Purification and Characterization of the Secondary Alcohol Dehydrogenase from Propane-utilizing *Mycobacterium vaccae* Strain JOB-5

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Mycobacterium vaccae strain JOB-5 cultured in the presence of propane contained an inducible secondary alcohol dehydrogenase. The enzyme was purified 198-fold using DEAE-cellulose, ω -aminopentyl agarose and NAD-agarose chromatography. The M_r of the enzyme was approximately 136000, with subunits of M_r 37000. The pH optimum for the reaction oxidizing propan-2-ol to propanone was 10–10.5 while the optimum for the reverse reaction was 7.5–8.5. The isoelectric point was 4.9. NAD but not NADP could serve as electron acceptor. The apparent K_m values for propan-2-ol and NAD were 4.9×10^{-5} M and 2.8×10^{-4} M, respectively. The enzyme was inhibited by thiol reagents and metal chelators. It appears to play an essential role in the metabolism of propane by this bacterium.

INTRODUCTION

The growth of *Mycobacterium vaccae* strain JOB-5 with propane as sole source of carbon and energy involves a subterminal oxidation of propane to propan-2-ol and propanone (Vestal & Perry, 1969). Subsequent reactions and a $C_2 + C_1$ cleavage produce acetate, which is assimilated through the glyoxylate cycle. The C_1 moiety is assimilated through the cellular reduced C_1 pool (Coleman & Perry, 1984). Little is known about the enzymes responsible for the series of reactions that convert propane to acetate in this organism. There have been reports on the intermediary metabolism of propanone in other organisms (Taylor *et al.*, 1980), and the pathway apparently differs from that in *M. vaccae*. In order to elucidate the pathway of propane oxidation in *M. vaccae* we have initiated studies on the enzymes involved. Among these is propan-2-ol dehydrogenase, synthesized during growth on propane, an enzyme first reported by Taylor *et al.* (1980). This report describes the partial purification and characterization of the enzyme. A preliminary report of this work has appeared (Coleman & Perry, 1983).

METHODS

Organism and growth conditions. *Mycobacterium vaccae* strain JOB-5 (ATCC 29678) was grown in a mineral salts medium containing substrate as previously described (Vestal & Perry, 1969). Large quantities of cells for enzyme purification were obtained by growth in 20-litre carboys with 0.2% propan-2-ol + 0.05% succinate (sodium salt) as substrate. Cells were grown to late-exponential phase, harvested by centrifugation at 4 °C and washed once in the mineral salts medium followed by centrifugation.

Enzyme purification. All steps were performed at 5 °C. To 80 g wet weight of cells grown on propan-2-ol was added 160 ml 50 mM-Tris/HCl buffer, pH 8.0. The cells were disrupted by two passes through an Aminco French pressure cell. The lysed cell suspension was homogenized in a blender for 10 s to decrease viscosity and then centrifuged at 10000 *g* for 20 min. To the supernatant fluid (crude extract) was added 0.05 vol. 1 M-MnCl₂ to precipitate nucleic acid. After centrifugation at 20000 *g* for 20 min, the supernatant fluid was brought to 45% of saturation by the addition of solid ammonium sulphate. After centrifugation at 12000 *g* for 15 min, the supernatant was discarded and the pellet was resuspended in 10 mM-Tris/HCl buffer pH 8.0 (buffer A) and dialysed overnight against 4 l of this buffer. The dialysate was centrifuged to remove any precipitate and then applied to a DEAE-cellulose column (40 × 5 cm) equilibrated with buffer A. After washing with 200 ml of buffer, the enzyme was eluted with a linear gradient (total volume 1 litre) from 0 to 0.8 M-KCl in buffer A. Fractions showing highest propan-2-ol dehydrogenase activity were combined and the protein was precipitated by adjusting

