Purification and characterization of benzaldehyde dehydrogenase I from *Acinetobacter calcoaceticus*

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Benzaldehyde dehydrogenase I was purified from *Acinetobacter calcoaceticus* by DEAE-Sephacel, phenyl-Sepharose and f.p.l.c. gel-filtration chromatography. The enzyme was homogeneous and completely free from the isofunctional enzyme benzaldehyde dehydrogenase II, as judged by denaturing and non-denaturing polyacrylamide-gel electrophoresis. The subunit M_r value was 56000 (determined by SDS/polyacrylamide-gel electrophoresis). Estimations of the native M_r value by gel-filtration chromatography gave values of 141000 with a f.p.l.c. Superose 6 column, but 219000 with Sephacryl S300. Chemical cross-linking of the enzyme subunits indicated that the enzyme is tetrameric. Benzaldehyde dehydrogenase I was activated more than 100-fold by K^+ , Rb^+ and NH_4^+ , and the apparent K_m for K^+ was 11.2 mM. The pH optimum in the presence of K^+ was 9.5 and the pI of the enzyme was 5.55. The apparent K_m values for benzaldehyde and NAD^+ were 0.69 μM and 96 μM respectively, and the maximum velocity was approx. 110 $\mu mol/min$ per mg of protein. Various substituted benzaldehydes were oxidized at significant rates, and $NADP^+$ was also used as cofactor, although much less effectively than NAD^+ . Benzaldehyde dehydrogenase I had an NAD^+ -activated esterase activity with 4-nitrophenol acetate as substrate, and the dehydrogenase activity was inhibited by a range of thiol-blocking reagents. The absorption spectrum indicated that there was no bound cofactor or prosthetic group. Some of the properties of the enzyme are compared with those of other aldehyde dehydrogenases, specifically the very similar isofunctional enzyme benzaldehyde dehydrogenase II from the same organism.

INTRODUCTION

Bacteria of the genera *Acinetobacter* and *Pseudomonas* can use a range of aromatic compounds as sources of carbon and energy for growth. So far as is known, the catabolic pathways involved in the breakdown of any particular compound are virtually identical in the two groups of organisms, but the patterns of regulation of gene expression may be different. For instance, in the 3-oxoadipate pathway, *Pseudomonas putida* (the best studied of the pseudomonads) has relatively small blocks of co-ordinately regulated enzymes, whereas *Acinetobacter calcoaceticus* shows more extensive co-ordination, with fewer compounds serving as inducers (Canovas & Stanier, 1967). As a consequence, *A. calcoaceticus* has isofunctional 3-oxoadipate enol-lactonases and 3-oxoadipate CoA-transferases just beyond the point of metabolic convergence where pairs of co-ordinately expressed blocks of enzymes overlap. By contrast, in *P. putida* there is no need for such isofunctional enzymes, because the smaller regulatory groups do not overlap, although this necessitates rather more compounds acting as inducers (Canovas & Stanier, 1967). Extensive characterization of this pathway both at the genomic and at the enzymic level has allowed Ornston and colleagues to outline its possible evolutionary history (Neidle *et al.*, 1988, and references therein). They suggested how duplicated genes might have been stabilized against elimination by homologous recombination. They also pointed out how

slipped-strand mismatch repair mechanisms may have stabilized the genes against genetic drift, and may also under special circumstances have generated genetic diversity far more quickly than is possible by point mutations (Neidle *et al.*, 1988). These arguments were developed chiefly to deal with the duplication of genes to provide enzymes with altered substrate specificities, but presumably they would equally apply to isofunctional enzymes that catalyse the same reaction but are induced under different conditions.

