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Purification of the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 of *Pseudomonas putida* MT53 and their preliminary comparison with benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases I and II from *Acinetobacter calcoaceticus*

RONALD M. CHALMERS, ALAN J. SCOTT and CHARLES A. FEWSON*

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, UK

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A procedure was developed for purifying both the benzyl alcohol dehydrogenase and the benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 from a single batch of *Pseudomonas putida* MT53. The procedure involved disruption of the bacteria in the French pressure cell and preparation of a high-speed supernate, followed by chromatography on DEAE-Sephacel, Matrex Gel Red A and Blue Sepharose CL-6B which separated the two enzymes, Phenyl Sepharose CL-4B and Matrex Gel Green A. The final preparations gave single bands on electrophoresis under denaturing and non-denaturing conditions. The subunit M_r values of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are 43000 and 56300 respectively. Cross-linking studies with dimethylsuberimidate indicate that both enzymes are probably tetramers, although they run anomalously through gel-filtration columns. The benzyl alcohol dehydrogenase was fairly specific for NAD^+ as cofactor but the benzaldehyde dehydrogenase had appreciable activity with $NADP^+$ as well as with NAD^+. The optimum pH values are 9·4 and 9·3 for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase respectively. Benzaldehyde dehydrogenase appears to require a monovalent cation for maximum activity. The apparent K_m and maximum velocity values of the two plasmid-encoded dehydrogenases and of the chromosomally encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases I and II from *Acinetobacter calcoaceticus* were determined for NAD^+ and for the unsubstituted substrates and for the monomethyl ring-substituted analogues. The corresponding apparent specificity constants were then calculated. All three benzaldehyde dehydrogenases had very similar substrate profiles, as did the two benzyl alcohol dehydrogenases. The plasmid-encoded benzaldehyde dehydrogenase resembles benzaldehyde dehydrogenase I from *A. calcoaceticus* (which also requires monovalent cations for activity) in being much more heat-stable than benzaldehyde dehydrogenase II. Overall, the plasmid-encoded benzyl alcohol dehydrogenase from *P. putida* appears to be remarkably similar to the chromosomally encoded benzyl alcohol dehydrogenase from *A. calcoaceticus*, and the plasmid-encoded benzaldehyde dehydrogenase is very similar to the two chromosomally encoded benzaldehyde dehydrogenases, particularly benzaldehyde dehydrogenase I.

Introduction

The degradation of toluene and of *m*- and *p*-xylenes by certain pseudomonads is catalysed by enzymes that are encoded by TOL plasmids which are probably the best-characterized of all known catabolic plasmids (Burlage *et al.*, 1989; Assinder & Williams, 1990). However, while many of the genetic aspects of these plasmids are fairly well understood, few of the enzymes which they encode have been purified and characterized. The flexibility of the upper part of the TOL-plasmid-encoded pathway is increased by the fact that the enzymes seem to be substrate-ambiguous and can tolerate certain substitutions on the aromatic ring. For instance, experiments with extracts of *Pseudomonas putida* mt-2(pWW0) have shown that the benzyl alcohol dehydrogenase (encoded by the *xylB* gene) and the benzaldehyde dehydrogenase (encoded by the *xylC* gene) can use as substrates either the unsubstituted compounds or the 3- or 4-monomethyl

Abbreviation: FPLC, fast protein liquid chromatography (Pharmacia system).

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analogues (Worsey & Williams, 1975). We have previously purified equivalent, but chromosomally encoded, dehydrogenases from *Acinetobacter calcoaceticus* and they also have relaxed substrate specificities (MacKintosh & Fewson, 1988*a*, *b*; Chalmers & Fewson, 1989). *A. calcoaceticus* benzaldehyde dehydrogenase I is involved in the oxidation of mandelate and certain ring-substituted mandelates to the corresponding benzoates, and benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are involved in the oxidation of benzyl alcohol and various ring-substituted benzyl alcohols. However, *A. calcoaceticus* cannot grow on monomethylmandelates or monomethylbenzyl alcohols because it has no way of dealing with the toluates that are formed by their oxidation (Fewson, 1988). In the present paper we describe a method for the purification of the plasmid-encoded benzyl alcohol and benzaldehyde dehydrogenases from *Pseudomonas putida* MT53(pWW53) and show that they have remarkably similar properties to the chromosomally encoded enzymes from *A. calcoaceticus*.

