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# Comparison of benzyl alcohol dehydrogenases and benzaldehyde dehydrogenases from the benzyl alcohol and mandelate pathways in *Acinetobacter calcoaceticus* and from the TOL-plasmid-encoded toluene pathway in *Pseudomonas putida*

## *N*-Terminal amino acid sequences, amino acid compositions and immunological cross-reactions

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1. *N*-Terminal sequences were determined for benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus* N.C.I.B. 8250, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 in *Pseudomonas putida* MT53 and yeast $K^+$-activated aldehyde dehydrogenase. Comprehensive details of the sequence determinations have been deposited as Supplementary Publication SUP 50161 (5 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1991) **273**, 5. The extent of sequence similarity suggests that the benzyl alcohol dehydrogenases are related to each other and also to established members of the family of long-chain $Zn^{2+}$-dependent alcohol dehydrogenases. Benzaldehyde dehydrogenase II from *Acinetobacter* appears to be related to the *Pseudomonas* TOL-plasmid-encoded benzaldehyde dehydrogenase. The yeast $K^+$-activated aldehyde dehydrogenase has similarity of sequence with the mammalian liver cytoplasmic class of aldehyde dehydrogenases but not with any of the *Acinetobacter* or *Pseudomonas* enzymes. 2. Antisera were raised in rabbits against the three *Acinetobacter* enzymes and both of the *Pseudomonas* enzymes, and the extents of the cross-reactions were determined by immunoprecipitation assays with native antigens and by immunoblotting with SDS-denatured antigens. Cross-reactions were detected between the alcohol dehydrogenases and also among the aldehyde dehydrogenases. This confirms the interpretation of the *N*-terminal sequence comparisons and also indicates that benzaldehyde dehydrogenase I from *Acinetobacter* may be related to the other two benzaldehyde dehydrogenases. 3. The amino acid compositions of the *Acinetobacter* and the *Pseudomonas* enzymes were determined and the numbers of amino acid residues per subunit were calculated to be: benzyl alcohol dehydrogenase and TOL-plasmid-encoded benzyl alcohol dehydrogenase, 381; benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II, 525; TOL-plasmid-encoded benzaldehyde dehydrogenase, 538.

## INTRODUCTION

Pathways for the catabolism of benzyl alcohol and mandelate are important in *Acinetobacter calcoaceticus* because they contribute to the metabolic versatility of the organism by channelling the flux of aromatic carbon into the 3-oxoadipate *ortho* ring-cleavage pathway (Kennedy & Fewson, 1968). The pathway encoded by the TOL plasmid *xylCAB* operon in *Pseudomonas putida* acts in a similar way by converting toluene and 3- and 4-methyltoluenes (xylenes) into substrates of the *meta* ring-cleavage pathway (Burlage *et al.*, 1989; Assinder & Williams, 1990). We have previously described the purification and preliminary characterization of the benzyl alcohol dehydrogenases and benzaldehyde dehydrogenases from the benzyl alcohol and mandelate pathways in *A. calcoaceticus* N.C.I.B. 8250 and from the toluene pathway encoded by the TOL plasmid pWW53 in *Ps. putida* MT53 (MacKintosh & Fewson, 1988*a*,*b*; Chalmers & Fewson, 1989*a*; Chalmers *et al.*, 1990). The properties of the two benzyl alcohol dehydrogenases and of the three benzaldehyde dehydrogenases are remarkably similar, and it is hard to avoid the conclusion that there are evolutionary links within each group of enzymes (Chalmers & Fewson, 1989*a*,*b*; Chalmers *et al.*, 1990).

Two hypotheses have been proposed to account for the great variety of catabolic capabilities of microbial species: (*a*) retrograde evolution, in which the enzymes of a pathway evolved from a single ancestral enzyme by the processes of gene duplication and mutation (Horowitz, 1945, 1965), and (*b*) gene recruitment, in which copies of genes for existing enzymes are recruited and integrated into novel pathways by the processes of gene duplication, mutation and/or translocation (e.g. Jensen, 1976). There are many examples of evolution by gene recruitment but very few well-founded examples of retrograde evolution. The detailed comparison of the alcohol dehydrogenases and aldehyde dehydrogenases from the mandelate, benzyl alcohol and toluene pathways in *A. calcoaceticus* and *Ps. putida* appeared to be a good opportunity to test the hypothesis of retrograde evolution because the enzymes share superficial characteristics that would be expected of enzymes that had evolved by retrograde evolution: all five enzymes share two common substrates ($NAD^+$ and benzaldehyde) and participate in peripheral metabolic pathways that may be more likely to undergo rapid evolution than the

Abbreviations used: TOL benzyl alcohol dehydrogenase, TOL-plasmid-encoded benzyl alcohol dehydrogenase; TOL benzaldehyde dehydrogenase, TOL-plasmid-encoded benzaldehyde dehydrogenase.

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central pathways, which are constrained because they are vital. The fundamental questions addressed by this work are as follows. (*a*) Did the isofunctional benzaldehyde dehydrogenases from the benzyl alcohol and mandelate pathways of *A. calcoaceticus* evolve from a common ancestor? (*b*) Are the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases in *Acinetobacter* related to the respective enzymes in *Pseudomonas*? (*c*) Did the benzyl alcohol dehydrogenases from either organism evolve from the benzaldehyde dehydrogenases by retrograde evolution or do their evolutionary histories pre-date those of the pathways in which they now reside? Answers to these questions will further our understanding of the mechanisms by which metabolic diversity was established and in particular they may help us to define the origins of catabolic plasmids.

## EXPERIMENTAL

### Materials

Materials were generally of the best quality available and most were obtained from the sources described by Chalmers & Fewson (1989*a*). $Na^{125}I$ in NaOH solution was from Amersham International, Little Chalfont, Bucks., U.K. Matrex Gel Green A and Matrex Gel Red A were from Amicon, Danvers, MA, U.S.A. $N_2$ gas was from British Oxygen Co., Guildford, Surrey, U.K. Urea was from BRL Ltd., Glasgow, U.K. $AgNO_3$ was from Johnson Matthey, Royston, Herts., U.K. Iodobeads were from Pierce Europe B.V., OUD-Beijerland, The Netherlands. Protein A, Tween 20 and yeast $K^+$-activated aldehyde dehydrogenase were from Sigma Chemical Co., Poole, Dorset, U.K. Freund's adjuvant (complete and incomplete) was from Difco Laboratories, Detroit, MI, U.S.A. Heat-inactivated horse serum was from Gibco, Paisley, Strathclyde, U.K. Nitrocellulose was from Schleicher und Schüll, Dassel, Germany. Rabbit normal serum was from Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.

