Partial Purification and Characterization of an Alcohol Dehydrogenase of *Mycobacterium tuberculosis* var. *bovis* (BCG)

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An alcohol dehydrogenase of broad specificity was purified 43-fold from extracts of *Mycobacterium tuberculosis* var. *bovis* (BCG) grown on Sauton medium. It was also present in cells grown in Dubos medium with Tween 80 and bovine serum albumin. The enzyme, which appeared to be soluble, acted as an oxidoreductase in the system butan-1-ol–NADP. It was eluted from Sephadex G-200, hydroxylapatite and DEAE-cellulose in a single peak. The molecular weight, as determined by gel filtration on Sephadex G-200, was about 75 000. Results of electrophoresis in sodium dodecyl sulphate–polyacrylamide gels were compatible with the existence of two subunits each of molecular weight 37 500. The optimum pH was about 8.5 when the enzyme catalysed the oxidation of butan-1-ol, and about 8.2 for the reverse reaction. The apparent K_m was 0.125 mM for butyraldehyde and 0.22 M for butan-1-ol. The dehydrogenase activity was maintained after heat treatment (40 min at 55 °C) in the presence of 30% (w/v) glycerol, but was abolished by heating (40 min at 55 °C) in the presence of 0.1 M-EDTA. The activity of enzyme inactivated by heat and EDTA could be fully restored at room temperature in the presence of 2 mM- Zn^{2+} .

INTRODUCTION

When *Mycobacterium tuberculosis* var. *bovis* (BCG) was grown under Zn^{2+} deficiency the cell composition and products excreted into the medium could to some extent be correlated with a decrease (85%) in specific activity of a soluble alcohol dehydrogenase (De Bruyn *et al.*, 1981). The present paper describes the partial purification of this enzyme and reports investigations on its requirement for Zn^{2+} , its preferred substrates, its presence and activity in relation to the growth medium used and its cellular location.

METHODS

Culture of bacteria. *Mycobacterium tuberculosis* var. *bovis* (BCG), French strain 1173P2, was usually grown on the surface of Sauton medium at 37.5 °C. When specified, aerated cultures in Dubos medium with bovine serum albumin and Tween 80 were used.

Preparation of extracts. The pellicles were harvested after 5, 7 or 14 d, using a Büchner funnel with Whatman no. 1 paper, and washed with one culture volume of phosphate-buffered saline. The cells were suspended in 0.05 M- KH_2PO_4 /NaOH buffer (pH 7) containing 1 mM-dithiothreitol (0.3 g wet wt cells per 1 ml buffer), homogenized with a Potter homogenizer and disrupted at 83 to 110 MPa in a French press. Deoxyribonuclease (0.1 mg, Boehringer) and 0.5 ml 0.2 M- $MgSO_4$ solution were added for each 10 ml crude extract, and the mixture was incubated at 4 °C for 15 min. The extract was clarified by centrifugation at 10 000 g for 30 min. After a second centrifugation at 100 000 g for 90 min the resulting supernatant was used for further purification.

Protein estimation. To avoid interference by glycerol and dithiothreitol in the Folin reaction, proteins were determined by the Coomassie Blue method (Spector, 1978) with the Bio-Rad reagent.

Enzyme assay. Alcohol dehydrogenase activity was measured by following the reduction of NADP at 340 nm. To measure the activity during the preparation of extracts and purification, butan-1,3-diol was routinely used as

substrate. The incubation mixture (total volume 2.5 ml) contained 0.5 mM-NADP, 0.13 M-KH₂PO₄/NaOH buffer (pH 7.5) and 0.8 M-butan-1,3-diol; the assay was initiated by addition of the substrate. In studies on the properties of the enzyme, butan-1-ol (0.7 M) or butyraldehyde (0.02 M) was used as substrate. One unit of activity is defined as the amount of enzyme catalysing the reduction of 1 nmol NADPH min⁻¹ at 22 °C.

Analytical sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Electrophoresis was performed as described by Laemmli (1970) with a 10 to 20% (w/v) acrylamide gradient. Just before each run the preparations were heated at 100 °C for 5 min in 0.03 M-Tris/HCl buffer (pH 6.8) containing 0.5% (w/v) SDS, 0.3 M-β-mercaptoethanol and 5% (w/v) glycerol.