to 75% of saturation with solid ammonium sulphate. The suspension was centrifuged at 12000 *g* for 20 min and the pellet was resuspended in 50 ml 10 mM-Tris/HCl buffer, pH 7.0, containing 0.5 mM-DTT (buffer B). After dialysis overnight against 4 vols buffer B, the solution was applied to an ω -aminopentyl agarose column (25 $\times$ 1.5 cm) equilibrated with buffer B. After washing with 100 ml buffer B, the enzyme was eluted with a linear gradient (total volume 600 ml) from 0 to 0.4 M-KCl in buffer B. The fractions containing the highest propan-2-ol dehydrogenase activity were combined and applied to a type 3 NAD-agarose column (NAD linked to agarose via the C8 of the purine ring). The column (1 ml bed volume) was washed with 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 mM-DTT and 0.5 M-KCl (buffer C) until no more protein (measured as A_{280}) was present in the effluent. Enzyme was then eluted with 1 mM-NAD in buffer C and the solution was passed through a column of Bio-Gel P-6DG (Bio-Rad) to remove NAD, using buffer C as eluent.

Enzyme assay. Propan-2-ol dehydrogenase was assayed spectrophotometrically by following the alcohol-dependent reduction of NAD at 340 nm. The standard reaction mixture contained, in a final volume of 1 ml: HEPES buffer, pH 8.5, 40 μ mol; NAD, 2 μ mol; propan-2-ol, 5 μ mol; and a suitable amount of enzyme. Reactions were initiated by the addition of substrate. One unit of activity is defined as that amount of enzyme required to reduce 1 μ mol NAD min^{-1} at 30 $^{\circ}\text{C}$. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoresis. Samples were taken from stages in the purification of the propan-2-ol dehydrogenase and subjected to electrophoresis in 7.5% (w/v) cylindrical polyacrylamide gels. The general procedure of Davis (1964) was used with the buffer system of Laemmli (1970), omitting SDS. Propan-2-ol dehydrogenase activity was located by incubating the gel for 30 min in the dark at room temperature in a solution containing the following (per 10 ml): 400 μ mol HEPES, pH 8.5; 10 μ mol propan-2-ol; 5 μ mol NAD; 6 μ mol nitroblue tetrazolium; and 1.2 μ mol phenazine methosulphate. Regions containing propan-2-ol dehydrogenase appeared as dark formazan bands on a clear background. Protein was stained by immersion of the gels in 0.25% Coomassie Brilliant Blue R-250 in 40% (v/v) methanol/10% (v/v) glacial acetic acid for at least 4 h. Gels were destained in 40% (v/v) methanol/10% (v/v) glacial acetic acid.

SDS-PAGE electrophoresis was done on 12% (w/v) polyacrylamide slab gels (150 $\times$ 1.5 mm) according to the procedure of Fairbanks *et al.* (1971).

Standard proteins of M_r 14400–92500 were used as markers and gels were stained as described above. Densitometer scans of stained gels were obtained using a Gilford spectrophotometer equipped with a gel scanning unit.

Isoelectric focusing. Polyacrylamide gel isoelectric focusing was performed as described by Righetti & Drysdale (1971) with the following modifications. Enzyme samples containing 1–5 μ g protein in a 1% (w/v) solution of carrier ampholyte containing 20% (w/v) sucrose were layered onto cylindrical 5% (w/v) polyacrylamide gels (100 $\times$ 5 mm) containing 2% (w/v) total carrier ampholytes (0.8% pH 3–10, 1.2% pH 5–7). The samples were overlaid with 100 μ l 10% (w/v) sucrose. The remaining space in each tube was filled with upper buffer solution. The upper and lower buffers were 10 mM-histidine and 10 mM-glutamic acid, respectively. A control tube loaded with horse spleen ferritin (pI range 4.2–4.3) was used to visually monitor the progress of the run. After electrophoresis at 8 $^{\circ}\text{C}$ for 6–8 h at a constant power of 5 W, the gels were removed from the tubes and sliced into 5 mm segments. Each segment was placed in a vial containing 1 ml degassed 5 mM-KCl and incubated at 4 $^{\circ}\text{C}$ overnight. The pH of each sample was determined with a combination electrode. Enzyme activity was located in gels prepared in an equivalent manner by incubating them in the dehydrogenase stain described.

