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

Location and regulation of expression

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(Received 11 September 1984/Accepted 11 December 1984)

Acinetobacter calcoaceticus possesses an L(+)-lactate dehydrogenase and a D(–)-lactate dehydrogenase. Results of experiments in which enzyme activities were measured after growth of bacteria in different media indicated that the two enzymes were co-ordinately induced by either enantiomer of lactate but not by pyruvate, and repressed by succinate or L-glutamate. The two lactate dehydrogenases have very similar properties to L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase. All four enzymes are NAD(P)-independent and were found to be integral components of the cytoplasmic membrane. The enzymes could be solubilized in active form by detergents; Triton X-100 or Lubrol PX were particularly effective. D(–)-Lactate dehydrogenase and D(–)-mandelate dehydrogenase could be selectively solubilized by the ionic detergents cholate, deoxycholate and sodium dodecyl sulphate.

Acinetobacter calcoaceticus N.C.I.B. 8250 has separable stereospecific dehydrogenases for L(+)-lactate, D(–)-lactate and L(+)-mandelate (Fewson & O'Donnell, 1981). In addition, some mutants possess a novel D(–)-mandelate dehydrogenase (Hills & Fewson, 1983a). All four enzymes are NAD(P)-independent and appear to be membrane-bound (Fewson & O'Donnell, 1981). Mandelate dehydrogenases seem to be rather uncommon in bacteria, but those from pseudomonads have also been shown to be NAD(P)-independent and membrane-bound or 'particulate' (Stanier *et al.*, 1953; Hegeman, 1966). There appears to have been no report of successful solubilization of active forms of mandelate dehydrogenase, but this is described in the present paper. Although NAD(P)-independent lactate dehydrogenases are probably more common in bacteria, they appear to have rather diverse properties (Garvie, 1980). Some are soluble and others are membrane-bound. In general the regulation of their expression has not been closely studied. Further, few of them have been characterized in any detail, although those from *Lactobacillus arabinosus* (Snoswell, 1963), *Escherichia coli* (Futai, 1973; Futai & Kimura, 1977; Pratt

et al., 1979) and *Megasphaera elsdenii* (Olson & Massey, 1979) have been purified and shown to be flavoproteins.

Preliminary experiments have indicated that in *A. calcoaceticus* the properties of the two lactate dehydrogenases closely resemble those of the two mandelate dehydrogenases. In particular, L(+)-lactate dehydrogenase is strikingly similar to L(+)-mandelate dehydrogenase, and D(–)-lactate dehydrogenase is equally similar to D(–)-mandelate dehydrogenase (Fewson & O'Donnell, 1981). The purpose of the present work was to extend the comparison of the properties of the four enzymes, with particular reference to their location, and to determine the extent to which the regulation of their expression is connected.

Preliminary accounts of parts of this work have been published (O'Donnell & Fewson, 1980; O'Donnell *et al.*, 1981).

Experimental

Materials

Chemicals were of the best quality commercially available and most of them were obtained from the sources described by Hills & Fewson (1983a). Lysozyme, 3-deoxy-2-keto-octonic acid, Triton X-100, Lubrol PX, Lubrol WX and the sodium salt of

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cholic acid were all obtained from Sigma Chemical Co., Poole, Dorset, U.K. Sodium deoxycholate and sodium dodecyl sulphate (specially purified for biochemical work) were from British Drug Houses, Poole, Dorset, U.K.

Organisms

Acinetobacter calcoaceticus wild-type strain 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 C1005 [constitutive L(+)-mandelate dehydrogenase, no D(-)-mandelate dehydrogenase], C1041 [inducible L(+)- and D(-)-mandelate dehydrogenases], C1123 [constitutive D(-)-mandelate dehydrogenase, no L(+)-mandelate dehydrogenase] and C1219 [constitutive L(+)- and D(-)-mandelate dehydrogenases] derived from *A. calcoaceticus* N.C.I.B. 8250 were described by Hills & Fewson (1983a) and have been deposited in the National Collection of Industrial Bacteria.

Growth of bacteria and preparation of extracts

Bacteria were maintained as described by Hills & Fewson (1983a) except that mutant strains C1005, C1123 and C1219 were maintained in 10 ml of complex medium plus DL-mandelate instead of nutrient broth because we have found it necessary to maintain the selection pressure during storage in order to avoid the outgrowth of revertants. The salts medium used in examining regulation of synthesis of L(+)- and D(-)-lactate dehydrogenases has been described previously (Hills & Fewson, 1983b). For all other experiments, bacteria were grown in a complex medium that contained 26 g of Oxoid no. 1 nutrient broth, 2 g of KH_2PO_4 , 1 g of $(\text{NH}_4)_2\text{SO}_4$, 0.9 g of L-glutamic acid hydrochloride and 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 litre of glass-distilled water. This medium was supplemented with DL-lactate (1.5 g/l) and/or DL-mandelate (1.5 g/l) as required, adjusted to pH 7.0 with NaOH and sterilized by autoclaving at 120°C.

