Purification and properties of L-mandelate dehydrogenase and comparison with other membrane-bound dehydrogenases from *Acinetobacter calcoaceticus*

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L-Mandelate dehydrogenase was purified from *Acinetobacter calcoaceticus* by Triton X-100 extraction from a 'wall + membrane' fraction, ion-exchange chromatography on DEAE-Sephacel, $(\text{NH}_4)_2\text{SO}_4$ fractionation and gel filtration followed by further ion-exchange chromatography. The purified enzyme was partially characterized with respect to its subunit M_r (44 000), pH optimum (7.5), pI value (4.2), substrate specificity and susceptibility to various potential inhibitors including thiol-blocking reagents. FMN was identified as the non-covalently bound cofactor. The properties of L-mandelate dehydrogenase are compared with those of D-mandelate dehydrogenase, D-lactate dehydrogenase and L-lactate dehydrogenase from *A. calcoaceticus*.

INTRODUCTION

Acinetobacter calcoaceticus N.C.I.B. 8250 can use mandelate as sole source of carbon and energy (Fewson, 1967). L(+)-Mandelate is oxidized to phenylglyoxylate by L-mandelate dehydrogenase in the wild-type strain, and some mutants possess a D-mandelate dehydrogenase and so can grow on D(–)-mandelate (Hills & Fewson, 1983a). Both mandelate dehydrogenases are co-ordinately synthesized with phenylglyoxylate acting as the inducer (Hills & Fewson, 1983b). In addition to the stereospecific mandelate dehydrogenases, a pair of stereospecific lactate dehydrogenases can be co-ordinately induced in *A. calcoaceticus* by either D(–)- or L(+)-lactate (Fewson & O'Donnell, 1981; Allison *et al.*, 1985a). Experiments with crude extracts showed that all four dehydrogenases, although separable, had several properties in common. D-Lactate dehydrogenase and D-mandelate dehydrogenase appeared to be particularly similar to each other, as did L-lactate dehydrogenase and L-mandelate dehydrogenase (Fewson & O'Donnell, 1981; Allison *et al.*, 1985a). The mandelate dehydrogenases and lactate dehydrogenases are all NAD(P)⁺-independent and they are integral components of the cytoplasmic membrane (Allison *et al.*, 1985a). The optimal conditions for their solubilization have been determined, and the detergent/protein ratio was found to be especially important since the enzymes were not released if insufficient detergent was added and the enzymes were inactivated if too much detergent was used (Allison *et al.*, 1985b). The similarities between the enzymes specific for the D-enantiomers of mandelate and lactate were again shown by the selective solubilization of these enzymes by the ionic detergents cholate, deoxycholate and SDS (Allison *et al.*, 1985b). D-Mandelate dehydrogenase and D-lactate dehydrogenase have been purified to homogeneity and have been shown to be monomeric proteins of similar M_r , containing non-covalently bound FAD as prosthetic group (Allison *et al.*, 1985b). L-Lactate dehydrogenase has also been

purified and contains non-covalently bound FMN (Allison & Fewson, 1986).

The present paper describes the purification and properties of L-mandelate dehydrogenase, thus allowing a detailed comparison of the properties of all four dehydrogenases from *A. calcoaceticus*.

EXPERIMENTAL

Materials

Chemicals were the best quality commercially available and most of them were obtained from the sources described by Hills & Fewson (1983a) and by Allison *et al.* (1985a,b).

Organism

Acinetobacter calcoaceticus mutant strain C1005 (N.C.I.B. 11339; constitutive L-mandelate dehydrogenase, no D-mandelate dehydrogenase) was derived from the wild-type strain (Fewson *et al.*, 1978).

Growth of bacteria

Bacteria were maintained and grown on complex medium as described by Allison *et al.* (1985a). Large amounts of bacteria were grown in a 10-litre fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewkesbury, Glos., U.K.) with the use of a draw-and-fill procedure. An inoculum was prepared by aseptically transferring 0.1 ml of a stock culture into 50 ml of the complex medium plus DL-mandelate (in a 250 ml conical flask) and shaking (120 rev./min) the mixture at 30 °C for 24 h. The whole of this culture was then aseptically transferred to 400 ml of complex medium plus DL-mandelate (in a 2-litre conical flask) and shaken (120 rev./min) at 30 °C for 24 h. The entire culture was used to inoculate the fermenter, which contained 10 litres of complex medium plus DL-mandelate and 50 ml of sterile 1% (v/v) poly(propylene glycol) 2025 as antifoam. A 20-litre reservoir containing the same medium was

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connected to the fermenter, which was operated at 30 °C with an aeration rate of 4 litres of sterile air/min and stirring at setting 2.5 (approx. 350 rev./min). After 16 h growth, when the apparent absorbance of the culture at 500 nm was at least 2.0, 9 litres of the culture were harvested, washed and stored at -20 °C (Hills & Fewson, 1983b), and 9 litres of fresh medium were added to the fermenter from the reservoir. After another period of growth this process was repeated, and finally the last 10 litres of culture were grown and harvested.

