Membrane-bound lactate dehydrogenases and mandelate dehydrogenases of *Acinetobacter calcoaceticus*

Purification and properties

Nigel ALLISON,* Michael J. O'DONNELL and Charles A. FEWSON

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Procedures were developed for the optimal solubilization of D-lactate dehydrogenase, D-mandelate dehydrogenase, L-lactate dehydrogenase and L-mandelate dehydrogenase from wall + membrane fractions of *Acinetobacter calcoaceticus*. D-Lactate dehydrogenase and D-mandelate dehydrogenase were co-eluted on gel filtration, as were L-lactate dehydrogenase and L-mandelate dehydrogenase. All four enzymes could be separated by ion-exchange chromatography. D-Lactate dehydrogenase and D-mandelate dehydrogenase were purified by cholate extraction, $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration, ion-exchange chromatography and chromatofocusing. The properties of D-lactate dehydrogenase and D-mandelate dehydrogenase were similar in several respects: they had relative molecular masses of 62800 and 59700 respectively, pI values of 5.8 and 5.5, considerable sensitivity to *p*-chloromercuribenzoate, little or no inhibition by chelating agents, and similar responses to pH. Both enzymes appeared to contain non-covalently bound FAD as cofactor.

INTRODUCTION

Acinetobacter calcoaceticus N.C.I.B. 8250 can use lactate and mandelate as sole sources of carbon and energy for growth (Scheme 1; Fewson, 1967). Lactate is converted into pyruvate by a pair of stereospecific lactate dehydrogenases, which are co-ordinately induced by either D(–) or L(+)–lactate (Allison *et al.*, 1985). L(+)–Mandelate is oxidized to phenylglyoxylate by L-mandelate dehydrogenase in the wild-type strain, and some mutants also have a D-mandelate dehydrogenase, which allows growth on D(–)-mandelate (Hills & Fewson, 1983*a*). Phenylglyoxylate induces both mandelate dehydrogenases (Hills & Fewson, 1983*b*).

D-Lactate dehydrogenase, L-lactate dehydrogenase, D-mandelate dehydrogenase and L-mandelate dehydrogenase have several features in common. They are all NAD(P)-independent and are integral components of the cytoplasmic membrane (Allison *et al.*, 1985). Experiments with crude extracts have indicated that D-lactate dehydrogenase and D-mandelate dehydrogenase are particularly similar to each other, as are L-lactate dehydrogenase and L-mandelate dehydrogenase; the enzymes oxidizing the D(–)-enantiomers are more easily solubilized from membranes, more heat-labile and more sensitive to inhibition by *p*-chloromercuribenzoate (Fewson & O'Donnell, 1981; Allison *et al.*, 1985).

Pure forms of all four enzymes are required in order to compare their properties further and to provide a platform for speculation about their possible common evolutionary origin. There appears to have been no previous report of the purification of a mandelate dehydrogenase, and those NAD(P)-independent lactate dehydrogenases that have been characterized (Garvie, 1980) were from organisms quite different from *A. calcoaceticus*. In the present paper we describe the

separation of the four dehydrogenases and the purification and partial characterization of D-lactate dehydrogenase and D-mandelate dehydrogenase.

EXPERIMENTAL

Materials

Chemicals were of the best quality commercially available, and most of them were obtained from the sources described by Hills & Fewson (1983*a*) and Allison *et al.* (1985). Materials for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and *N*-methylphenazonium methosulphate were obtained from BDH Chemicals, Poole, Dorset, U.K. DEAE-Sephacel, Sephadex G-150 (superfine grade), Polybuffer exchanger PBE 94 and Polybuffer PB74 were from Pharmacia, Milton Keynes, Bucks., U.K. Ultrogel AcA-34 was obtained from LKB Instruments, South Croydon, Surrey, U.K. Molecular-mass markers for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were obtained from BDH Chemicals (cytochrome *c*, M_r 12300; myoglobin, M_r 17200; chymotrypsinogen A, M_r 25700; ovalbumin, M_r 45000; albumin, M_r 66200; ovotransferrin, M_r 77000) and Pharmacia (α -lactalbumin, M_r 14000; trypsin inhibitor, M_r 20100; carbonic anhydrase, M_r 30000; ovalbumin, M_r 43000; albumin, M_r 67000; phosphorylase *b*, M_r 94000). Molecular-mass markers for gel filtration were obtained from Pharmacia (thyroglobulin, M_r 669000; ferritin, M_r 440000; catalase, M_r 232000; fructose biphosphate aldolase, M_r 158000), Sigma Chemical Co., Poole, Dorset, U.K. (ovalbumin, M_r 45000; chymotrypsinogen A, M_r 25700; cytochrome *c*, M_r 12300), Boehringer Mannheim, Lewes, East Sussex, U.K. (alkaline phosphatase, M_r 100000) and Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. (bovine

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

* Present address: Department of Microbiology, University of Sheffield, Sheffield S10 2TN, U.K.

Scheme 1. Metabolism of lactate and mandelate by *A. calcoaceticus*

Key to enzymes: 1, L-lactate dehydrogenase; 2, D-lactate dehydrogenase; 3, L-mandelate dehydrogenase; 4, D-mandelate dehydrogenase.

serum albumin, M_r 66200). *Naja naja* venom, the lithium salt of D(–)-lactic acid, the free acid form (grade L-1) of L(+)–lactic acid and *N*-methylphenazonium ethosulphate were obtained from Sigma Chemical Co.