Inocula were prepared by aseptically transferring 0.1 ml of a stock culture into 50 ml of complex medium (in 250 ml flasks) and growing at 23°C with shaking for 24 h. These cultures were used as inocula (2.5%, v/v) for 400 ml of complex media (in 2-litre flasks), which were then incubated on an orbital shaker at 120 rev./min for 16–24 h at 23°C. When the A_{500} of the culture reached approx. 2.0, the bacteria were harvested, washed and used immediately or stored at -20°C. For the experiments on enzyme induction, inocula (10 ml of 24 h nutrient-broth cultures) were added to 70 ml of media in 250 ml flasks. The cultures were shaken at 120 rev./min at 23°C for 2.5 or 5 h (see the legend to

Fig. 1). The bacteria were then harvested, washed and stored at -20°C (Hills & Fewson, 1983b).

Extracts were prepared by using ultrasonic treatment as described by Hills & Fewson (1983b) except that a 20-fold dilution of the cell suspension used had an A_{500} of 0.5 rather than 0.25.

Preparation of membrane fractions

Bacteria were suspended in 0.05 M- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.5, (0.25 g wet wt./ml) and disrupted by four passages through a cooled French pressure cell (98 MPa). The broken-cell suspension was centrifuged (165 000g for 150 min) at 4°C, and the pellet washed twice (by resuspension and centrifugation) and resuspended in 20 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.0, (0.25 g original wet wt./ml). This crude 'wall + membrane' fraction was stored at -23°C.

Outer-membrane and inner-membrane fractions were prepared from *A. calcoaceticus* mutant strain C1219 grown in complex medium plus DL-mandelate and DL-lactate by a method slightly modified from that of Scott *et al.* (1976). Freshly harvested bacteria were suspended (50 mg wet wt./ml) in about 200 ml of 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.6 M-sucrose. The suspension was incubated with lysozyme (10 mg/g wet wt.) for 150 min at 23°C before lysis of the spheroplasts by the addition of 10 vol. of cold distilled water. Deoxyribonuclease (2 mg/g original wet wt.) was added to decrease the viscosity. After the mixture had been stirred for 90 min at 4°C, intact bacteria were removed by centrifugation (15 000g for 20 min) at 4°C. Membranes were then collected from the resulting supernatant by centrifuging at 65 000g for 120 min, then washed twice by resuspension and centrifugation, and then resuspended in a small volume (approx. 12 ml) of 0.05 M- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.5, containing 20 µg of chloramphenicol/ml. Centrifugation of this crude membrane fraction at top speed (approx. 15 000g) in an Eppendorf 3200 centrifuge for 10 min resulted in the sedimentation of the cytoplasmic membranes, the outer membranes remaining in the supernatant fraction. The cytoplasmic membranes were washed once by resuspension and centrifugation, and then resuspended in 0.05 M- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.5, containing 20 µg of chloramphenicol/ml. The outer membranes were recovered from the pooled supernatant fractions by centrifuging at 65 000g for 120 min and resuspended in 0.05 M- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.5, containing 20 µg of chloramphenicol/ml.

Enzyme assays

L(+)-Mandelate dehydrogenase and D(-)-mandelate dehydrogenase activities were assayed by

following the *N*-methylphenazonium methosulphate-dependent reduction of 2,6-dichloroindophenol at 600 nm as described by Hills & Fewson (1983a). D(-)-Lactate dehydrogenase activity was measured in the same way except that 1.5 μ mol of D(-)-lactate was used as substrate. The assay procedure for L(+)-mandelate dehydrogenase activity was modified to measure L(+)-lactate dehydrogenase by replacing L(+)-mandelate with 1.5 μ mol of L(+)-lactate as substrate and lowering the amount of bovine serum albumin in the cuvette from 10 mg to 1 mg in order to obtain maximal activity. NADH oxidase activity was determined by measuring the oxidation of NADH at 340 nm (Kennedy & Fewson, 1968). NADP-dependent isocitrate dehydrogenase activity was assayed by the method of Borthwick *et al.* (1984).

All enzyme activities are expressed as enzyme units; 1 unit is defined as the amount of enzyme catalysing the conversion of 1 μ mol of substrate into product/min.

Analytical methods

3-Deoxy-2-keto-octonic acid was measured by the thiobarbituric acid method of Keleti & Lederer (1974) as described by McDougall *et al.* (1983). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. Samples containing detergent were assayed with the use of standards containing the same amount of detergent.