Purification of L-mandelate dehydrogenase

All steps in the purification were carried out at 4 °C.

Step 1. A 'wall + membrane' fraction was prepared as described by Allison *et al.* (1985a) from 140 g wet wt. of frozen bacteria of mutant strain C1005 that had been grown in complex medium plus DL-mandelate.

Step 2. The 'wall + membrane' fraction was diluted to 20 mg of protein/ml, and 10% (w/v) Triton X-100 was added to a final concentration of 0.5 mg of detergent/mg of protein. The suspension was stirred slowly for 30 min and then centrifuged at 165 000 $g_{av.}$ for 120 min. Ethanediol was added to the supernatant ('Triton extract') to a final concentration of 20% (v/v) and the Triton extract was then either used immediately or stored at -20 °C.

Step 3. The Triton extract was applied to a DEAE-Sephacel column (20 cm long $\times$ 5.2 cm diam.) that had been equilibrated with 20 mM-potassium phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 0.1 mM-dithiothreitol and 20% (v/v) ethanediol. The column was washed (2-3 column volumes) with the same buffer containing 0.1 M-NaCl to remove unbound material. Protein was eluted by applying a linear gradient of 0.1-0.25 M-NaCl in the same buffer (total volume 2 litres) at 60 ml/h, and fractions were collected at 6 min intervals. The A_{280} of the eluate was monitored and the fractions were assayed for L-mandelate dehydrogenase activity. Fractions containing more than 25% of the activity of the peak fraction were pooled.

Step 4. Solid $(NH_4)_2SO_4$ (specially low in heavy-metal ions for enzyme work) was added to the pooled ion-exchange fractions to give 15% saturation. When all the $(NH_4)_2SO_4$ had dissolved, the solution was stirred for 30 min before being centrifuged at 165 000 $g_{av.}$ for 30 min. More $(NH_4)_2SO_4$ was added to the resulting supernatant to give 30% saturation and the solution was again stirred for 30 min and centrifuged as before. The floating material was then resuspended in 20 mM-potassium phosphate buffer, pH 7.5, containing 0.05% Triton X-100, 0.1 mM-dithiothreitol and 10% (v/v) ethanediol.

Step 5. The 15-30%-satn. $(NH_4)_2SO_4$ fraction of the ion-exchange pool was applied to an Ultrogel AcA-34 gel-filtration column (71 cm $\times$ 5.2 cm diam.) that had been equilibrated with 20 mM-potassium phosphate buffer, pH 7.5, containing 0.05% Triton X-100, 0.1 mM-dithiothreitol and 10% (v/v) ethanediol. Protein was eluted from the column with the same buffer at 60 ml/h, and fractions were collected every 10 min. Fractions

containing L-mandelate dehydrogenase activity were pooled.

Step 6. The pooled fractions from the gel-filtration column were applied to a second DEAE-Sephacel ion-exchange column (5 cm long $\times$ 2.6 cm diam.) pre-equilibrated with 20 mM-potassium phosphate buffer, pH 7.5, containing 0.05% Triton X-100, 0.1 mM-dithiothreitol and 10% (v/v) ethanediol. The column was washed (2-3 column volumes) with the same buffer containing 0.1 M-NaCl. A linear gradient of 0.1-0.25 M-NaCl was applied (total volume 400 ml) at 20 ml/h, and fractions were collected every 6 min. Fractions containing L-mandelate dehydrogenase activity were pooled, ethanediol was added to a final concentration of 20% (v/v) and the pool was stored at -20 °C.

Enzyme assays

L-Mandelate dehydrogenase activities were assayed by measuring the reduction of 2,6-dichloroindophenol at 600 nm in the presence of *N*-methylphenazonium methosulphate and bovine serum albumin as described previously (Hills & Fewson, 1983a). A ΔA_{600} of -7.18 was taken to correspond to the oxidation of 1 μ mol of substrate at pH 7.5 (Allison *et al.*, 1985a). All enzyme activities are expressed as enzyme units, where 1 unit is the amount of enzyme catalysing the oxidation of 1 μ mol of substrate/min.