M_r determination. The M_r of the purified propan-2-ol dehydrogenase was estimated by gel filtration according to the method of Andrews (1964) using a Sephadex G-200 column (74 $\times$ 2.5 cm). The elution buffer was 0.1 M-NaCl, 0.5 M-Tris/HCl, pH 7.5. Blue Dextran 2000 was used to determine the void volume of the column. The marker proteins were catalase, aldolase, bovine serum albumin and ovalbumin. Eluent was monitored at 280 nm to determine marker protein position and assayed for enzyme activity to locate the propan-2-ol dehydrogenase.

Identification of reaction products. A Hewlett-Packard 5880A gas chromatograph equipped with a flame-ionization detector was used to identify and quantify the product of the dehydrogenase reaction. Samples (1 μ l) from the standard reaction mix (propan-2-ol as substrate) were removed at selected time intervals and injected directly onto a glass column (6.5 mm $\times$ 3 m) packed with 5% Carbowax 20 M on 80/120 Carbopack B (Supelco). Product retention times and peak areas were compared with those of standard compounds. Column temperature was 100 $^{\circ}\text{C}$. Injection and detector temperature were 225 $^{\circ}\text{C}$. Carrier gas (He) flow rate was 25 ml min^{-1} . The amount of NADH present at sampling time was estimated from the absorbance at 340 nm ($\epsilon = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

RESULTS

Effect of growth substrate on activity of the propan-2-ol dehydrogenase. The propan-2-ol dehydrogenase was present at very low specific activities (0.008–0.015 units per mg protein) in extracts of cells following growth on propan-1-ol, succinate, propionate or acetate. Growth with

Table 1. *Purification of propan-2-ol dehydrogenase of M. vaccae JOB-5*

The starting material was 80 g (wet weight) of cells grown on 0.2% propan-2-ol + 0.05% sodium succinate.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity [units (mg protein) ⁻¹]	Purification factor	Yield (%)
Crude extract	192	160	1800	0.089	1.0	100
Ammonium sulphate (0–45% sat.)	60	104	804	0.196	2.2	65
DEAE-cellulose	41	66	208	0.278	3.1	41
ω -Aminopentyl agarose	61	47	69	0.792	8.9	20
NAD-agarose		35.1	2	17.6	198.0	15

propan-2-ol, propanone or propane as substrate resulted in 10-fold higher levels of propan-2-ol dehydrogenase.

Purification of enzyme. The final enzyme preparation had a specific activity 198-fold higher than that in crude extracts and represented a yield of 15%. A loss of enzyme after DEAE-cellulose chromatography was tolerated since this step removed a large amount of uncharacterized contaminating material which interfered with the binding of the enzyme to the ω -aminopentyl agarose column. The material eluting from the ω -aminopentyl agarose column at salt concentrations between 85 and 135 mM contained enzyme activity. A large protein peak eluting before the propan-2-ol dehydrogenase and partially overlapping it was the major contaminant in the combined active fractions. This protein was separated from the propan-2-ol dehydrogenase using an NAD-agarose column. The purification steps and yields are presented in Table 1. Preliminary screening experiments with affinity supports such as Cibacron Blue F3G-A agarose and Procion Red agarose indicated that the enzyme did not bind to these ligands.

PAGE at pH 8.8 was used to assess enzyme purity. The major protein band corresponded to alcohol dehydrogenase activity. Scans of stained gels at 595 nm indicated the presence of two minor bands, both migrating more slowly than alcohol dehydrogenase and representing approximately 5% of the total protein, as judged by the peak areas of the stained gel scans. Thus the final preparation of alcohol dehydrogenase appeared to be approximately 95% homogeneous.

