Mutant strains of *Acinetobacter calcoaceticus* possessing additional mandelate dehydrogenases

Identification and preliminary characterization of the enzymes

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Acinetobacter calcoaceticus wild-type strain N.C.I.B. 8250 can grow on only the L(+)-isomer of mandelate but mutant strains have been isolated that can grow on D(–)-mandelate. These mutants contain a novel D(–)-mandelate dehydrogenase in addition to the original L(+)-mandelate dehydrogenase. A second wild-type strain, EBF 65/65, shows the opposite pattern and can grow on D(–)-mandelate but not on L(+)-mandelate; mutants have been isolated that possess an L(+)-mandelate dehydrogenase in addition to the original D(–)-mandelate dehydrogenase and can thus grow on L(+)-mandelate. Both L(+)- and D(–)-mandelate dehydrogenases, whether originally present or evolved, are very similar in many respects: they are membrane-bound and NAD(P)⁺-independent; their activities have similar dependence on temperature and pH; they are inhibited by oxalate but not by several metal-chelating agents; they are stereospecific in their action and are inhibited by the opposite stereoisomers. D(–)-Mandelate dehydrogenase is much more susceptible than L(+)-mandelate dehydrogenase to inhibition by HgCl₂ and *p*-chloromercuribenzoate and is much more heat-labile.

Several bacteria and fungi have inducible enzymes for the metabolism of mandelate; most strains use only one of the stereoisomers but a few can use both (Kenyon & Hegeman, 1979; Fig. 1). Mandelate is generally oxidized to phenylglyoxylate (benzoylformate). There seems to be no known case of an organism possessing a mandelate dehydrogenase

that is not stereospecific and most organisms that can use both L(+)- and D(–)-mandelate have either a

Abbreviations used: BSA, bovine serum albumin; DCIP, 2,6-dichloroindophenol; PMS, *N*-methylphenazonium methosulphate.

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Fig. 1. Alternative pathways for the metabolism of mandelate by micro-organisms
1, D(–)-Mandelate dehydrogenase; 2, L(+)-mandelate dehydrogenase; 3, mandelate racemase (EC 5.1.2.2); 4, L(+)-mandelate 4-hydroxylase.

pair of stereospecific dehydrogenases or a racemase and a single dehydrogenase (Kenyon & Hegeman, 1979). *Pseudomonas convexa* employs a different strategy and converts D(–)-mandelate into L(+)-mandelate and then carries out a ring-hydroxylation (Bhat & Vaidyanathan, 1976; Fig. 1).

Acinetobacter calcoaceticus N.C.I.B. 8250 can grow on L(+)-mandelate but not on D(–)-mandelate and has only an L(+)-mandelate dehydrogenase (Fewson, 1967; Kennedy & Fewson, 1968). However, we isolated some mutant strains that could grow on both isomers and in the present paper we show that this is the result of the expression of a novel D(–)-mandelate dehydrogenase. Parts of this work have been published in a preliminary form (Fewson *et al.*, 1976), but it is only more recently that we have obtained high rates of D(–)-mandelate dehydrogenase activity, since we found that the enzyme is heat-labile and requires PMS and BSA for maximum activity. We also describe in the present paper how another wild-type strain of *A. calcoaceticus*, EBF 65/65, shows the opposite pattern: it has only a D(–)-mandelate dehydrogenase but it gives rise to mutants that have a novel L(+)-mandelate dehydrogenase. Very little is known about bacterial mandelate dehydrogenases beyond the facts that they are NAD(P)⁺-independent and appear to be membrane-bound (Stanier *et al.*, 1953; Hegeman, 1966; Kennedy & Fewson, 1968) and we include a comparison of some of the properties of the enzymes from *A. calcoaceticus*.

Experimental

Materials

Chemicals were of the best quality available commercially and most of them were obtained from

the sources summarized by Beggs & Fewson (1977). Mandelic acids (Puriss.) were from Fluka (Fluorochem, Glossop, Derbyshire, U.K.) and measurements of their melting points and specific optical rotations confirmed the manufacturer's description of greater than 99% purity. BSA (fraction V) was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; some samples were delipidated by charcoal treatment (Chen, 1967). BSA ('essentially fatty acid-free') was obtained from Sigma, Poole, Dorset, U.K. Aldolase and glyceraldehyde 3-phosphate dehydrogenase (both from rabbit muscle; Sigma) were dialysed and concentrated in a B15 Minicon macrosolute concentrator (Amicon Corp., Lexington, MA, U.S.A.) before use.