Methods

Materials. Chemicals were generally of the best quality commercially available and most of them were from the sources listed by MacKintosh & Fewson (1988*a*) and Chalmers & Fewson (1989). Matrex Gel Green A was obtained from Amicon.

Buffers. These were prepared at room temperature by adjusting the pH value of approximately five-fourths strength solutions with the appropriate acid or base and then making to volume.

Growth of bacteria. Pseudomonas putida MT53(pWW53) (Keil *et al.*, 1985) was obtained from Professor P. A. Williams, Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Gwynedd, UK. Stock cultures were maintained at 4 °C on agar slopes containing minimum salts medium and 5 mM-*m*-toluate (Worsey & Williams, 1975). A loopful of bacteria from the stock culture was used to inoculate 74 ml of Luria broth (10 g tryptone l^{-1}, 5 g yeast extract l^{-1} and 0·5 g NaCl l^{-1}) in a 250 ml Erlenmeyer flask and grown at 30 °C with shaking (180 oscillations min^{-1}) for 17 h. This culture was used to inoculate 3·7 l of Luria broth in a 10 l flask and then grown at 30 °C with aeration (500 ml min^{-1}) and magnetic stirring (Livingstone *et al.*, 1972). A portion of the culture (3·3 l) was used to inoculate 6·7 l of three-halfs strength minimum medium (Murray *et al.*, 1972) containing 7·5 mM-3-methylbenzyl alcohol as carbon source. The culture was grown for 160 min in a 10 l fermenter under the conditions described by Hoey *et al.* (1987). This procedure gave a good yield of bacteria with fairly high specific activities of both benzyl alcohol and benzaldehyde dehydrogenases. The bacteria were harvested and washed as described by Hills & Fewson (1983) and used immediately or stored at −20 °C.

Preparation of extract. Bacteria (about 30 g wet wt) were resuspended in about 70 ml of ice-cold 100 mM-potassium phosphate buffer, pH 7·5, and disrupted by four passages through the French pressure cell (Baker & Fewson, 1989) and centrifuged at 105000 ***g*** for 150 min at 4 °C. The supernate (extract) was dialysed overnight against 2 l of buffer A (50 mM-potassium phosphate, pH 7·5, containing 2 mM-DTT).

Purification of plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase. All steps were done in a cold room set at 4 °C and in addition some of the buffers used for the hydrophobic-interaction chromatography were maintained at 0 °C in an ice-water slurry.

Chromatography on DEAE-Sephacel. Dialysed extract (85 ml) was applied at 40 ml h^{-1} to a DEAE-Sephacel column (5·0 × 4·0 cm) which had been pre-equilibrated with buffer A. After washing with 750 ml of buffer A at 80 ml h^{-1}, the two enzymes were eluted with a linear 50–350 mM-potassium phosphate buffer gradient (pH 7·5) containing 2 mM-DTT. The gradient volume was 1·0 l and the flow rate was 26 ml h^{-1}. Both enzymes were eluted after approximately 180 ml and fractions with peak activity were pooled. The pooled fractions (68 ml) were dialysed for a total of 4 h against eight changes (500–750 ml each) of buffer B (100 mM-potassium phosphate, pH 7·5, containing 2 mM-DTT).