Chromatography. Approximately 40 ml of 100 000 g supernatant of an extract of BCG cells from a 7 d-old culture was applied to a Sephadex G-50 column (2.6 × 45 cm) equilibrated with 0.01 M-KH₂PO₄/NaOH buffer (pH 7). All buffers used in the purification procedure contained 1 mM-dithiothreitol and 30% (w/v) glycerol. The most active fractions were collected and applied to a hydroxylapatite column (2.6 × 24 cm), equilibrated with the same buffer. The column was then washed with 40 ml of this equilibration buffer and eluted with a 300 ml linear gradient of 10 to 65 mM-KH₂PO₄/NaOH buffer (pH 7). At the end of the gradient, 65 mM-KH₂PO₄/NaOH buffer (pH 7) was applied. After the KCl concentration had been adjusted to 0.1 M by dilution with 0.05 M-KH₂PO₄/NaOH buffer (pH 7) containing 2.4 M-KCl, the most active fractions were applied to a DEAE-cellulose DE52 column (2.6 × 27 cm) equilibrated with 0.05 M-KH₂PO₄/NaOH buffer (pH 7) containing 0.1 M-KCl. The column was washed with 20 ml of this buffer and eluted with a 480 ml linear gradient of 0.1 to 0.6 M-KCl in the same buffer.

Molecular weight was estimated by gel filtration using a Sephadex G-200 column (2.5 × 45 cm) equilibrated with 0.05 M-KH₂PO₄/NaOH buffer (pH 7.5) and eluted with the same buffer. The column was calibrated with proteins of known molecular weight.

RESULTS

Enzyme stability. The activity of a 100 000 g supernatant fraction (10 to 12 mg protein ml⁻¹) in 0.05 M-KH₂PO₄/NaOH buffer (pH 7) containing 1 mM-dithiothreitol had decreased by 10 to 15% after 7 d at 4 °C. Stability was no better in 0.05 M-Tris/HCl buffer (pH 7.5) containing 5 mM-zinc acetate and 1 mM-dithiothreitol. After 16 h at 4 °C in 0.05 M-sodium citrate buffer (pH 5.5), the enzyme activity was 43% lower than in 0.05 M-KH₂PO₄/NaOH buffer (pH 7.5). Purification or storage of purified preparations led to considerable loss of activity.

The stability of the enzyme during purification was improved by including glycerol (30%, w/v) in all buffers (Guerillot & Vandecasteele, 1977). Conservation of the activity of partially purified enzyme was better in 80% (w/v) glycerol. The most purified preparation [specific activity 1888 units (mg protein)⁻¹] lost 15 to 20% of its activity after 4 weeks at -30 °C in 80% (w/v) glycerol.

Enzyme activity. The activity of the alcohol dehydrogenase was maximal in cultures grown for 5 d. The activity decreased to 80% of the maximum after 7 d, and to 75% after 14 d. The specific activity of the enzyme in the 100 000 g supernatant from cells grown in Dubos medium for 7 d was two to three times higher than in that from cells grown on Sauton medium.

Enzyme purification. The results of the purification are given in Table 1. The enzyme was eluted from hydroxylapatite, and subsequently from DEAE-cellulose, in a single peak.

Properties of the partially purified enzyme. The molecular weight was estimated to be about 75 000 by gel filtration on Sephadex G-200 using beef liver catalase, beef heart lactic dehydrogenase, *Escherichia coli* alkaline phosphatase and yeast hexokinase as markers. Three different enzyme preparations were compared by SDS-polyacrylamide gel electrophoresis. One major band remained in the most purified preparation (Fig. 1) and this had an apparent molecular weight of 37 500.

The enzyme was highly active in the formation of butan-1-ol from butyraldehyde over a broad pH range from 7.5 to 8.5; the optimum pH was around 8.2. The enzyme was also active in the formation of butyraldehyde from butan-1-ol over a broad pH range from 7.5 to

Table 1. *Purification of alcohol dehydrogenase from BCG*

The enzyme was assayed using butan-1,3-diol as substrate as described in Methods.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Purification factor
Supernatant, 30 min, 10000 g	50	645	27 735	43	—
Supernatant, 90 min, 100000 g	40	400	20 800	52	1.2
Sephadex G-50	36	302	16 610	55	1.2
Hydroxylapatite	26	22.9	9 183	401	9.3
DEAE-cellulose DE52	16	2.2	4 153	1888	43.5

Fig. 1. Scan of SDS-polyacrylamide gel after electrophoresis of BCG alcohol dehydrogenase [9 μ g protein, specific activity 1888 units (mg protein)⁻¹]. The positions of reference protein markers (in a gel run simultaneously) are indicated: 1, bovine serum albumin (mol. wt 68 000); 2, ovalbumin (45 000); 3, *E. coli* asparaginase (33 000); 4, tobacco mosaic virus (17 000); 5, *E. coli* lysozyme (14 400).

9.0; the optimum was around 8.5. With glycine/NaOH buffer, activities from pH 8.2 to 9.0 were higher than with KH_2PO_4 /NaOH buffer, probably because of a Schiff's base reaction of the aldehyde formed with the glycine of the buffer.

Apparent K_m values were determined with butan-1-ol and butyraldehyde as substrates using 23 units of enzyme [specific activity 600 units (mg protein)⁻¹]. For butyraldehyde in 0.13 M- KH_2PO_4 /NaOH buffer (pH 7.5) with 0.25 mM-NADPH, the apparent K_m was 0.125 mM. For butan-1-ol in the same buffer with 0.5 mM-NADP, the apparent K_m was much higher, 0.22 M.