Bacteria

Pseudomonas putida N.C.I.B. 9494 (A 3.12) and *A. calcoaceticus* N.C.I.B. 8250 were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K. *A. calcoaceticus* C48 (a double auxotroph *ile-1 met-1* derived from strain EBF 65/65 by Towner & Vivian, 1976) was provided by Dr. A. Vivian, Thames Polytechnic, London, U.K. Mutants of wild-type strain N.C.I.B. 8250 are listed in Table 1.

Growth of organisms and preparation of extracts

Bacteria were maintained, grown, harvested, stored and extracts prepared as described previously (Livingstone & Fewson, 1972; Cook *et al.*, 1975). The only significant modifications were that cells were generally grown at 23°C, stored at –18°C, resuspended in 0.1M-KH₂PO₄/K₂HPO₄ buffer, pH 7.5, and the current used for ultrasonic disruption was 3 A. In a few experiments where we

Table 1. Mutant strains derived from *A. calcoaceticus* N.C.I.B. 8250

Mutant	Accession number and previous designation	D(–)-Mandelate dehydrogenase	L(+)-Mandelate dehydrogenase	Isolation procedure
C1005	N.C.I.B. 11339; HMM5	Absent	Constitutive	Growth of N.C.I.B. 8250 in the presence of 2-phenylpropionate (Fewson <i>et al.</i> , 1978)
C1038	N.C.I.B. 11210; D40G	Inducible	Absent	Transformation of C1408 with DNA from C1041 followed by selection for growth on D(–) but not on L(+)-mandelate
C1039		Inducible	Absent	
C1040		Inducible	Absent	
C1041	N.C.I.B. 11209; 41	Inducible	Inducible	Growth of N.C.I.B. 8250 on D(–)-mandelate
C1042	41Z	Inducible	Inducible	Transformation of C1408 with DNA from C1041; able to grow on both D(–) and L(+)-mandelate
C1043		Inducible	Inducible	
C1123	N.C.I.B. 11456	Constitutive	Absent	Growth of C1040 in the presence of 2-phenylpropionate
C1219	N.C.I.B. 11457	Constitutive	Constitutive	Growth of C1041 in the presence of 2-phenylpropionate
C1408	N.C.I.B. 10789; NF1408	Absent	Absent	Beggs & Fewson (1977)

wanted to avoid too much damage to membranes, bacteria were disrupted by passage through a cooled French pressure cell (see below). The salts media used for the growth of strain C48 and its derivatives contained DL-isoleucine and L-methionine (each at a final concentration of 50 mg/litre).

Isolation of mutants able to grow on D(-) or L(+) mandelate

Various volumes (0.1 to 4.0 ml) of nutrient broth cultures of wild-type strain N.C.I.B. 8250 were inoculated into 250 ml Erlenmeyer flasks containing 5 mM-D(-)-mandelate + 0.25 mM-phenylglyoxylate/salts medium (50 ml). Some of the inocula had been treated with *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine as described by Livingstone & Fewson (1972). The flasks were shaken at 30°C for several days and samples from any that showed growth were streaked on plates of 5 mM-D(-)-mandelate/salts medium containing 1.5% (w/v) agar. A representative fast-growing colony derived from each flask that had shown growth was purified and stored. The first set of these experiments was done by Dr. J. D. Beggs. Mutants of strain C48 able to grow on L(+)-mandelate were isolated in a similar way except that enrichment was in 5 mM-L(+)-mandelate/salts medium containing isoleucine and methionine.

Isolation of mutants with constitutive enzymes

Mutants with constitutive mandelate dehydrogenase activities were isolated on 2 mM-2-phenylpropionate (as anti-inducer) + 2 mM-phenylglyoxylate/salts medium containing 1.5% (w/v) agar (Fewson *et al.*, 1978).

Genetic transformation

The experiments followed the general procedure described previously (Ahlquist *et al.*, 1980) and were done by Dr. E. F. Ahlquist. DNA isolated from mutant C1041 was used to transform mutant C1408; plates of 5 mM-D(-)-mandelate + 50 μ M-phenylglyoxylate/salts medium containing 1.5% (w/v) agar were used to select for growth.