Organisms

Acinetobacter calcoaceticus N.C.I.B. 8250 (inducible L-mandelate dehydrogenase, no D-mandelate dehydrogenase) was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K. Mutant strains C1123 (N.C.I.B. 11456; constitutive D-mandelate dehydrogenase, no L-mandelate dehydrogenase) and C1219 (N.C.I.B. 11457, constitutive D- and L-mandelate dehydrogenases) were derived from the wild-type strain N.C.I.B. 8250 and were described by Hills & Fewson (1983a).

Growth of bacteria

Bacteria were maintained as described previously (Allison *et al.*, 1985). For experiments in which all four dehydrogenases were examined, mutant strain C1219 was grown in complex medium plus DL-mandelate and DL-lactate as described by Allison *et al.* (1985). When large amounts of bacteria were required for the purification of D-lactate dehydrogenase and D-mandelate dehydrogenase, mutant strain C1213 was grown in complex medium plus DL-mandelate and DL-lactate in a 10-litre fermenter (Braun Biostat V; F. T. Scientific Instruments, Tewkesbury, Glos., U.K.). An inoculum was prepared by aseptically transferring 0.1 ml of a stock culture into 50 ml of the complex medium (in a 250 ml conical flask) and shaking (120 rev./min) at 23 °C for 24 h. The whole of this culture was then aseptically transferred to 400 ml of complex medium (in a 2-litre conical flask) and shaken (120 rev./min) at 23 °C for 24 h. The entire culture was used to inoculate the fermenter, which contained 10 litres of complex medium and 50 ml of sterile 1% poly(propylene glycol) 2025 (BDH Chemicals) as antifoam. The fermenter was operated at 23 °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, the bacteria were harvested, washed and stored at –20 °C (Hills & Fewson, 1983b).

Preparation of a Triton extract containing all four dehydrogenases

A 'wall + membrane' fraction was prepared from 35 g wet wt. of frozen bacteria (mutant strain C1219 grown in complex medium plus DL-mandelate and DL-lactate) as described by Allison *et al.* (1985) and stirred gently for 30 min at 4 °C with Triton X-100 added to a final concentration of 0.5 mg of detergent/mg of protein. The suspension was centrifuged (113000 *g* for 120 min at 4 °C) and the supernatant ('Triton extract') was carefully decanted and stored at –20 °C until required.

Purification of D-lactate dehydrogenase and D-mandelate dehydrogenase

Step 1. A 'wall + membrane' fraction was prepared as described by Allison *et al.* (1985) from 70 g wet wt. of frozen bacteria of mutant strain C1123 that had been grown in complex medium plus DL-lactate and DL-mandelate.

Step 2. Sodium cholate (10%, w/v) was added to the 'wall + membrane' fraction to a final concentration of 1.5 mg/mg of protein. The suspension was stirred gently for 30 min at 4 °C and then centrifuged at 113000 *g* for 120 min at 4 °C. The supernatant ('cholate extract') was stored at –20 °C until needed.

Steps 1 and 2 were repeated, with a further 70 g of bacteria, and the two cholate extracts were combined for the following step.

Step 3. Solid $(\text{NH}_4)_2\text{SO}_4$ (specially low in heavy metals for enzyme work) was slowly added to the combined cholate extracts, while gently stirring at 4 °C, to give 30% saturation. When all the $(\text{NH}_4)_2\text{SO}_4$ had dissolved, the solution was stirred for another 30 min before being centrifuged at 113000 *g* for 30 min at 4 °C. More $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give 40% saturation, and the solution was stirred and centrifuged as before. The pellet was dissolved in 20 mM-potassium

phosphate buffer, pH 7.5, containing 0.1% (w/v) Triton X-100 and 100 μ M-dithiothreitol.

All buffers and solutions used in subsequent steps contained 0.1% (w/v) Triton X-100 and 100 μ M-dithiothreitol, and all procedures were carried out at 4 °C unless stated otherwise.

Step 4. The 30–40%-(NH₄)₂SO₄ fraction was applied to a column of Ultrogel AcA-34 (bed volume 1400 ml, 71 cm long $\times$ 5.2 cm diam.). Protein was eluted with 20 mM-potassium phosphate buffer, pH 7.5, at a rate of 60 ml/h, fractions being collected every 10 min. The A_{280} of the effluent was monitored and fractions were assayed for dehydrogenase activity. D-Lactate dehydrogenase and D-mandelate dehydrogenase were co-eluted from this column, and fractions containing more than 25% of the activity of the peak fraction were pooled.

Step 5. The dehydrogenase pool after gel filtration was applied to a column of DEAE-Sephacel (15 cm long $\times$ 2.5 cm diam.) pre-equilibrated with 20 mM-potassium phosphate buffer, pH 7.5. The column was washed (approx. 1–2 column volumes) with this buffer to remove unbound material before elution of protein with a linear gradient of 20–260 mM-potassium phosphate buffer, pH 7.5 (total volume 450 ml), at 20 ml/h. Fractions were collected at 5 min intervals. D-Lactate dehydrogenase was eluted (70–80 mM-potassium phosphate) before D-mandelate dehydrogenase (100–110 mM-potassium phosphate). Separate pools of D-lactate dehydrogenase and D-mandelate dehydrogenase were made by combining appropriate fractions.