Effects of inhibitors were measured after pre-incubation with the enzyme and phosphate buffer (total volume 2.5 ml) at 27 °C for 10 min before addition of 2,6-dichloroindophenol, *N*-methylphenazonium methosulphate, bovine serum albumin and L-mandelate as substrate (to a final volume 3.0 ml).

Electrophoresis

The purity of samples from various stages of the purification was monitored by using SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Slab gels containing 10% or 12.5% (w/v) polyacrylamide were stained and destained as described by Fairbanks *et al.* (1971) and then scanned with an LKB 2202 UltroScan laser densitometer.

Analysis of flavin

Non-covalently bound flavin was removed from the purified enzyme by using trichloroacetic acid as described by Allison *et al.* (1985b). Emission and excitation spectra of neutralized extracts were recorded before and after addition of *Naja naja* snake venom, with a Perkin-Elmer 3000 fluorescence spectrophotometer, and were compared with spectra of standard FMN and FAD.

Analytical methods

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Conductivities were measured with a Radiometer (Copenhagen, Denmark) type CDM2e conductivity meter. Isoelectric points were determined as described by Allison *et al.* (1985b).

RESULTS AND DISCUSSION

Purification of L-mandelate dehydrogenase

In preliminary work various attempts at purification consistently gave a multi-component system containing

Table 1. Purification of L-mandelate dehydrogenase from *A. calcoaceticus* mutant strain C1005

See the text for experimental details.

	Volume (ml)	Protein concentration (mg/ml)	Total activity (units)	Specific activity (units/mg of protein)	Purification factor	Yield (%)
'Wall + membrane' fraction	180	20.0	1502	0.417	1	100
Triton extract	208	3.23	1154	1.72	4	77
First ion-exchange fraction	194	0.25	283	5.84	14	19
(NH ₄) ₂ SO ₄ (15–30% satn.) fraction	31	0.23	75	10.5	25	5.0
Gel-filtration fraction	136	0.034	66	14.2	34	4.4
Second ion-exchange fraction	103	0.01	17	16.1	39	1.1

L-mandelate dehydrogenase activity. SDS/polyacrylamide-gel electrophoresis showed three major proteins of almost equal intensity, with apparent M_r values of 66000, 44000 and 27000. However, it was possible to obtain an almost homogeneous single protein (apparent M_r 44000) with L-mandelate dehydrogenase activity by the procedure described in the Experimental section. A typical purification is summarized in Table 1. Triton X-100 solubilized 60–80% of the starting L-mandelate dehydrogenase activity, giving about a 4-fold purification. The recovery of L-mandelate dehydrogenase activity from the 15–30%-satn.-(NH₄)₂SO₄ fraction was variable (25–80%). Nonetheless, a 2–3-fold purification was obtained at this stage, which allowed the sample to be concentrated before gel filtration. L-Mandelate dehydrogenase activity was eluted at a position corresponding to an apparent M_r of 300000. The last stage of the purification involved a second ion-exchange step, and the gel scan obtained following SDS/polyacrylamide-gel electrophoresis of the purified L-mandelate dehydrogenase is shown in Fig. 1. It can be seen that the final L-mandelate dehydrogenase sample was contaminated with small amounts of the two polypeptides with M_r values of 66000 and 27000. Experiments in which extraction and purification were done in the presence of various concentrations of EDTA, phenylmethanesulphonyl fluoride and benzamidine gave very similar results, so there was no indication that the low yields were due to proteolysis. Although the yield was low there was sufficient enzyme for it to be partially characterized.

Properties of L-mandelate dehydrogenase

Stability. The enzyme retained its activity for several weeks provided it was kept at -20°C in the presence of dithiothreitol, ethanediol and Triton X-100.

Relative molecular mass. Analysis of the purified L-mandelate dehydrogenase by SDS/polyacrylamide-gel electrophoresis gave an apparent M_r value of 44000 (± 800 s.d. for 20 determinations). Gel filtration of the purified sample on a column (30 cm long $\times$ 0.7 cm diam.) of Ultrogel AcA-34 that had been calibrated with proteins of known M_r gave an apparent M_r of 64000 (average of two determinations). This indicates that the enzyme exists as a monomer, because a dimer would have a significantly higher M_r value (approx. 88000). Simple subtraction then suggests that L-mandelate dehydrogenase binds about 0.5 mg of Triton X-100/mg of protein, which is well within the range of the amounts of

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified L-mandelate dehydrogenase

Slab gels containing 12.5% (w/v) polyacrylamide were electrophoresed, stained with Coomassie Blue, destained and scanned as described in the Experimental section. The profile shows the position of the Pharmacia M_r markers ($\downarrow$) and of the purified L-mandelate dehydrogenase (approx. $4 \mu\text{g}$ of protein). The start of the gel ($\downarrow$) and the position of the tracker dye front ($\downarrow$) are also indicated.