Measurement of mandelate racemization

Washed suspensions of bacteria (6–22 mg dry wt./ml) were incubated in 20 mM-L(+) or -D(-)-mandelate/salts medium (10 ml total volume) in Thunberg tubes that had been thoroughly flushed with O₂-free N₂ before addition of the mandelate to the rest of the reaction mixture. After 2–5 h at 30°C, 1.0 ml of 1 M-HCl was added to each tube and the contents were centrifuged at 12000 g for 30 min at 4°C. The supernatants were extracted with 3 $\times$ 10 ml of peroxide-free diethyl ether and the extracts were then combined and evaporated to dryness. Chrysanthemate esters of the methyl mandelates were prepared and the diastereoisomers distinguished by

g.l.c. (Brooks *et al.*, 1973). The analyses were done by Mr. I. C. Matson with the assistance of Mr. C. G. Edmonds in the laboratory of Professor C. J. W. Brooks, Department of Chemistry, University of Glasgow.

Enzyme assays

D(-)-Mandelate dehydrogenase activity was measured in reaction mixtures (3.0 ml) containing 200 μ mol of KH₂PO₄/K₂HPO₄ buffer, pH 7.5, 10 mg of BSA (dissolved in 0.1 M-KH₂PO₄/K₂HPO₄ buffer, pH 7.5), 200 nmol of DCIP, 1 μ mol of PMS, extract (usually 100 μ l) and 1.5 μ mol of D(-)-mandelate (0.1 ml of a solution that had been adjusted to pH 7.5) to initiate the reaction. Reduction of DCIP was followed at 27°C. Oxidation of 1 μ mol of D(-)-mandelate per assay corresponds to an *A*₆₀₀ of -6.87. Usually the absorbances of four cuvettes were followed in a spectrophotometer fitted with an automatic sample changer (triplicate cuvettes with mandelate and one cuvette with no substrate to serve as a control).

L(+)-Mandelate dehydrogenase was measured in the same way, except that L(+)-mandelate was used as substrate.

Phenylglyoxylate decarboxylase (EC 4.1.1.7) and benzaldehyde dehydrogenase I activities were measured as described by Livingstone & Fewson (1972).

Oxygen uptake

Clark-type oxygen electrodes (Rank Brothers, Cambridge, U.K.) were maintained at 27°C. The total volume of reaction mixture was 3.0 ml. Endogenous rates of O₂ uptake were followed for about 5 min and then the substrate was added in a volume of 0.1 ml.

Chromatography

Free acids were separated by t.l.c. on Serva Feinbiochemica Silufol (UV254; Micro-Bio Laboratories, London S.W.6, U.K.) using solvent A [butan-1-ol saturated with (NH₄)₂CO₃ buffer, pH 9.7 (Fewster & Hall, 1951)]. 2,4-Dinitrophenylhydrazones were prepared by standard procedures and separated by ascending chromatography on Whatman no. 1 paper using solvent B {butan-1-ol/ethanol/(NH₄)₂CO₃ buffer [7.2 g of (NH₄)₂CO₃ + 5.2 ml of NH₃ (specific gravity 0.88) per litre] (40:11:19, by vol.)}. Solvents were freshly prepared for each experiment and chromatograms were developed at room temperature.

Analytical methods

Protein contents of suspensions of intact bacteria were estimated on the basis that a suspension whose turbidity at 500 nm was 1 contained 168 μ g of protein/ml. Protein contents of extracts were determined by the method of Lowry *et al.* (1951) with

BSA as standard; wall plus membrane fractions were suspended in 0.66M-NaOH and incubated overnight at 30°C before analysis.

Phenylglyoxylate concentrations were measured after chloroform extraction as described by Cook *et al.* (1975). The identity of the product of D(-)-mandelate oxidation was confirmed by examining acidified chloroform extracts of reaction mixtures. These had the same absorption spectra ($\lambda_{\text{max.}} = 253 \text{ nm}$; $\lambda_{\text{min.}} = 222 \text{ nm}$) as a solution of authentic phenylglyoxylate. T.l.c. in solvent A showed a single spot with an R_F value (0.35) characteristic of phenylglyoxylate. The 2,4-dinitrophenylhydrazone derivative of the product had the same absorption spectrum as that of authentic phenylglyoxylate ($\lambda_{\text{max.}} = 388 \text{ nm}$; $\lambda_{\text{min.}} = 310 \text{ nm}$) and it gave the same R_F value (0.80) as authentic material after separation by paper chromatography in solvent B.