M_r and subunit composition. After three independent determinations, the M_r of the enzyme was estimated to be 136000 ± 5000 by gel filtration. SDS-PAGE of the enzyme showed a single protein band corresponding to an M_r of 37000, suggesting that the enzyme is a tetramer, constructed from identical subunits.

Isoelectric point. A single band of activity, corresponding to an isoelectric point of 4.9 ± 0.1 , appeared after staining isoelectric focusing polyacrylamide gels for propan-2-ol dehydrogenase.

Reaction products. Gas chromatographic analyses of the standard reaction mixture (propan-2-ol as substrate) at timed intervals indicated that propanone accumulated concomitantly with the formation of equimolar amounts of NADH. No other products were detected.

pH dependence of the reaction rate. The initial velocity of both the forward (propan-2-ol $\rightarrow$ propanone) and reverse reactions over a range of pH values was tested using the purified enzyme. Both reactions occurred to some extent over a fairly broad pH range with the optimum for the forward reaction between 10 and 10.5 and for the reverse reaction between 7.5 and 8.5.

Substrate specificity. The purified enzyme oxidized both primary and secondary alcohols (Table 2). The primary alcohols had K_m values at least two orders of magnitude higher than those for secondary alcohols. In this regard the enzyme can best be described as a secondary alcohol dehydrogenase. Among the secondary alcohols, the highest V_{max} was seen with butan-2-ol and the lowest K_m with propan-2-ol. The following substrates were tested at concentrations of 30 mM (or at their highest solubility if lower than 30 mM) and were not oxidized: methanol,

Table 2. *Specificity of the purified propan-2-ol dehydrogenase from M. vaccae strain JOB-5 for various alcohol substrates*

Assay mixtures contained, in a final volume of 1 ml, HEPES buffer, pH 8.5, 40 μ mol; NAD, 2 μ mol; and 0.1 μ g enzyme protein. Substrates were tested in duplicate at each of five different concentrations from 0.5 to 5 times the approximated K_m . The K_m and V_{max} values were calculated from double reciprocal plots (Lineweaver & Burk, 1934).

Substrate	Apparent K_m	Relative V_{max}
Propan-2-ol	0.05	1.0
Butan-2-ol	0.08	1.25
Pentan-2-ol	2.0	1.1
Butan-2,3-diol	2.7	0.5
Cyclohexanol	5.9	0.7
Pentan-1-ol	8.1	0.6
Propan-1,2-diol	13.0	0.7
Butan-1-ol	15.4	0.5
Propan-1-ol	18.0	0.4
3-Methyl-1-butanol	18.6	0.2

Table 3. *Kinetic parameters of the dehydrogenase reaction*

Kinetic parameters are shown for the forward and reverse reactions. Reaction mixtures contained, in a final volume of 1 ml: HEPES buffer, pH 8.5, 40 μ mol; enzyme, 0.1 μ g; NAD⁺ or NADH; propan-2-ol or propanone. Reactions were initiated by addition of propan-2-ol or propanone and the rate of NAD⁺ reduction or NADH oxidation was monitored spectrophotometrically at 340 nm, 30 °C.

Substrate	K_m (M)	Dissociation constant (M)
Propan-2-ol	4.9×10^{-5}	4.8×10^{-5}
Propanone	3.0×10^{-4}	3.1×10^{-4}
NAD	2.8×10^{-4}	2.2×10^{-4}
NADH	1.5×10^{-4}	1.7×10^{-4}

ethanol, ethylene glycol, 1,3-propanediol, 1,3-dichloro-2-propanol, glycerol, 3-hydroxypyruvate, 3-hydroxybutyrate, *tert*-butanol, 2-hexanol, 3-hexanol, 2-heptanol, 4-heptanol, benzyl alcohol, phenylethyl alcohol, acetol, cyclopentanol, cyclooctanol, cyclodecanol, cyclohexanediols (1,3-, 1,2- and 1,4-); methylcyclohexanols (2-, 3- and 4-); dimethylcyclohexanols (2,3- and 2,6-); dicyclopentyl methanol; and 3-cyclohexyl-1-propanol.