Control proteins for immunoblotting were obtained from Sigma Chemical Co., except for horse liver alcohol dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, which were from Boehringer Corp., Lewes, East Sussex, U.K., and BSA hen's-egg ovalbumin, rabbit muscle fructose-bisphosphate aldolase, horse spleen ferritin and bovine thyroid thyroglobulin, which were obtained as components of the Pharmacia (Uppsala, Sweden) gel-filtration calibration kit (high $M_r$).

### Bacteria

Bacteria were from the sources described by Chalmers & Fewson (1989*a*) and Chalmers *et al.* (1990).

### General methods

Denaturing and non-denaturing PAGE, protein concentration determinations and pH measurements were carried out as described by Chalmers & Fewson (1989*a*). Polyacrylamide gels were stained for activity and also for protein with Coomassie Brilliant Blue as described by Chalmers & Fewson (1989*a*) or with silver as described by Wray *et al.* (1981). Enzyme assays were carried out by monitoring the reduction of $NAD^+$ at 340 nm as described by Chalmers *et al.* (1990). Small samples were centrifuged in Eppendorf tubes at 15000 *g* in an Eppendorf 3200 centrifuge.

### Enzyme purification

Benzaldehyde dehydrogenase I was purified as described by Chalmers & Fewson (1989*a*). Benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase were purified by a modification of the method of MacKintosh & Fewson (1988*a*) as described by Chalmers & Fewson (1989*a*). The TOL-plasmid-encoded enzymes (TOL benzaldehyde dehydrogenase and TOL benzyl alcohol dehydrogenase) were purified as described by Chalmers *et al.* (1990)

### Electro-elution of enzymes from polyacrylamide gels

Purified benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase were electrophoresed on non-denaturing polyacrylamide gels. Each of the proteins was separated into two bands (Chalmers & Fewson, 1989*a*), and these were electro-eluted from the gels and prepared for protein sequencing as described by Findlay *et al.* (1989). Yeast $K^+$-activated aldehyde dehydrogenase was also prepared by this method except that it was electro-eluted from SDS/polyacrylamide gels.

### *N*-Terminal sequence analysis

Most of the protein sequencing was carried out in the Department of Biochemistry, University of Leeds, by automated solid-phase Edman degradation (Laursen, 1971) using the microsequence facility built by the Protein Sequence Unit. Amino acid phenylthiohydantoin derivatives were identified off-line by reverse-phase ($C_{18}$) micro-bore h.p.l.c. A full description of the sequencing protocol has been given by Findlay *et al.* (1989). The yeast $K^+$-activated aldehyde dehydrogenase *N*-terminal sequence was determined initially on a MilliGen 6600 ProSequencer with on-line h.p.l.c. with the protein covalently attached to a Sequelon–DITC membrane disc, and was then confirmed by the above method. Other *N*-terminal sequences were determined by Mr. B. Dunbar at the Department of Biochemistry, University of Aberdeen, using an Applied Biosystems model 470A/477A protein sequencer. Amino acid phenylthiohydantoin derivatives were identified off-line by reverse-phase h.p.l.c. using a Waters Associates chromatography system with an Apex Cyano column (4.6 mm × 25 cm), and elution was carried out with a gradient of acetate/acetonitrile.

Purified proteins were carboxymethylated as described by Lumsden & Coggins (1978). Proteins sequenced at the University of Aberdeen were dialysed for 4 days against eight changes of at least 1000 vol. of water, and those sequenced at the University of Leeds were dialysed first against 0.1% SDS and finally against water. Proteins that were eluted from polyacrylamide gels were sequenced without further treatment.

Purified benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase each showed two major bands after non-denaturing PAGE (Chalmers & Fewson, 1989*a*; Chalmers *et al.*, 1990), even though both enzymes were pure as judged by several criteria (MacKintosh & Fewson, 1988*a*,*b*). The relative amounts of protein and activity in each of the major bands were determined by using an LKB 2202 Ultroscan laser densitometer to scan non-denaturing gels that had been stained either for protein or for enzyme activity. The less mobile band of benzaldehyde dehydrogenase II had 9% of the protein and 26% of the activity whereas the more mobile band had 91% of the protein and only 74% of the activity. The less mobile band of benzyl alcohol dehydrogenase had 35% of the protein and 56% of the activity whereas the more mobile band had 65% of the protein and only 44% of the activity. The *N*-terminal sequence of each of the major bands from each of the enzyme preparations was determined after the proteins had been electro-eluted from non-denaturing gels. There were no discrepancies between the *N*-terminal sequences of the proteins from either of the pairs of bands and the corresponding purified proteins at positions where amino acid residues were identified, and the most likely explanation for the double bands is that there are two populations of molecules that each have different net charges. Other workers have ascribed similar effects to the deamidation of asparagine

and glutamine residues (Julia *et al.*, 1988) or to differences in the oxidation states of thiol groups (Jörnvall, 1973).

**Production of antisera**

Before the enzymes were injected into the rabbits the aldehyde dehydrogenases were assayed for the presence of alcohol dehydrogenase activity and vice versa. The upper limits of contamination were calculated to be approximately 1 part in 600 for alcohol dehydrogenase contaminating aldehyde dehydrogenase and approximately 1 part in 200 for aldehyde dehydrogenase contaminating alcohol dehydrogenase; however, the actual extents of contamination were possibly much lower than this. In order to quantify the extent of any possible cross-contamination of the antigens by inactive enzyme, different amounts of each sample were subjected to SDS/PAGE and the extent of any possible cross-contamination was estimated by making careful comparisons between tracks of the gel lightly loaded with one enzyme and adjacent tracks heavily loaded with a second enzyme. Benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II were not separated by SDS/PAGE (Chalmers & Fewson, 1989*a*), and the extent of any possible cross-contamination was estimated by using non-denaturing PAGE followed by protein staining or activity staining. There was apparently no cross-contamination of the enzyme preparations, insofar as the resolution of this method allows. The possible extents of cross-contaminations between the *Acinetobacter* and the *Pseudomonas* enzymes were not estimated because they were purified from different sources.

