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Simple Efficient Methods for the Isolation of Malate Dehydrogenase from Thermophilic and Mesophilic Bacteria

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Malate dehydrogenase from a number of bacteria drawn from several genera and representing the mesophilic, moderately thermophilic and extremely thermophilic classes was isolated by procedures which involve only a small number of steps (in most cases only two), of which the key one is affinity chromatography on 5'-AMP–Sepharose and/or on NAD^+–hexane–agarose. Electrophoretic analysis of the native enzymes in polyacrylamide gel and of the denatured enzymes in sodium dodecyl sulphate/polyacrylamide gel revealed no significant protein impurity in the purified preparations. The yields ranged from about 40% to over 80%. The malate dehydrogenases from the extreme thermophiles and from some of the moderate thermophiles are appreciably less efficient catalytically than their mesophilic homologues.

Malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37), a key enzyme in the tricarboxylic acid cycle, is widely distributed in living organisms and has been studied extensively in animal systems. However, malate dehydrogenases from bacteria are much less well investigated, and unlike the enzyme species from animals, they appear to exhibit some diversity in characteristics such as molecular size and subunit structure (You & Kaplan, 1975). Thus the *Bacillus subtilis* malate dehydrogenase is a tetramer of total mol.wt. 117000–148000 (Murphey *et al.*, 1967*a*; Yoshida, 1965), whereas the enzymes from *Escherichia coli* (Murphey *et al.*, 1967*a*,*b*) and *Pseudomonas testosteroni* (You & Kaplan, 1975) are dimers of total mol.wt. 60000–74000 and the malate dehydrogenase from another Pseudomonad (Kohn & Jakoby, 1968) has been reported to have a mol.wt. of 43000. Information on malate dehydrogenases from thermophilic bacteria is even more scanty. Comparative studies of thermophile malate dehydrogenases and their mesophilic counterparts, directed at an understanding of thermophilism in biochemical terms, should be facilitated by an appreciation of the variations as well as of similarities manifested among different members of each of the two groups, thermophile and mesophile. As the first stage of such an investigation, we have developed easy methods for the isolation of malate dehydrogenase from a number of thermophilic (both moderately thermophilic and extremely thermophilic) and mesophilic bacteria. We describe here these methods, which produce the various enzyme species in apparently homogeneous state and in high yield.

Materials and Methods

Organisms and growth conditions

The following organisms were used: moderate thermophiles: a prototrophic *Bacillus* isolated by Epstein & Grossowicz (1969), referred to here as BI, *Bacillus stearothermophilus* N.C.A. 1518 Ra2 (provided by Dr. M. L. Fields), *Bacillus stearothermophilus* N.C.A. 2184 (provided by Dr. R. E. Gordon), *Bacillus stearothermophilus* N.C.A. 1503 and *Thermomonospora fuscha* N.C.I.B. 11185 and *Thermoactinomyces sacchari* N.C.I.B. 10486 (both obtained from National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K.); extreme thermophiles: *Bacillus caldotenax* (provided by Dr. A. Atkinson) and *Thermus aquaticus* YT-1; mesophiles: *Bacillus subtilis* Marburg and *Bacillus licheniformis* A5 from laboratory stock and *Pseudomonas indigofera* (provided by Dr. B. McFadden). *B. stearothermophilus* N.C.A. 1503 and *T. aquaticus* were grown in a tryptone/yeast extract/sucrose/salts medium (Sargeant *et al.*, 1971) at 60°C and a tryptone/yeast extract/salts medium (Ramaley & Hixson, 1970) at 70°C respectively at the Microbiological Research Establishment, Porton, Wilts., U.K., and supplied to us as frozen cell pastes. Growth conditions for the other bacteria were: strain BI at 55°C in a salts/succinate or salts/acetate medium (Sundaram *et al.*, 1969), *B. stearothermophilus* N.C.A. 1518 Ra2 and N.C.A. 2184 at 55°C in a salts/acetate medium (Humbert *et al.*, 1972) supplemented with 0.4% nutrient broth (Oxoid, London E.C.4, U.K.), *B. subtilis* at 37°C in a salts/glucose medium (Yoshida,

1965), *P. indigofera* and *B. licheniformis* at 37°C and *T. fuscha* and *T. sacchari* at 55°C in 2.5% nutrient broth, and *B. caldotenax* at 70°C in 2.5% nutrient broth supplemented with 1% yeast extract. All cultures were vigorously aerated during growth. Cells, harvested from late-exponential-phase cultures, were stored frozen until used.

