Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus*

Purification and preliminary characterization

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A quick, reliable, purification procedure was developed for purifying both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from a single batch of *Acinetobacter calcoaceticus* N.C.I.B. 8250. The procedure involved disruption of the bacteria in the French pressure cell and preparation of a high-speed supernatant, followed by chromatography on DEAE-Sephacel, affinity chromatography on Blue Sepharose CL-6B and Matrex Gel Red A, and finally gel filtration through a Superose 12 fast-protein-liquid-chromatography column. The enzymes co-purified as far as the Blue Sepharose CL-6B step were separated on the Matrex Gel Red A column. The final preparations of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II gave single bands on electrophoresis under non-denaturing conditions or on SDS/polyacrylamide-gel electrophoresis. The enzymes are tetramers, as judged by comparison of their subunit (benzyl alcohol dehydrogenase, 39 700; benzaldehyde dehydrogenase II, 55 000) and native (benzyl alcohol dehydrogenase, 155 000; benzaldehyde dehydrogenase II, 222 500) M_r values, estimated by SDS/polyacrylamide-gel electrophoresis and gel filtration respectively. The optimum pH values for the oxidation reactions were 9.2 for benzyl alcohol dehydrogenase and 9.5 for benzaldehyde dehydrogenase II. The pH optimum for the reduction reaction for benzyl alcohol dehydrogenase was 8.9. The equilibrium constant for oxidation of benzyl alcohol to benzaldehyde by benzyl alcohol dehydrogenase was determined to be 3.08×10^{-11} M; the ready reversibility of the reaction catalysed by benzyl alcohol dehydrogenase necessitated the development of an assay procedure in which hydrazine was used to trap the benzaldehyde formed by the NAD^+ -dependent oxidation of benzyl alcohol. The oxidation reaction catalysed by benzaldehyde dehydrogenase II was essentially irreversible. The maximum velocities for the oxidation reactions catalysed by benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were 231 and 76 $\mu\text{mol/min}$ per mg of protein respectively; the maximum velocity of the reduction reaction of benzyl alcohol dehydrogenase was 366 $\mu\text{mol/min}$ per mg of protein. The pI values were 5.0 for benzyl alcohol dehydrogenase and 4.6 for benzaldehyde dehydrogenase II. Neither enzyme activity was affected when assayed in the presence of a range of salts. Absorption spectra of the two enzymes showed no evidence that they contain any cofactors such as cytochrome, flavin, or pyrroloquinoline quinone. The kinetic coefficients of the purified enzymes with benzyl alcohol, benzaldehyde, NAD^+ and NADH are also presented.

INTRODUCTION

Acinetobacter calcoaceticus N.C.I.B. 8250 can grow on benzyl alcohol or L(+)-mandelate as sole sources of carbon and energy and both compounds are converted into benzoate by a convergent pathway (Scheme 1) (Fewson, 1967; Livingstone & Fewson, 1972). Benzyl alcohol is oxidized by benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II, which are co-ordinately induced by benzaldehyde or benzyl alcohol. L(+)-Mandelate is metabolized by L(+)-mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I, and these three enzymes are co-ordinately induced by phenylglyoxylate (Livingstone & Fewson, 1972; Beggs & Fewson, 1977). In the present paper we describe the purification and preliminary characterization of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II. Isolation of these two

enzymes forms part of an overall comparison of the soluble NAD^+ -dependent aromatic alcohol and aldehyde dehydrogenases that are present in *A. calcoaceticus* and other bacteria (MacKintosh & Fewson, 1987a).

Preliminary accounts of parts of this work have been published (MacKintosh & Fewson, 1987a,b).

EXPERIMENTAL

Materials

Chemicals were of the best quality commercially available, and most of them were obtained from the sources described by Allison *et al.* (1985a,b).

Matrex Gel Red A was from Amicon, Stonehouse, Glos., U.K. Bicine, Coomassie Brilliant Blue G250, hydrazine hydrate, Nitro Blue Tetrazolium and *N*-methylphenazonium methosulphate were from BDH

Abbreviation used: f.p.l.c., fast protein liquid chromatography (Pharmacia system).