The mandelate and benzyl alcohol pathway of *A. calcoaceticus* contains a further example of isofunctional enzymes, and again they occur immediately after a point of metabolic convergence. *A. calcoaceticus* metabolizes mandelate and benzyl alcohol to benzoate and then, by the 3-oxoadipate pathway, to succinate and acetyl-CoA. During growth on mandelate, benzaldehyde dehydrogenase I is induced by phenylglyoxylate, whereas the isofunctional enzyme benzaldehyde dehydrogenase II, together with benzyl alcohol dehydrogenase, is induced by benzaldehyde or benzyl alcohol (Livingstone *et al.*, 1972). During certain phases of growth on mandelate both benzaldehyde dehydrogenases are induced, possibly because of the accumulation of benzaldehyde in the medium (Cook *et al.*, 1975). We have previously described the purification and characterization of benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase (MacKintosh & Fewson, 1988a,b). In the present paper we complete this work by describing the purification and

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

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characterization of benzaldehyde dehydrogenase I, and we are therefore now in a position to compare the properties of the two isofunctional enzymes. This comparison should provide the foundation for subsequent studies of the evolutionary history of the two benzaldehyde dehydrogenases, and these in turn will help to clarify the relationship between them. For example, what is the selective advantage in maintaining two distinct enzymes with the consequential genetic burden? Are they derived from a common ancestor and, if so, how were the genes stabilized against elimination by recombination? Answers to these sorts of questions will help to test the generality of Ornston's conclusions (Neidle *et al.*, 1988) about the origin, function and stabilization of duplicated genes.

Parts of this work have been described in a preliminary form (Chalmers & Fewson, 1988, 1989).

EXPERIMENTAL

Materials

All of the chemicals were from BDH Chemicals, Poole, Dorset, U.K., except for the following: bovine serum albumin from W. Smith Ltd., Edgware, Middx., U.K.; acrylamide, bisacrylamide and SDS from FSA Laboratory Supplies, Loughborough, Leics., U.K.; dithiothreitol, NAD⁺, NADP⁺, Tris and gel-filtration standard proteins from Boehringer Corp., Lewes, Sussex, U.K.; Polybuffer PB 74, gel-filtration standard proteins, electrophoresis standard proteins, DEAE-Sephacel, phenyl-Sepharose CL-4B, Sephacryl S300 and f.p.l.c. pre-packed columns from Pharmacia, Milton Keynes, Bucks., U.K.; Centricon 30 microconcentrators from Amicon, Danvers, MA, U.S.A.; 4-nitrophenol acetate from Sigma Chemical Co., Poole, Dorset, U.K.; pyronin Y from G. T. Gurr Ltd., London S.W.6, U.K. Dimethylsuberimide was synthesized in this Institute by Professor J. R. Coggins. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were purified essentially as described by MacKintosh & Fewson (1988a). Column washing was increased to 10 column vol. at each stage in the purification, and samples were concentrated after the dye-ligand affinity steps by using a small DEAE-Sephacel column rather than by vacuum dialysis.

Growth of bacteria and preparation of extracts

A. calcoaceticus N.C.I.B. 8250 was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. *A. calcoaceticus* mutant strain C1005 (N.C.I.B. 11339, previously HMM 5, constitutive for benzaldehyde dehydrogenase I) was derived from *A. calcoaceticus* N.C.I.B. 8250 (Fewson *et al.*, 1978).

Bacteria were maintained as described by Allison *et al.* (1985). Large quantities of mutant strain C1005 were grown and harvested by a draw-and-fill procedure using a 10-litre fermenter with a 20-litre reservoir, containing a complex medium plus 1.5 g of DL-mandelate/l and 0.005% poly(propylene glycol) 2025 (Allison *et al.*, 1985; Hoey *et al.*, 1987).

A broken-cell suspension was prepared at 0–10 °C by passage through a French pressure cell and centrifuged at 105 000 *g* for 150 min at 4 °C (Allison *et al.*, 1985). The supernatant (extract) was dialysed overnight at 4 °C against 40 vol. of 20 mM-potassium phosphate buffer

(pH 7.5)/2 mM-dithiothreitol and used immediately or stored frozen at –20 °C. In either case the extract was centrifuged at 29 000 *g* for 20 min at 4 °C immediately before the first purification step.

Purification of benzaldehyde dehydrogenase I

Extract was prepared from approx. 20 g wet wt. of *A. calcoaceticus* C1005 grown on a complex medium plus 1.5 g of DL-mandelate/l. Steps 1, 2 and 4 were carried out at 0–4 °C, and step 3 was done at room temperature. Enzyme assays used 100 μ M-benzaldehyde as substrate (see below).