Chromatography on Matrex Gel Red A/Blue Sepharose CL-6B. The dialysed DEAE-Sephacel pool was applied to a Matrex Gel Red A column (2·6 × 8·0 cm; these dimensions appear to be critical for the success of this stage) connected in series with a Blue Sepharose CL-6B column (2·6 × 11·5 cm). Both columns were pre-equilibrated with buffer B, and during application the flow (50 ml h^{-1}) was stopped for 20 min every 20 min. The flow rate was lowered to 25 ml h^{-1} and the columns were washed with 400 ml of buffer B. The benzyl alcohol dehydrogenase passed through both columns and fractions with peak activity were pooled and taken on to the hydrophobic-interaction chromatography. The Matrex Gel Red A column was disconnected and the Blue Sepharose CL-6B column, which contained benzaldehyde dehydrogenase, was washed at 85 ml h^{-1} with 300 ml of buffer C (110 mM-potassium phosphate, pH 7·5, containing 2 mM-DTT). The flow through the column was then reversed and benzaldehyde dehydrogenase was eluted at 44 ml h^{-1} with buffer C containing 0·2 mM-NAD^+. As soon as enzyme activity was detected in the eluent, the NAD^+ concentration was raised to 0·5 mM. Fractions with peak activity containing homogeneous benzaldehyde dehydrogenase were pooled.

Chromatography on Phenyl Sepharose CL-4B. The pooled fractions (69 ml) containing benzyl alcohol dehydrogenase eluted from the two dye columns were applied to a Phenyl Sepharose CL-4B column (2·6 × 11·0 cm) pre-equilibrated with buffer B. The column was washed with the following buffers in turn: 50 ml of buffer B, 200 ml of buffer D (5 mM-potassium phosphate, pH 7·5, containing 2 mM-DTT), 200 ml of ice-cold buffer D containing 20% (v/v) ethanediol and finally 250 ml of ice-cold buffer D containing 50% (v/v) ethanediol. The flow rate was lowered from 50 to 20 ml h^{-1} and benzyl alcohol dehydrogenase was eluted from the column with 250 ml of buffer D containing 65% (v/v) ethanediol. The eluent was passed through a small (1·6 × 3·5 cm) column of DEAE-Sephacel pre-equilibrated with buffer D. The DEAE-Sephacel column was washed with the following buffers in turn: 15 ml of buffer D containing 50% (v/v) ethanediol, 15 ml of buffer D containing 20% (v/v) ethanediol, 15 ml of buffer D and finally 35 ml of buffer A. The flow rate was reduced from 20 to 9·5 ml h^{-1} and benzyl alcohol dehydrogenase was eluted with 300 mM-potassium phosphate buffer, pH 7·5, containing 2 mM-DTT. Fractions with peak activity were pooled and dialysed for a total of 3 h against six changes (500 ml each) of 10 mM-potassium phosphate buffer, pH 6·5, containing 2 mM-DTT and 5 mM-$MgCl_2$.

Chromatography on Matrex Gel Green A. The dialysed pooled fractions (12·2 ml) were applied at 80 ml h^{-1} to a Matrex Gel Green A column (4·5 × 5·0 cm) pre-equilibrated with buffer E (20 mM-potassium phosphate, pH 6·5, containing 2 mM-DTT and 5 mM-$MgCl_2$). The column was washed with 15 ml of buffer E and the flow was then stopped for 30 min. The column was washed with a further 300 ml of buffer E before the flow rate was lowered to 30 ml h^{-1} and homogeneous benzyl alcohol dehydrogenase was eluted with

50 mM-potassium phosphate buffer, pH 6·5, containing 2 mM-DTT, 5 mM-$MgCl_2$ and 1 mM-NAD^+. Fractions with peak activity were pooled.

Concentration and storage of the enzymes. The final pooled fractions containing the two homogeneous enzymes were concentrated by repeated use of Amicon Centriprep concentrators. The concentrated enzymes were each mixed with an equal volume of ethanediol and stored at −20 °C (see Table 1).

Purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases I and II from A. calcoaceticus. Benzaldehyde dehydrogenase I was purified as described by Chalmers & Fewson (1989). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were purified essentially as described by MacKintosh & Fewson (1988*a*) except that column washing was increased to ten column volumes at each stage and samples were concentrated after the dye-ligand chromatography steps using a small DEAE-Sephacel column rather than by vacuum dialysis.

Enzyme assays. Benzyl alcohol dehydrogenase activity was determined in a Bicine/hydrazine buffer as described by MacKintosh & Fewson (1988*a*). Benzaldehyde dehydrogenase activity was measured as described by Chalmers & Fewson (1989).

Electrophoresis, determination of kinetic parameters and M_r values and chemical cross-linking of enzyme subunits, and analytical methods. These were all as described by Chalmers & Fewson (1989).

Results and Discussion

Purification of plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase

The procedure described in Methods was developed for the purification of milligram amounts of both enzymes from a single batch of bacteria (Table 1). Dialysis of the extract was necessary to ensure reproducible behaviour on the ion-exchange column, from which both enzymes were eluted at such similar salt concentrations that a single pool of both activities could be collected for the next stage. In most experiments there was a subsidiary peak of benzaldehyde dehydrogense activity on ion-exchange chromatography and this may correspond to the chromosomally encoded enzyme mentioned by Worsey & Williams (1975). The arrangement of a pair of columns connected in series, first Matrex Gel Red A and then Blue Sepharose CL-6B, served to separate the enzymes and yielded homogeneous benzaldehyde dehydrogenase after elution of the Blue Sepharose column with NAD^+ (Fig. 1, lane G). Many proteins, including benzyl alcohol dehydrogenase, were washed through both dye columns (Fig. 1, lane D). Benzyl alcohol dehydrogenase was then purified to homogeneity by hydrophobic-interaction chromatography followed by removal of a few remaining contaminants by chromatography on Matrex Gel Green A (Fig. 1, lanes E and F). Both enzymes lost a little activity on concentration and mixing with ethanediol (Table 1), but they were then stable when stored at −20 °C. The purified enzymes were both homogeneous as judged by denaturing PAGE (Fig. 1) and by non-denaturing PAGE followed by staining for protein and for enzyme activity. The enzymes have been purified twice by this procedure and several times by very similar procedures.

Table 1. *Purification of plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from P. putida MT53*

The results are presented for a purification starting with 28 g wet wt of cells. ND, Not determined.

	Vol. (ml)	Total activity (units)	Total protein (mg)	Specific activity [units (mg protein)$^{-1}$]	Purification (-fold)	Yield (%)
(a) Benzyl alcohol dehydrogenase						
Extract	85	5372	2601	2·07	1	100
DEAE-Sephacel	68	5265	340	15·5	7·5	98
Matrex Gel Red/ Blue Sepharose	70	3367	92	36·8	17·8	63
Phenyl Sepharose	7·3	2520	9·8	257	133	47
Matrex Gel Green	103	2050	ND	ND	ND	38
Concentration and ethanediol	3·0	1503	4·0	376	181	28
(b) Benzaldehyde dehydrogenase						
Extract	85	1262	2601	0·49	1	100
DEAE-Sephacel	68	1074	340	3·16	6·5	85
Matrex Gel Red/ Blue Sepharose	115	506	6·9	73·7	150	40
Concentration and ethanediol	8·3	415	6·9	60·2	123	33