Chemicals

These were obtained as indicated below: oxaloacetic acid from Calbiochem, Bishop's Stortford, Herts., U.K.; NAD^+ and NADH from Boehringer Corp., Lewes, East Sussex, U.K.; phenylmethanesulphonyl fluoride, phenazine methosulphate, Nitro Blue Tetrazolium and L-malic acid from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.; 5'-AMP–Sepharose 4B from Pharmacia, London W5 5SS, U.K.; NAD^+–hexane–agarose type I from P-L Biochemicals, Milwaukee, WI, U.S.A.; bovine serum albumin from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; acrylamide monomer and sodium dodecyl sulphate from BDH Chemicals, Poole, Dorset, U.K.; *NNN'N'*-tetramethylethylenediamine for polyacrylamide-gel electrophoresis from Kodak, Kirkby, Liverpool, U.K., and DEAE-cellulose (DE 52) from Whatman, Maidstone, Kent, U.K. Hydroxyapatite was prepared by the method of Atkinson *et al.* (1973). Other chemicals were purchased from commercial sources at the highest purity available.

Protein and enzyme assays

Protein was determined by the procedure of Warburg & Christian (1941), or more accurately by a micro-biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard. Malate dehydrogenase was assayed at 30°C by the method of Murphey *et al.* (1967*a*). One unit of enzyme activity catalyses the oxidation of 1 μmol of NADH/min at 30°C.

Polyacrylamide-gel electrophoresis

Native proteins were electrophoresed by the method of Davis & Ornstein (1961). The acrylamide concentration in the running gel was 7.5% (w/v) and the gels were made in 0.5cm × 7.5cm tubes. A current strength of 2mA/gel was maintained during the electrophoresis. After electrophoresis the gels were stained for protein with 1% (w/v) Amido Black in 7% (v/v) acetic acid; they were destained free of excess dye with 7% acetic acid. Staining of the gels for malate dehydrogenase activity was done by incubating them in a buffer mixture containing L-malate, NAD^+, Nitro Blue Tetrazolium and phenazine methosulphate (Gabriel, 1971). Electrophoresis of denatured proteins in polyacrylamide gels containing 0.2% sodium dodecyl sulphate, prepared in 0.5cm × 15cm tubes, was carried out by the method described by Fairbanks *et al.* (1971). The proteins were denatured in a boiling sodium dodecyl sulphate/2-mercaptoethanol buffer mixture (Laemmli, 1970). After electrophoresis at 5mA/gel, the polypeptides were located in the gels by staining with Coomassie Brilliant Blue; excess dye was removed with 10% (v/v) acetic acid (Fairbanks *et al.*, 1971).

Purification of malate dehydrogenases from strain BI and B. stearothermophilus strains N.C.A. 1518 *Ra*2, *N.C.A.* 2184 *and N.C.A.* 1503

Preparation of cell-free extract and DEAE-cellulose chromatography. Cells of these moderate thermophiles were suspended in 20mM-Tris/HCl buffer, pH7.6, or 50mM-sodium/potassium phosphate buffer, pH7.5, containing 1mM-EDTA. BI cells were digested with lysozyme (Sundaram, 1973) and cells of the three *B. stearothermophilus* strains were disrupted by either passage through a French press or shaking with glass beads in a Braun homogenizer. The cell homogenates were centrifuged at 4°C and 40000*g* for 30min and the supernatant cell-free extracts were collected. The cell-free extract containing 0.7–1.5g of protein was applied to a column (2cm × 25cm) of DEAE-cellulose equilibrated with 10mM-Tris/HCl buffer, pH8, containing 1mM-EDTA and 1mM-2-mercaptoethanol and the enzyme was eluted at 4°C with 600ml of the same buffer mixture containing a linear gradient of KCl from 0 to 0.2M, or, in the case of strain BI, from 0 to 0.3M. The active fractions were pooled.