Triton bound by other membrane proteins (Helenius & Simons, 1975). The apparent M_r value of the purified L-mandelate dehydrogenase is very different from the apparent M_r obtained on gel filtration of a Triton X-100 extract (M_r 430000; Allison *et al.*, 1985b) and at the gel-filtration step during the purification (M_r 300000), presumably because more proteins were present in the Triton X-100 micelles at the earlier stages of the purification.

pH optimum and isoelectric point. L-Mandelate dehydrogenase has a fairly broad pH range and an optimum in phosphate buffer of pH 7.5, which is similar to that obtained for the unpurified enzyme (Table 2). The isoelectric point was approx. 4.2.

Effect of substrates on L-mandelate dehydrogenase activity. L-Mandelate dehydrogenase was found to be

Table 2. Comparison of the properties of purified L-mandelate dehydrogenase with those of L-mandelate dehydrogenase in extracts of bacteria disrupted by ultrasonic treatment

	Pure enzyme	Bacterial extract*
K_m value for L-mandelate (μM)	186 ± 19 (3)	207 ± 12 (3)
pH value for maximum activity (with pH value for half-maximum activity)	7.5 (5.2, 8.95)	7.8 (6.0, 8.9)
Inhibition obtained with 20 mM-D-mandelate in the presence of 100 μM -L-mandelate (%)	60	69
Decrease in activity if bovine serum albumin was omitted from the reaction mixture (%)	45	43
Decrease in activity if <i>N</i> -methylphenazonium methosulphate was omitted from the reaction mixture (%)	74	76
Decrease in activity if both bovine serum albumin and <i>N</i> -methylphenazonium methosulphate were omitted from the reaction mixture (%)	51	54

* From Hills (1979) and Hills & Fewson (1983a).

completely stereospecific for L-mandelate, and D-mandelate was not oxidized by this enzyme. The K_m for L-mandelate (186 μM) was virtually unaltered from that obtained in crude bacterial homogenates (Table 2). D-Mandelate inhibited L-mandelate dehydrogenase activity only at high concentrations (> 10 mM) and only when L-mandelate was present at a low concentration (Table 2). L-Lactate and D-lactate gave no detectable rate of 2,6-dichloroindophenol reduction, even at a final concentration of 2 mM, and they did not affect L-mandelate dehydrogenase activity even at concentrations of 20 mM in the presence of 100 μM -L-mandelate. The inclusion of bovine serum albumin and *N*-phenazine methosulphate in the reaction mixture gave about double the activity of L-mandelate dehydrogenase, which contrasts with their greater effects on the activity of D-mandelate dehydrogenase (Allison *et al.*, 1985b) but which is very similar to their effects on L-mandelate dehydrogenase activity in bacterial homogenates (Table 2). Triton X-100 could replace bovine serum albumin in the assay of D-mandelate dehydrogenase (Allison *et al.*, 1985b) but not in that of L-mandelate dehydrogenase.

Effects of inhibitors. Purified L-mandelate dehydrogenase was sensitive to most thiol-blocking reagents. HgCl_2 (10 μM) was completely inhibitory. Iodoacetamide (10 mM) or *N*-ethylmaleimide (5 mM) inhibited about three-quarters of the activity, and 50% of the activity was lost after incubation with from 30 μM - to 125 μM -*p*-chloromercuribenzoate, depending on the concentration of enzyme. Iodoacetate (10 mM) did not inhibit. L-Mandelate dehydrogenase was unaffected by 10 mM-EDTA, 4 mM-2,2'-bipyridyl, 10 mM-KCN or 10 mM- NaN_3 . Mepacrine (10 mM) inhibited about half the activity. Oxalate inhibited activity, as it does other NAD(P)^+ -independent bacterial lactate dehydrogenases and mandelate dehydrogenases (Garvie, 1980; Allison *et al.*, 1985b).