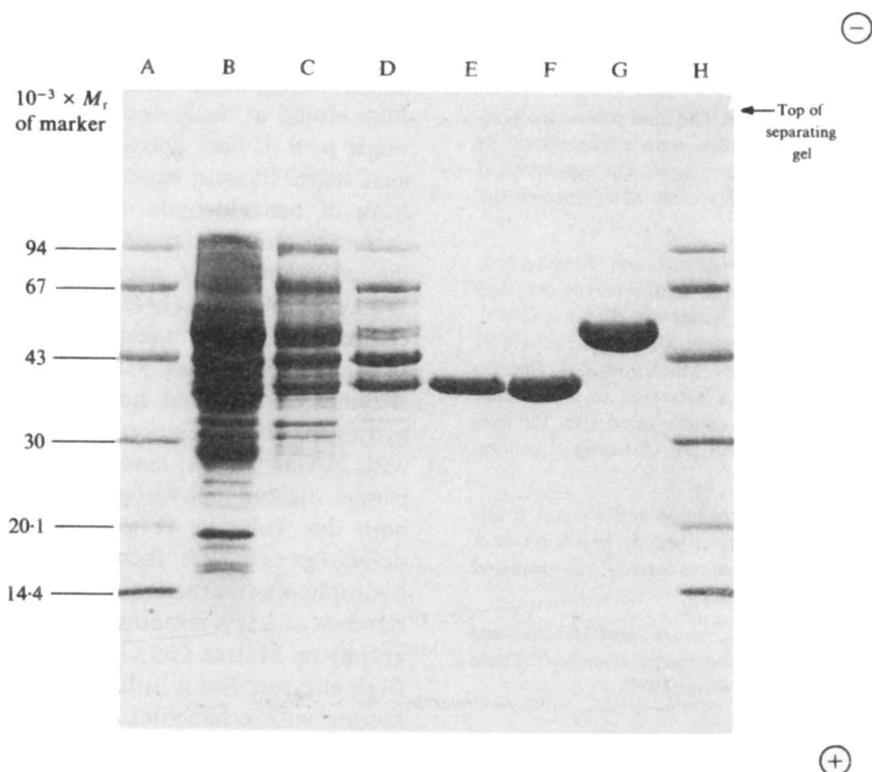

Fig. 1. Purification of plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase monitored by SDS-PAGE. A 12·5% (w/v) SDS-polyacrylamide gel with a 5·3% (w/v) stacking gel was run and stained for protein. Lanes: A and H, M_r markers; B, extract (122 μg protein); C, DEAE-Sephacel pool (50 μg); D, Matrex Gel Red A/Blue Sepharose wash pool (32 μg); E, Phenyl Sepharose pool (16 μg); F, Matrex Gel Green A pool (17 μg); G, Blue Sepharose pool (26 μg).

Determination of M_r

The subunit M_r was estimated to be 43000 ± 680 SD (n = 15) for the benzyl alcohol dehydrogenase and 56300 ± 600 SD (n = 13) for the benzaldehyde dehydrogenase. These values are very similar to those for the equivalent enzymes from *A. calcoaceticus* [benzyl alcohol dehydrogenase, 39700 (MacKintosh & Fewson, 1988*a*); benzaldehyde dehydrogenase I, 56000 (Chalmers & Fewson, 1989); benzaldehyde dehydrogenase II, 55000 (MacKintosh & Fewson, 1988*a*)]. The apparent native M_r values estimated by gel filtration through an FPLC Superose 12 column were 82000 and 122000 for the benzyl alcohol dehydrogenase and the benzaldehyde dehydrogenase respectively. However, when the enzymes were cross-linked by treatment with dimethylsuberimidate and then analysed using SDS–PAGE, four major bands appeared in both preparations and these corresponded in M_r value to monomer, dimer, trimer and tetramer. In both enzyme preparations the major bands corresponding to trimer and tetramer each appeared as a group of minor bands and the results were more difficult to interpret than for the enzymes from *A. calcoaceticus* (Chalmers & Fewson, 1989). It is most likely that both of the plasmid-encoded enzymes, like the *A. calcoaceticus* enzymes (MacKintosh & Fewson, 1988*a*; Chalmers & Fewson, 1989) are tetrameric, but that like benzaldehyde dehydrogenase I from *A. calcoaceticus* (Chalmers & Fewson, 1989) they run anomalously through Superose gel filtration columns.

Effects of pH and cations

The pH optima for the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are 9·4 and 9·3 respectively, almost exactly the same as for the *A. calcoaceticus* enzymes (MacKintosh & Fewson, 1988*a*; Chalmers & Fewson, 1989).