Step 6. The final step of the purification procedure for each enzyme involved the use of f.p.l.c. chromatofocusing. Each enzyme pool was dialysed for 16 h against 20 vol. of 25 mM-Bis-Tris/HCl buffer, pH 6.7, containing 5% (v/v) ethanediol. The non-diffusible material and all the solutions used for f.p.l.c. were filtered (0.22 μ m-pore-size Millipore membrane filter) before use. The f.p.l.c. Mono P column (5 ml bed volume) was equilibrated with 25 mM-Bis-Tris/HCl buffer, pH 6.7, containing 5% (v/v) ethanediol before application of a portion of the dialysed and filtered dehydrogenase pool. A pH gradient was set up in the column by using Polybuffer PB74 diluted 1:10 with water, adjusted to pH 4.8 with 0.1 M-HCl and containing 5% (v/v) ethanediol. Fractions (1 ml) were collected every minute, and those containing peak dehydrogenase activity were combined. D-Mandelate dehydrogenase was very unstable at its isoelectric point (pH 5.5), so, to minimize loss of enzyme activity, 0.1 ml of 1 M-potassium phosphate buffer, pH 7.5, was added to all the tubes before the fractions were collected. Further ethanediol was added to each enzyme pool after f.p.l.c. to give a final concentration of 10% (v/v), and the pure enzymes were stored at –20 °C.

If necessary, pools from chromatofocusing columns were precipitated with (NH₄)₂SO₄ (80% satn.), dissolved in 20 mM-potassium phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 100 μ M-dithiothreitol and either 10% or 20% (v/v) ethanediol and then dialysed overnight against 3 litres of the same buffer before storage at –20 °C.

Determination of isoelectric points

This was done with conventional column chromatofocusing. D-Lactate dehydrogenase or D-mandelate dehydrogenase samples after ion-exchange chromatography were dialysed for 16 h against 20 vol. of 25 mM-

imidazole/HCl buffer, pH 6.9, before application to a 12 ml column of Polybuffer exchanger PBE 94 pre-equilibrated with the same buffer. Protein was eluted with Polybuffer PB 74 diluted 1:8 with water and adjusted to pH 4.8 with HCl, at a rate of 20 ml/h. Fractions were collected every 5 min and assayed for dehydrogenase activity. The A_{280} of the effluent was monitored and the pH value of each fraction was determined. The pI values quoted are averages for at least three different experiments.

Preparation of buffers and determination of pH optima

Unless otherwise stated, buffers were prepared at room temperature by adjusting the pH values of approximately five-fourths-strength solutions with HCl or NaOH and then making to volume.

The activities of pure dehydrogenases were measured in cuvettes containing appropriate buffers (final concentrations 66.7 mM) at different pH values in the range 5.8–9.0. In each case the pH value of the final reaction mixture was measured shortly after the completion of the spectrophotometric assay.

Enzyme assays

L-Lactate dehydrogenase, L-mandelate dehydrogenase, D-lactate dehydrogenase and D-mandelate dehydrogenase activities were assayed by measuring the reduction of 2,6-dichloroindophenol at 600 nm in the presence of *N*-methylphenazonium methosulphate. This was done as described previously (Hills & Fewson, 1983a; Allison *et al.*, 1985), except that the amount of D-lactate and D-mandelate used in the assays of the respective dehydrogenases was increased to 6 μ mol in order to ensure conditions close to substrate saturation. In the present work an A_{600} of –7.18 was taken to correspond to the oxidation of 1 μ mol of substrate at pH 7.5 (see Armstrong, 1964).

All enzyme activities are expressed as enzyme units, where 1 unit is defined as the amount of enzyme catalysing the oxidation of 1 μ mol of substrate/min.

Electrophoresis

The purity of samples from various stages of the purification procedure was monitored by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970). Tube gels containing 10% (w/v) polyacrylamide were stained, destained and scanned as described by Lumsden & Coggins (1977). Measurements of relative molecular masses were done in the same way except that in some of the later experiments we used 7.5%, 10% or 12.5% (w/v) polyacrylamide slab gels. These were stained and destained as described by Fairbanks *et al.* (1971) and then scanned with an LKB 2202 UltroScan laser densitometer.

Non-denaturing gel electrophoresis was done in tube gels as above except that sodium dodecyl sulphate was replaced by 0.1% Triton X-100 in all buffers and gels, and the samples were not boiled. After electrophoresis, gels were stained either for protein with Coomassie Brilliant Blue G-250 or for dehydrogenase activity by incubation for 30 min at 30 °C in the dark in a mixture containing 80 mM-potassium phosphate buffer, pH 7.5, 4 mg of bovine serum albumin/ml, 0.4 mM-*N*-methylphenazonium methosulphate, 0.24 mM-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 2.4 mM substrate (either D-lactate or D-mandelate).

Fig. 1. Gel filtration of an extract of *A. calcoaceticus* mutant strain C1219 solubilized with Triton X-100

A Triton extract (25 ml), prepared as described in the Experimental section, containing 136 units of D-lactate dehydrogenase, 39 units of L-lactate dehydrogenase, 96 units of D-mandelate dehydrogenase and 136 units of L-mandelate dehydrogenase was applied to a column (71 cm long $\times$ 5.2 cm diam.) of Ultrogel AcA-34. The proteins were eluted with 20 mM-potassium phosphate buffer, pH 7.5, containing 0.1% Triton X-100 and 100 μ M-dithiothreitol at a flow rate of 60 ml/h. Fractions (5.15 ml) were assayed for protein by the Bradford (1976) method (■) and for D-lactate dehydrogenase (▲), L-lactate dehydrogenase (●), D-mandelate dehydrogenase (△) and L-mandelate dehydrogenase (○) activities.