Step 1: chromatography on DEAE-Sephacel. Dialysed extract (50 ml) was applied at 20 ml/h to a DEAE-Sephacel column (2.6 cm $\times$ 9 cm) which had been pre-equilibrated with buffer A [25 mM-potassium phosphate (pH 7.5)/2 mM-dithiothreitol]. After washing with 10 column vol. of buffer A at 80 ml/h, benzaldehyde dehydrogenase I was eluted with a linear 25–200 mM-potassium phosphate buffer gradient (pH 7.5) containing 2 mM-dithiothreitol. The gradient volume was 500 ml and the flow rate was 20 ml/h. Benzaldehyde dehydrogenase I was eluted after approx. 160 ml and fractions with peak activity were pooled.

Step 2: chromatography on phenyl-Sepharose CL-4B. The pooled fractions (45 ml) from step 1 were applied to a phenyl-Sepharose column (2.6 cm $\times$ 9.8 cm) pre-equilibrated with buffer A. The column was washed with 50 ml of buffer A, followed by 50 ml of buffer B [5 mM-potassium phosphate (pH 7.5)/2 mM-dithiothreitol] and finally with 500 ml of buffer B containing 30% (v/v) ethanediol. The flow rate was decreased from 50 ml/h to 20 ml/h, and benzaldehyde dehydrogenase I was eluted from the column with 300 ml of buffer B containing 40% ethanediol. The column eluate was passed through a DEAE-Sephacel column (1.6 cm $\times$ 3 cm) pre-equilibrated with buffer B. The DEAE-Sephacel column was washed at 20 ml/h with 20 ml of buffer B and then with 60 ml of 50 mM-potassium phosphate buffer (pH 7.5)/2 mM-dithiothreitol before benzaldehyde dehydrogenase I was finally eluted with buffer C [100 mM-potassium phosphate (pH 7.5)/2 mM-dithiothreitol] at 6 ml/h. Fractions with peak activity were pooled.

Step 3: gel filtration through f.p.l.c. Superose 12. The pooled fractions (5.6 ml) from step 2 were concentrated to 180 μ l in a Centricon 30 micro-concentrator and applied to a f.p.l.c. Superose 12 column (1 cm $\times$ 30 cm) pre-equilibrated with buffer C. The flow rate was 0.3 ml/min throughout. Fractions containing benzaldehyde dehydrogenase I activity were checked for homogeneity by SDS/polyacrylamide-gel electrophoresis before they were pooled.

Step 4: storage. The pooled fractions (1.0 ml) from step 3 were mixed with an equal volume of ethanediol and stored at –20 °C.

Enzyme assays

All enzyme assays were carried out at 27 °C, and redistilled benzaldehyde was used for the determination of kinetic parameters.

Dehydrogenase activity. Benzaldehyde dehydrogenase I activity was routinely measured in 1 ml assay mixtures

containing 0.15 M-glycine/KOH buffer, pH 9.5, 2 mM-NAD⁺ and up to 50 μ l of enzyme stock solution, and the reaction was initiated by benzaldehyde (final concn. 100 μ M). The reduction of NAD⁺ was monitored at 340 nm, and the light path was 1 cm. Under these conditions the progress curve of the reaction was not linear; however, the rate was proportional to the amount of enzyme added when the change in absorbance was 12×10^{-3} – 82×10^{-3} /min. Linear progress curves were obtained if the initial benzaldehyde concentration in the assay mixture was lowered to 3–12 μ M, and under these conditions the rate was proportional to the amount of enzyme added when the change in absorbance was 1.5×10^{-3} – 9.5×10^{-3} /min. One enzyme unit is defined as 1 μ mol of substrate converted/min, and the molar absorption coefficient of NAD⁺ was assumed to be 6.3×10^3 M⁻¹·cm⁻¹. Dehydrogenase activity was also measured fluorimetrically in the same reaction mixture scaled up to 3 ml by monitoring the reduction of NAD⁺ at 340 nm (excitation)/460 nm (emission). Reaction velocities determined fluorimetrically are expressed in arbitrary units/100 ng of protein, and may be approximately converted into specific activity (units/mg of protein) by dividing by 2.70.