We have obtained preliminary evidence that the benzaldehyde dehydrogenase requires K^+, NH_4^+ or Rb^+ (but not Na^+) for maximum activity when assayed in ethanolamine/HCl, Tris/HCl or glycine buffers. However, we have not been able to demonstrate an absolute requirement for a monovalent cation, even after prolonged dialysis against various buffers. Benzaldehyde dehydrogenase I from *A. calcoaceticus* has an almost absolute dependence on the presence of K^+, NH_4^+ or Rb^+

Table 2. *Comparison of the kinetic coefficients of selected substrates for the plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from P. putida MT53 with the coefficients for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases I and II from A. calcoaceticus*

The apparent K_m and V values for the substrates 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, but otherwise using the standard assay procedures. Benzaldehyde dehydrogenase activity was measured spectrofluorometrically. The second substrate was generally present at high concentration, except for when the apparent $K_m^{NAD^+}$ for benzaldehyde dehydrogenase I was determined, in which case the benzaldehyde concentration had to be restricted because of the pronounced substrate inhibition (Chalmers & Fewson, 1989). The concentration of benzaldehyde used in each of the apparent $K_m^{NAD^+}$ determinations is given in parentheses. The concentrations of substrates were calculated from the ΔA_{340} after their complete enzymic oxidation or reduction in the presence of excess amounts of the second substrate. The kinetic parameters were calculated from initial velocity measurements by the Direct Linear method (Eisenthal & Cornish-Bowden, 1974) using the Enzpack computer program (Williams, 1985) and the 68% confidence limits are given in parentheses.

Enzyme	Substrate	Apparent K_m (μM)	Apparent V [units (mg protein)$^{-1}$]	Apparent specificity constant V/K_m [l·min^{-1} (mg protein)$^{-1}$]
Plasmid-encoded benzyl alcohol dehydrogenase (*P. putida*)	NAD+	219 (209–229)	90·8 (87·7–92·8)	0·42
	Benzyl alcohol	233 (209–259)	96·4 (92·2–106)	0·41
	2-Methylbenzyl alcohol	605 (578–617)	55·4 (53·8–59·9)	0·09
	3-Methylbenzyl alcohol	81·2 (76·8–85·7)	83·6 (82·4–85·2)	1·03
	4-Methylbenzyl alcohol	106 (99·6–113)	90·5 (87·1–93·0)	0·85
Benzyl alcohol dehydrogenase (*A. calcoaceticus*)	NAD+	40·6 (38·6–40·8)	357 (372–379)	8·79
	Benzyl alcohol	121 (115–127)	351 (337–356)	2·90
	2-Methylbenzyl alcohol	992 (920–1080)	405 (387–418)	0·41
	3-Methylbenzyl alcohol	146 (136–152)	498 (485–507)	3·41
	4-Methylbenzyl alcohol	118 (110–126)	593 (564–620)	5·02
Plasmid-encoded benzaldehyde dehydrogenase (*P. putida*)	NAD+ (22·2 μM-benzaldehyde)	79·0 (74·3–84·4)	96·2 (93·2–98·9)	1·22
	Benzaldehyde	0·79 (0·71–0·98)	104 (97·7–110)	132
	2-Methylbenzaldehyde	88·3 (80·4–123)	3·92 (3·70–4·90)	0·05
	3-Methylbenzaldehyde	0·60 (0·56–0·65)	46·8 (45·6–47·1)	78
	4-Methylbenzaldehyde	1·06 (1·00–1·13)	94·7 (93·6–97·0)	89·3
Benzaldehyde dehydrogenase I (*A. calcoaceticus*)	NAD+ (3·7 μM-benzaldehyde)	96 (73–106)	110 (99·6–117)	1·15
	Benzaldehyde	0·69 (0·53–0·80)	106 (95·4–120)	154
	2-Methylbenzaldehyde	26·8 (24·8–28·4)	8·59 (8·40–8·70)	0·32
	3-Methylbenzaldehyde	1·22 (1·11–1·32)	100 (93·1–106)	82·0
	4-Methylbenzaldehyde	3·02 (2·67–3·35)	49·1 (47·5–50·6)	16·3
Benzaldehyde dehydrogenase II (*A. calcoaceticus*)	NAD+ (11·2 μM-benzaldehyde)	121 (113–133)	60·8 (58·9–63·1)	0·50
	Benzaldehyde	0·63 (0·56–0·73)	63·5 (60·1–63·1)	101
	2-Methylbenzaldehyde	28·2 (22·8–33·0)	17·5 (15·8–18·3)	0·62
	3-Methylbenzaldehyde	0·38 (0·33–0·41)	22·3 (21·6–22·9)	58·7
	4-Methylbenzaldehyde	0·97 (0·82–1·13)	54·0 (51·3–58·2)	55·6