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Scheme 1. Convergent pathways for the catabolism of benzyl alcohol and mandelate by *A. calcoaceticus* N.C.I.B. 8250

Chemicals, Poole, Dorset, U.K. Dithiothreitol, NAD^+ , NADH and the gel-filtration calibration standards were from Boehringer Corp, Lewes, Sussex, U.K. DEAE-Sephacel, Blue Sepharose CL-6B, Polybuffer PB 74 and pre-packed Superose 12 and Mono P columns were from Pharmacia, Milton Keynes, Bucks., U.K. Benzyl alcohol and benzaldehyde were redistilled before use.

Organism

Acinetobacter calcoaceticus N.C.I.B. 8250, the wild-type strain, was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

Growth of bacteria

Bacteria were maintained as described previously (Allison *et al.*, 1985a). To obtain large amounts of bacteria, *A. calcoaceticus* N.C.I.B. 8250 was grown, by a draw-and-fill procedure, in complex medium (Allison *et al.*, 1985a) plus 20 mM-benzyl alcohol in a 10-litre fermenter with an attached 20-litre reservoir. An inoculum was prepared in a conical flask (250 ml) containing 50 ml of nutrient broth, which was inoculated (0.1%, v/v) with stock culture and shaken (120 rev./min) at 30 °C for 24 h. The whole of this culture was then aseptically transferred to 400 ml of complex medium containing 20 mM-benzyl alcohol in a 2-litre conical flask and shaken (120 rev./min) at 30 °C for 24 h. The entire culture was used to inoculate the fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewkesbury,

Glos, U.K.), which contained 10 litres of complex medium plus 20 mM-benzyl alcohol, and 50 ml of sterile 1% poly(propylene glycol) 2025 was added as antifoam (Allison *et al.*, 1985b). The fermenter was operated at 30 °C with an aeration rate of 4 litres of sterile air/min and stirring at setting 2.5 (approx. 250 rev./min). After growth for 16 h, 9 litres of culture were removed for harvesting, and the washed bacteria were stored at -20 °C; 9 litres of complex medium containing 20 mM-benzyl alcohol were aseptically transferred into the fermenter from the reservoir. The bacteria were grown for a further 9 h, harvested, and more fresh medium was added. The bacteria were then grown for 16 h, 9 litres of medium were removed for harvesting, and the remaining 2 litres of medium in the reservoir aseptically transferred into the fermenter. After a further 4 h of growth, the fermenter was emptied and the last of the bacteria were harvested, and stored at -20 °C.

Preparation of cell-free extracts

Frozen pelleted cells were resuspended in 4 vol. of 75 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol (the final volume was usually 45 ml) and disrupted by four passages through the French pressure cell (4-3398A; American Instrument Co., Silver Spring, MD, U.S.A.) at a pressure of 98 MPa (14 300 lb/in²) (Allison *et al.*, 1985a). The homogenate was centrifuged at 64 000 *g* for 90 min at 4 °C. The supernatant was used as the starting material for the purification of benzyl

alcohol dehydrogenase and benzaldehyde dehydrogenase II as described below.

Measurement of enzyme activities

The oxidation reaction of benzyl alcohol dehydrogenase was measured in reaction mixtures (3.0 ml) containing 100 mM-Bicine/360 mM-hydrazine buffer, pH adjusted with NaOH to 9.2, 2 mM-NAD⁺ and enzyme (usually 20 μ l), and the reaction was initiated with benzyl alcohol (final concn. 200 μ M). Hydrazine was included in the reaction mixture to prevent non-linearity in the progress curve caused by the reverse action. The reduction reaction of benzyl alcohol dehydrogenase was measured in a reaction mixture (3.0 ml) containing 100 mM-Bicine/NaOH buffer, pH 8.9, 0.25 mM-NADH and enzyme (usually 20 μ l), and the reaction was initiated with benzaldehyde (final concn. 100 μ M). The oxidation reaction of benzaldehyde dehydrogenase II was measured in a reaction mixture (3.0 ml) containing 100 mM-Bicine/NaOH buffer, pH 9.5, 2 mM-NAD⁺ and enzyme (usually 20 μ l), and the reaction was initiated with benzaldehyde (final concn. 10 μ M). For these assays the reduction of NAD⁺ or oxidation of NADH was monitored at 340 nm, 27 °C. One unit of enzyme activity is defined as 1 μ mol of substrate converted/min; specific activities are given as units/mg of protein. The absorption coefficient of NADH at 340 nm is $6.30 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Boehringer, 1976).