Cation-independent dehydrogenase activity. Benzaldehyde dehydrogenase I was generally changed into K⁺-free buffer by gel-filtration chromatography through an f.p.l.c. desalting column (1 cm $\times$ 10 cm) that had been pre-equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/2 mM-dithiothreitol. The activation of benzaldehyde dehydrogenase I by various cations was determined in 3 ml assay mixtures containing 0.15 M-ethanolamine/HCl buffer, pH 9.5, 2 mM-NAD⁺ and up to 50 μ l of enzyme stock solution, and the reaction was initiated by benzaldehyde (final concn. 100 μ M).

Esterase activity. The rate was measured in 1 ml assay mixtures containing 980 μ l of 0.15 M-sodium pyrophosphate buffer (pH 8.5)/100 mM-KCl, 10 μ l of 18 mM-4-nitrophenol acetate in acetone and various amounts of NAD⁺, and the reaction was initiated by up to 10 μ l of enzyme. The hydrolysis of 4-nitrophenol acetate was monitored at 400 nm, and the molar absorption coefficient of 4-nitrophenol was assumed to be 14.69×10^3 M⁻¹·cm⁻¹ at pH 8.5. For comparison, benzaldehyde dehydrogenase activity was also measured in 990 μ l of the same pyrophosphate buffer containing 100 mM-KCl, 2 mM-NAD⁺, 100 μ M-benzaldehyde plus 10 μ l of enzyme stock solution.

Determination of kinetic parameters

The apparent K_m and maximum velocities for substrates of benzaldehyde dehydrogenase I were determined by measuring the initial reaction rates at various non-saturating concentrations of the first substrate in the presence of a fixed concentration of the second substrate. When the concentration of benzaldehyde was varied, NAD⁺ was used at a close to saturating concentration of 2 mM. When the concentration of NAD⁺ was varied, the benzaldehyde concentration was only 3.7 μ M because of substrate inhibition at higher concentrations. The kinetic parameters were calculated from initial-velocity measurements by the Direct Linear method (Eisenthal & Cornish-Bowden, 1974) by using the Enzpack computer program (Williams, 1985). Each of the sets of data used

to calculate the apparent K_m values quoted in the Results and discussion section had the form of a rectangular hyperbola. The concentrations of the substrate stock solutions were calculated from the ΔA_{340} after the complete enzymic oxidation or reduction of one substrate in the presence of excess amounts of the second substrate.

Polyacrylamide-gel electrophoresis

The purification of benzaldehyde dehydrogenase I was monitored with the SDS/polyacrylamide-gel system of Laemmli (1970), except that 96 mM-Tris/HCl buffer replaced 125 mM-Tris/HCl buffer in the stacking gel. Slab gels (19.0 cm $\times$ 9.5 cm $\times$ 0.15 cm) consisted of a 5.3 % (w/v) polyacrylamide stacking gel and a 12.5 % (w/v) polyacrylamide separating gel. Samples (40 μ l, diluted with water if necessary) were prepared for electrophoresis by boiling for 2 min in a mixture with 25 μ l of 0.2 M-dithiothreitol and 10 μ l of tracker-dye mixture. The tracker-dye mixture was 10 ml of 0.5 M-Tris/HCl (pH 6.8), 1.5 g of SDS, 8 g of sucrose and 100 μ g of pyronin Y. Gels were stained with Coomassie Brilliant Blue G250 with the reagents described by MacKintosh & Fewson (1988a).

Non-denaturing polyacrylamide gels were prepared exactly as above, except that SDS was omitted from all of the solutions and the separating gel was only 7.5 % polyacrylamide. The samples were loaded directly on to the gel without any treatment, or were first made to 2 % (v/v) in glycerol. Non-denaturing gels were electrophoresed at 20 mA (constant current), and the apparatus was cooled by water circulating from an ice bath. Non-denaturing gels were either stained for protein as above, or they were activity-stained by the method of MacKintosh & Fewson (1988a), except that the gels were first incubated in staining buffer (without staining reagents) for 30 min at 4 °C.

After the chemical cross-linking of enzyme subunits, the quaternary structure was examined by using a phosphate-buffered gel system in which 7.5 %-polyacrylamide slab gels (19.0 cm $\times$ 9.5 cm $\times$ 0.15 cm) were prepared and used essentially as described by Shapiro & Maizel (1969). The gels were silver-stained by the method of Wray *et al.* (1981).