Benzaldehyde concentrations were measured after extraction with n-hexane ($\lambda_{\text{max.}} = 240 \text{ nm}$; Cook *et al.*, 1975).

Results and discussion

The mechanism of D(-)-mandelate metabolism

We considered five possible explanations that could account for the ability of mutants of *A. calcoaceticus* N.C.I.B. 8250 to grow on D(-)-mandelate: (i) that there had been a change in the stereospecificity of L(+)-mandelate dehydrogenase so that it now used D(-)-mandelate [but not L(+)-mandelate] as substrate; (ii) that L(+)-mandelate dehydrogenase had lost its stereospecificity and could oxidize either L(+)- or D(-)-mandelate; (iii) that there was a mandelate racemase; (iv) that there was a new D(-)-mandelate dehydrogenase in addition to the existing L(+)-mandelate dehydrogenase; (v) that D(-)-mandelate was metabolized by a pathway that did not involve the formation of phenylglyoxylate.

We examined twelve independently isolated mutants of *A. calcoaceticus* N.C.I.B. 8250 able to grow on D(-)-mandelate; seven of them were spontaneous mutants and five (including strain C1041) were isolated after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. All of these mutants could still grow on L(+)-mandelate. Furthermore, they all had both L(+)- and D(-)-mandelate dehydrogenase activities after growth in the presence of phenylglyoxylate, L(+)-mandelate or D(-)-mandelate. These results rule out possibility (i).

When strain C1408 (a blocked mutant unable to grow on either isomer of mandelate) was transformed with DNA from strain C1041, 306 transformants were isolated as a result of selection for the ability to grow on D(-)-mandelate/salts agar. Replica plating experiments showed that 270 of

them could also grow on L(+)-mandelate; two of these strains (C1042 and C1043) were examined further and shown to have inducible L(+)- and D(-)-mandelate dehydrogenase activities. Three strains (C1038, C1039 and C1040) were chosen at random from the 36 strains unable to grow on L(+)-mandelate and were found to have an inducible D(-)-mandelate dehydrogenase; however, no L(+)-mandelate dehydrogenase activity could be detected after growth in the presence of phenylglyoxylate, L(+)-mandelate or D(-)-mandelate. The large proportion of strains able to grow on both isomers after selection on D(-)-mandelate suggests that the appropriate genes may be close together on the chromosome. More important, the existence of strains (derived from C1041) that can grow on D(-)-mandelate but have no L(+)-mandelate dehydrogenase activity rules out possibility (ii), a non-specific mandelate dehydrogenase.

The properties of transformants such as strain C1038 also rule out (iii), since a racemase would serve no purpose unless followed by an L(+)-mandelate dehydrogenase. Further evidence for the absence of a racemase was obtained when intact cells of strain C1041 were incubated with D(-)- or L(+)-mandelate under anaerobic conditions (to prevent substrate oxidation) and the isomeric composition of the products examined. No racemization was detected, although under the same conditions *P. putida* N.C.I.B. 9494, which is known to possess a mandelate racemase (Kenyon & Hegeman, 1979), formed equal amounts of each isomer from either L(+)- or D(-)-mandelate.

When D(-)-mandelate-utilizing strains were grown in D(-)-mandelate/salts medium, the cultures produced benzaldehyde, presumably formed by enzymic decarboxylation of phenylglyoxylate (Beggs *et al.*, 1976). Further indirect evidence for the formation of phenylglyoxylate was provided by the observation that L(+)-mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I were all induced when strain C1041 was grown in D(-)-mandelate + L-glutamate/salts medium (specific activities 90, 597 and 27 nmol/min per mg of protein respectively) and it is known that phenylglyoxylate is the inducer of this regulon (Livingstone & Fewson, 1972). Confirmation that D(-)-mandelate is oxidized to phenylglyoxylate was provided by the discovery of a D(-)-mandelate dehydrogenase as described below. The rates of D(-)-mandelate dehydrogenase activity measured in extracts of bacteria that had been grown in D(-)-mandelate/salts medium (at least 100 nmol/min per mg of protein) were compatible with (a) the rates of O_2 uptake by washed suspensions of similar cells [approx. 500 nmol of O_2 /min per mg of protein, which is equivalent to approx. 80 nmol of D(-)-mandelate oxidized/min per mg of protein assuming

almost complete oxidation] and with (b) the rates of substrate utilization (approx. 100 nmol/min per mg of protein) calculated from the growth rate.