Determination of kinetic parameters

To obtain K_m and maximum-velocity (V) values for the substrates and cofactors of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II, initial velocities were measured at several non-saturating concentrations of each compound. The kinetic coefficients for the substrates were determined by the construction of primary and, where necessary, secondary plots as described by Engel (1977). Initial velocities were analysed by using the Enzpack computer program (Williams, 1985), which allows determination of the kinetic coefficients by the Direct Linear method (Eisenthal & Cornish-Bowden, 1974). For the primary double-reciprocal plots, lines were fitted by using the K_m and V values obtained by Direct Linear analysis. K_m and V values were obtained for one substrate either at a fixed concentration of the second substrate or when the concentration of the second substrate has been extrapolated to infinity, by the two-substrate method described by Engel (1977). For double-reciprocal plots, each initial-velocity determination was done in duplicate, and all experimental points are shown in the Figures; where a single point is given, the replicate values were identical. The concentrations of stock solutions of benzyl alcohol, benzaldehyde and NAD⁺ were standardized enzymically by using either benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase II. The stock substrate was made limiting in the assay while the second substrate was in excess; the complete ΔA_{340} corresponded to the amount of stock substrate initially added. The concentration of stock solutions of NADH was determined by measuring the A_{340} of diluted solutions.

Calculation of equilibrium constant

Benzyl alcohol dehydrogenase catalyses both the oxidation of benzyl alcohol and the reduction of benzaldehyde. The equilibrium constant, K'_{eq} , of the

reaction was calculated from the progress curve of benzyl alcohol oxidation in the absence of hydrazine. The progress curve reached a plateau when the concentration of substrates and products reached equilibrium. Assuming 1:1 stoichiometry of NADH and benzaldehyde production, the K'_{eq} value was calculated from the equation:

$$K'_{eq} = \frac{[\text{benzaldehyde}][\text{NADH}]}{[\text{benzyl alcohol}][\text{NAD}^+]} \times \frac{1}{\text{antilog pH}}$$

Analytical methods

Protein concentrations were determined by the procedure of Bradford (1976). Bovine serum albumin was used as the standard, and it was assumed that a solution containing 1 mg/ml had an A_{280} of 0.65 (Janatova *et al.*, 1968). During the purification procedure the A_{280} was measured with an LKB Uvicord monitor. Conductivities were measured with a Radiometer (Copenhagen, Denmark) type CDM2e conductivity meter.

Preparation of buffers and determination of pH optima

Buffers were prepared at room temperature by adjusting the pH values of approximately five-fourths strength solutions with HCl, NaOH or KOH and then making to volume.

The activities of the pure dehydrogenases were measured in cuvettes containing appropriate buffers (final concns. 100 mM). In each case, the pH value of the final reaction mixture was measured shortly after the completion of the spectrophotometric assay.

Purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II

An extract of *A. calcoaceticus* N.C.I.B. 8250 grown in complex medium plus 20 mM-benzyl alcohol was prepared as described above from 9 g (wet wt.) of cells. Steps 1–3 and 5 were carried out at 0–4 °C, and step 4 was at room temperature.

Step 1: chromatography on DEAE-Sephacel. The extract (approx. 38 ml) was applied to a DEAE-Sephacel column (3.8 cm $\times$ 2.6 cm) pre-equilibrated in Buffer A [75 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol]. The column was washed in Buffer A until the A_{280} of the effluent decreased to approx. 0.05. The buffer was then changed to Buffer B [110 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol] to elute both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II. The flow rate throughout was 50 ml/h. The fractions containing enzyme activities were pooled (approx. 45 ml) and dialysed overnight against 3×1000 ml of Buffer C [10 mM-potassium phosphate buffer (pH 6.0)/2 mM-dithiothreitol/5 mM-MgCl₂].

Step 2: chromatography on Blue Sepharose CL-6B. The dialysed pool (approx. 50 ml) was applied to a Blue Sepharose CL-6B column (9.5 cm $\times$ 2.6 cm) pre-equilibrated in Buffer C. After application the pump was stopped for 30 min, and then the column was washed with Buffer C. Once the A_{280} of the effluent had returned to the baseline, the washing was continued until a further 100 ml (i.e. 2 column vol.) had passed through the column. The buffer was then changed to Buffer C containing 0.12 mM-NAD⁺ and the buffer flow direction reversed to elute the enzymes. The flow rate throughout

Table 1. Purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus* N.C.I.B. 8250

For full experimental details see the text.