Absorption spectra and analysis of flavin

Enzyme solutions were filtered (0.22 μ m-pore-size Millipore filters) before absorption spectra were recorded. The absorption spectra of pure enzymes (200–300 μ g of protein) in 20 mM-potassium phosphate buffer, pH 7.5, containing 0.1% Triton X-100 were determined in 1 ml masked quartz cuvettes with a Cary 219 (Varian) scanning spectrophotometer.

Non-covalently bound flavin was removed from pure enzyme (150–200 μ g of protein) by the addition of ice-cold trichloroacetic acid to a final concentration of 10% (w/v). After standing at 4 °C for 15 min the protein precipitate was removed by centrifugation (15000 *g* for 10 min) and re-extracted by a second identical treatment with trichloroacetic acid. The supernatants were combined and extracted twice with 4 vol. of diethyl ether. Residual ether was removed under a stream of N₂. The trichloroacetic acid extract was neutralized by adding 0.5 ml of 1 M-potassium phosphate buffer, pH 7.5, and made up to a final volume of 3 ml with 0.1 M-potassium phosphate buffer, pH 7.5. The emission and excitation spectra of neutralized extracts were recorded in 3 ml quartz cuvettes with a Perkin-Elmer 3000 fluorescence spectrometer. The flavin content was quantified by comparing the fluorescence of the neutralized extract at 540 nm (exciting at 450 nm) with those of standard FMN and FAD solutions (Bessey *et al.*, 1949).

Analytical methods

Protein was usually measured by the procedure of Lowry *et al.* (1951), except in the case of enzyme pools obtained by chromatofocusing, when the Bradford (1976) method was used in order to minimize interference from Polybuffer. Bovine serum albumin was used as the standard for both methods.

Conductivities were measured with a Radiometer (Copenhagen, Denmark) type CDM2e conductivity meter.

RESULTS AND DISCUSSION

Optimal conditions for solubilization of the enzymes

Previous work showed that active forms of all four enzymes were solubilized by non-ionic detergents such as Triton X-100 and that D-lactate dehydrogenase and D-mandelate dehydrogenase were selectively solubilized by ionic detergents such as sodium cholate (Allison *et al.*, 1985). Optimal conditions for enzyme solubilization have now been defined. The success of solubilization was dependent on the concentration of detergents and especially on the detergent/protein ratio; too little detergent failed to release the enzymes, whereas too much caused loss of activity.

Maximum release of D-mandelate dehydrogenase and D-lactate dehydrogenase was achieved by using about 0.2–0.3 mg of Triton X-100/mg of protein. A somewhat higher detergent/protein ratio of about 0.3–0.5 mg of Triton X-100/mg of protein was required effectively to solubilize L-mandelate dehydrogenase or L-lactate dehydrogenase and the extent of solubilization of these two enzymes was rather variable from experiment to experiment. Inclusion of 0.4 M-NaClO₄ did not improve the solubilization, nor did 0.15 M-KCl. None of the dehydrogenases showed any significant loss of activity in Triton X-100-solubilized extracts during overnight storage at 4 °C or for 9 days at –20 °C. Nonidet P-40 behaved very like Triton X-100. Lubrol PX also yielded good solubilization of all four dehydrogenases; however, 65–85% of L-lactate dehydrogenase or L-mandelate dehydrogenase activity was lost during storage as a Lubrol PX-solubilized extract for 9 days at –20 °C. Lubrol WX was less effective at solubilizing the enzymes than was Lubrol PX, but resulted in less loss of activity during subsequent storage.

Sodium cholate solubilized less protein per mg of detergent than did Triton X-100. Although D-lactate dehydrogenase and D-mandelate dehydrogenase were solubilized by cholate, L-lactate dehydrogenase and

Fig. 2. Separation of the four dehydrogenases by ion-exchange chromatography

A Triton extract (9 ml) prepared from mutant strain C1219 and containing 35.2 units of D-lactate dehydrogenase, 7.6 units of L-lactate dehydrogenase, 20.2 units of D-mandelate dehydrogenase and 17.4 units of L-mandelate dehydrogenase was applied to a column (15.5 cm long $\times$ 2.5 cm diam.) of DEAE-Sephacel, pre-equilibrated with 20 mM-potassium phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 100 μ M-dithiothreitol and 10% (v/v) ethanediol. The column was eluted with a linear gradient of 0–0.5 M-NaCl (total volume 900 ml) prepared in the same buffer. The flow rate was 20 ml/h. Fractions (2.5 ml) were collected and assayed for D-lactate dehydrogenase (▲), L-lactate dehydrogenase (●), D-mandelate dehydrogenase (△) and L-mandelate dehydrogenase (○) activities. The conductivities (□) were also measured.

L-mandelate dehydrogenase were left associated with the membrane, still in active forms. These results contrast with those reported by Futai & Kimura (1977), who found that L-lactate dehydrogenase could be solubilized from *Escherichia coli* membranes by sodium cholate in amounts sufficient for purification. Attempts to solubilize L-lactate dehydrogenase and L-mandelate dehydrogenase from membranes after cholate extraction, by using 0.5 mg of Triton X-100/mg of protein, were unsuccessful. The sodium cholate/protein ratio yielding the optimal release of D-lactate dehydrogenase and D-mandelate dehydrogenase was found to be about 1.5 mg of cholate/mg of protein. Both of these enzymes were stable during storage of cholate-solubilized extracts overnight at 4 °C or for 9 days at –20 °C.

Treatment of intact bacteria with Triton X-100 or sodium cholate solubilized substantial amounts of D-lactate dehydrogenase and D-mandelate dehydrogenase, but not the other two enzymes. Unfortunately, the yield was not sufficiently high to allow the disruption step to be omitted from the purification procedure.

