthroline were relatively inefficient inhibitors of the enzyme, giving approx. 15% inhibition when added at a concentration of 3 mM.

Substrate specificity

Various other diols were tested as possible substrates for *cis*-toluene dihydrodiol dehydrogenase. Reaction mixtures were those routinely used to assay *cis*-toluene dihydrodiol dehydrogenase, except that *cis*-toluene dihydrodiol was replaced by the test substrate at a final concentration of 0.5 mM. The compounds tested included pentane-1,4-diol, propane-1,3-diol, butan-2-ol, butan-1-ol, glycerol and ethanol. No activity was detected with these substrates. None of these compounds, when added at 10 mM to standard assay mixtures, was found to inhibit *cis*-toluene dihydrodiol dehydrogenase. Activity was observed when either *cis*-naphthalene dihydrodiol or *cis*-benzene dihydrodiol was used as substrate. The enzyme was specific for NAD^+. No activity was detected when $NADP^+$, phenazine methosulphate, cytochrome *c*, potassium ferricyanide or 2,6-dichlorophenolindophenol was substituted as electron acceptor for the enzyme.

Michaelis constants

The K_m of *cis*-toluene dihydrodiol dehydrogenase for *cis*-toluene dihydrodiol was 92 μM, as determined by means of double-reciprocal plots (Fig. 2). To determine

Table 2. Comparison of purified *cis*-diol dehydrogenases

Substrate	Organism	Reference	Characteristics of enzyme				
			$10^{-3} \times M_r$	Subunits		K_m (μM)	
				No.	$10^{-3} \times M_r$	Diol	NAD^+
cis-Toluene dihydrodiol	*Bacillus* sp.	The present work	172	6	29.5	92	80
cis-Toluene dihydrodiol	*Pseudomonas putida*	Rogers & Gibson, 1977	104	4	27	<2	660
cis-Benzene dihydrodiol	*Pseudomonas putida*	Axcell & Geary, 1973	440	4	110	286	43.5
cis-Naphthalene dihydrodiol	*Pseudomonas putida*	Patel & Gibson, 1974	102	4	25.5	29	800
Chloridazo-dihydrodiol	Unidentified bacterium	Eberspächer & Lingens, 1978	220	4	50	250, 100*	200, 250*
cis-1,2-Benzoic acid diol	*Alcaligenes eutrophus*	Reiner, 1972	94.6	4	24	200	150

* Two isoenzymes exist which differ in K_m values for substrate.

the apparent K_m of *cis*-naphthalene dihydrodiol the reactions were performed at pH 7, since at higher pH values the autoxidation of the reaction product interfered with the enzyme assay. The apparent K_m values of *cis*-toluene dihydrodiol dehydrogenase for *cis*-benzene dihydrodiol and *cis*-napthalene dihydrodiol were 330 μM and 51 μM respectively. This suggests that substrates with side groups have greater affinity for the enzyme.

Product inhibition

Non-competitive or 'mixed' inhibition was observed with either 3-methylcatechol [K_{iE} (slope) = 3.6 mM; K_{iES} (intercept) = 1.7 mM] or NADH (K_{iE} = 0.21 mM; K_{iES} = 0.14 mM) using *cis*-toluene dihydrodiol concentrations ranging from 0.05 to 0.5 mM. When the NAD^+ concentration was varied from 0.25 to 2.5 mM, mixed inhibition was observed with 3-methylcatechol and the K_{iE} and K_{iES} values were 2.7 mM and 5.2 mM respectively. However, at saturating concentrations of the diol, uncompetitive inhibition was observed (K_i = 1.2 mM). Competitive inhibition was observed with NADH (Fig. 3), and a K_i of 80 μM was determined. Since the K_i for NADH is comparable with the K_m for NAD^+, it suggests that NADH may have a physiological role in the control of the activity of the enzyme. The dioxygenase which converts toluene into *cis*-toluene dihydrodiol requires NADH as a substrate and, therefore, intracellular accumulation of NADH would indicate a lowered activity of toluene dioxygenase.

Enzyme mechanism

The primary Lineweaver–Burk plots intersect in the third quadrant, which suggests that the mechanism is sequential. However, the product-inhibition pattern indicates the mechanism is ordered Bi Bi. The K_m values for NAD^+ using either *cis*-toluene dihydrodiol or *cis*-benzene dihydrodiol were very similar (80 μM and 90 μM respectively), suggesting that NAD^+ must bind first in an ordered Bi Bi mechanism. Inhibition by 3-methylcatechol with respect to NAD^+ became uncompetitive at saturating concentrations of the diol and is further evidence for an ordered Bi Bi mechanism.

DISCUSSION

Dehydrogenases that catalyse the oxidation of the *cis*-diols from benzoic acid (Reiner, 1972), benzene (Axcell & Geary, 1973), naphthalene (Patel & Gibson, 1974), toluene (Rogers & Gibson, 1977) and chloridazon (Eberspächer & Lingens, 1978) have been purified. A comparison of these enzymes is summarized in Table 2. Apart from the enzyme reported here, all the other *cis*-dihydrodiol dehydrogenases described were isolated from mesophilic organisms, and most work has been carried out with *Pseudomonas putida*. The enzyme described in the present paper is unique in that it has been isolated from a thermotolerant *Bacillus* species and exhibits some unusual characteristics, including remarkable thermostability. The enzyme exists as a hexamer and not, as in the case of previously reported enzymes, as a tetramer. Furthermore, although very little work on the kinetics of the other dehydrogenases has been carried out, it is notable that the K_m values all differ significantly. The K_m values of the *cis*-toluene dihydrodiol dehydrogenase reported here were 51 μM, 92 μM and 330 μM for *cis*-naphthalene dihydrodiol, *cis*-toluene dihydrodiol and *cis*-benzene dihydrodiol respectively. This suggests that substrates with side groups which contribute towards increased hydrophobicity have greater affinity for the enzyme.

