Affinity Chromatography of Bacterial Lactate Dehydrogenases

By NIALL KELLY, MICHAEL DELANEY and PÁDRAIG O'CARRA

Department of Biochemistry, University College, Galway, Ireland

(Received 20 September 1977)

The affinity system used was the immobilized oxamate derivative previously used to purify mammalian lactate dehydrogenases. The bacterial dehydrogenases specific for the L-stereoisomer of lactate behaved in the same way as the mammalian enzymes, binding strongly in the presence of NADH. The D-lactate-specific enzymes, however, did not show any biospecific affinity for this gel. The L-specific enzymes could be purified to homogeneity in one affinity-chromatographic step. The D-specific enzymes could be efficiently separated from the L-specific ones and could then be further purified on an immobilized NAD derivative. The mechanism of activation of the lactate dehydrogenase from *Streptococcus faecalis* by fructose 1,6-bisphosphate was investigated by using the immobilized oxamate gel.

In mammals, the product of anaerobic glycolysis is invariably the L-isomer of lactate. In other lactate-producing organisms the isomer formed may be either the L- or the D-form, or a mixture of both. These variations are due to differences in the stereospecificity of the lactate dehydrogenases responsible for the terminal lactate-forming step. Bacteria display all these variations and some organisms also contain allosterically modulated lactate dehydrogenases.

The isolation of these bacterial enzymes has hitherto depended on 'conventional' protein-fractionation techniques, which make for tedious purification procedures and usually result in low yields of the enzymes. We have applied affinity chromatography to the isolation of a selection of bacterial lactate dehydrogenases and report here highly efficient purifications of the L-specific enzymes. We also show how purification of the D-specific enzymes can be simplified by using affinity chromatography. Affinity chromatography has also proved useful in studying the kinetic mechanism of an allosterically modulated lactate dehydrogenase.

Materials and Methods

Biochemicals were obtained as follows: Sepharose 4B from Pharmacia (G.B.) Ltd., London W.5, U.K.; 1,6-diaminohexane from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; 1,3-diaminopropanol from Ralph Emanuel, Wembley, Middx., U.K.; 1-ethyl-3-(3-diethylaminopropyl)carbodi-imide from Sigma (London) Chemical Co., London S.W.6, U.K.; β -NADH from Boehringer Corp. (London) Ltd., London W.5, U.K.

Organisms and culture conditions

The bacteria studied were grown in the following media in 2-litre batches. The cells were harvested in the early stationary phase, about 36 h after inoculation. *Bacillus subtilis* (Marburg strain, A.T.C.C. 6051) was grown anaerobically at 30°C in minimal glucose medium. *Leuconostoc mesenteroides* (U.Q.M. 99) was grown and supplied by Dr. O. Ward, Institute for Industrial Research and Standards, Galway, Ireland. *Lactobacillus plantarum* (A.T.C.C. 8041) was grown anaerobically at 37°C in a medium containing 0.3% beef extract, 3% yeast extract, 0.5% glucose and tomato-juice supernatant added to a final concentration of 10%. The pH of the medium was 7.0. *Streptococcus faecalis* (A.T.C.C. 19433) was grown anaerobically at 37°C in a medium containing 0.5% K_2HPO_4 , 0.25% Tryptone, 0.25% yeast extract and 0.5% glucose. The pH of the medium was 7.5.

Preparation of cell extracts

After being harvested by centrifugation, the cells were suspended in 0.02M- KH_2PO_4 , pH 6.8, containing 0.5M-KCl and disrupted in a Brawn blender. The cell debris was then centrifuged and the supernatant retained.

Lactate dehydrogenase from *B. subtilis* was assayed as described by Yoshida & Freese (1964), that from *L. mesenteroides* as described by Gordon & Doelle (1974), that from *Lact. plantarum* as described by Dennis & Kaplan (1960) and that from *S. faecalis* as described by Wittenberger & Angelo (1970).