Antisera were raised against the enzymes in their native states and also against the *Acinetobacter* enzymes after they had been denatured by the reduction and carboxymethylation of their cysteine residues. Before inoculation, samples of the denatured enzymes were subjected to amino acid analysis and all of the cysteine expected in each of the enzyme preparations was detected as carboxymethylcysteine. The amount of protein in the carboxymethylated samples was estimated from the density of bands after SDS/PAGE and staining with Coomassie Brilliant Blue.

Antisera were raised with the assistance of Dr. I. D. Hamilton. The purified enzymes were each mixed with 1.0 ml of Freund's complete adjuvant. The mixtures were made up to 2.0 ml with water and emulsified by sonication for several seconds. The mixtures were injected subcutaneously into New Zealand White rabbits at six sites. After 6 weeks the animals were boosted with further injections as described above except that Freund's incomplete adjuvant was used. After a further 2 weeks the animals were bled. The blood was kept overnight at 4 °C and clotted material was removed from the antiserum. The antisera were stored at −20 °C. Each rabbit was identified with a code number and the amount of antigen in the inoculation and the boost were as follows: benzyl alcohol dehydrogenase, 392, 640 μg and 320 μg; carboxymethylated benzyl alcohol dehydrogenase, 417, 900 μg and 450 μg; TOL benzyl alcohol dehydrogenase, 1077, 310 μg and 155 μg, and 1078, 620 μg and 310 μg; benzaldehyde dehydrogenase I, 022, 100 μg and 50 μg, 023, 500 μg and 250 μg, and 254, 350 μg and 250 μg; carboxymethylated benzaldehyde dehydrogenase I, 389, 900 μg and 450 μg; benzaldehyde dehydrogenase II, 314, 500 μg and 250 μg, and 251, 500 μg and 250 μg; carboxymethylated benzaldehyde dehydrogenase II, 390, 900 μg and 450 μg; TOL benzaldehyde dehydrogenase, 1079, 300 μg and 150 μg, and 1080, 600 μg and 300 μg.

**Immunoprecipitation assay**

Immunoprecipitation assays were carried out at 0–4 °C. A serial dilution of antigen was prepared and a volume of each dilution was mixed with an equal volume of antiserum. The antiserum was used either undiluted or after dilution, depending upon the strength of the cross-reaction to be determined. After incubating for 1 h the mixtures were centrifuged for 5 min. The supernatants, along with samples of the mixtures saved before centrifugation, were assayed for enzyme activity. The point of equivalence was the amount of antigen added when the activity first reached a minimum (usually zero). The titre of an antiserum (μg of protein/ml of antiserum) is defined as the amount of antigen added at the point of equivalence multiplied by the dilution of the antiserum in the mixture.

***Staphylococcus aureus* immunoprecipitation assay**

*S. aureus* cells (Cowan I strain) were prepared as described by Kessler (1975). The cells were washed three times by centrifugation and resuspension to their original volume in 25 mM-potassium phosphate buffer, pH 7.5, containing 2 mM-dithiothreitol.

The *S. aureus* immunoprecipitation assay was carried out at 0–4 °C. A small amount of enzyme was diluted to 50 μl with 25 mM-potassium phosphate buffer, pH 7.5 containing 2 mM-dithiothreitol and mixed with an equal volume of undiluted antiserum. The amount of enzyme used was as small as possible, considering the need to assay the activity at the end of the procedure. The mixture was incubated for 1 h and 100 μl of *S. aureus* cells were added. After a further 45 min, with occasional mixing, the samples were centrifuged for 5 min and the supernatants were assayed for enzyme activity.

**Quantitative immunoblotting**

Quantitative immunoblotting was based on the method of Nimmo *et al.* (1986). Up to 3 mg of Protein A was iodinated by using up to $3.7 \times 10^7$ Bq of $Na^{125}I$ per mg of Protein A and five Iodobeads, according to the manufacturer's instructions. The preparation was finally diluted so that 1 ml contained 100 μg of Protein A (assuming no losses) and up to $2.0 \times 10^6$ Bq of $^{125}I$. The purified proteins were made 2% (w/v) in SDS by the addition of the appropriate amount of 20% (w/v) SDS and then boiled for 2 min after they had been made 70 mM in dithiothreitol by the addition of the appropriate amount of 2 M-dithiothreitol. The protein solutions were diluted with buffer A [190 mM-glycine/25 mM-Tris (base)] until each contained 0.02% (w/v) SDS. The proteins were then ready for application to the nitrocellulose membranes; however, they were usually diluted with buffer B (buffer A plus 0.02% SDS) so that 200 μl of the solution contained 0.8 μg of protein.

Nitrocellulose membranes were wetted in buffer B and placed in the Bio-Rad Dot Blot SF apparatus. Up to 200 μl of protein solution (0.8 μg of protein) was placed in each well, and if less than this amount was used the volume was made up to 200 μl with buffer B. A slight vacuum was applied to the apparatus so that the samples were drawn through the nitrocellulose membrane over a period of several minutes. The wells were then washed twice with 200 μl of buffer B and the membrane was quickly removed from the apparatus.

Non-specific protein binding sites were blocked by incubating the membranes in buffer C [10 mM-Tris/HCl buffer, pH 7.2, containing 0.15 M-NaCl and 0.5% (w/v) Tween 20] for at least 6 h at 4 °C. Antiserum (0.4 ml) in 40 ml of buffer C containing 5% (v/v) heat-inactivated horse serum was used to probe each membrane. After incubation overnight with gentle agitation at 23 °C, the membranes were washed five times in buffer C (15 min each) to remove any unbound antiserum. The membranes were then incubated for 2 h with gentle agitation in 40 ml of buffer C containing $^{125}I$-Protein A (100 μg; $1.2 \times 10^6$ Bq). Washing was then repeated as described above and the membranes were dried at 30 °C between sheets of filter paper. Sections of the nitrocellulose membranes, corresponding in position to the wells in

the Dot Blot SF apparatus, were cut out and their radioactivities counted with an LKB 1275 Minigamma. Blank sections of each membrane were also cut out and their radioactivities counted to determine the background count rates, and these were subtracted from the count rates in each band.