The enzyme could use NAD as an electron acceptor but not NADP. The following compounds were also tested and none could serve as electron acceptor: FMN, FAD, cytochrome *c* (horse heart), phenazine methosulphate and 2,6-dichlorophenolindophenol.

Kinetic parameters. The kinetic parameters of both the forward (NAD reduction) and reverse reactions were evaluated with propan-2-ol and propanone as the respective substrates. All conditions except the substrate concentrations were as described for the standard assay. Data obtained from duplicate assays at five different subsaturating concentrations of each of the reactants in both the forward and reverse directions were plotted according to Lineweaver & Burk (1934). The intercepts of the family of intersecting lines obtained were replotted according to Dalziel (1957). The K_m values and dissociation constants for the two reactions are reported in Table 3. The K_m values and dissociation constants are quantitatively indistinguishable given the error involved in the measurements. An ordered mechanism is apparent, but other tests are required to ascertain the particular class of reaction.

Effect of mono- and divalent cations on propan-2-ol dehydrogenase activity. No stimulation of enzyme activity was observed in the presence of the following cations, each at 1 mM, added to the standard reaction mixture as their sulphate salts: Na⁺, K⁺, NH₄⁺, Ca²⁺, Mg²⁺, Co²⁺, Cu²⁺, Mn²⁺ or Zn²⁺.

Table 4. *Effect of various compounds on the activity of the purified propan-2-ol dehydrogenase from M. vaccae strain JOB-5*

The standard assay was used with 0.1 µg enzyme protein per assay. The reaction mixture containing all components except NAD and propan-2-ol was incubated in the dark for 10 min at 30 °C. The reaction was started by the addition of NAD and propan-2-ol and the rates were compared with those of a control with no inhibitor present.

Compound	Final concn (mM)	Percentage inhibition
1,10-Phenanthroline	0.05	32
	0.1	45
	1.0	100
2,2'-Dipyridyl	0.5	3
	1.0	22
	3.0	43
8-Hydroxyquinoline	0.5	51
	1.0	77
EDTA	20.0	0
KCN	0.01	0
	0.1	6
NaN ₃	300.0	8
Semicarbazide	0.1	0
	1.0	27
	3.0	55
4-Hydroxymercuribenzoic acid	1.0×10^{-3}	10
	3.0×10^{-3}	63
	1.0×10^{-2}	100
Iodoacetate	1.0	27
	10.0	47
	17.5	77
Blue Dextran 2000	2.5×10^{-2} *	0

* Final dye concentration, using $\epsilon = 13.6 \text{ mm}^{-1} \text{ cm}^{-1}$ for Cibacron Blue F3GA at 610 nm (Ticha *et al.*, 1978).

Inhibition of enzyme. The enzyme was strongly inhibited by metal-complexing agents (1,10-phenanthroline, 2,2'-dipyridyl, and 8-hydroxyquinoline) and thiol reagents (4-hydroxymercuribenzoic acid and iodoacetate), as well as by the carbonyl reagent semicarbazide (Table 4). No inhibition was seen in the presence of Blue Dextran 2000 even at NAD concentrations of one-fifth its K_m .

DISCUSSION

The presence of a secondary alcohol dehydrogenase in crude extracts of *M. vaccae* strain JOB-5 following growth on propane supports the proposed pathway for propane metabolism via propan-2-ol (Vestal & Perry, 1969). The data indicate that the enzyme is inducible, appearing at significant levels only after growth of the organism on propane, propan-2-ol, or propanone. The low activity of this enzyme in extracts of cells following growth in the presence of propan-1-ol also suggests that the enzyme is not involved in the metabolism of propan-1-ol by *M. vaccae* strain JOB-5.