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Disc-gel electrophoresis was carried out in 5% (w/v) polyacrylamide gels at pH 8.3 by the method of Ornstein (1964). Protein on gels was detected by fixing in 12% (w/v) trichloroacetic acid and staining in 0.25% Coomassie Brilliant Blue. Lactate dehydrogenase activity was located in the electrophoretic gels by the method of Epstein *et al.* (1971).

Preparation of affinity gels and chromatographic procedures

The immobilized oxamate gel was prepared by the method of O'Carra & Barry (1972) as modified by O'Carra & Barry (1974).

Unless otherwise stated chromatography was carried out at 4°C in columns of 10ml bed volume run at a flow rate of 1 column volume per 10min. The standard irrigant was 0.02M-KH₂PO₄, pH 6.8, containing 0.5M-KCl, which contained further additions as indicated. Extracts applied to the column were adjusted to 0.02M-KH₂PO₄, pH 6.8, containing 0.5M-KCl and 200µM-NADH before application.

In most of the experiments performed in the present study, the extract from 1g of bacterial cells was applied to the 10ml columns. However, this quantity of affinity gel is capable of handling much larger volumes of extract in a single run, the capacity being similar to that reported originally by O'Carra & Barry (1974) for mammalian lactate dehydrogenases, i.e. milligram quantities of lactate dehydrogenase can be purified in a single run on one 10ml column. Column size and other details of the preparations can be scaled-up without difficulty.

Results and Discussion

Affinity-chromatographic approaches to the purification of vertebrate lactate dehydrogenases have been based on either the use of immobilized NAD (the 'general ligand' or 'group ligand' for dehydrogenases) or the use of immobilized analogues of the specific ligands, pyruvate and lactate (O'Carra & Barry, 1972). Immobilized NAD or AMP analogues bind a variety of dehydrogenases, a fact that militates against the efficient purification of an individual

enzyme such as lactate dehydrogenase. Such was our experience with the bacterial enzymes. The immobilized NAD analogues described by Barry & O'Carra (1973) adsorbed most of the bacterial lactate dehydrogenases investigated and these could be eluted with soluble NADH (A. Cullina, T. Griffin & P. O'Carra, unpublished work). Although considerable purification was achievable in this way, the eluted enzymes were invariably contaminated with other proteins. Far more efficient purifications were achieved by the specific-ligand approach using the immobilized oxamate derivative shown in Fig. 1. This is an analogue of the specific substrate pyruvate and was originally used by O'Carra & Barry (1972) for purification of mammalian lactate dehydrogenases. The ordered kinetic mechanism of mammalian lactate dehydrogenase ensures that the enzyme must bind

Fig. 1. Structure of the Sepharose-linked oxamate derivative

The hatched area to the left represents the agarose matrix.

Fig. 2. Affinity chromatography of bacterial lactate dehydrogenases on the Sepharose-linked oxamate derivative (a) *B. subtilis*; (b) *L. mesenteroides*; (c) *Lact. plantarium*. ●, Protein; ○, enzyme activity; Δ, D-specific enzyme activity from *Lact. plantarium*; □, L-specific enzyme activity from *Lact. plantarium*. Irrigant throughout was 0.02M-KH₂PO₄, pH 6.8, containing 0.5M-KCl. Chromatography was carried out at 4°C.

NADH first before it binds pyruvate or its oxamate analogue. The mammalian lactate dehydrogenases were adsorbed on columns of the immobilized oxamate derivative only in the presence of NADH, which must therefore be added to the loading and irrigating buffer. The lactate dehydrogenase is eluted spontaneously when NADH is omitted from the buffer.

Fig. 2(a) shows that a similar mechanism operates in the L-specific lactate dehydrogenase from *B. subtilis*. This enzyme behaves on this affinity system in exactly the same way as described by O'Carra & Barry (1972) for mammalian lactate dehydrogenase 1.