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
(a) Benzyl alcohol dehydrogenase						
Crude extract	38	1056	704	1.5	100	1
Ion exchange	45	938	53	17.7	89	11.8
Dialysis	50	793	50	15.9	75	10.4
Blue Sepharose CL-6B	48	380	2.83	134	36	89
Matrex Gel Red A	0.2	178	0.81	220	17	147
Superose 12 f.p.l.c.	0.9	171	0.72	238	16	159
Dialysis	0.32	171	0.72	238	16	159
(b) Benzaldehyde dehydrogenase II						
Crude extract	38	388	704	0.55	100	1
Ion exchange	45	285	53	5.4	73	9.8
Dialysis	50	238	50	4.8	61	8.7
Blue Sepharose CL-6B	48	190	2.83	67	49	121
Matrex Gel Red A	1.2	143	1.84	78	37	142
Dialysis	0.41	143	1.80	79	37	144

was 99 ml/h. The fractions containing the enzyme activities were pooled (approx. 50 ml).

Step 3: chromatography on Matrex Gel Red A. The Blue Sepharose CL-6B elution pool (approx. 50 ml) was applied to a Matrex Gel Red A column (3.8 cm × 2.6 cm) pre-equilibrated in Buffer C containing 0.12 mM-NAD⁺. After application of the pool the pump was stopped for 30 min, and then the column was washed with Buffer C containing 0.12 mM-NAD⁺. The benzaldehyde dehydrogenase II activity was located in the wash fractions, which were pooled (approx. 50 ml). Once the effluent A_{280} had returned to the baseline, the washing was continued until a further 80 ml (i.e. 4 column vol.) had passed through the column. The benzyl alcohol dehydrogenase activity was then eluted by increasing the NAD⁺ concentration in Buffer C to 2.75 mM, and reversing the buffer flow direction. The flow rate throughout was 99 ml/h. The fractions containing the benzyl alcohol dehydrogenase activity were pooled (approx. 30 ml). Both the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II pools were concentrated overnight by vacuum dialysis. The benzyl alcohol dehydrogenase pool was further concentrated to 0.2 ml and its buffer changed to Buffer D [50 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol] using a Centricon-30 microconcentrator (Amicon).

Step 4: gel filtration through f.p.l.c. Superose 12. The concentrated benzyl alcohol dehydrogenase (0.2 ml) was applied to an f.p.l.c. Superose 12 gel-filtration column (30 cm × 1 cm) pre-equilibrated in Buffer D. The flow rate throughout was 0.3 ml/min. Two peaks of A_{280} were observed, the major peak corresponding to the benzyl alcohol dehydrogenase activity. The peak fraction, containing most of the enzyme activity, plus the fractions immediately adjacent to the peak were pooled (approx. 0.9 ml).

Step 5: dialysis and storage. The benzyl alcohol dehydrogenase pool and the concentrated benzaldehyde dehydrogenase II (approx. 1.0 ml) were then individually dialysed overnight against Buffer E [100 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol/1 mM-EDTA/1 mM-benzamidine/1.2 mM-phenylmethanesulphonyl fluoride/40 % (v/v) glycerol]. The dialysed pools (both approx. 0.3 ml) were then stored at -20 °C.

Determination of isoelectric points

Samples of purified dehydrogenases in 20 mM-piperidine/HCl buffer, pH 5.7, were applied to an f.p.l.c. Mono P chromatofocusing column (20 cm × 0.5 cm) pre-equilibrated in the same buffer. Protein was eluted with Polybuffer PB 74 diluted 1:8 with water and adjusted to pH 3.6 with HCl. The flow rate was 1 ml/min, and 0.75 ml fractions were collected and assayed for enzyme activities. The A_{280} of the effluent was monitored, and the pH value of each fraction was determined. The pI values quoted are averages of two different experiments.

Absorption spectra

The absorption spectra of purified enzymes in 100 mM-potassium phosphate buffer, pH 7.5, were determined in 1 ml quartz cuvettes in a Pye Unicam SP 8-100 spectrophotometer. The scan speed was 1 nm/s.