Determination of M_r values

The subunit M_r value for benzaldehyde dehydrogenase I under denaturing conditions was determined by SDS/polyacrylamide-gel electrophoresis. The calibration proteins were (M_r values in parentheses) 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). Gels were stained for protein (as above) and then scanned with an LKB 2202 Ultrosan laser densitometer.

The native M_r value for benzaldehyde dehydrogenase I was determined in duplicate by gel filtration through an f.p.l.c. Superose 6 column (1 cm $\times$ 30 cm) at a flow rate of 0.3 ml/min. The column buffer was 100 mM-potassium phosphate (pH 7.5)/2 mM-dithiothreitol, and the calibration proteins were thyroglobulin (M_r 669 000), ferritin (440 000), catalase (232 000), aldolase (158 000) and albumin (68 000). Standards and samples were injected in 200 μ l, and the proteins were detected by continuously monitoring the A_{280} of the eluate. The column void volume was assumed to be the elution volume of thyroglobulin. The native M_r value was also determined

in duplicate by gel filtration through a Sephacryl S300 column (1.6 cm $\times$ 90 cm) by using the same column buffer and calibration proteins as above. Three additional calibration proteins were also used: ovalbumin (45000), chymotrypsinogen A (25000) and cytochrome *c* (12500). The flow rate was 20 ml/h, and the column void volume was taken as the elution volume of Blue Dextran. Standards and samples were applied in 2 ml, and the proteins were detected in the eluent by continuously monitoring the A_{280} and by enzyme activity.

Chemical cross-linking of enzyme subunits

The quaternary structures of benzaldehyde dehydrogenase I, benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase were investigated by using chemical cross-linking with dimethylsuberimide, essentially as described by Lumsden & Coggins (1977). The enzyme samples (1 mg of protein/ml) were treated with only 6 mM-dimethylsuberimide, and after 30 min incubation they were treated again.

Determination of pI

Purified benzaldehyde dehydrogenase I in 25 mM-Bistris/HCl buffer, pH 6.7, was applied to an f.p.l.c. Mono P chromatofocusing column (0.5 cm $\times$ 20 cm) pre-equilibrated with the same buffer. Protein was eluted with Polybuffer PB 74 diluted 1+10 with water and adjusted to pH 4.8 with HCl. The flow rate was 0.5 ml/min during sample application and 1.0 ml/min during elution. Benzaldehyde dehydrogenase I was located in the eluate by monitoring the A_{280} and by enzyme assay. The pI value quoted is the pH value of the fraction with peak enzyme activity, averaged over two determinations.

Analytical methods

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The presence of protein in the column eluents was monitored with an LKB Uvicord monitor or a Pharmacia UV-1 monitor. Fine pH measurements were made with a Radiometer (Copenhagen, Denmark) type M26 pH monitor fitted with a GK 2302 micro pH electrode.

RESULTS AND DISCUSSION

Purification of benzaldehyde dehydrogenase I

Table 1 summarizes the results of a typical purification of benzaldehyde dehydrogenase I in three steps by ion-

exchange, hydrophobic-interaction and gel-filtration chromatography. The eluate from the phenyl-Sepharose column was passed through a small DEAE-Sepharose column to concentrate the benzaldehyde dehydrogenase I activity and to remove ethanediol from the buffer. Benzaldehyde dehydrogenase I was purified 116-fold with a 24% yield. It was homogeneous and completely free from benzaldehyde dehydrogenase II, as judged by denaturing and non-denaturing polyacrylamide-gel electrophoresis (Fig. 1). The sample of benzaldehyde dehydrogenase II used for comparison was pure as judged by several criteria (MacKintosh & Fewson, 1988b), and the multiple bands seen after non-denaturing electrophoresis (tracks C, D and F of Fig. 1) are probably caused by minor charge or other heterogeneities. The specific activity of the purified enzyme was quite constant among independent preparations (25.5 units/mg of protein, s.d. = 2.5, n = 9), but the fold purification was dependent on the specific activity of the extract.