The purified *cis*-toluene dihydrodiol dehydrogenase was inhibited by mercurials, which infers the involvement of thiol groups at the active site. The inefficiency of metal-ion chelators on the enzyme suggests that the activity does not depend on metal ions. Conversely, Axcell & Geary (1973) observed that purified *cis*-benzene dihydrodiol dehydrogenase was inhibited by 1,10-phenanthroline and that the enzyme contained loosely bound ferrous ions that were essential for full enzyme activity. It is noteworthy that the dioxygenase that catalyses the conversion of benzene into *cis*-benzene dihydrodiol also requires ferrous ions for activity (Yeh *et al.*, 1977) and NADH as a substrate. Since NADH is a common product of the dehydrogenases, there is a possibility that a dioxygenase–dehydrogenase complex may exist in the intact organism, permitting a recycling

of electrons from one enzyme to the other. There is evidence of a coupled aryl mono-oxygenase–epoxide hydrase system in mammalian microsomes (microsomal fractions) (Oesch & Daly, 1972). Also it has been suggested that, at least under certain growth conditions, there is direct coupling between methane mono-oxygenase and methanol dehydrogenase (Leak & Dalton, 1986).

The enzyme described here was isolated from a *Bacillus* species which grew optimally at 50 °C. The enzyme exhibited a temperature optimum of 80 °C and was thermostable, with a half-life of 110 min at 80 °C. Indeed, it was consistently observed that, after 5 min at this temperature, the activity increased slightly. When the incubation at 80 °C was carried out in the presence of *cis*-toluene dihydrodiol or NAD^+, then the stability of the enzyme was decreased. This finding suggests that, on binding substrate or cofactor, a conformational change of the enzyme makes it more susceptible to inactivation. The activation energy of the reaction was 36 $kJ \cdot mol^{-1}$, which is relatively high for an enzymic reaction (Morris, 1974). The Arrhenius plot was linear up to 65 °C, at which point the curve levelled off, suggesting some type of conformational change above 65 °C which lowers the activation energy to approx. 4.41 $kJ \cdot mol^{-1}$. It has been shown by Matsunaga & Nosoh (1974) that a conformational change occurs in glutamine synthetase of *B. stearothermophilus*, well below the temperature at which the catalytic site is destroyed.

The first intracellular enzyme crystallized from a thermophilic bacterium was glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (Amelunxen, 1966). This enzyme also exhibited marked thermostability, and after heating at 80 °C for 10 min, only 1% inactivation occurred. It was observed that this enzyme, and several other enzymes from thermophilic organisms, had a high content of acidic amino acids and exhibited acidic pI values (Singleton & Amelunxen, 1973). After further investigations, Amelunxen & Murdock (1978) concluded that there was no direct correlation between thermostability of proteins and acidic isoelectric points, since more data were required. This is borne out by the enzyme described here, which exhibited the fairly high pI of 6.4.

We gratefully acknowledge Dr. Gillian Stephens and Dr. Richard Jenkins for helpful advice and discussions. This work was supported by a Science and Engineering Research Council Biotechnology Directorate studentship to H.D.S.

REFERENCES

Amelunxen, R. E. (1966) Biochim. Biophys. Acta **122**, 175–181

Amelunxen, R. E. & Murdock, A. L. (1978) in Microbial Life in Extreme Environments (Kushner, D. J., ed.), pp. 217–278, Academic Press, New York

Axcell, B. C. & Geary, P. J. (1973) Biochem. J. **136**, 927–934

Eberspächer, J. & Lingens, F. (1978) Hoppe-Seyler's Z. Physiol. Chem. **359**, 1323–1334

Green, R. L. & Street, G. B. (1984) Science **226**, 651–656

Jenkins, R. O. & Dalton, H. (1985) FEMS Microbiol. Lett. **30**, 227–231

Laemmli, U. K. (1970) Nature (London) **227**, 680–689

Leak, D. J. & Dalton, H. (1986) Appl. Microbiol. Biotechnol. **23**, 477–481

Matsunaga, A. & Nosoh, Y. (1974) Biochim. Biophys. Acta **365**, 208–211

Morris, J. G. (1974) in A Biologist's Physical Chemistry (Barrington, E. J. W. & Willis, A. J., eds.), pp. 313–317, Arnold, London

Oesch, F. & Daly, J. W. (1972) Biochem. Biophys. Res. Commun. **46**, 1713–1720

O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021

Patel, T. R. & Gibson, D. D. (1974) J. Bacteriol. **119**, 879–888

Reiner, A. M. (1972) J. Biol. Chem. **247**, 4960–4965

Rogers, J. E. & Gibson, D. T. (1977) J. Bacteriol. **130**, 1117–1124

Singleton, R. & Amelunxen, R. E. (1973) Bacteriol. Rev. **37**, 320–342

Sonnleitner, B. & Fiechter, A. (1983) Trends Biotechnol. **1**, 74–80

Vishniac, W. & Santer, M. (1957) Bacteriol. Rev. **21**, 195–213

Yeh, W. K., Gibson, D. T. & Te-Ning Liu (1977) Biochem. Biophys. Res. Commun. **78**, 401–410

Received 29 October 1986/2 February 1987; accepted 25 February 1987