There seems no reason to doubt that in strains such as C1041 or C1219, D(–)-mandelate is entirely metabolized by oxidation to phenylglyoxylate in a reaction catalysed by a new D(–)-mandelate dehydrogenase. Phenylglyoxylate can then be oxidized as though it has been formed from L(+)-mandelate.

D(–)-Mandelate dehydrogenase

A wall plus membrane fraction of mutant C1219, which had no detectable phenylglyoxylate decarboxylase activity in the absence of added thiamin pyrophosphate, oxidized D(–)-mandelate to give an equimolar concentration of phenylglyoxylate.

A similar wall plus membrane fraction from mutant C1219 was used to measure rates of O₂ uptake under a range of conditions using either L(+) or D(–)-mandelate as substrate (Table 2). Inclusion of catalase (38 750 units) in the reaction mixture had no effect on the rate or extent of O₂ uptake, so presumably the product was water rather than H₂O₂. The results showed that the presence of both PMS and BSA was necessary to obtain the maximum rates of O₂ uptake and suggested that electron transfer to O₂, rather than dehydrogenase activity, was rate-limiting in the unsupplemented extracts. Experiments of this sort, as well as spectrophotometric measurements of rates of reduction of DCIP, led to the development of the routine method for measuring D(–)-mandelate dehydrogenase activity that is described in the Experimental

section. Spectrophotometric assay of D(–)-mandelate and L(+)-mandelate dehydrogenase activities of the wall plus membrane fraction used for the experiments described in Table 1 gave values of 141 and 338 nmol/min per mg of protein respectively; these agree well with the rates of 153 and 346 calculated from the rates of O₂ consumption for the same conditions (Table 1, PMS + BSA + DCIP; 1 mol of O₂ is equivalent to the dehydrogenation of 2 mol of mandelate).

Inclusion of BSA in the spectrophotometric reaction mixture was necessary to obtain appreciable D(–)-mandelate dehydrogenase activity but it inhibited L(+)-mandelate dehydrogenase activity (Table 3). Provided that BSA was also present, PMS further increased the D(–)-mandelate dehydrogenase activity and it also increased the rate of L(+)-mandelate dehydrogenase (Table 3). Inclusion of both BSA and PMS [optimal concentrations approx. 0.1–0.9% (w/v) and 0.3–2 mM respectively; higher concentrations were inhibitory] in the reaction mixtures improved the linearity of the assays with time (for at least 5 min), but introduced a slow substrate-independent reduction of DCIP, which had to be corrected for by the inclusion of an appropriate control (Table 3; see the Experimental section).

BSA is often included in dehydrogenase assays of this type (e.g. succinate dehydrogenase; Hatefi, 1978) but its role is not clear, although it is sometimes thought to be involved in the protection of essential thiol groups or in some sort of protein–protein interaction. However, the following results suggest that neither of these explanations is

Table 2. Oxygen uptake by a wall plus membrane fraction of mutant C1219 using D(–)-mandelate or L(+)-mandelate as substrates

Mutant strain C1219 was disrupted by ultrasonic treatment as described in the Experimental section and then centrifuged at 100 000 *g* for 2.5 h at 4°C. The precipitate ('wall plus membrane') was resuspended in 0.1 M-phosphate buffer, pH 7.5, to give a protein concentration of 5.9 mg/ml. Rates of O₂ uptake were measured in various reaction mixtures containing 0.2 ml of the wall plus membrane fraction as described in the Experimental section and are given as means ± s.d., with the numbers of determinations shown in parentheses. Rates have been corrected by subtraction of the endogenous rate, which was 1.2 ± 1.0 (9) nmol of O₂/min per mg of protein.