Determination of M_r values and subunit structure

The subunit M_r value for the enzyme determined by SDS/polyacrylamide-gel electrophoresis was 56000 (s.d. = 363, n = 14). When benzaldehyde dehydrogenase I, benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase (the last two enzymes have subunit M_r values of 55000 and 39700 respectively; MacKintosh & Fewson, 1988a) were cross-linked by treatment with dimethylsuberimide and then analysed by SDS/polyacrylamide-gel electrophoresis (results not shown), four major bands appeared in all three enzyme preparations, corresponding in M_r value to monomer, dimer, trimer and tetramer. The M_r of native benzaldehyde dehydrogenase I was determined to be 141000 (two identical determinations) by gel filtration through an f.p.l.c. Superose 6 column. It was eluted at exactly the same volume as aldolase (M_r = 158000). The native M_r appeared to be 219000 as judged by gel filtration through Sephacryl S300. It is most likely that all three dehydrogenases are tetrameric (see also MacKintosh & Fewson, 1988a), but that benzaldehyde dehydrogenase I runs anomalously through the Superose 6 gel-filtration column.

Effect of pH and cations on benzaldehyde dehydrogenase I

The maximum rate of benzaldehyde oxidation occurred at pH 9.5 in the presence of approx. 70 mM- K^+ . This pH optimum is almost identical with that for

Table 1. Purification of benzaldehyde dehydrogenase I from *A. calcoaceticus* C1005

Full details of the purification are given in the Experimental section. Enzyme assays contained 100 μ M-benzaldehyde.

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Dialysed extract	50	197	895	0.22	100	1
Ion exchange	45	110	75	1.47	56	6.7
Phenyl-Sepharose	5.6	51	2.0	25.5	26	116
Superose 12	1.0	47	1.85	25.4	24	116
Ethanediol added	2.0	47	1.85	25.4	24	116

Fig. 1. Electrophoresis of benzaldehyde dehydrogenase I from *A. calcoaceticus* C1005

(a) The purification of benzaldehyde dehydrogenase I was monitored on a 12.5%-polyacrylamide slab gel containing 0.1% SDS. Tracks: A and F, M_r markers (approx. 1 μ g of each); B, extract (42 μ g of protein); C, ion-exchange elution pool (23 μ g of protein); D, phenyl-Sepharose elution pool (7.3 μ g of protein); E, Superose 12 elution pool (8 μ g of protein). (b) Non-denaturing 7.5%-polyacrylamide slab gel with activity stain. Tracks: A, *A. calcoaceticus* C1005 extract (89 μ g of protein); B, pure benzaldehyde dehydrogenase I (1.6 μ g of protein); C, *A. calcoaceticus* N.C.I.B. 8250 extract (benzaldehyde dehydrogenase II induced) (180 μ g of protein); D, pure benzaldehyde dehydrogenase II (14 μ g of protein); E, pure benzaldehyde dehydrogenase I (40 μ g of protein); F, pure benzaldehyde dehydrogenase II (60 μ g of protein). (c) Non-denaturing 7.5%-polyacrylamide slab gel with protein stain; 18 μ g of pure benzaldehyde dehydrogenase I.

benzaldehyde dehydrogenase II (MacKintosh & Fewson, 1988a) and the benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 in *P. putida* (R. M. Chalmers, A. J. Scott & C. A. Fewson, unpublished work); however, it is much higher than the optimum for the yeast K^+ -activated aldehyde dehydrogenase (Bostian & Betts, 1978a).

The pI of benzaldehyde dehydrogenase I was 5.55. The

Fig. 2. Activation of purified benzaldehyde dehydrogenase I by K^+ and NH_4^+

The effects of KCl ($\square$) and NH_4 Cl ($\triangle$) on the enzyme activity were determined as described in the Experimental section, except that in this case the enzyme was not first gel-filtered into K^+ -free buffer, so that all of the assay mixtures contained 890 μ M- K^+ added in the enzyme stock solution. Assays were carried out in duplicate for KCl.

homogeneity of the enzyme was confirmed by the observation of a single A_{280} peak in the column eluate.