Results and discussion

Co-ordinacy of induction and repression of the lactate dehydrogenases

When the wild-type strain N.C.I.B. 8250 was grown in different media the specific activities of the two lactate dehydrogenases were in constant proportion to each other. Correlation coefficients for results of experiments such as that illustrated in Fig. 1 were between 0.94 and 0.98. Neither enzyme was constitutive; both enantiomers of lactate induced both enzymes; the product, pyruvate, did not induce; succinate and L-glutamate repressed. Similar results (not shown) were obtained with mutants C1005, C1041, C1123 and C1219. These results indicate that in *A. calcoaceticus* the two lactate dehydrogenases are induced co-ordinately; this contrasts with the situation in *E. coli*, where the NAD(P)-independent D(-)-lactate dehydrogenase is constitutive whereas the L(+)-lactate dehydrogenase is inducible (Kline & Mahler, 1965; Barnes & Kaback, 1971; Garvie, 1980).

There was no correlation between the specific activities of the lactate dehydrogenases and of the two mandelate dehydrogenases (which are co-ordinately regulated and are induced by phenyl-

Fig. 1. Co-ordinate regulation of D(-)-lactate dehydrogenase and L(+)-lactate dehydrogenase of *A. calcoaceticus*. *A. calcoaceticus* wild-type strain N.C.I.B. 8250 was grown in nutrient broth, complex medium or salts media containing various carbon sources, which were all at 5 mM unless otherwise stated. The bacteria were harvested after 2.5 h (●) or 5 h (○) growth, and the enzyme activities were measured in extracts prepared by ultrasonic treatment. Carbon sources were: 1, L-glutamate; 2, succinate; 3, DL-lactate; 4, D-lactate; 5, L-lactate; 6, DL-lactate + L-glutamate; 7, D-lactate + L-glutamate; 8, L-lactate + L-glutamate; 9, DL-lactate + L-glutamate + succinate; 10, DL-lactate + succinate; 11, pyruvate + L-glutamate; 12, pyruvate; 13, DL-lactate + 20 mM-succinate; 14, nutrient broth; 15, complex medium + 1.5 g of DL-lactate/l.

glyoxylate; Hills & Fewson, 1983b). Mutant C1041, which has a novel D(-)-mandelate dehydrogenase (Hills & Fewson, 1983a), still had wild-type activity of D(-)-lactate dehydrogenase, thus ruling out any notion that D(-)-mandelate dehydrogenase activity might have arisen in mutants as a result of changes in substrate- or inducer-specificity of the D(-)-lactate dehydrogenase.

No lactate dehydrogenase activity was detected in extracts when NAD⁺ and NADP⁺ were tested as potential electron acceptors in glycine/hydrazine buffer, pH 9.5.

Location of lactate dehydrogenases and mandelate dehydrogenases

Hills & Fewson (1983a) suggested on the basis of sedimentation experiments that both L(+)-mandelate dehydrogenase and D(-)-mandelate dehydrogenase are membrane-bound. We have now examined the location of all four dehydrogenases by using suitable marker enzymes: NADP-dependent

isocitrate dehydrogenase as a cytoplasmic marker and NADH oxidase as a characteristic component of the cytoplasmic membrane. High-speed centrifugation (165000*g* for 90 min) of a homogenate prepared by passage through a French pressure cell as described in the Experimental section sedimented 90–100% of the total NADH oxidase, L(+)-lactate dehydrogenase and L(+)-mandelate

dehydrogenase and 80–90% of the D(–)-lactate dehydrogenase and D(–)-mandelate dehydrogenase activities. More than 90% of the isocitrate dehydrogenase activity remained in the soluble fraction. About half of the protein was in the precipitate and half in the 'soluble' fraction.

A more precise location of the dehydrogenases was found by examining inner and outer mem-

Table 1. *Distribution of lactate dehydrogenase and mandelate dehydrogenase activities in the inner and outer membranes of A. calcoaceticus*

Membrane fractions of *A. calcoaceticus* mutant strain C1219 were prepared as described in the Experimental section. A portion was then centrifuged to separate inner and outer membranes, and these were resuspended to 1.5 ml and 1.4 ml respectively.