FMN as the prosthetic group

The absorption difference spectrum for the pure L-mandelate dehydrogenase with and without L-mandelate showed a peak at 400 nm with a shoulder between 420 and 460 nm. Addition of dithionite produced almost

exactly the same bleaching effect as substrate. These difference spectra were not exactly the same as those of soluble flavoproteins, but may have been affected by the environment within the Triton micelles. Treatment of the purified L-mandelate dehydrogenase with cold trichloroacetic acid resulted in an extract whose emission and excitation fluorescence spectra were identical with those of standard solutions of FAD and FMN. The fluorescence emission at 520 nm (exciting at 450 nm) of the standard solutions and of the trichloroacetic acid extract of L-mandelate dehydrogenase was abolished by the addition of dithionite. The addition of a small crystal of *Naja naja* snake venom to the standard solution of FAD resulted in a 9-fold increase in the fluorescence at 520 nm. However, the snake venom had no effect on the emission fluorescence spectrum of the standard solution of FMN or of the trichloroacetic acid extract of L-mandelate dehydrogenase. This suggests that FMN is the flavin prosthetic group of L-mandelate dehydrogenase.

Comparison of the properties of the four dehydrogenases

The partial characterization of L-mandelate dehydrogenase allows us to complete the comparison of the properties of the two mandelate dehydrogenases and the two lactate dehydrogenases that are present in *A. calcoaceticus* (Table 3). The four dehydrogenases are similar in many respects, and the striking similarities of the two D(−)-specific enzymes and the two L(+)-specific enzymes that were first observed in the crude extracts (Fewson & O'Donnell, 1981; Hills & Fewson, 1983a) prevail in the purified enzymes (Tables 2 and 3; Allison *et al.*, 1985b). The properties summarized in Table 2 indicate that L-mandelate dehydrogenase has been purified in its native conformation, as has been shown for the other enzymes (Allison *et al.*, 1985b; Allison & Fewson, 1986); this is in contrast with some other membrane-bound dehydrogenases, which have shown substantial changes in pH optima, K_m values and substrate specificities on purification (e.g. Futai, 1973; Kohn & Kaback, 1973; Jones & Venables, 1983).

The subunit M_r of L-mandelate dehydrogenase is very similar to that of L-lactate dehydrogenase from *A. calcoaceticus* (Table 2) and to that of the membrane-bound L-lactate dehydrogenase from *Escherichia coli*

Table 3. Comparison of the four purified dehydrogenases

Information is taken from the present paper and from Allison *et al.* (1985*a,b*) and Allison & Fewson (1986).

	L-Mandelate dehydrogenase	L-Lactate dehydrogenase	D-Mandelate dehydrogenase	D-Lactate dehydrogenase
Inducer	Phenylglyoxylate	D- or L-Lactate	Phenylglyoxylate	D- or L-Lactate
Location	All are integral components of the cytoplasmic membrane			
Solubilization by non-ionic detergents (e.g. Triton-X-100 or Lubrol) (% of activity released)	43-72	55-76	87-91	83-98
Solubilization by ionic detergents (e.g. cholate or deoxycholate) (% of activity released)	4-42	13-32	78-91	63-87
M_r (monomer)	44000	40000	60000	63000
pI value	4.2	< 4.0	5.5	5.8
pH optimum	7.5	7.5	8.0	7.7
Specific activity (units/mg of protein)	16.1	27.6	322	338
Stereospecificity	All are stereospecific for their substrate			
K_m value for substrate (μM)	186	83	385 (in addition D-lactate is a substrate but $K_m = 4.8$ mM)	308
Assay conditions	2,6-Dichloroindophenol reduction measured in the presence of <i>N</i> -methylphenazonium methosulphate or <i>N</i> -methylphenazonium ethosulphate. All require bovine serum albumin (or Triton X-100 in the case of D-mandelate dehydrogenase and D-lactate dehydrogenase) in the reaction mixture for full activity			
Inhibitors	None is affected by a wide range of chelating agents. All are moderately susceptible to several thiol-blocking reagents. All are inhibited by oxalate			
Inhibition by <i>p</i> -chloro-mercuribenzoate (concn. giving 50% inhibition)	Moderate (> 30 μM)	Moderate	Very severe (< 1 μM)	Severe (approx. 5 μM)
Cofactor (non-covalently bound)	FMN	FMN	FAD	FAD

(M_r 43000; Futai & Kimura, 1977). The latter two enzymes behave as oligomeric proteins on gel filtration, e.g. L-lactate dehydrogenase from *A. calcoaceticus* has an apparent M_r of 670000 (Allison & Fewson, 1986); however, L-mandelate dehydrogenase seems to exist as a monomer. Purified D-mandelate dehydrogenase and D-lactate dehydrogenase from *A. calcoaceticus* as well as the D-lactate dehydrogenase from *E. coli* are also monomeric proteins, but their M_r values are 60000, 63000 and 65000 respectively (Futai, 1973; Kohn & Kaback, 1973; Campbell *et al.*, 1984; Allison *et al.*, 1985*b*).