Addition to reaction mixture*	Substrate ...	Rate of O ₂ uptake (nmol/min per mg of protein)	
		D(–)-Mandelate	L(+)-Mandelate
None		15.6 ± 2.7 (4)	39.5 ± 1.4 (5)
DCIP		7.8 (1)†	21.9 (1)†
PMS		85.1 ± 1.0 (2)	174.9 ± 5.8 (2)
DCIP + PMS		43.6 ± 6.1 (2)†	120.3 ± 21.0 (2)†
DCIP + PMS + BSA		76.3 ± 13.9 (5)†	172.9 ± 4.7 (3)†
BSA		20.3 ± 3.2 (2)	43.4 ± 0.7 (2)
BSA + PMS		105.1 ± 4.9 (3)	208.1 ± 44.9 (3)

* Concentration as in the standard reaction mixture used for measuring D(–)-mandelate dehydrogenase activity (see the Experimental section).

† There was an initial period when virtually no O₂ uptake was observed and the reaction mixtures became colourless, i.e. these rates were established only after all the DCIP had been reduced.

Table 3. *Effects of components of the reaction mixture on the activities of D(–)-mandelate dehydrogenase and L(+)-mandelate dehydrogenase*

An extract of mutant strain C1219 was prepared as described in the Experimental section and the enzyme activities were measured spectrophotometrically in various reaction mixtures.

Addition to reaction mixture	Substrate added ...	Enzyme specific activity (nmol/min per mg of protein)		
		D(–)-Mandellate	L(+)-Mandellate	None
DCIP, PMS, BSA		146	269	24
DCIP, PMS		11	154	7
DCIP, BSA		41	65	9
DCIP only		5	124	0

sufficient in the present case. BSA could be partially replaced by the thiol-containing proteins glyceraldehyde 3-phosphate dehydrogenase (at its best, half as effective as BSA at 1% in the reaction mixture) and rabbit muscle aldolase (18% as effective at 0.27%), but not by lysozyme, ovalbumin or ribonuclease (0.001–3%) or by dithiothreitol, reduced glutathione or 2-mercaptoethanol at concentrations below those that caused significant non-enzymic reduction of DCIP (0.03–10 μ M). Neither glycerol nor ethylene glycol (0.5–30%) could replace BSA. Addition of an extract of non-induced cells could substitute for BSA to some extent; this protective effect was confirmed by the fact that without any addition the apparent specific activity of D(–)-mandelate dehydrogenase increased with increasing amounts of extract in the reaction mixture, whereas in the presence of the optimal concentration of BSA the specific activity was independent of the amount of extract used. Commercial BSA, delipidated BSA and commercial delipidated BSA all had identical effects, so there is no hint that BSA is providing lipid that might be required for these membrane-bound enzymes.

Neither mammalian cytochrome *c* (0.1–0.4%) nor potassium ferricyanide (0.1 to 0.5 mM) could substitute for DCIP as electron acceptor. In the presence of PMS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.23 mM) gave about 80% of the activity with DCIP. Addition of NAD⁺ (0.1–4 mM), NADP⁺ (0.03–0.2 mM), FAD (0.02–0.2 mM) or FMN (0.02–0.2 mM) did not increase D(–)-mandelate dehydrogenase activity in the presence of DCIP, and there was no activity with NAD⁺ or NADP⁺ in the absence of DCIP. Similar results were obtained for L(+)-mandelate dehydrogenase.

Rates of D(–)-mandelate dehydrogenation were identical when measured under the standard aerobic conditions or in Thunberg cuvettes flushed with O₂-free N₂. This is consistent with the observation that no O₂ uptake occurred while DCIP was being reduced (see the footnote to Table 2). Addition of catalase to the standard assay had no effect on the rate of DCIP reduction in the presence of D(–)-mandelate. H₂O₂ production could not be detected

with the Boehringer Perid reagent [peroxidase + 2,2'-diao-di-(3-ethylbenzthiazoline 6-sulphonic acid)]. There was no evidence for the involvement of superoxide since 10 μ M-tetranitromethane had no effect on the rate; superoxide dismutase did not increase the rate and was inhibitory when added at relatively high concentration (0.1 mg/assay). We found no combination of catalase and/or superoxide dismutase that could substitute for BSA in the assay.