Protection of enzyme activities

A low concentration (0.1–0.2%) of detergent was always included in solutions in order to prevent precipitation of the enzymes. Both D-lactate dehydrogenase and D-mandelate dehydrogenase are extremely susceptible to inactivation by certain thiol-blocking reagents, so dithiothreitol was added to all solutions. Glycerol and ethanediol were found to stabilize L-lactate dehydrogenase and L-mandelate dehydrogenase during chromatographic procedures.

Gel-filtration chromatography of a Triton X-100 extract

L-Lactate dehydrogenase and L-mandelate dehydrogenase were co-eluted on gel filtration (apparent M_r 430000), as were D-lactate dehydrogenase and D-

mandelate dehydrogenase (apparent M_r 210000) (Fig. 1). The elution pattern was virtually the same when the buffer described in the legend to Fig. 1 was replaced by 0.1 M-Tris/HCl, pH 7.5, containing 0.2% Triton X-100, 100 μ M-dithiothreitol and 30% (v/v) glycerol.

Ion-exchange chromatography of a Triton X-100 extract

All four activities could be separated by chromatography on DEAE-Sephacel (Fig. 2), proving that there are indeed four separate enzymes. Trailing of D-lactate dehydrogenase activity into the D-mandelate dehydrogenase peak can be explained by the fact that D-mandelate dehydrogenase has some activity towards D-lactate as substrate (see below).

L-Lactate dehydrogenase and L-mandelate dehydrogenase

Neither of these enzymes has yet been purified to homogeneity, partly because of problems that we have encountered as a result of their sensitivity to high salt concentrations and low pH values.

Purification of D-lactate dehydrogenase and D-mandelate dehydrogenase

Both enzymes were purified from mutant strain C1123, which has no L-mandelate dehydrogenase activity (Hills & Fewson, 1983a). Typical results are shown in Table 1.

Sodium cholate was used for solubilization in preference to Triton X-100 because it selectively solubilized these two enzymes. Over 90% of the total membrane activities of D-lactate dehydrogenase and D-mandelate dehydrogenase were usually released into the 'soluble' fraction.

Treatment with $(\text{NH}_4)_2\text{SO}_4$ gave some purification and allowed the material to be concentrated before gel filtration. The detergent was changed to Triton X-100 at this stage. This increased the stability of the enzyme

Table 1. Purification of D-lactate dehydrogenase and D-mandelate dehydrogenase from *A. calcoaceticus* mutant strain C1213

For full experimental details see the text.

	D-Lactate dehydrogenase						D-Mandelate dehydrogenase					
	Volume (ml)	Concn. of protein (mg/ml)	Total activity (units)	Specific activity (units/ mg of protein)	Purification factor	Yield (%)	Volume (ml)	Concn. of protein (mg/ml)	Total activity (units)	Specific activity (units/ mg of protein)	Purification factor	Yield (%)
'Wall + membrane' fraction*	(a) 60 (b) 70	24.5 23.9	593 605	0.40 0.36	1†	100†	60 70	24.5 23.9	620 633	0.42 0.38	1†	100†
Cholate extract*	(a) 65.5 (b) 71.5	4.50 3.59	667 643	2.26 2.51	6.2†	109†	65.5 71.5	4.50 3.59	591 643	2.00 2.51	5.6†	99†
(NH ₄) ₂ SO ₄ (30-40% satn.)	61	1.94	722	6.10	16	60	61	1.94	722	6.10	15	58
Gel filtration	165	0.24	554	14.0	36	46	165	0.24	636	16.1	40	51
DEAE-Sephacel	59	0.15	271	30.6	80	23	72	0.14	263	26.1	65	21
Chromatofocusing	2.4	0.058	47	338	880	4‡	6.7	0.032	69	322	800	5‡

*The first two steps were carried out twice and the cholate extracts then bulked (see the Experimental section).

†Values are calculated from the sums of the results for the individual fractions.

‡Only about half the pool from ion-exchange chromatography was chromatofocused.

Fig. 3. Chromatofocusing of D-lactate dehydrogenase

A sample from a dialysed pool of D-lactate dehydrogenase after ion-exchange chromatography (see step 5 of the purification procedure in the Experimental section) was applied to a 12 ml column of PBE 94 and chromatofocused as described in the Experimental section for the measurement of isoelectric points. Fractions were collected every 5 min (20 ml/h) and the A_{280} (○), pH (□) and D-lactate dehydrogenase activity (▲) were measured.

activities over prolonged periods. More importantly, Triton X-100 was a better detergent to use in subsequent purification procedures, probably because it has a lower critical micellar concentration than cholate, is not susceptible to precipitation below pH 7 and has no net charge (Helenius & Simons, 1975; Tanford & Reynolds, 1976).

D-Lactate dehydrogenase and D-mandelate dehydrogenase were co-eluted from the Ultrogel AcA-34 column in a single peak of activity (apparent M_r 130 000). The pattern was much as previously observed for gel filtration of a Triton X-100 extract of strain C1219 (Fig. 1) except that the enzyme activity peak was broader and the apparent M_r was lower (presumably because the micelles now contained less of other membrane components). There was usually more than 75% recovery of the activities of both enzymes after gel filtration.

Ion-exchange chromatography of the pooled fractions from gel filtration separated the two enzymes. D-Lactate dehydrogenase was eluted at a lower phosphate concentration (70–80 mM) than was D-mandelate dehydrogenase (100–110 mM). The overall recovery of both enzyme activities from this column was 60–70%. However, in order to achieve a better purification at the expense of enzyme yield, only about 70% of each recovered activity was taken through to the next stage.