Electrophoresis

The purity of samples from various stages of the purification procedure was monitored by using SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Slab gels (19.0 cm × 9.5 cm × 0.15 cm) containing 10 % (w/v) polyacrylamide were stained, after electrophoresis, in 0.1 % (w/v) Coomassie Brilliant Blue G250/50 % (v/v) methanol/10 % (v/v) acetic acid for 1 h at 40 °C and then destained in 10 % methanol/10 % acetic acid, also at 40 °C.

Non-denaturing gel electrophoresis was conducted as above, except that SDS was omitted, and the samples

Fig. 1. Chromatography of the elution pool from a Blue Sepharose CL-6B column on Matrex Gel Red A

The elution pool (approx. 48 ml) from a Blue Sepharose CL-6B column was applied to a column of Matrex Gel Red A (3.8 cm $\times$ 2.6 cm) pre-equilibrated in Buffer C [10 mM-potassium phosphate buffer (pH 6.0)/2 mM-dithiothreitol/5 mM-MgCl₂] plus 0.12 mM-NAD⁺. After loading, the pump was stopped for 30 min ($\downarrow$), and then the column was washed with Buffer C plus 0.12 mM-NAD⁺ for 4 column vol. after the A_{280} of the effluent had returned to the baseline. The buffer was changed to Buffer C plus 2.75 mM-NAD⁺, and the flow direction reversed ($\uparrow$) to elute the enzyme activities. The flow rate was 99 ml/h, and 5 ml fractions were collected. $\circ$, A_{280} ; $\blacktriangle$, benzyl alcohol dehydrogenase activity; $\bullet$, benzaldehyde dehydrogenase II activity.

Fig. 2. Chromatography of a concentrated elution pool from a Matrex Gel Red A column on an f.p.l.c. Superose 12 gel-filtration column

The elution pool (approx. 30 ml) from a Matrex Gel Red A column was concentrated to 0.2 ml and applied to an f.p.l.c. Superose 12 gel filtration column (30 cm $\times$ 1 cm) pre-equilibrated in Buffer D [50 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol]. The flow rate was 0.3 ml/min, and 0.3 ml fractions were collected. $\circ$, A_{280} ; $\blacktriangle$, benzyl alcohol dehydrogenase activity.

were not boiled. After electrophoresis, gels were stained either for protein as above, or for dehydrogenase activity by incubation for 10 min at 30 °C in the dark in a mixture containing 66.7 mM-sodium pyrophosphate buffer, pH 9.0, 2 mM-NAD⁺, 550 μ M-*N*-methylphenazonium methosulphate, 55 μ M-Nitro Blue Tetrazolium and substrate (either 0.2 mM-benzyl alcohol or 0.1 mM-benzaldehyde).

Determination of M_r values

Native M_r values were determined in duplicate with a Sephacryl S-300 (superfine grade) column (75 cm $\times$ 1.6 cm) equilibrated with 100 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol, and calibrated (M_r values in parentheses) with ferritin (450 000), catalase (240 000), fumarase (194 000), aldolase (158 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 500). The flow rate was 20 ml/h. The void volume was established with Blue Dextran.

M_r values of the enzymes under denaturing conditions were estimated by SDS/polyacrylamide-gel electrophoresis. The protein standards were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α -lactalbumin (14 400).

RESULTS AND DISCUSSION

Purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II

Details of a typical purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus* N.C.I.B. 8250 are given in Table 1.

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II co-purified through chromatography on DEAE-Sephacel and Blue Sepharose CL-6B. The enzymes were separated at the Matrex Gel Red A chromatography step (Fig. 1), resulting in homogeneous benzaldehyde dehydrogenase II. Homogeneous benzyl alcohol dehydrogenase was obtained from the application of the concentrated elution pool after Matrex Gel Red A chromatography to an f.p.l.c. Superose 12 column (Fig. 2). A third homogeneous protein, identified as having glycerol-3-phosphate dehydrogenase activity, was also obtained after chromatography on the Superose 12 column (Fig. 3a, track I). The purification procedure is convenient, rapid (approx. 60 h), and reproducible. Both enzymes were isolated in good yield, e.g. 0.7 mg of benzyl alcohol dehydrogenase and 1.8 mg of benzaldehyde dehydrogenase II from 9 g wet wt. of bacteria. The purified enzymes retained more than 90% of their original activity over a 3-month period at -20 °C in Buffer E.