Reproducibility of the assay

The reproducibility of the assay procedure was tested with strain C1219 because it is susceptible to lysis (C. A. Hills & C. A. Fewson, unpublished work) and might be feared to give the least reproducible results of all our strains.

(a) Cultures were grown from different inocula on five separate days, harvested and frozen; extracts were prepared and assayed in triplicate five times. The mean ($\pm$ S.D.) specific activity was 142 ± 15 ($n = 5$) nmol/min per mg of protein.

(b) To test for variability of disruption and centrifugation, a single bacterial suspension of known low activity was divided into eight portions and each disrupted, centrifuged and then assayed in triplicate. The mean ($\pm$ S.D.) specific activity was 44.2 ± 2.8 ($n = 8$) nmol/min per mg of protein.

(c) To check variation amongst assays, a single sample of an extract prepared for (b) was assayed 36 times and gave a mean ($\pm$ S.D.) specific activity of 44.8 ± 2.6 ($n = 36$) nmol/min per mg of protein.

There was practically no loss of D(–)-mandelate dehydrogenase activity on freezing or during subsequent storage at -18°C .

The standard procedure therefore gives a reasonably accurate and reproducible measure of D(–)-mandelate dehydrogenase specific activity.

Comparison of the properties of D(–)-mandelate dehydrogenase and L(+)-mandelate dehydrogenase

Whenever possible, comparative studies were carried out with strain C1219, which possesses both

Table 4. Comparison of some properties of D(–)-mandelate dehydrogenase and L(+)-mandelate dehydrogenase

The proportion of each enzyme in the wall plus membrane fraction was determined by disrupting mutant C1219 by three passages through the French press (98 MPa) and then centrifuging the homogenate at 165 000 g for 90 min at 4°C; activities were measured in the precipitate ('wall plus membrane') and supernatant fractions. For the other experiments, bacteria were disrupted by ultrasonic treatment and then centrifuged at 12 000 g for 30 min at 4°C. K_m and K_i values were determined with extracts prepared from strains C1005 and C1123, which possess only L(+)-mandelate dehydrogenase or D(–)-mandelate dehydrogenase activity respectively; other experiments were done with mutant C1219. K_m values were calculated from linear sections of Lineweaver–Burk plots; both enzymes showed signs of substrate inhibition at concentrations above about 0.5 mM. K_i values were calculated from measurements of activity of D(–)-mandelate dehydrogenase in the presence of 0, 1 or 2.5 mM-L(+)-mandelate and of L(+)-mandelate dehydrogenase in the presence of 0, 2.5 or 10 mM-D(–)-mandelate. Activation energies were calculated from Arrhenius plots, which were approximately linear between 10 and 25°C. Response to pH was measured in K_2HPO_4/KH_2PO_4 buffers. Inhibitors were pre-incubated with the extract for 10 min at 27°C before initiating the reaction with substrate. Results are means of from two to four experiments.

	D(–)-Mandelate dehydrogenase	L(+)-Mandelate dehydrogenase
Activity (% of total) in the wall plus membrane fraction	83	92
K_m value (μM) for D(–)-mandelate	545	No activity
K_m value (μM) for L(+)-mandelate	No activity	207
K_i value (μM) for inhibition by the opposite stereoisomer	720	4080
Activation energy (kJ/mol)	42	54
pH value for maximum activity (and pH values for half-maximum activity)	7.8 (6.6, 8.6)	7.8 (6.0, 8.9)
Inhibition (%) by 25 μM -oxalate	50	52
Concentration (μM) of $HgCl_2$ giving 50% inhibition	0.7	3.0
Concentration (μM) of <i>p</i> -chloromercuribenzoate giving 50% inhibition	0.5	200
Activity (%) remaining after heating an extract (approx. 0.5 mg of protein/ml) at 30°C for 200 min	15	92

enzymes, so that the same extract could be used to study both activities.

Both enzymes appear to be membrane-bound. Most of the activities were precipitated after high-speed centrifugation (Table 4) under conditions where approximately half of the total protein remained in the supernatant fraction. Both activities could be solubilized by detergents (O'Donnell *et al.*, 1981).