for maximum activity (Chalmers & Fewson, 1989). The plasmid-encoded benzyl alcohol dehydrogenase, like the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus* (MacKintosh & Fewson, 1988*a*), appears not to require cations for activity.

Cofactor specificity

Benzaldehyde dehydrogenase, as originally shown by Worsey & Williams (1975) using crude extracts of *P. putida* mt-2, had appreciable activity when NAD+ was replaced by NADP+. The other dehydrogenases are more specific for NAD+. The average activities with 2 mM-NADP+ relative to the activities with 2 mM-NAD+ were: plasmid-encoded benzyl alcohol dehydrogenase, 1·9%; benzyl alcohol dehydrogenase, 7·8%; plasmid-encoded benzaldehyde dehydrogenase, 27%; benzaldehyde dehydrogenases I and II, 4·9% and 4·3% respectively. The K+-activated yeast aldehyde dehydrogenase also uses both NAD+ and NADP+ as cofactors (Bostian & Betts, 1978).

Substrate specificity

Apparent K_m and V values for all five enzymes were determined for NAD+, benzaldehyde or benzyl alcohol and the three monomethyl-substituted benzyl alcohols or benzaldehydes (Table 2). The results were then used to calculate 'apparent specificity constants' (apparent V/ apparent K_m) which are analogous to 'specificity constants' (k_{cat}/K_m) except that instead of relating the activity to the number of active sites, the activity is related to the amount of enzyme protein. We chose this

approach because our aim was to compare the relative specificities of the enzymes for each of the substrates and any attempt to incorporate the estimated number of active sites into the calculations could only increase the error. A high value for the apparent specificity constant presumably indicates complementarity between the active site of the enzyme and the transition-state analogue of the substrate (Fersht, 1985). In so far as they overlap, the present measurements are in good agreement with previous estimates (MacKintosh & Fewson, 1988*a*, *b*; Chalmers & Fewson, 1989) except for the inexplicable report that *A. calcoaceticus* benzaldehyde dehydrogenase II did not oxidize 2- or 3-methylbenzaldehyde (MacKintosh & Fewson, 1988*b*). In experiments with crude extracts, Worsey & Williams (1975) found that TOL plasmid pWW0-encoded dehydrogenases had a little more than half as much activity with 3- or 4-methyl-substituted compounds as with the unsubstituted substrates.

The apparent K_m benzaldehyde value for the plasmid-encoded benzaldehyde dehydrogenase was only 0·79 μM, very close to the value for the two *A. calcoaceticus* benzaldehyde dehydrogenases. Benzaldehyde dehydrogenase was substrate-inhibited in the presence of more than about 10 μM-benzaldehyde. Both benzaldehyde dehydrogenases I and II from *A. calcoaceticus* are substrate-inhibited (MacKintosh & Fewson, 1988*b*; Chalmers & Fewson, 1989), as are many other aldehyde dehydrogenases (Bostian & Betts, 1978).