Dithiothreitol (2 mM) and MgCl₂ (5 mM) were required to stabilize both enzyme activities. MgCl₂ was particularly important during dialysis; it also aided binding of the enzymes to the Blue Sepharose CL-6B and Matrex Gel Red A affinity columns.

The purified enzymes were homogeneous as judged by the criteria of denaturing (Fig. 3a) and non-denaturing (Fig. 3b) polyacrylamide-gel electrophoresis.

Determination of M_r values

The subunit M_r values of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were 39 700

Fig. 3. Electrophoresis of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II

(a) The purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus* N.C.I.B. 8250 as monitored by SDS/polyacrylamide-gel electrophoresis. A 10% (w/v) polyacrylamide slab gel containing 0.1% (w/v) SDS was used. Tracks: B, crude extract (20 μ g of protein); C, ion-exchange elution pool (10 μ g of protein); D, dialysed ion-exchange elution pool (10 μ g of protein); E, Blue Sepharose CL-6B elution pool (2 μ g of protein); F, Matrex Gel Red A wash pool (1 μ g of protein); G, Matrex Gel Red A elution pool (1 μ g of protein); H, f.p.l.c. Superose 12 major protein peak (1 μ g of protein); I, f.p.l.c. Superose 12 minor protein peak (0.4 μ g of protein); A and J, M_r standard proteins (approx. 1 μ g of protein each). (b) Non-denaturing polyacrylamide gel and activity stain of purified benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus* N.C.I.B. 8250. A 7% (w/v) polyacrylamide slab gel was used. Tracks: A, B and C, crude extract (20 μ g of protein); D and E, purified benzaldehyde dehydrogenase II (2 μ g of protein); F and G, purified benzyl alcohol dehydrogenase (2 μ g of protein). Tracks A, D and F were stained for protein. Tracks B and E were stained for benzaldehyde dehydrogenase II activity, and tracks C and G were stained for benzyl alcohol dehydrogenase activity, as described in the Experimental section.

± 400 ($n = 24$) and 55000 ± 900 ($n = 33$) respectively. The M_r values of the enzymes under non-denaturing conditions were 155000 (from two identical values) for benzyl alcohol dehydrogenase and 222500 (216000, 229000) for benzaldehyde dehydrogenase II. Comparison of the subunit and native M_r values of the enzymes suggests that both are tetrameric under the conditions

used for the native M_r estimations. The tetrameric nature of the native enzymes has been confirmed by cross-linking experiments *in vitro* using dimethylsuberimide (R. M. Chalmers, personal communication).

Effect of pH on enzyme activity

The maximum activities for the oxidation reactions of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II, and for the reduction reaction of benzyl alcohol dehydrogenase (no detectable reductase reaction for benzaldehyde dehydrogenase II could be observed at any pH), were estimated to be at the following pH values: benzyl alcohol oxidation by benzyl alcohol dehydrogenase, pH 9.2; benzaldehyde reduction of benzyl alcohol dehydrogenase, pH 8.9; benzaldehyde oxidation by benzaldehyde dehydrogenase II, pH 9.5. At any particular pH the nature of the buffer (Hepes, glycine, phosphate, Bicine) did not appear to affect the initial rate, and neither did the presence of hydrazine.

Equilibrium constants

Benzyl alcohol dehydrogenase catalyses both the oxidation of benzyl alcohol and the reduction of benzaldehyde. The equilibrium constant, K'_{eq} , of the reaction was calculated from the progress curve of benzyl alcohol oxidation in the absence of hydrazine. The average K'_{eq} value for the oxidation of benzyl alcohol was estimated to be 3.08×10^{-11} M. This value compares well with that (2.98×10^{-11} M) calculated from the redox potentials of benzyl alcohol and NAD^+ (Scharschmidt *et al.*, 1984). At the assay pH of 9.2 the reduction of aldehyde to alcohol is therefore thermodynamically favoured, and the inclusion of hydrazine in the assay mixture is required to prevent non-linearity in the progress curve for alcohol oxidation. Benzaldehyde dehydrogenase II did not reduce benzoate (1 mM) with $NADH$ (0.25 mM) to benzaldehyde at a detectable rate.

Other aromatic alcohol dehydrogenases exhibit reductase activity; 3-aminobenzyl alcohol dehydrogenase from *Mycobacterium tuberculosis* has a reductase activity which is 3 times the dehydrogenase activity (Sloane, 1973), and 3-hydroxybenzyl alcohol dehydrogenase from *Penicillium urticae* reduces aldehyde 6 times faster than it oxidizes alcohol (Forrester & Gaucher, 1972).