Chromatofocusing was used as the final purification step for both enzymes. D-Lactate dehydrogenase proved to be extremely stable at its isoelectric point (pH 5.8); a sample from the pool after gel filtration lost no activity when stored at 4 °C for 24 h in Polybuffer, pH 4.0. D-Mandelate dehydrogenase activity of this material was, however, adversely affected by low pH; about 80% of the activity was lost after 24 h storage at the isoelectric point

(pH 5.5). This explains why the recovery of D-mandelate dehydrogenase activity from conventional column chromatofocusing experiments was so poor (< 20%) compared with D-lactate dehydrogenase (> 60%). The recovery of D-mandelate dehydrogenase from chromatofocusing was improved by: (a) using f.p.l.c., which lowered the separation time from 3–4 h for conventional columns to about 45 min; (b) including 5% (v/v) ethanediol in all the buffers; (c) adding potassium phosphate buffer, pH 7.5, to all the collecting tubes, thus raising the pH well above the isoelectric point as soon as possible. D-Lactate dehydrogenase could be satisfactorily purified by using conventional chromatofocusing (Fig. 3); however, the experiment summarized in Table 1 involved f.p.l.c. for both enzymes so that their purification could be compared.

The degree of purification at each step, as well as the overall purification, were similar for both enzymes (Table 1). The results suggest that each enzyme constitutes only about 0.1% of the total protein of the 'wall + membrane' fraction.

D-Lactate dehydrogenase was also purified from the wild-type strain N.C.I.B. 8250 after growth on complex medium containing DL-lactate, and its properties appeared to be exactly the same as those of the enzyme from mutant C1213.

Homogeneity and relative molecular masses of the purified enzymes

The elution profile of D-lactate dehydrogenase activity from the chromatofocusing column coincided with a peak in the A_{280} trace (Fig. 3). Similar results were obtained for D-mandelate dehydrogenase. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the D-lactate dehydrogenase and D-mandelate dehydrogenase pools after chromatofocusing revealed, in each case, a single band after staining with Coomassie Blue (Fig. 4). M_r values of 62 812 ($\pm$ 1986 s.d. for ten determinations on four batches of enzyme) and 59 720 ($\pm$ 1457 s.d. for 16 determinations on three batches of enzyme) were obtained for D-lactate dehydrogenase and D-mandelate dehydrogenase respectively. The apparent M_r values were the same whether measured in 7.5%, 10% or 12.5% (w/v) polyacrylamide gels.

Non-denaturing polyacrylamide-gel electrophoresis of the purified D-lactate dehydrogenase in the presence of 0.1% Triton X-100 revealed a single protein band coincident with D-lactate dehydrogenase activity (R_F 0.138). Non-denaturing electrophoresis of the purified D-mandelate dehydrogenase also gave a single band of protein and enzyme activity (R_F 0.125).

Gel filtration of samples that contained both D-lactate dehydrogenase and D-mandelate dehydrogenase activities always showed co-elution of the enzymes (e.g. Fig. 1). Gel filtration of purified D-lactate dehydrogenase through a Sephadex G-150 column that had been calibrated with molecular-mass standards indicated an apparent M_r of 101 000. This implies that the enzyme exists as a monomer, because a dimer would have a significantly higher M_r value (approx. 126 000). Simple subtraction then suggests that D-lactate dehydrogenase binds about 0.6 mg of Triton X-100/mg of protein, which is well within the range of the amounts of Triton bound by other membrane proteins (Helenius & Simons, 1975); however, there must be some uncertainty about the interpretation of molecular sizing of protein-Triton complexes by using

Fig. 4. Polyacrylamide-gel electrophoresis of purified D-lactate dehydrogenase and D-mandelate dehydrogenase

Slab gels containing 12.5% (w/v) polyacrylamide were electrophoresed, stained with Coomassie Blue, destained and scanned with an LKB densitometer as described in the Experimental section. The profiles show: (a) molecular-mass markers; (b) and (c) D-lactate dehydrogenase and D-mandelate dehydrogenase (approx. 0.25 μ g of each) respectively; (d) control (unloaded) track. The tops of the gels are aligned ($\downarrow$) and the position of the Bromophenol Blue tracker dye is indicated ($\downarrow$).

gel filtration, even though the elution patterns of the molecular-mass standards were not significantly affected by the presence of 0.1% Triton X-100.

The D-lactate dehydrogenase of *A. calcoaceticus* appears to be similar in size to the membrane-bound NAD(P)-independent D-lactate dehydrogenase of *E. coli*, which has an M_r of 64613, based on the nucleotide sequence (Campbell *et al.*, 1984), and is also monomeric (Futai, 1973; Kohn & Kaback, 1973). By contrast, the membrane-bound L-lactate dehydrogenase of *E. coli* appears to be multimeric, and has subunits of M_r 43000 (Futai & Kimura, 1977).

Properties of D-lactate dehydrogenase and D-mandelate dehydrogenase

Stability. Both enzymes were stable for many months provided that they were kept at -20°C in the presence of 10% (v/v) ethanediol at neutral pH.

Isoelectric points and pH optima. The pI values of D-lactate dehydrogenase and D-mandelate dehydrogenase were 5.8 and 5.5 respectively.