The reaction velocities of enzyme samples [9 units, specific activity 733 units (mg protein)⁻¹] with butan-1-ol or butyraldehyde as substrate and NADP or NADPH as cofactor were arbitrarily assigned a value of 100. With 0.5 M-crotyl alcohol as substrate the relative reaction velocity compared with butan-1-ol was 120. With other alcohols, each used at a concentration of 1 M, the relative velocities were: butan-1,3-diol, 70; propan-1,3-diol, 31; butan-1,4-diol, 26; ethanol, 16; propan-2-ol, 8; ethylene glycol, 7; allyl alcohol, 7; butan-2,2-diol and glycerol, no detectable activity. With 0.02 M-anisaldehyde the reaction velocity relative to butyraldehyde was 35, and with 0.02 M-glycolaldehyde it was 33, whereas with 0.2 M-cinnamaldehyde the relative velocity was 120.

The reaction velocities of enzyme samples [6.5 units, specific activity 600 units (mg protein)⁻¹] were also compared with 0.02 M-butyraldehyde as substrate and NADPH or NADH at the same final concentration (0.25 mM). The reaction velocity obtained with NADH was only 10 to 15 % of that obtained with NADPH.

Since many alcohol dehydrogenases are zinc-requiring enzymes (Dickinson & Dickinson, 1976; Rudolph *et al.*, 1978), the metal chelators 1,10-phenanthroline and EDTA were tested as possible inhibitors. Enzyme [10 units, specific activity 977 units (mg protein)⁻¹] was incubated in 0.15 M-KH₂PO₄/NaOH buffer (pH 7.5) with or without inhibitor for 20 min at 22 °C prior to addition of substrate: 0.1 M-EDTA and 6 mM-1,10-phenanthroline gave 14 % and 31 % inhibition, respectively.

To examine the effect of heat treatment, enzyme [36 units, specific activity 977 units (mg protein)⁻¹] was incubated at 55 °C in 0.05 M-KH₂PO₄/NaOH buffer (pH 7) containing 1 mM-dithiothreitol and 30 % (w/v) glycerol with or without EDTA. Samples (about 10 units of enzyme) were taken before incubation and after 20 and 40 min incubation and assayed with butyraldehyde as substrate. Heating in the absence of EDTA resulted in a small increase in activity: from 11 units before heating to 12 and 13 units after 20 and 40 min respectively. Heating in the presence of 0.1 M-EDTA led to almost complete inactivation: after 20 min the activity was only 0.5 units and after 40 min, 0.3 units. When incubated at 55 °C in the absence of glycerol and EDTA, the enzyme was inactivated by 50 % after 40 min.

The activity of enzyme inactivated by heat and EDTA in the presence of glycerol could be fully restored by passing the solution through a Sephadex G-25 column pre-equilibrated with 0.02 M-triethanolamine/HCl buffer (pH 7.5) containing 2 mM-zinc acetate at room temperature and allowing the enzyme fractions to stand for 45 min at room temperature before assay.

Enzyme location. Attempts to establish a periplasmic location for this enzyme have been unsuccessful, although 2 to 4 % of the activity was released when suspensions of cell from 7 d-old cultures were incubated for 30 min at 37 °C in 0.05 M-KH₂PO₄/NaOH with glycerol and dithiothreitol. These conditions did not release cytoplasmic glutamic-oxaloacetic transaminase activity, which is very high in these cells. Use of deoxycholate (0.5 %, w/v), Triton X-100 (0.5 to 5 %, w/v) or lysozyme [1.5 mg (g wet wt)⁻¹] plus EDTA (20 mM), with or without prior delipidation by acetone or acetone/EDTA at 4 °C (Nason, 1955), did not give better results, possibly because of enzyme denaturation.

Activity in other species. The enzyme was present in *Mycobacterium fortuitum* and *Mycobacterium phlei* grown in Dubos medium, but with much lower activity: 10 and 18 units (mg protein)⁻¹, respectively.

DISCUSSION

The BCG alcohol dehydrogenase described here has a broad specificity and is present in BCG grown on Sauton medium (glycerol as carbon source) as well as in Dubos medium with Tween 80 and serum albumin (amino acids as carbon source). The apparent K_m value is so high for butanol that only the K_m value for butyraldehyde may have physiological significance. This suggests that the enzyme uses aldehydes as substrates *in vivo*. An NADP-alcohol dehydrogenase activity catalysing the reduction of palmitaldehyde to palmityl alcohol, involved in wax biosynthesis, is known to occur in particulate fractions of *Mycobacterium tuberculosis* H37Ra (Wang *et al.*, 1972).

The BCG alcohol dehydrogenase needs Zn²⁺ for its activity, but more experiments are needed to determine whether Zn²⁺ is involved at the catalytic site or is acting as a stabilizing factor, possibly to maintain the apparent dimeric form of the enzyme.

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