Determination of isoelectric points

The average pI values for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were determined to be 5.0 and 4.6 respectively.

Effect of salts on enzyme activity

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were neither activated nor inhibited when assayed in the presence of the following salts: 1 mM- $MgCl_2$, 1 mM- $NaCl$, 1 mM- KCl , 1 mM- Na_2SO_4 or 1 mM- NH_4Cl . These results are consistent with those of Livingstone *et al.* (1972), who showed that in bacterial extracts the activity of benzaldehyde dehydrogenase I, but not benzaldehyde dehydrogenase II, was stimulated by certain univalent cations.

Absorption spectra

The absorption maxima for both benzyl alcohol dehydrogenase and benzaldehyde II were at 214 nm and 280 nm. No other maxima were observed, and there was no absorbance above 340 nm, suggesting that neither

Table 2. Kinetic coefficients of benzyl alcohol dehydrogenase with benzyl alcohol, benzaldehyde, NAD⁺ and NADH

For typical plots see Fig. 4. The analytical procedures are described in the Experimental section. The numbers of individual experiments are given in parentheses, except where only two experiments were done, in which case the individual values are given in parentheses.

Reaction	Kinetic parameter	Estimated value
Benzyl alcohol + NAD ⁺ ↓ benzaldehyde + NADH + H ⁺	K_m for benzyl alcohol (μM)	17.3 ± 0.65 (3)* 17.5 ± 0.75 (3)†
	K_m for NAD ⁺ (μM)	15.2 ± 2.2 (3)* 19.3 ± 0.61 (3)†
	V (units/mg of protein)	231 ± 8.7 (3)* 192 ± 21.4 (3)†
Benzaldehyde + NADH + H ⁺ ↓ benzyl alcohol + NAD ⁺	Apparent K_m for benzaldehyde (μM)	4.0 (3.1, 4.9)
	Apparent K_m for NADH (μM)	39.8 (33.3, 46.3)
	V (units/mg of protein)	366 ± 40 (4)

* Assayed in the presence of hydrazine.
† Assayed in the absence of hydrazine.

Fig. 4. Dependence of the activity of benzyl alcohol dehydrogenase on the concentration of substrate and cofactor

(a) Double-reciprocal plot of initial velocities of the forward reaction against benzyl alcohol concentration at a series of fixed NAD⁺ concentrations: ○, 9.75 μM ; ●, 11.7 μM ; □, 15.6 μM ; ■, 23.4 μM ; △, 46.8 μM . Inset: replot of slopes (▲) and intercepts (▽) against the reciprocal of the NAD⁺ concentration. The enzyme was assayed in the presence of hydrazine. (b) Double-reciprocal plots of the initial velocities of the reverse reaction of benzyl alcohol dehydrogenase against (top) benzaldehyde concentration at a fixed NADH concentration of 0.25 mM, and (bottom) NADH concentration at a fixed benzaldehyde concentration of 0.1 mM.

enzyme contained bound cofactor or prosthetic group such as cytochrome, flavin, or pyrroloquinoline quinone, which have been found in some other bacterial alcohol and aldehyde dehydrogenases (MacKintosh & Fewson, 1987a). Both enzymes behaved as entirely soluble

proteins (e.g. when enzyme distribution was measured after centrifuging bacterial extracts at various speeds), and of the electron acceptors tested (NAD⁺, NADP⁺ and *N*-methylphenazonium methosulphate) NAD⁺ was the only one that was reduced by either enzyme. Therefore,

Fig. 5. Double-reciprocal plots of the initial velocities of the reaction of benzaldehyde dehydrogenase II with benzaldehyde and NAD⁺

Double-reciprocal plots of initial velocities against: (a) benzaldehyde concentration at a fixed NAD⁺ concentration of 2.0 mM, and (b) NAD⁺ concentration at a fixed benzaldehyde concentration of 0.01 mM. Up to four initial-rate measurements were obtained for the lower benzaldehyde concentrations in plot (a).

although some bacterial alcohol and aldehyde dehydrogenases are membrane-bound (MacKintosh & Fewson, 1987a), both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *A. calcoaceticus* are clearly cytoplasmic, NAD⁺-linked, dehydrogenases.