The results in Table 2 show that the plasmid-encoded dehydrogenases are little or no better than the *A. calcoaceticus* enzymes in dealing with 3- or 4-methyl-substituted substrates and none of the enzymes is very effective at oxidizing 2-methyl-substituted compounds. For all three benzaldehyde dehydrogenases, the substrate with the highest apparent specificity constant was benzaldehyde. Benzaldehyde dehydrogenase I had a relatively low specificity constant for 4-methylbenzaldehyde and the plasmid-encoded benzaldehyde dehydrogenase differed from the other two benzaldehyde dehydrogenases in that the specificity constant for 2-methylbenzaldehyde was particularly low.

Heat stabilities

Benzaldehyde dehydrogenase I from *A. calcoaceticus* is much more resistant to thermal inactivation than is benzaldehyde dehydrogenase II when bacterial extracts are incubated at 37 °C (Livingstone *et al.*, 1972). We have now shown that this different susceptibility to heat persists after the enzymes have been purified and that the plasmid-encoded benzaldehyde dehydrogenase is even more thermostable than is benzaldehyde dehydrogenase I (Table 3). All three enzymes are to some extent protected from heat inactivation by benzaldehyde (Table 3).

Conclusions

The results in this paper taken together with those described previously (MacKintosh & Fewson, 1988*a*, *b*; Chalmers & Fewson, 1989) show that the plasmid-encoded benzyl alcohol and benzaldehyde dehydrogenases from *P. putida* are remarkably similar to the equivalent chromosomally encoded enzymes from

Table 3. *Comparison of the heat stability of the plasmid-encoded benzaldehyde dehydrogenase from P. putida with the heat stabilities of benzaldehyde dehydrogenases I and II from A. calcoaceticus*

The purified enzymes were diluted in 100 mM-potassium phosphate buffer, pH 7·5, containing 5 mM-DTT and incubated in sealed Eppendorf tubes in water-baths at 50 °C or at 60 °C. Samples were also incubated under the same conditions with buffer/DTT plus either 2 mM-NAD^+ or 1 mM-benzaldehyde. Samples were removed at various times, cooled on ice for 2 min, centrifuged for 2 min, and the supernates then assayed for activity. Plots of enzyme activity against time were constructed and the times to reach 50% inactivation were estimated. Results are shown for a single representative experiment and several other experiments gave similar results. ND, Not determined.

Temperature	Addition to phosphate/DTT buffer	Time to reach 50% inactivation (min)		
		Plasmid-encoded benzaldehyde dehydrogenase	Benzaldehyde dehydrogenase I	Benzaldehyde dehydrogenase II
50 °C	None	ND	75	2
60 °C	None	50	19	1
60 °C	NAD^+	62	15	3
60 °C	Benzaldehyde	80	33	21

A. calcoaceticus. Both benzyl alcohol dehydrogenases are soluble, NAD^+-dependent enzymes with similar subunit M_r values, pH optima and substrate specificities. Similarly, all three benzaldehyde dehydrogenases have very similar M_r values, pH optima and substrate specificities. The plasmid-encoded benzaldehyde dehydrogenase is particularly similar to benzaldehyde dehydrogenase I from *A. calcoaceticus* in its apparent requirement for a monovalent cation and in its relative heat stability. It is hard to avoid the conclusion that there are evolutionary links amongst these plasmid-encoded and chromosomally encoded enzymes in two quite different genera. The development of a convenient method for purifying the two plasmid-encoded dehydrogenases, together with their preliminary characterization, should complement work on the molecular biology of TOL plasmids.

Note added in proof

J. P. Shaw and S. Harayama (personal communication) have obtained similar results for the benzyl alcohol dehydrogenase and the benzaldehyde dehydrogenase encoded by the pWW0 TOL plasmid of *Pseudomonas putida*. The pWW0-encoded benzaldehyde dehydrogenase appears to have a much higher apparent K_m for benzaldehyde and a lower V value than the pWW53-encoded enzyme, and to constitute a greater proportion of the cell protein.

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