The purified propan-2-ol dehydrogenase from strain JOB-5 differs in several respects from previously described secondary alcohol dehydrogenases. The NAD-dependent secondary alcohol dehydrogenases from *Pseudomonas* sp. ATCC 21439 (Hou *et al.*, 1979) and a *Pichia* sp. (Patel *et al.*, 1979) are both smaller in size and are composed of two subunits of equal molecular weight. The enzyme from strain JOB-5, with four subunits, appears to be structurally similar to the NAD-linked secondary alcohol dehydrogenases from *Candida boidinii* (Schutte *et al.*, 1982), *Pseudomonas fluorescens* NRRL B-1244 (Hou *et al.*, 1983), the NADP-linked secondary alcohol

dehydrogenase from *Thermoanaerobium brockii* (Lamed & Zeikus, 1981), and the alcohol dehydrogenase from bakers' yeast (Hayes & Velick, 1954). The isoelectric point of the enzyme from *C. boidinii* (Schutte *et al.*, 1982) is identical to that reported here for the enzyme from strain JOB-5.

The substrate specificities of the previously reported enzymes, however, are different from that of the propan-2-ol dehydrogenase from strain JOB-5. The enzyme from *C. boidinii* is highly specific for secondary alcohols, with no activity on short-chain primary alcohols (Schutte *et al.*, 1982). The enzyme from *T. brockii* oxidizes primary alcohols but at much lower $V_{\max}$ values than those calculated for the enzyme from strain JOB-5. In addition, the enzyme from *T. brockii* is specific for NADP as electron acceptor. Both the enzyme from strain JOB-5 and that from *Ps. fluorescens* (Hou *et al.*, 1983) oxidize a narrow range of secondary alcohols and several primary alcohols. The enzyme from *M. vaccae*, however, does not oxidize methanol and ethanol. A difference is also observed in the pH optimum for the forward reaction catalysed by these two enzymes. Although chemically this reaction should be favoured at higher pH values, the enzyme from *Ps. fluorescens* has a broad yet distinct optimum between pH 7 and pH 9, whereas the enzyme from strain JOB-5 has an optimum between pH 10 and pH 10.5.

With the exception of the enzyme from *T. brockii*, the enzyme from strain JOB-5 is similar to all of the above-mentioned enzymes in its sensitivity to thiol reagents and metal-chelating agents. The enzyme from *T. brockii* (Lamed & Zeikus, 1981) was inhibited by thiol reagents but not by metal chelators.

A final distinguishing property of the propan-2-ol dehydrogenase from *M. vaccae* strain JOB-5, which became apparent during the preliminary screening of potential purification procedures, was the absence of binding to Cibacron Blue F3GA-agarose. This type of affinity ligand has found general use in the purification of NAD-linked enzymes and was used in the purification of the enzymes from *C. boidinii*, *T. brockii*, *Pichia* sp., *Pseudomonas* sp. ATCC 21439 and *Ps. fluorescens*. No binding of the enzyme from *M. vaccae* strain JOB-5 could be demonstrated using a variety of buffers, pH values, ionic strengths and flow rates. This observation was further corroborated by the absence of inhibition of the enzyme by Blue Dextran 2000. Possible explanations for these observations are that the Cibacron Blue F3GA moiety either cannot gain access to the dinucleotide fold responsible for NAD binding or that the fold is not present. According to Wilson (1976), 'the absence of marked interaction with Blue Dextran might reasonably be taken as an indication that the dinucleotide fold is not present in the enzymes'. In view of the structural similarities between the enzyme from strain JOB-5 and the other four-subunit enzymes, especially that from bakers' yeast, the absence of a dinucleotide fold would be an interesting feature, considering the evolutionary conservation of the structure (Rosemann *et al.*, 1974; Schultz & Schirmer, 1974). More specific structural studies are required to resolve this apparent inconsistency.

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