The pH optimum for D-lactate dehydrogenase activity in potassium phosphate buffer was found to be 7.7, with

half-maximal activity occurring at pH 6.8 and pH 8.8. When the phosphate buffer was replaced by equimolar concentrations of Mes, 2-(cyclohexylamino)ethanesulphonic acid or Tris buffers, similar enzyme rates were obtained over the pH ranges where there was effective buffering. D-Mandelate dehydrogenase activity was maximal at pH 8.0 with phosphate buffer, with 50% of the maximum rate occurring at pH 7.0 and pH 9.0. Mes and Tricine buffers gave essentially the same activity as phosphate buffer over the common pH ranges.

The pH optima observed for the pure enzymes did not differ greatly from the value of pH 7.8 obtained with crude ultrasonic extracts of bacteria, by Hills & Fewson (1983a) for D-mandelate dehydrogenase or in the present work for D-lactate dehydrogenase.

These experiments did not involve determinations of maximum velocities at each pH value; nevertheless the results are useful for comparative purposes and show the similarities between the two enzymes and between the purified and crude preparations. Changes of at least 1 pH unit have been reported for the pH optima of some membrane-bound dehydrogenases after solubilization (Futai, 1973; Kohn & Kaback, 1973; Jones & Venables, 1983).

Effects of Triton, substrates and electron acceptors. Under standard assay conditions, both D-lactate dehydrogenase and D-mandelate dehydrogenase gave linear progress curves for several minutes. If bovine serum albumin was omitted from the reaction mixture for either enzyme there was a 5–10-fold decrease in the rate and a pronounced deceleration; addition of bovine serum albumin back to such assays only partially restored the rate. Triton X-100 (10 mg/3 ml of reaction mixture) could completely replace bovine serum albumin for both enzymes with respect to rate and to linearity of the reactions. Furthermore, addition of Triton X-100 to an assay mixture containing neither bovine serum albumin nor added detergent resulted in the immediate restoration of the full rate and a linear progress curve. Pratt *et al.* (1979) noted that, when Triton X-100 was omitted from the assay solution for the D-lactate dehydrogenase from *E. coli*, the activity was 3–5-fold lower than in the presence of 1% Triton.

When *N*-methylphenazonium methosulphate was omitted from the reaction mixture, D-mandelate dehydrogenase activity was decreased by approx. 70% and D-lactate dehydrogenase activity was decreased by 90%. Ghosh & Quayle (1979) have discussed the advantages of using *N*-methylphenazonium ethosulphate in dye-coupled assays, and certainly we found that the use of this compound lowered the 'blank' (minus substrate) rates for both enzymes. However, the apparent maximum velocity for D-mandelate dehydrogenase was almost identical with either compound, and the apparent maximum velocity for D-lactate dehydrogenase was lowered by about 30% if *N*-methylphenazonium methosulphate was replaced by the ethosulphate. Further, both enzymes were inhibited by high concentrations (> 1 mM) of *N*-methylphenazonium ethosulphate, whereas *N*-methylphenazonium methosulphate had no inhibitory effect at these concentrations. The concentrations of *N*-methylphenazonium methosulphate and *N*-methylphenazonium ethosulphate supporting half-maximal activities were respectively about 60 μM and 100 μM for D-lactate dehydrogenase and both about 20 μM for D-mandelate dehydrogenase.

Table 2. Apparent Michaelis constants for various preparations of D-lactate dehydrogenase and D-mandelate dehydrogenase

Apparent K_m values were obtained from Eadie-Hofstee plots. Rates are given as means $\pm$ S.D. with the numbers of determinations in brackets; in the case of duplicate measurements the means are listed with the individual values in parentheses.

Preparation	K_m (μ M)	
	D-Lactate dehydrogenase	D-Mandelate dehydrogenase
Sonic extract	302 (265, 340)	200 $\pm$ 18 [3]
'Wall + membrane'	264 (255, 273)	240 (213, 267)
Pure enzyme	308 $\pm$ 24 [4]	385 $\pm$ 63 [5]

Concentrations of 2,6-dichloroindophenol between 26 μ M and 133 μ M supported the same initial rates of enzyme activity.

The apparent K_m value of D-lactate dehydrogenase for D-lactate as substrate appeared to be unchanged after solubilization and purification (Table 2). Purified D-mandelate dehydrogenase showed a slight increase in its apparent K_m value for D-mandelate as substrate (Table 2), but this is a rather small change compared with some that have been reported for membrane-bound dehydrogenases. Thus pure D-lactate dehydrogenase of *E. coli* had a 3-fold lower K_m value (0.72 mM) than that measured for the enzyme in the membrane (2.2 mM) (Futai, 1973), and solubilization of D-alanine dehydrogenase from *E. coli* gave a decrease in K_m values for various substrates of from 2- to 25-fold (Jones & Venables, 1983).

Both dehydrogenases appear to be completely stereospecific for their substrates, and neither of them oxidized L-mandelate or L-lactate. D-Mandelate was not a substrate for D-lactate dehydrogenase. D-Lactate was a substrate for D-mandelate dehydrogenase, but with a high K_m value (4.8 mM) and only about 20% of the V_{max} for D-mandelate; this activity was abolished after treatment of the enzyme with antibodies raised against D-mandelate dehydrogenase, but not after treatment with antibodies against D-lactate dehydrogenase.

Effects of inhibitors. Both enzymes were sensitive to most thiol-blocking reagents. 10 mM-Iodoacetamide, 10 μ M-HgCl₂ or 10 μ M *p*-chloromercuribenzoate almost completely inhibited both enzymes; 1 mM-*N*-ethylmaleimide gave about 50% inhibition; 10 mM-iodoacetate, however, was not inhibitory. The great sensitivity to *p*-chloromercuribenzoate is consistent with results of experiments with crude extracts (Fewson & O'Donnell, 1981; Hills & Fewson, 1983a).