### Amino acid compositions

Amino acid compositions were determined essentially as described by Baker & Fewson (1989). Amino acid mixtures were analysed by using an LKB 4400 amino acid analyser, except for those prepared from the *Pseudomonas* enzymes, which were analysed by Dr. D. G. Campbell (Protein Phosphorylation Group, Department of Biochemistry, University of Dundee) using a Waters PICO TAG amino acid analyser. The amino acid composition of each sample was normalized to one amino acid and the percentage composition of each amino acid was calculated. There was very little difference between the results obtained after normalizing to glutamate or phenylalanine, and the results presented here were obtained after normalizing to glutamate.

## RESULTS AND DISCUSSION

### *N*-Terminal sequence analysis

The *N*-terminal sequences of the purified *Acinetobacter* and *Pseudomonas* enzymes were determined after they had been reduced and carboxymethylated. The *N*-terminal sequence of the yeast $K^+$-activated aldehyde dehydrogenase was determined after electro-elution from an SDS/polyacrylamide gel. All sequences were determined at least twice, and comprehensive details of the determinations have been deposited as Supplementary Publication SUP 50161 at the British Library Document Supply Centre.

The *N*-terminal sequences of benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase were used to search the U.K. Protein Engineering Club's OWL (Version 6.0) data base (Akrigg *et al.*, 1988) with the use of the SWEEP program (Version 3.02). The sequences were also used to search the GenBank and the NBRF data bases with the use of the GCG Wordsearch program. The search, and reference to the relevant literature, revealed that benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase are apparently members of the long-chain $Zn^{2+}$-dependent family of alcohol dehydrogenases, of which the horse liver cytoplasmic enzyme is the archetypal example (Fig. 1*a*; Jörnvall *et al.*, 1987). The *N*-terminal sequences of benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase each have 26% sequence identity with the horse liver enzyme. At present the family contains at least 17 eukaryotic and three bacterial enzymes (Fig. 1; Jörnvall *et al.*, 1987) in addition to benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase. The first 50 positions of the horse liver cytoplasmic alcohol dehydrogenase sequence contain four resi-