D-Mandelate dehydrogenase is rather susceptible to inhibition by L-mandelate and can use D-lactate as a substrate (Table 3; Allison *et al.*, 1985*b*). L-Mandelate dehydrogenase is less susceptible to inhibition by the other enantiomer and cannot dehydrogenate L-lactate (the present paper). The ability of D-mandelate dehydrogenase to dehydrogenate D-lactate may be an evolutionary relic or simply a consequence of the similarity in the chemical structures.

NAD(P)⁺-independent lactate dehydrogenases from some other bacteria have been shown to require bivalent metal ion for activity and are inhibited by metal-ion-chelating reagents (Olson & Massey, 1979), but L-mandelate dehydrogenase, like D-mandelate dehydrogenase, D-lactate dehydrogenase and L-lactate dehydrogenase (Allison *et al.*, 1985*b*; Allison & Fewson, 1986), is not affected by metal-ion chelators and therefore presumably has no metal ion requirement, unless the chelators are sterically prevented from removing the metal ion.

All four dehydrogenases are susceptible to thiol-blocking reagents such as HgCl₂ (Allison *et al.*, 1985*b*; Allison & Fewson, 1986; Table 3), and experiments with the purified enzymes have confirmed the observations first made with crude homogenates (Hills & Fewson, 1983*a*) that D-lactate dehydrogenase and D-mandelate dehydrogenase are much more sensitive to *p*-chloro-mercuribenzoate than are L-mandelate dehydrogenase and L-lactate dehydrogenase (Table 3).

FMN has been identified as the non-covalently bound prosthetic group of L-mandelate dehydrogenase and L-lactate dehydrogenase from *A. calcoaceticus* (Allison & Fewson, 1986; Table 3) and of L-lactate dehydrogenase from *E. coli* (Futai & Kimura, 1977). By contrast, FAD was identified as the prosthetic group of both D-mandelate dehydrogenase and D-lactate dehydrogenase from *A. calcoaceticus* (Allison *et al.*, 1985*b*), and in this respect these latter two enzymes resemble the membrane-bound D-lactate dehydrogenase from *E. coli* (Futai, 1973) and the soluble D-lactate dehydrogenase from *Megasphaera elsdenii* (Brockman & Wood, 1975), both of which contain FAD.

The striking similarities between the L-mandelate dehydrogenase and L-lactate dehydrogenase and between D-mandelate dehydrogenase and D-lactate dehydrogenase with reference to their ease of extraction by detergents, subunit M_r , pH optima, pI values, susceptibility to thiol-blocking reagents and presence of FMN or FAD as cofactors (Table 3) may suggest that each of these pairs of enzymes had a common evolutionary origin. It is much less easy to imagine that all four

enzymes had a common origin in view of the fairly substantial differences between the two D(−)-specific and the two L(+)-specific enzymes (Table 3), despite the original experiments with crude extracts that indicated they were all rather similar in nature (Fewson & O'Donnell, 1981; Hills & Fewson, 1983*a*). D-Mandelate dehydrogenase and L-mandelate dehydrogenase appear to be genetically co-regulated (Hills & Fewson, 1983*b*), as do D-lactate dehydrogenase and L-lactate dehydrogenase (Allison *et al.*, 1985*a*), and the genes responsible for D-mandelate dehydrogenase and L-mandelate dehydrogenase activities appear to map close to each other (Vakeria *et al.*, 1984), and so, whether or not D-mandelate dehydrogenase has arisen as a result of expression of a previously silent gene (Hills & Fewson, 1983*b*; Vakeria *et al.*, 1984), the regulatory systems for the pair of mandelate enzymes and the pair of lactate enzymes appear to have evolved in different directions. The close resemblance of the pair of L(+)-specific enzymes and the pair of D(−)-specific enzymes from *A. calcoaceticus* to the L- and D-lactate dehydrogenases respectively of *E. coli* suggests that this would be another fruitful set of enzymes in which to look for sequence homologies.

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