Benzaldehyde dehydrogenase I, in contrast with benzaldehyde dehydrogenase II (MacKintosh & Fewson, 1988a), was activated by univalent cations, and maximum activity occurred at approx. 70 mM- K^+ (Fig. 2). The apparent K_m for K^+ was 11.2 mM (68% confidence limits = 10.8–12.1 mM), and this is similar to the value determined for the yeast K^+ -activated aldehyde dehydrogenase (Dickinson & Haywood, 1987). In common with the yeast enzyme (Dickinson & Haywood, 1987) and the NADP $^+$ -specific aldehyde dehydrogenase from *P. putida* (Stachow *et al.*, 1967), Rb^+ and NH_4^+ were good substitutes for K^+ . The activation curves for K^+ and Rb^+ were each in the form of a rectangular hyperbola. The response to NH_4^+ was sigmoidal (Fig. 2), but we have no explanation for this observation, and we know of no other documented cases of this type. In assay mixtures containing 70 mM-salt the activation by K^+ , Rb^+ and NH_4^+ was 164-, 135- and 129-fold respectively. There was no evidence from experiments using various buffers that either Tris or ethanolamine give any significant activation of benzaldehyde dehydrogenase I. In contrast with the yeast enzyme (Dickinson & Haywood, 1987) and the *P. putida* enzyme (Stachow *et al.*, 1967), which were very unstable in the absence of K^+ , the stability of benzaldehyde dehydrogenase I (at 4 $^{\circ}$ C) was not lowered in the absence of K^+ , nor was there a detectable lag phase (< 30 s) when K^+ -free enzyme was assayed in the presence of K^+ .

Substrates and cofactors

Benzaldehyde dehydrogenase I was inhibited by low concentrations of benzaldehyde, with maximum activity

Fig. 3. Substrate inhibition of benzaldehyde dehydrogenase I by benzaldehyde

Enzyme activity was measured at a close-to-saturating concentration of NAD^+ (2 mM) by monitoring the fluorescence of NADH as described in the Experimental section.

at approx. 4–5 μM -benzaldehyde (Fig. 3). The apparent K_m for benzaldehyde was 0.69 μM (68% confidence limits = 0.53–0.80 μM) and the maximum velocity was 278 arbitrary units (approx. 106 units/mg of protein) (68% confidence limits = 251–313 arbitrary units). The apparent K_m for NAD^+ was measured in the presence of only 3.7 μM -benzaldehyde and was found to be 96 μM (68% confidence limits = 79.3–106 μM). The maximum velocity was 290 arbitrary units (approx. 110 units/mg of protein) (68% confidence limits = 262–307 arbitrary units). Several other aldehyde dehydrogenases have apparent substrate K_m values below 1 μM and are substrate-inhibited; these include benzaldehyde dehydrogenase II (MacKintosh & Fewson, 1988a), the NADP^+ -specific aldehyde dehydrogenase from *P. putida* (Stachow *et al.*, 1967) and the benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 (R. M. Chalmers, A. J. Scott & C. A. Fewson, unpublished work).

The following substituted benzaldehydes were oxidized by benzaldehyde dehydrogenase I (all were tested at 100 μM , and the percentage of the rate with benzaldehyde is given in parentheses): 2-fluorobenzaldehyde (41), 3-fluorobenzaldehyde (98), 4-fluorobenzaldehyde (210), 2-methylbenzaldehyde (13), 3-methylbenzaldehyde (119), 4-methylbenzaldehyde (240), 2-hydroxybenzaldehyde (39), 3-hydroxybenzaldehyde (222) and 3-methoxybenzaldehyde (235). The following compounds were oxidized at detectable rates: 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 2-methoxybenzaldehyde, 3-methoxy-4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 2-bromobenzaldehyde, perillaldehyde, hexan-1-al and octan-1-al. Except for 4-hydroxybenzaldehyde, the enzyme has a higher tolerance for substitutions at the *meta* and *para* positions of the aromatic ring than at the *ortho* position. The results are broadly similar to those for benzaldehyde dehydrogenase II (MacKintosh & Fewson, 1988b), except that benzaldehyde dehydrogenase I did not oxidize either of the aliphatic aldehydes tested. Many aldehyde dehydrogenases, including the yeast K^+ -activated enzyme (Bostian & Betts, 1978b), accept both aromatic and aliphatic aldehydes as substrates.

When 2 mM- NADP^+ was used as cofactor, benzaldehyde dehydrogenase I oxidized benzaldehyde at only 6% of the rate achieved with 2 mM- NAD^+ .

The absorption maximum for benzaldehyde dehydrogenase I was at 280 nm, and there was no absorbance above 340 nm, indicating that the enzyme had no bound cofactor or prosthetic group. The only unusual feature of the absorption spectrum was a small but distinct blip at 285 nm.