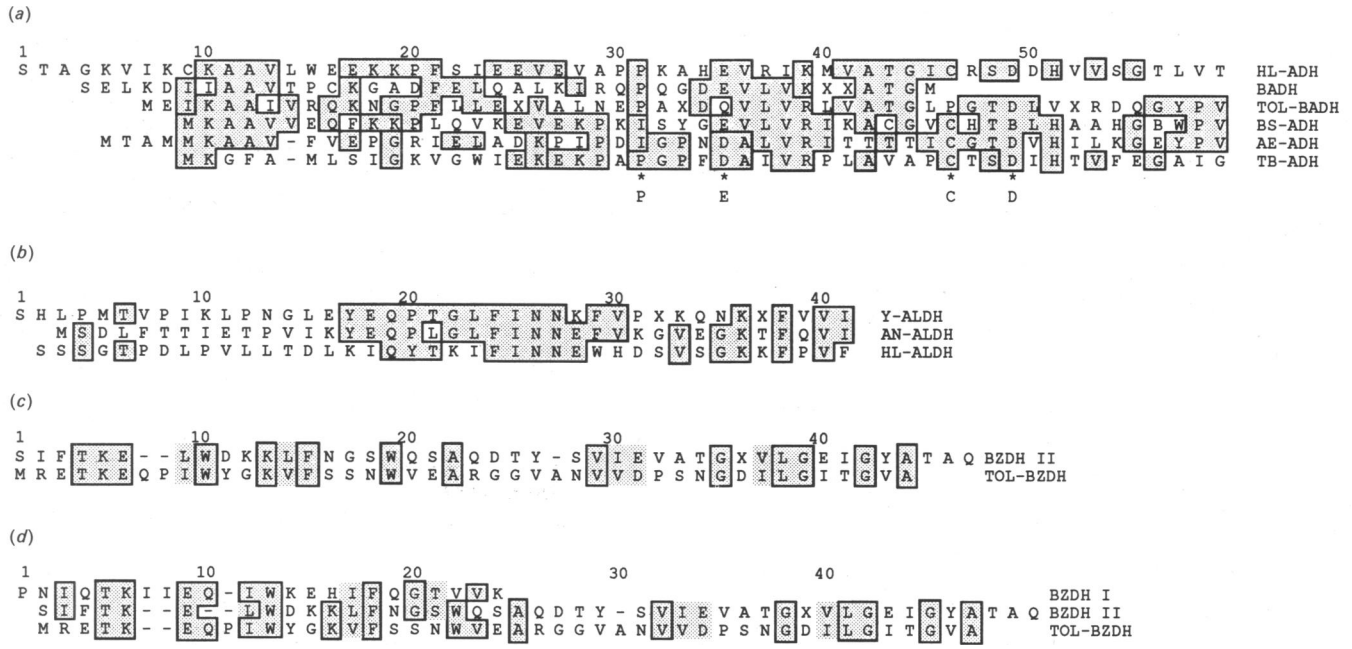


**Fig. 1. *N*-Terminal sequence alignments of alcohol dehydrogenases and aldehyde dehydrogenases**

(*a*) The sequences of the alcohol dehydrogenases are presented in an alignment that gives the maximum number of identities (boxed and shaded residues) and the minimum number of gaps. Amino acids B and D are counted as identities. The residues indicated by asterisks are conserved among 17 enzymes from mammals, higher plants and yeasts (Jörnvall *et al.*, 1987). Abbreviations: BADH, benzyl alcohol dehydrogenase; TOL-BADH, TOL benzyl alcohol dehydrogenase; HL-ADH, horse liver alcohol dehydrogenase [sequence from Jörnvall *et al.* (1987)]; BS-ADH, *Bacillus stearothermophilus* alcohol dehydrogenase [sequence from Bridgen *et al.* (1973) and Jeck *et al.* (1979)]; AE-ADH, *Alcaligenes eutrophus* alcohol dehydrogenase [sequence from Jendrossek *et al.* (1988)]; TB-ADH, *Thermoanaerobium brockii* alcohol dehydrogenase [sequence from Peretz & Burstein (1989)]. (*b*) The sequences of the aldehyde dehydrogenases are presented in an alignment that gives the maximum number of identities (boxed and shaded residues). Abbreviations: Y-ALDH, yeast $K^+$-activated aldehyde dehydrogenase; AN-ALDH, *Aspergillus nidulans* aldehyde dehydrogenase [sequence from Pickett *et al.* (1987)]; HL-ALDH, horse liver cytoplasmic aldehyde dehydrogenase [sequence from von Bahr-Lindström *et al.* (1984)]. (*c* and *d*) The sequences of the benzaldehyde dehydrogenases are presented in an alignment that is consistent throughout both of the comparisons and gives the maximum number of identities (boxed and shaded residues), the maximum number of conservative replacements (shaded residues) and the minimum number of gaps. Conservative replacements are: I/L/V; D/E; K/R; T/S. Abbreviations: BZDH I, benzaldehyde dehydrogenase I; BZDH II, benzaldehyde dehydrogenase II; TOL-BZDH, TOL benzaldehyde dehydrogenase. The following residues were tentative assignments: TOL benzyl alcohol dehydrogenase (*a*), positions 46 and 60; yeast $K^+$-activated aldehyde dehydrogenase (*b*), positions 36 and 39; TOL benzaldehyde dehydrogenase (*c* and *d*), positions 40 and 41.

dues that are conserved in 17 members of the family from mammals, higher plants and yeasts (Fig. 1*a*; Jörnvall *et al.*, 1987). When the sequences of benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase are aligned with those of the three other bacterial members of the family (Fig. 1*a*) it appears that none of the four residues is conserved in all five proteins. Proline-31 and glutamic acid-35 are both conserved in benzyl alcohol dehydrogenase; however, TOL benzyl alcohol dehydrogenase has glutamine at position 35. Cysteine-46 is one of the three amino acid residues that ligates the catalytic $Zn^{2+}$ ion (Eklund *et al.*, 1976) and it is conserved in all of the members of the family for which there are unambiguous sequence data available (Fig. 1; Jörnvall *et al.*, 1987). The residues corresponding to position 46 in the alignment in Fig. 1(*a*) were not identified conclusively for benzyl alcohol dehydrogenase or TOL benzyl alcohol dehydrogenase; however, the extensive sequence identities with other members of the family between positions 34 and 44 are consistent with the expectation of a cysteine residue at position 46 (Fig. 1*a*).

Overall, benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase appear to be members of the long-chain $Zn^{2+}$-dependent family of alcohol dehydrogenases, although there is no evidence from experiments with metal-ion-chelating agents that the putative active-site $Zn^{2+}$ ion can be removed easily (MacKintosh & Fewson, 1988*b*; C. A. Fewson, unpublished work). Benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase appear to be unrelated to either the $Fe^{2+}$-dependent group (e.g. Youngleson *et al.*, 1989) or the metal-ion-independent group of alcohol dehydrogenases (e.g. Jörnvall *et al.*, 1984).

The *N*-terminal sequences of benzaldehyde dehydrogenase I, benzaldehyde dehydrogenase II, TOL benzaldehyde dehydrogenase and the yeast $K^+$-activated aldehyde dehydrogenase were used to search the OWL, GenBank and the NBRF data bases in ways identical with those used for the alcohol dehydrogenase sequences (see above). The only biologically meaningful relationship found was between the yeast $K^+$-activated aldehyde dehydrogenase and the aldehyde dehydrogenase of *Aspergillus nidulans*, which is in turn related to the mammalian cytoplasmic class of aldehyde dehydrogenases (Fig. 1*b*: Pickett *et al.*, 1987). None of the three aldehyde dehydrogenases from *Acinetobacter* or *Pseudomonas* appeared to be related to the yeast enzyme. This is perhaps surprising, because benzaldehyde dehydrogenase I and the yeast enzyme have a number of similar features, including a requirement for $K^+$ (Bostian & Betts, 1978; Chalmers & Fewson, 1989*a*). The *N*-terminal sequences of benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase were more similar to each other than to the *N*-terminal sequence of benzaldehyde dehydrogenase I, and when they were aligned three gaps were required to maximize the number of identical residues (Fig. 1*c*). When all three aldehyde dehydrogenase sequences were aligned a total of eight gaps was required to maximize the number of identical residues (Fig. 1*d*). The large number of gaps introduced into the sequences complicated the assessment of whether or not the similarities are consistent with evolutionary relationships among the proteins. In order to eliminate the uncertain influence of so many gaps, residues 1–11 of the alignment in Fig. 1(*d*) were excluded from statistical analysis. Although considerably shorter, the remaining sequences contain only one gap. The sequence alignments were analysed by using the Monte Carlo shuffle analysis provided by the computer program RDF2 (Pearson & Lipman, 1988). The alignment of the benzaldehyde dehydrogenase II sequence with that of TOL benzaldehyde dehydrogenase had a score almost 9 standard deviations above the mean of the shuffled scores, and the highest of the shuffled scores was considerably lower than the alignment score. The pairwise alignments of the benzaldehyde dehydrogenase I sequence with those of benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase had scores that were between 2 and 3 standard deviations above the mean of the shuffled alignments; however, in both cases the highest shuffled score exceeded the alignment score. Overall, these results indicate that there is probably an evolutionary relationship between benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase, but that the sequence similarity between benzaldehyde dehydrogenase I and each of the other two enzymes may be due to chance.