However, the lactate dehydrogenase from *L. mesenteroides*, which is D-stereospecific, behaves quite differently and is not significantly retarded in the presence of NADH (Fig. 2b), nor did this enzyme show any detectable biospecific adsorption when a number of operating parameters (e.g. temperature, pH) and the nature of the spacer arm were varied.

The immobilized oxamate thus seems to behave as a pyruvate analogue only for the L-stereospecific lactate dehydrogenase and is not a ligand for the D-stereospecific enzyme. This is consistent with reports that oxamate does not competitively inhibit D-stereospecific lactate dehydrogenase. The reason for the differential inhibition of the L-specific enzymes is unclear, since oxamate, like pyruvate, and unlike lactate, is optically inactive (see Dennis & Kaplan, 1960).

Lact. plantarum contains variable proportions of both D- and L-specific lactate dehydrogenases. When extracts from this organism were chromatographed on immobilized oxamate in the presence of NADH (Fig. 2c), the D-specific enzyme was not retarded and emerged with the 'breakthrough' protein peak, whereas the L-specific enzyme remained strongly adsorbed and emerged only when the NADH in the irrigant was discontinued.

Results with extracts from other bacteria and from higher organisms containing D- and L-specific enzymes indicate that this pattern of behaviour of the D- and L-specific lactate dehydrogenases is a general phenomenon.

This immobilized oxamate system provides an excellent method for purifying the L-specific enzyme. As in the mammalian enzymes the method can be applied directly to crude extracts, though preferably the extract should be subjected to a preliminary $(\text{NH}_4)_2\text{SO}_4$ -fractionation step. When the chromatographic procedure indicated in Fig. 2 was followed, the L-specific enzyme was recovered in each case in high yield (>95%) and essentially pure (as judged by disc-gel electrophoresis) after the single affinity-chromatographic step.

To achieve a similar degree of purity by 'conventional' techniques, procedures must be followed involving five to seven time-consuming purification steps, yielding variable recoveries of

Table 1. *Affinity chromatography of lactate dehydrogenase from S. faecalis on immobilized oxamate*

Chromatography was carried out at 15°C, the irrigant throughout being 0.02M- KH_2PO_4 , pH 6.2, containing 0.5M-KCl. Other additions were as stated. Abbreviation: Fru-1,6- P_2 , fructose 1,6-bisphosphate.

Additions to applied samples and irrigant	Strength of biospecific adsorption*
None	Zero
200 μM -NADH	Strong
30 μM -NADH	Weak
1 μM -NADH	Undetectable
30 μM -NADH+	Strong
4mM-Fru-1,6- P_2	

* Adsorption is regarded as strong if the enzyme remains fully adsorbed while the column is irrigated with 10 column volumes or more of the irrigating buffer (without altering the indicated additions). 'Weak' adsorption refers to spontaneous elution of the enzyme within 2-3 column volumes without change in the irrigating conditions.

enzyme (see, e.g., Dennis & Kaplan, 1960; Dennis, 1962; Yoshida & Freese, 1964; Wittenberger & Angelo, 1970). Further, such 'conventional' procedures must be separately developed or modified on a trial-and-error basis for each species, whereas the affinity-chromatographic method seems to be applicable to a variety of species without modification.

Although the immobilized oxamate system does not adsorb the D-specific enzyme, it nonetheless provides a useful step in the purification of the D-specific enzyme from species such as *Lact. plantarum* containing a mixture of both D- and L-specific enzymes. Separation of the two types of lactate dehydrogenase generally constitutes one of the more difficult aspects of purification by 'conventional' methods (see Dennis & Kaplan, 1960; Dennis, 1962), but is achieved cleanly and in high yield (>95%) by this affinity system.