Esterase activity and the effects of thiol-blocking reagents

Both benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II had esterase activity with 4-nitrophenol acetate as substrate. The rate was approx. 2% of the dehydrogenase activity, and the enzymes were both activated by low concentrations of NAD^+ (Fig. 4). It was impossible to measure esterase activity accurately at the pH optimum of the dehydrogenase activities (pH 9.5) because of the spontaneous hydrolysis of the substrate. Activation of the esterase function by NAD^+ is consistent with a single active site for both the esterase and the dehydrogenase activities.

Many aldehyde dehydrogenases have esterase activity with 4-nitrophenol acetate, and there has been some debate as to whether a single active-site thiol group is involved in both the esterase and the dehydrogenase activities (Tu & Weiner, 1988). The strongest evidence supports the existence of a single active site with a common thiol group forming the thioester acyl-intermediate in both reaction mechanisms. In addition, certain aldehyde dehydrogenases may have a second active site which is involved only in ester hydrolysis. However, this may consist of no more than a single reactive thiol group or the remnant of an ancestral active site (Tu & Weiner, 1988).

Benzaldehyde dehydrogenase I was inhibited by a number of thiol-blocking reagents (Table 2). It was similar to benzaldehyde dehydrogenase II in being far more sensitive towards 4-chloromercuribenzoate and *N*-ethylmaleimide than towards iodoacetate or iodoacetamide (MacKintosh & Fewson, 1988b). Benzaldehyde dehydrogenase I also resembled benzaldehyde dehydrogenase II in that considerable protection from inhibition

Fig. 4. NAD^+ -activated esterase activity of benzaldehyde dehydrogenase I ($\square$) and benzaldehyde dehydrogenase II ($\triangle$)

Esterase activity was measured as described in the Experimental section with 4-nitrophenol acetate as substrate.

Table 2. Inhibition of benzaldehyde dehydrogenase I by thiol-blocking reagents

Benzaldehyde dehydrogenase I was diluted in 25 mM-potassium phosphate buffer, pH 7.5, containing various concentrations of thiol-blocking reagents. The mixtures were incubated on ice, and at intervals samples were taken for assay. There was a background concentration of 33 μ M-dithiothreitol in the incubation mixture from the enzyme storage buffer. Plots were constructed of activity against time, and from these the time to reach 50% inhibition was estimated. The concentrations of benzaldehyde dehydrogenase I in the incubation mixture and the assay were 11.9 μ g/ml and 597 ng/ml respectively.

Inhibitor	Inhibitor concn. (mM)	Time to reach 50% inhibition (min)
Iodoacetate	1	> 30
	5	23
	10	11
Iodoacetamide	0.5	17
	1	10
	10	< 1
4-Chloromercuribenzoate	0.05	4
	0.1	< 1
N-Ethylmaleimide	0.05	4
	0.1	< 1

was afforded by the presence of benzaldehyde (results not shown).

Jacoby (1963) proposed that the reaction mechanism of aldehyde dehydrogenases involved a thioester acyl-intermediate formed by a nucleophilic attack on the aldehyde substrate. The protection of benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II by substrate from inhibition by thiol-blocking reagents and their NAD⁺-activated esterase activities is consistent with this type of mechanism.

Conclusions

Benzaldehyde dehydrogenase I has several features in common with other aldehyde dehydrogenases, and in particular it is almost identical with benzaldehyde dehydrogenase II. Both enzymes are tetrameric and have similar subunit M_r values, sensitivity to thiol-blocking reagents, pH optima, substrate specificity, esterase activity and K_m values for benzaldehyde, and both are inhibited by modest concentrations of benzaldehyde. The chief difference discovered so far is that benzaldehyde dehydrogenase I is activated by K⁺, Rb⁺ and NH₄⁺, as are the yeast K⁺-activated aldehyde dehydrogenase (Dickinson & Haywood, 1987) and the NADP⁺-specific aldehyde dehydrogenase of *P. putida* (Stachow *et al.*, 1967).

The numerous points of similarity between benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II prompt us to suggest that they were created by the duplication and divergence of a single gene coding for an ancestral aldehyde dehydrogenase. An immunological investigation in this laboratory is trying to establish whether or not this was the case (Chalmers & Fewson, 1989).

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