### Immunological cross-reactions among the enzymes

Antisera were raised in rabbits against all five enzymes in their native states and also against the *Acinetobacter* enzymes after they had been denatured by the reduction and carboxy-

**Table 1. Immunoprecipitation titrations of benzaldehyde dehydrogenases and benzyl alcohol dehydrogenases**

All of the titrations were carried out at least twice (as described in the Experimental section) and the largest difference between duplicate determinations was 50%. The lower values of duplicate determinations are recorded. Abbreviation: N.D., not determined.

| | | Titre (μg of antigen bound/ml of antiserum) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum... | Benzaldehyde dehydrogenase I | | | | Benzaldehyde dehydrogenase II | | | TOL benzaldehyde dehydrogenase | | Benzyl alcohol dehydrogenase | | TOL benzyl alcohol dehydrogenase | |
| Antigen | Rabbit code no.... | 022 | 023 | 254 | 389* | 314 | 251 | 390* | 1079 | 1080 | 392 | 417* | 1077 | 1078 |
| Benzaldehyde dehydrogenase I | | 493 | 243 | 1440 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Benzaldehyde dehydrogenase II | | 75 | 18 | 5 | 0 | 30 | 64 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| TOL benzaldehyde dehydrogenase | | 0 | 0 | 0 | 0 | 0 | 0 | N.D. | 230 | 230 | 0 | N.D. | 0 | 0 |
| Benzyl alcohol dehydrogenase | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 52 | 52 |
| TOL benzyl alcohol dehydrogenase | | 0 | 0 | 0 | 0 | 0 | 0 | N.D. | 0 | 0 | 0 | N.D. | 152 | 152 |

* Antiserum raised against reduced and carboxymethylated antigen.

**Table 2. Quantitative immunoblotting of benzaldehyde dehydrogenases and benzyl alcohol dehydrogenases**

Antigen (0.8 $\mu$g) was applied to nitrocellulose membranes by using the Bio-Rad Dot Blot SF apparatus, and after probing with antiserum and $^{125}$I-Protein A (as described in the Experimental section) appropriate sections of the membrane were cut out and their radioactivities determined. The results are from representative experiments and all of the cross-reactions were determined at least twice. Cross-reactions of less than 1 % of that with the homologous antigen have been recorded as zero and control proteins that did not cross-react with any of the antisera are not shown in the Table. The amounts of radioactivity associated with bands containing homologous antigen were quite constant in different experiments using the same antiserum; however, they varied between 20000 and 80000 c.p.m. depending upon which antiserum was used. Control proteins were BSA, hen's-egg ovalbumin, rabbit muscle fructose-bisphosphate aldolase, horse spleen ferritin, bovine thyroglobulin, pig heart fumarase, bovine liver catalase, bovine erythrocyte carbonic anhydrase, *Cellulomonas* glycerol dehydrogenase, *Thermoanaerobium brockii* alcohol dehydrogenase, horse liver alcohol dehydrogenase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, yeast alcohol dehydrogenase and *Pseudomonas putida* formaldehyde dehydrogenase.

| | | Cross-reaction (% of reaction with homologous antigen) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum... | Benzaldehyde dehydrogenase I | | | | Benzaldehyde dehydrogenase II | | | TOL benzaldehyde dehydrogenase | | Benzyl alcohol dehydrogenase | | TOL benzyl alcohol dehydrogenase | |
| Antigen | Rabbit code no.... | 022 | 023 | 254 | 389* | 314 | 251 | 390* | 1079 | 1080 | 392 | 417* | 1077 | 1078 |
| Benzaldehyde dehydrogenase I | | 100 | 100 | 100 | 100 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Benzaldehyde dehydrogenase II | | 4 | 20 | 18 | 27 | 100 | 100 | 100 | 23 | 40 | 0 | 0 | 4 | 0 |
| TOL benzaldehyde dehydrogenase | | 14 | 14 | 54 | 15 | 7 | 8 | 22 | 100 | 100 | 0 | 0 | 0 | 0 |
| Benzyl alcohol dehydrogenase | | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 38 | 24 |
| TOL benzyl alcohol dehydrogenase | | 1 | 0 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 94 | 69 | 100 | 100 |
| Horse spleen ferritin | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| Bovine liver catalase | | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| *Thermoanaerobium brockii* alcohol dehydrogenase | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| Horse liver alcohol dehydrogenase | | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 |
| Yeast alcohol dehydrogenase | | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 1 |

* Antiserum raised against reduced and carboxymethylated antigen.

methylation of their cysteine residues. The extents of the cross-reactions between each of the enzymes and each of the antisera were determined by using immunoprecipitation assays with the antigens in their native states and by using quantitative immunoblotting with SDS-denatured antigens.

In immunoprecipitation assays all of the antisera raised against native enzymes reacted with their respective homologous antigens (Table 1). However, the antiserum raised against denatured benzaldehyde dehydrogenase I was the only one of the three antisera raised against denatured enzymes to react with the native homologous antigen. The titre of this antiserum was between 50- and 300-fold less than the titres of the three antisera raised against native benzaldehyde dehydrogenase I (Table 1). Only two heterologous cross-reactions were observed. These were between benzyl alcohol dehydrogenase and anti-(TOL benzyl alcohol dehydrogenase) serum and between benzaldehyde dehydrogenase II and anti-(benzaldehyde dehydrogenase I) serum (Table 1).

*S. aureus* immunoprecipitation assays confirmed the results of the immunoprecipitation experiments. These assays were not quantitative and they were used only to confirm the presence or absence of immunological reactions.

The contamination of enzyme preparations used to raise antisera can be a substantial problem because the response of an experimental animal is not necessarily proportional to the amount of antigen administered but may also depend on the antigenicity of the substance. Anti-(benzaldehyde dehydrogenase I) serum (code no. 022) was treated with an amount of benzaldehyde dehydrogenase I equal to its titre (493 $\mu$g/ml; Table 1), and after incubation and centrifugation the supernatant had no titre towards benzaldehyde dehydrogenase II. This result indicates that anti-(benzaldehyde dehydrogenase I) serum contained a single population of antibodies some of which were directed against epitopes shared by benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II. If the original benzaldehyde dehydrogenase I inoculum had been cross-contaminated with benzaldehyde dehydrogenase II one would expect to detect a sub-population of antibodies, directed against benzaldehyde dehydrogenase II, that were not removed from solution by pretreatment of the antiserum with benzaldehyde dehydrogenase I.