Kinetic properties

Table 2 gives the kinetic coefficients for benzyl alcohol dehydrogenase obtained from primary and secondary plots (Figs. 4a and 4b) with benzyl alcohol, benzaldehyde, NAD⁺ and NADH. The maximum velocity of the reverse reaction (aldehyde to alcohol) is about 1.6 times that of the forward reaction. The apparent K_m for benzaldehyde ($4.0 \mu M$) is over 4 times lower than the K_m for benzyl alcohol ($17.3 \mu M$), and the apparent K_m for NADH ($39.8 \mu M$) is approximately twice that for NAD⁺ ($15.2 \mu M$). The presence of hydrazine in the assays appeared to have little effect on the kinetic coefficients obtained for benzyl alcohol and NAD⁺.

Fig. 5 shows typical double-reciprocal plots of the

initial velocities of benzaldehyde dehydrogenase II against benzaldehyde and NAD⁺ concentration at a fixed concentration of the second substrate. The apparent K_m for benzaldehyde was determined to be $0.68 \mu M$ (0.57, 0.80) and that for NAD⁺ $250 \mu M$ (221, 284). The maximum velocity of the reaction was 76 ± 16 ($n = 4$) units/mg of protein.

Conclusions

When *A. calcoaceticus* is grown on mandelate, there is a transient accumulation of benzaldehyde and then of benzyl alcohol (Cook *et al.*, 1975). The low activity of benzaldehyde dehydrogenase I induced during growth on mandelate seems to be responsible for the accumulation of its substrate. As mandelate and phenylglyoxylate concentrations in the medium fall, the benzaldehyde induces benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II, and it is the reductase activity of the former enzyme that results in the accumulation of benzyl alcohol in the medium. The reduction of the aldehyde to the alcohol is favoured even at high pH, and presumably under the conditions inside the cell the equilibrium of the reaction will even more favour accumulation of benzyl alcohol.

Previous results (e.g. Livingstone *et al.*, 1972) did not rule out the possibility that dehydrogenase activities for both benzyl alcohol and benzaldehyde might reside on the same polypeptide. However, although the two activities co-purified during ion-exchange chromatography and affinity chromatography on Blue Sepharose CL-6B, it is clear that there are two separable enzymes (Table 1).

There is a great diversity of microbial alcohol and aldehyde dehydrogenases, but only a small proportion of them have been characterized in detail (MacKintosh & Fewson, 1987a). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *A. calcoaceticus* are rather similar to each other in terms of overall properties, but appear to be different from other microbial dehydrogenases with respect to one or more of the following features: cofactor specificity, location within the cell, substrate specificity, or genetic regulation. For instance, different strains of *A. calcoaceticus* possess a wide range of other alcohol and aldehyde dehydrogenases: some are membrane-bound and NAD(P)⁺-independent (e.g. Aurich *et al.*, 1987), some are NADP⁺-dependent (e.g. Fixter & Nagi, 1984), some are specific for normal or secondary aliphatic alcohols (e.g. Fewson, 1966; Beardmore-Gray & Anthony, 1983), and benzaldehyde dehydrogenase I is K⁺-dependent (Livingstone *et al.*, 1972). Amongst the pseudomonads, *Pseudomonas putida* strain A 3.12 grows very much more slowly on benzyl alcohol than does *A. calcoaceticus* and contains an NAD⁺-dependent alcohol dehydrogenase that is constitutive and has a specificity that seems to be directed against short-chain normal alcohols (Collins & Hegeman, 1984). Other strains of *P. putida* have more active benzyl alcohol and benzaldehyde dehydrogenases, but they are encoded by the TOL plasmid (e.g. Williams, 1981). Even the benzyl alcohol dehydrogenases of *P. putida* N.C.I.B. 9869 (Keat & Hopper, 1978) and *P. putida* T-2 (Suhara *et al.*, 1969), which most closely resemble the benzyl alcohol dehydrogenase from *A. calcoaceticus*, display clear differences of inducer specificity, kinetic constants and stability. The results reported in the present paper provide the starting point for a detailed molecular

comparison of the two aromatic dehydrogenases with benzaldehyde dehydrogenase I from the same organism, and this in turn will allow speculation as to whether gene duplication was involved in the evolution of these apparently similar dehydrogenases.

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