Each enzyme showed absolute stereospecificity for its substrate and was inhibited by the opposite isomer, although D(–)-mandelate dehydrogenase was much more sensitive to inhibition by L(+)-mandelate than was L(+)-mandelate dehydrogenase to inhibition by D(–)-mandelate (Table 4).

The effects of temperature and pH on the activities of both enzymes were very similar (Table 4).

Both enzymes were strongly inhibited by oxalate (Table 4). Neither enzyme was significantly affected by EDTA (up to 10 mM), *o*-phenanthroline or 8-hydroxyquinoline (up to 0.1 mM), 2,2-bipyridyl (up to 1 mM), KCN or NaN_3 (up to 1 mM). Iodoacetate (up to 10 mM) had little effect on either enzyme but iodoacetamide (10 mM) and *N*-ethylmaleimide (10 mM) inhibited both activities by about 90% and 80% respectively. Both enzymes were inhibited by low concentrations of $HgCl_2$ and *p*-chloromercuribenzoate, although in the latter case L(+)-mandelate

dehydrogenase was much less sensitive than D(–)-mandelate dehydrogenase (Table 4).

The most obvious difference between the two enzymes was their contrasting heat-stabilities (Table 4). The heat-lability of D(–)-mandelate dehydrogenase was observed in washed suspensions of intact bacteria and also in growing bacteria and was one of the reasons for our failure to obtain high rates of D(–)-mandelate dehydrogenase activity in our early experiments, which routinely used bacteria grown at 30°C. In a typical experiment, when mutant C1219 was grown at 30°C, the specific activities of L(+)- and D(–)-mandelate dehydrogenase were 144 and 36 nmol/min per mg of protein respectively, whereas when the same strain was grown at 23°C the specific activities were 113 and 130.

Glycerol or ethylene glycol (30%, v/v) protected D(–)-mandelate dehydrogenase from inactivation when extracts were incubated at 30°C but BSA (0.06–3.8%, w/v) and dithiothreitol (0.1–1 mM) had no effect.

Mutants of A. calcoaceticus EBF 65/65 able to grow on L(+)-mandelate

We found that wild-type strain EBF 65/65 can grow on D(–)-mandelate but not on L(+)-mandelate and so shows the reverse pattern to wild-type strain N.C.I.B. 8250. We then started this set of experiments with the doubly auxotrophic strain C48 to

facilitate future genetic work. Mutants of strain C48 were isolated on L(+)-mandelate/salts medium and, in experiments similar to those described above, were shown to possess a novel L(+)-mandelate dehydrogenase in addition to the original D(-)-mandelate dehydrogenase.

So far as we have looked at them, the properties of the L(+)-mandelate dehydrogenase and D(-)-mandelate dehydrogenase of mutants derived from EBF 65/65 have proved to be strikingly similar to the properties of the corresponding enzymes from mutants derived from wild-type N.C.I.B. 8250 (C. A. Hills & C. A. Fewson, unpublished work). In particular, the D(-)-mandelate dehydrogenase was much more sensitive to inhibition by *p*-chloromercuribenzoate than was the L(+)-mandelate dehydrogenase and the D(-)-mandelate dehydrogenase was very much more heat-labile. The latter observation undermined our original idea that the heat-lability of D(-)-mandelate dehydrogenase from strains such as C1219 was a result of it being an 'evolved' enzyme (see Clarke, 1974; Hartley, 1974), since, in the case of mutants derived from wild-type strain EBF 65/65, it is the new enzyme that is the more stable at high temperatures.

Conclusions

Apart from the obvious differences in their stereospecificities and their different susceptibilities to heat and to some thiol-specific reagents, L(+)-mandelate dehydrogenase and D(-)-mandelate dehydrogenase appear to have remarkably similar properties. This applies whether they are 'original' or 'evolved'. The similarities between the original L(+)-mandelate dehydrogenase of wild-type strain N.C.I.B. 8250 and the evolved L(+)-mandelate dehydrogenase of strain EBF 65/65 are even more striking, as are those between the evolved D(-)-mandelate dehydrogenase of N.C.I.B. 8250 and the original D(-)-mandelate dehydrogenase of EBF 65/65.

The simplest explanation for the origin of mutants able to grow on the second stereoisomer is that the new dehydrogenases result from the expression of previously 'silent' genes.

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