Component	Inner membranes		Outer membranes	
	Specific activity (munits/mg of protein)	Percentage of total enzyme units, 3-deoxy-2-keto-octonic acid or protein	Specific activity (munits/mg of protein)	Percentage of total enzyme units, 3-deoxy-2-keto-octonic acid or protein
L(+)-Lactate dehydrogenase	469	67	151	33
D(–)-Lactate dehydrogenase	890	68	281	32
L(+)-Mandelate dehydrogenase	1078	66	358	34
D(–)-Mandelate dehydrogenase	252	74	59	26
NADH oxidase	183	65	66	35
3-Deoxy-2-keto-octonic acid	(5.4 µg/ml)	5	(104 µg/ml)	95
Protein	(3.11 mg/ml)	40	(5.08 mg/ml)	60

Table 2. *Solubilization of lactate dehydrogenases and mandelate dehydrogenases*

Samples of crude 'wall + membrane' fractions of mutant strain C1219 (5 mg of protein in 1 ml) were stirred gently for 30 min at 4°C in buffer A (20 mM-KH₂PO₄/K₂HPO₄, pH 7.5) with appropriate additions before centrifuging at 165000*g* for 120 min at 4°C. Detergents were added to a concentration of 1 mg/mg of protein. Supernatant fractions were decanted and the pellets resuspended in buffer A. The samples were assayed for all four dehydrogenase activities, and the percentage of the total activity of each enzyme appearing in the supernatant was calculated. Of all the conditions tested only deoxycholate and sodium dodecyl sulphate caused any marked loss of activity, 40–50% of the total L(+)-mandelate dehydrogenase activities being lost during the course of the experiment in these two cases.

Percentage of the total activity in the supernatant fraction

Condition	L(+)-Lactate dehydrogenase	D(–)-Lactate dehydrogenase	L(+)-Mandelate dehydrogenase	D(–)-Mandelate dehydrogenase
Buffer A (control)	0	0.7	1.3	1.8
Buffer A + 200 mM-NaCl	0	0	0.6	0
Buffer A + 500 mM-KCl	0	0	0	0
Buffer A + 200 mM-MgCl ₂	1.6	0	0	0
Buffer A + 20 mM-EDTA	1.8	4.3	1.9	3.3
Buffer A + 20 mM-EGTA	0	0	0	0
200 mM-KH ₂ PO ₄ /K ₂ HPO ₄ buffer, pH 6.0	6.3	2.7	2.7	4.3
200 mM-KH ₂ PO ₄ /K ₂ HPO ₄ buffer, pH 7.5	1.5	2.8	0	2.3
200 mM-KH ₂ PO ₄ /K ₂ HPO ₄ buffer, pH 8.5	5.8	2.7	2.5	4.6
Buffer A + Triton X-100	70	88	71	89
Buffer A + Lubrol PX	76	87	72	91
Buffer A + Lubrol WX	55	83	43	87
Buffer A + sodium cholate	14	63	3.9	70
Buffer A + sodium deoxycholate	13	87	32	91
Buffer A + sodium dodecyl sulphate	32	68	42	78

branes. NADH oxidase was used as a marker for the inner (cytoplasmic) membrane and 3-deoxy-2-keto-octonic acid was used as the outer-membrane marker. The results (Table 1) demonstrate that the inner membranes were quite pure; there was very little contamination with outer membranes, as judged by the low content of 3-deoxy-2-keto-octonic acid. The outer-membrane fraction, however, clearly contained some inner membranes. Electron microscopy and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the inner-membrane and outer-membrane fractions gave results very similar to those obtained by Scott *et al.* (1976) for *A. calcoaceticus* strain HO1-N; there was a major protein band of M_r 44000 in the outer membranes. All four dehydrogenases showed the same distribution of activity as NADH oxidase and appear to be located in the inner (cytoplasmic) membrane of *A. calcoaceticus*.

Solubilization of lactate dehydrogenases and mandelate dehydrogenases

None of the dehydrogenases was significantly solubilized by methods commonly used to remove peripheral membrane proteins, such as increased ionic strengths, pH shifts, increased Mg^{2+} concentration or chelating agents (Table 2). The chaotropic agents KSCN and $NaClO_4$ (0.1 to 2M) did not release enzyme activity into the soluble fraction, and after treatment with either of these two compounds there was a substantial loss of activity of L(+)-lactate dehydrogenase and the two mandelate dehydrogenases. All four dehydrogenases were solubilized by detergents (Table 2). D(-)-Mandelate dehydrogenase and D(-)-lactate dehydrogenase were more readily solubilized than were L(+)-mandelate dehydrogenase or L(+)-lactate dehydrogenase, and this was particularly apparent for the ionic detergents tested: sodium cholate, sodium deoxycholate and sodium dodecyl sulphate (Table 2). The differential solubilization of the dehydrogenases adds to the list of the similarities between D(-)-lactate dehydrogenase and D(-)-mandelate dehydrogenase and between L(+)-lactate dehydrogenase and L(+)-mandelate dehydrogenase.

These results indicate that all four enzymes are integral membrane proteins located in the cytoplasmic membrane.

This work was supported by grants from the Medical Research Council and the Science and Engineering Research Council. Mr G. W. W. Cameron did some of the experiments on regulation of enzyme synthesis and Mr. A. Scott gave excellent technical assistance.

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