The azo-linked NAD analogue described by Barry & O'Carra (1973) has been useful in the purification of the D-specific enzyme from *L. mesenteroides*. The enzyme binds to this gel in 0.05M- KH_2PO_4 , pH 7.4, containing 2M-KCl and 1mM-EDTA, and can be cleanly eluted by addition of 1mM-NADH to the irrigating buffer. A considerable purification is achieved in this way, although disc-gel electrophoresis showed contaminating proteins (A. Cullina, T. Griffin & P. O'Carra, unpublished work).

Fructose 1,6-bisphosphate-activated lactate dehydrogenase

In common with other L-specific lactate dehydrogenases, the enzyme from *S. faecalis* shows no biospecific adsorption on the immobilized oxamate in the

Fig. 3. Suggested kinetic mechanism of lactate dehydrogenase from *S. faecalis*
Abbreviation: Fru-1,6- P_2 , fructose 1,6-bisphosphate.

Fig. 4. Affinity chromatography of lactate dehydrogenase from *S. faecalis* on immobilized oxamate
●, Protein; ○, lactate dehydrogenase activity. Chromatography was carried out at 15°C, the irrigant throughout being 0.02M-KH₂PO₄, pH6.2, containing 0.5M-KCl.

absence of NADH, but binds in its presence. This is consistent with an ordered kinetic mechanism similar to that proposed for mammalian lactate dehydrogenase (see, e.g., O'Carra & Barry, 1972). However, about 200 μ M-NADH is required to promote strong adsorption of the *S. faecalis* enzyme, this being much higher than that required for the enzyme from other organisms (about 1 μ M-NADH). The inclusion of fructose 1,6-bisphosphate in the irrigant decreases the NADH concentration required for strong adsorption of the *S. faecalis* enzyme to about 30 μ M. This suggests that the affinity of the enzyme for NADH is increased by almost an order of magnitude by prior binding of fructose 1,6-bisphosphate. These

results are consistent with the ordered substrate-binding mechanism illustrated in Fig. 3.

These above mechanistic parameters provide a number of possible strategies for the affinity-chromatographic purification of this enzyme. The enzyme can be adsorbed at a low concentration of NADH (about 30 μ M) in the presence of fructose 1,6-bisphosphate and can be eluted by omitting either the NADH or the fructose 1,6-bisphosphate, or it can be further retained by changing to a high NADH concentration (about 200 μ M) in the absence of fructose 1,6-bisphosphate and then eluted by decreasing the NADH concentration back to 30 μ M. We have found in practice that the enzyme is obtained seemingly in homogeneous form by a single-step elution programme discontinuing either the NADH or the NADH and fructose 1,6-bisphosphate simultaneously (Fig. 4). However, the more complex elution programmes outlined above may prove useful in the purification of these enzymes from other bacterial species.

We thank Dr. O. Ward, Institute for Industrial Research and Standards, Galway, Ireland, for culturing the *L. mesenteroides*. This work was supported by a grant-in-aid from the Medical Research Council. Thanks are also due to the Irish Department of Education for Research Maintenance Grants to N. K. and M. D.

References

- Barry, S. & O'Carra, P. (1973) *Biochem. J.* **135**, 595-607
- Dennis, D. (1962) *Methods Enzymol.* **5**, 426-430
- Dennis, D. & Kaplan, N. O. (1960) *J. Biol. Chem.* **235**, 810-818

- Epstein, C. J., Kwok, L. & Smith, S. (1971) *FEBS Lett.* **13**, 45–48
- Gordon, G. L. & Doelle, H. W. (1974) *Microbios* **9**, 199–215
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- O'Carra, P. & Barry, S. (1972) *FEBS Lett.* **21**, 281–285
- O'Carra, P. & Barry, S. (1974) *Methods Enzymol.* **34B**, 598–605
- Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
- Wittenberger, C. L. & Angelo, N. (1970) *J. Bacteriol.* **101**, 717–724
- Yoshida, A. & Freese, E. (1964) *Biochim. Biophys. Acta* **92**, 33–43