Immunoblotting of the enzymes from SDS/polyacrylamide gels confirmed that cross-reactions were located in a single band (results not shown), corresponding in $M_r$ value to the respective enzymes. For this reason there was no advantage in blotting the enzymes from SDS/polyacrylamide gels when application of the enzymes directly to the nitrocellulose membranes was more economical in terms of the materials used and the effort required. When the enzymes were applied directly to the membranes the relationship between the amount of antigen applied and the amount of radioactivity was positive and usually approximately linear between 0.05 and 0.8 $\mu$g of antigen.

A set of control proteins was selected (Table 2), i.e. proteins that were thought to be unlikely to have any very close evolutionary relationship with the aldehyde dehydrogenases and alcohol dehydrogenases that were the subjects of this investigation. Several alcohol dehydrogenases and aldehyde dehydrogenases were included in order to show that any cross-reactions between the subject proteins were not due to general features of sequence or structure shared by all dehydrogenase enzymes.

Immunoblotting confirmed the cross-reactions detected in the immunoprecipitation experiments (Table 2): benzyl alcohol dehydrogenase cross-reacted with antisera raised against TOL benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase II cross-reacted with antisera raised against benzaldehyde dehydrogenase I. Additional cross-reactions were also detected (Table 2). TOL benzyl alcohol dehydrogenase cross-reacted with antisera raised against benzyl alcohol dehydrogenase. TOL benzaldehyde dehydrogenase cross-reacted with antisera raised against benzaldehyde dehydrogenase II, and the reciprocal cross-reactions were also detected. TOL benzaldehyde dehydrogenase also cross-reacted with antisera raised against benzaldehyde dehydrogenase I; however, there were no significant reciprocal cross-reactions among benzaldehyde dehydrogenase I and antisera raised against TOL benzaldehyde dehydrogenase.

In immunoprecipitation assays antisera raised against the carboxymethylated proteins had relatively low or undetectable titres for their native homologous antigens (Table 1), but overall they were little different from the antisera raised against the native enzymes in their cross-reactivity towards SDS-denatured enzymes (Table 2). This indicates that the protocol for the preparation of the inoculum possibly denatures some significant proportion of the sample and elicits the production of antibodies against both native and denatured forms of the antigen. Alternatively, the protocol for probing the nitrocellulose membranes may allow some proportion of the bound antigen to regain its native conformation.

Immunological studies have often been used to demonstrate homology within families of isoenzymes (e.g. Arnon & Neurath, 1969; Arnheim *et al.*, 1971; Prager & Wilson, 1971*a*,*b*). In general, immunological cross-reactions are detectable between native antigens that have greater than about 60% sequence identity (Prager & Wilson, 1971*a*,*b*) and between denatured antigens that have greater than about 40% sequence identity (Arnheim *et al.*, 1971; Katinka *et al.*, 1980; Zakin *et al.*, 1983; Cassan *et al.*, 1986). The results of the immunological experiments with both native and SDS-denatured antigens indicate that the respective groups of alcohol dehydrogenases and aldehyde dehydrogenases have between 40% and 60% sequence identity. The *N*-terminal sequence identity between benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase is 36% (Fig. 1*a*).

None of the antisera raised against benzyl alcohol dehydrogenase or TOL benzyl alcohol dehydrogenase cross-reacted with either of the alcohol dehydrogenase enzymes from horse liver or *Thermoanaerobium brockii* that were used as control proteins for immunoblotting (Table 2). At first sight this might seem to be surprising because benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase appear to belong to the same family of $Zn^{2+}$-dependent alcohol dehydrogenases as these control proteins (Fig. 1*a*). Benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase have 8% and 16% *N*-terminal sequence identity respectively with the *Thermoanaerobium* enzyme, and both have 26% identity with the horse liver enzyme (Fig. 1*a*). These levels of sequence identities are thus considerably lower than the range detectable with immunological techniques (see above), and this may explain why cross-reactions were not detected.

### Subunit $M_r$ values, amino acid compositions and numbers of residues per enzyme subunit

Benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase have been reported to have subunit $M_r$ values of 39700 and 43000 respectively (MacKintosh & Fewson, 1988*a*; Chalmers *et al.*, 1990), yet they appear to run in almost identical positions ahead of the ovalbumin standard protein ($M_r$ 43000) when they are electrophoresed together on SDS/12.5%-polyacrylamide gels (result not shown). The discrepancy between the estimates of the $M_r$ values appears to be due to the fact that the value for benzyl alcohol dehydrogenase was determined with 10%-polyacrylamide gels (MacKintosh & Fewson, 1988*a*), whereas that for TOL benzyl alcohol dehydrogenase was de-

**Table 3. Amino acid compositions of benzaldehyde dehydrogenases and benzyl alcohol dehydrogenases**

The amino acid composition of apomyoglobin and each of the *Acinetobacter* enzymes was determined (as described in the Experimental section) twice in completely independent experiments. In one of the experiments the amino acid composition of the hydrolysed protein was determined in duplicate. The results of the duplicate determinations were first averaged together and then this composition was averaged with the independent determination to give the results presented here. The amino acid compositions of single samples of each of the TOL-plasmid-encoded enzymes were determined in duplicate and the mean values are recorded. In general the duplicate and the independent amino acid compositions for each enzyme were in very good agreement and in some cases the compositions differed only by a fraction of 1%. Abbreviation: CM, carboxymethyl.