There is no evidence that membrane-bound lactate dehydrogenases of *E. coli* contain metals, but Olson & Massey (1979) found that Zn(II) was necessary for the activity of an NAD(P)-independent D-lactate dehydrogenase from *Megasphaera* (formerly *Peptostreptococcus*) *elsdenii* and the enzyme was inhibited by *o*-phenanthroline and other chelating agents. However, neither D-lactate dehydrogenase nor D-mandelate dehydrogenase from *A. calcoaceticus* was inhibited by 10 mM-EDTA, 4 mM-2,2-bipyridyl, 0.4 mM-8-hydroxyquinoline or 0.4 mM-*o*-phenanthroline. Azide and cyanide inhibited the enzymes only at concentrations of about 10 mM.

Oxalate is a competitive inhibitor of NAD(P)-independent lactate dehydrogenases from various bacteria (Kohn & Kaback, 1973; Garvie, 1980), and it inhibited

Fig. 5. Absorption spectra and D-lactate-induced difference spectrum (inset) of pure D-lactate dehydrogenase

The absorption spectrum of D-lactate dehydrogenase (250 μ g) was measured in 20 mM-potassium phosphate buffer, pH 7.5, containing 0.1% Triton X-100 before (—) and 15 min after (----) the addition of 6 μ mol of D-lactate to the cuvette. The difference spectrum was calculated from the absorption spectra.

both D-lactate dehydrogenase and D-mandelate dehydrogenase from *A. calcoaceticus*.

Rather surprisingly, the D-mandelate dehydrogenase was inhibited by the non-substrate enantiomer of mandelate. Thus oxidation of either D-mandelate or D-lactate by pure D-mandelate dehydrogenase was inhibited by 80% in the presence of an equimolar concentration (2 mM) of L-mandelate. A similar concentration of L-lactate had no effect. D-Lactate dehydrogenase activity was not affected by equimolar concentrations of L-lactate, L-mandelate or D-mandelate.

Flavin as the possible prosthetic group

The absorption spectrum of pure D-lactate dehydrogenase had a distinct shoulder between 420 and 450 nm and this was abolished by addition of substrate or

$\text{Na}_2\text{S}_2\text{O}_4$ (Fig. 5). The D-lactate-induced difference spectrum (Fig. 5 inset) had peaks at 370 and 455 nm, suggesting the presence of a flavin group. D-Mandelate dehydrogenase has almost identical absorption spectra under these conditions. Treatment of pure D-lactate dehydrogenase or D-mandelate dehydrogenase with cold trichloroacetic acid yielded an extract that had emission and excitation fluorescence spectra identical with those of standard solutions of FMN or FAD. Addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the extract completely quenched fluorescence at 540 nm (exciting at 450 nm), as it did with standard FMN or FAD solutions. When a small crystal of *Naja naja* venom was added to the trichloroacetic acid extract a 7–8-fold increase in fluorescence was observed at 540 nm. Similar results were obtained with authentic FAD, corresponding to stoichiometric conversion of FAD into FMN by the phosphodiesterase in the venom; no change in fluorescence was observed when venom was added to a solution of FMN. Preliminary quantitative analysis of the flavin content suggests that there is between 0.5 and 1 mol of FAD/mol of enzyme, depending on whether protein concentration was measured by the method of Bradford (1976) or Lowry *et al.* (1951) respectively. [Rule *et al.* (1985) noted that estimates of the amounts of D-lactate dehydrogenase from *E. coli* varied by a factor of 2-fold depending on the method of protein estimation.] A tryptic digest of the trichloroacetic acid precipitate contained no detectable flavin, suggesting that the FAD in these enzymes is all non-covalently bound.

Other NAD(P)-independent lactate dehydrogenases have also been found to contain non-covalently bound flavin. Membrane-bound D-lactate dehydrogenase from *E. coli* (Futai, 1973) and the soluble D-lactate dehydrogenase from *M. elsdenii* (Brockman & Wood, 1975) both contain FAD and the membrane-bound L-lactate dehydrogenase from *E. coli* contains FMN as the prosthetic group (Futai & Kimura, 1977).

Conclusions

The behaviour of the purified forms of D-lactate dehydrogenase and D-mandelate dehydrogenase appears very similar to that of the enzymes within their membrane environment with respect to pH optima, K_m values and sensitivity to *p*-chloromercuribenzoate. This suggests that the solubilization and purification procedures did not greatly affect the activity and conformation of the enzymes.

Further work will be needed to determine the nature of the electron-transfer compounds involved between the flavin of the dehydrogenases and the terminal oxidases [which are presumably cytochromes *o* or *a*₂ (*d*) (Hardy & Dawes, 1985)].

The present work strengthens the view that D-lactate dehydrogenase and D-mandelate dehydrogenase of *A.*

calcoaceticus are very similar to each other. They co-purify through several stages. They have similar subunit molecular masses, isoelectric points, apparent K_m values for their substrates, pH optima and susceptibility to a range of inhibitors or potential inhibitors. Both contain non-covalently bound FAD. In view of these similarities in properties and the reactions that they catalyse, as well as the observation that D-lactate is a substrate for D-mandelate dehydrogenase, it is tempting to speculate that the pair of enzymes may have had a common evolutionary origin and that independent regulation of their biosynthesis was a more recent event.

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