| Amino acid | Amino acid composition (mol %) | | | | |
|---|---|---|---|---|---|
| | Benzaldehyde dehydrogenase I | Benzaldehyde dehydrogenase II | TOL benzaldehyde dehydrogenase | Benzyl alcohol dehydrogenase | TOL benzyl alcohol dehydrogenase |
| Asx | 9.68 | 9.09 | 10.3 | 8.61 | 10.3 |
| Glx | 14.4 | 15.7 | 10.4 | 11.5 | 9.55 |
| CM-Cys | 1.46 | 0.80 | 0.13 | 2.61 | 0.10 |
| Met | 2.52 | 0.46 | 2.3 | 1.32 | 1.18 |
| Thr | 4.74 | 5.19 | 3.76 | 5.28 | 4.54 |
| Ser | 7.61 | 5.53 | 4.75 | 4.84 | 6.45 |
| Gly | 8.33 | 9.90 | 10.4 | 11.0 | 10.6 |
| Ala | 7.35 | 10.9 | 12.0 | 9.51 | 8.79 |
| Val | 7.17 | 8.16 | 9.23 | 8.00 | 9.60 |
| Leu | 6.95 | 9.35 | 7.72 | 9.04 | 9.55 |
| Ile | 9.47 | 6.24 | 5.53 | 6.77 | 5.97 |
| Pro | 4.24 | 3.93 | 5.79 | 4.63 | 6.40 |
| Tyr | 1.53 | 1.91 | 1.25 | 2.08 | 1.62 |
| Phe | 4.21 | 4.33 | 3.55 | 4.72 | 4.54 |
| His | 2.46 | 1.50 | 2.09 | 2.26 | 1.96 |
| Lys | 5.78 | 4.69 | 4.96 | 5.86 | 5.11 |
| Arg | 2.14 | 2.32 | 5.79 | 1.93 | 3.77 |

termined with 12.5%-polyacrylamide gels (Chalmers *et al.*, 1990). The plots of mobility against $\ln M_r$ for the calibration proteins electrophoresed on 10%- and 12.5%-polyacrylamide gels are sufficiently different to lead to this discrepancy. We believe that 39700 represents the best estimate of the $M_r$ value for each of the enzymes, partly because this value is in good agreement with the subunit $M_r$ (40000) determined for the benzyl alcohol dehydrogenase encoded by the archetypal TOL plasmid pWWO, with either *Escherichia coli* maxicells (Harayama *et al.*, 1989) or the purified enzyme (S. Harayama, personal communication).

The amino acid compositions of the *Acinetobacter* and *Pseudomonas* enzymes were determined (Table 3) and, together with the subunit $M_r$ values of the enzymes, used to calculate the numbers of amino acid residues per enzyme subunit: benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase, 381; benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II, 525; TOL benzaldehyde dehydrogenase, 538.

Pairwise comparisons were carried out between the amino acid compositions of the alcohol dehydrogenases and among those of the aldehyde dehydrogenases by using the methods of Marchalonis & Weltman (1971) and Cornish-Bowden (1979). The empirical method of Marchalonis & Weltman indicated that the alcohol dehydrogenases show a degree of homology with each other, as do the three aldehyde dehydrogenases. In contrast, the Cornish-Bowden method, which satisfies a formal 95% significance test, indicated that none of the alcohol dehydrogenases or aldehyde dehydrogenases has a homologous relationship to each other. However, the index of similarity for the pair of alcohol dehydrogenases was closer to its threshold value than the duplicate amino acid composition determinations for benzyl alcohol dehydrogenase.

Both methods of comparing amino acid compositions indicated that there is a closer relationship between the alcohol dehydrogenases than among any of the aldehyde dehydrogenases, and that benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase are more closely related to each other than to benzaldehyde dehydrogenase I. This result is entirely consistent with the extents of *N*-terminal sequence identity among the enzymes.

## CONCLUSIONS

The *N*-terminal sequences of benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase show that they are related to each other and also that they are members of the family of long-chain $Zn^{2+}$-dependent alcohol dehydrogenases (Fig. 1*a*). The interpretation of the sequence similarities among the aldehyde dehydrogenase *N*-terminal sequences is more complicated than for those between the alcohol dehydrogenases because a number of gaps were introduced into the sequences (Figs. 1*c* and 1*d*). However, there does appear to be a significant degree of homology between benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase. The relationships, indicated by the *N*-terminal sequence comparisons, between the alcohol dehydrogenases and among the aldehyde dehydrogenases were confirmed by the immunological cross-reactions detected between benzyl alcohol dehydrogenase and TOL benzyl alcohol dehydrogenase and also between benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase (Tables 1 and 2). Additional cross-reactions were observed that helped to clarify one of the more tentative conclusions drawn from the *N*-terminal sequence comparisons. Although it was unclear whether the similarities among the *N*-terminal sequences of benzaldehyde dehydrogenase I and those of the other aldehyde dehydrogenases were significant, the cross-reactions detected between benzaldehyde dehydrogenase II and antisera raised against benzaldehyde dehydrogenase I, taken together with the cross-reactions between benzaldehyde dehydrogenase II and TOL benzaldehyde dehydrogenase, are consistent with evolutionary relationships among all three aldehyde dehydrogenases. However, reciprocal

cross-reactions between benzaldehyde dehydrogenase I and any of the antisera raised against benzaldehyde dehydrogenase II or TOL benzaldehyde dehydrogenase were not detected, and so this remains a tentative conclusion.

One of the original aims of this work was to test whether or not the alcohol dehydrogenases evolved from the aldehyde dehydrogenases by the process of retrograde evolution (see the Introduction). Neither the *N*-terminal sequence comparisons nor the immunological experiments provided evidence of an evolutionary relationship between the aldehyde dehydrogenase and the alcohol dehydrogenase groups of enzymes. It therefore appears that the alcohol dehydrogenases and aldehyde dehydrogenases in the benzyl alcohol and toluene pathways are associated because of gene recruitment and not because of retrograde evolution. If the alcohol dehydrogenases did in fact evolve from the aldehyde dehydrogenases by retrograde evolution then the relationships have been obscured by the effects of different selective pressures and genetic drift.

The most worthwhile extension of this work would be the cloning and sequencing of the genes for all five enzymes. This would firmly establish whether or not benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II evolved from a common ancestor, and if so it should help us to understand the mechanism by which the putative duplicate genes were stabilized against elimination by homologous recombination or by unequal crossing over. Furthermore, it would help to redress the imbalance between the large number of complete sequences available for eukaryotic members of the long-chain $Zn^{2+}$-dependent family of alcohol dehydrogenases and the small number of sequences available for prokaryotic members of the family.

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