Affinity chromatography on 5'-AMP–Sepharose 4B. A column (1cm × 4cm) of AMP–Sepharose was equilibrated with 0.1M-sodium/potassium phosphate buffer, pH7, containing 0.4M-NaCl. The enzyme preparation from the previous step was slowly passed through the column, which was then left for 30min before being washed with the phosphate/NaCl buffer until the effluent was protein-free, as indicated by the A_{280}. A linear gradient of 0–1mM-NADH in 150ml of the phosphate/NaCl buffer was applied to elute the enzyme. The malate dehydrogenase emerged early in the gradient over six or seven fractions (Fig. 1). The most active fractions were pooled.

Purification of B. subtilis and B. licheniformis malate dehydrogenase

The methods for purifying these malate dehydrogenase species were similar to those described above, but with the following variations. Cell-free extracts were prepared in 50mM-Tris/HCl buffer, pH8, containing 1mM-EDTA and 1mM-2-mercaptoethanol by lysozyme digestion at 37°C. After the incubation with lysozyme, brief homogenization with an Ultra-Turrax homogenizer produced a smooth lysate, which was centrifuged at 40000*g* for 30min to yield the cell-free

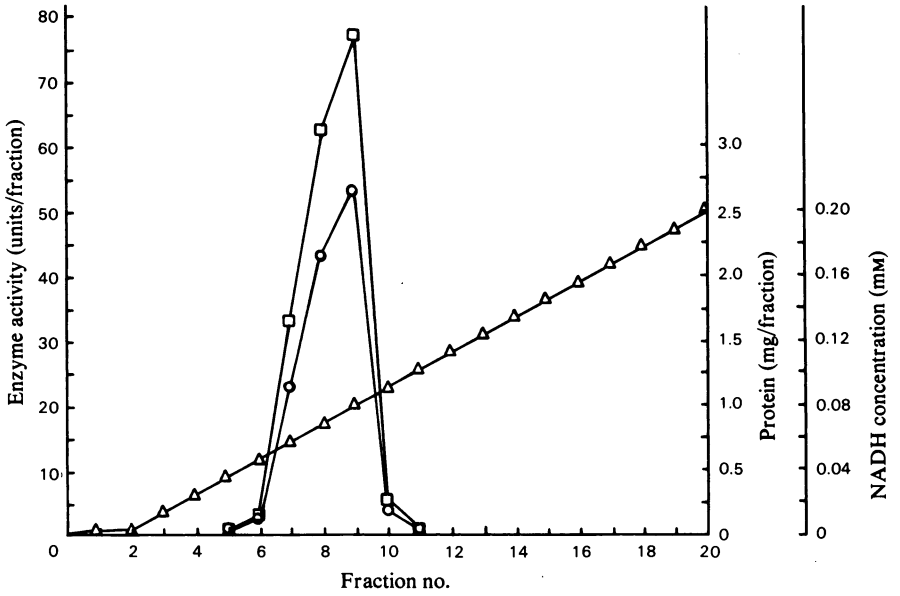

Fig. 1. *Fractionation of strain-BI malate dehydrogenase on AMP-Sepharose column*
A gradient of NADH was applied and 2ml fractions were collected. The NADH concentration in the fractions was determined from their A_{340}. □, Enzyme activity per fraction; ○, protein per fraction; △, NADH concentration.

extract. In the DEAE-cellulose chromatography step, the elution buffer (600ml) contained a linear gradient of NaCl from 0 to 0.5M for the *B. subtilis* malate dehydrogenase and from 0 to 0.3M for the *B. licheniformis* malate dehydrogenase. The protein in the active fractions was precipitated with $(NH_4)_2SO_4$ (70% saturation), dissolved in 10mM-sodium/potassium phosphate buffer, pH7, containing 1mM-EDTA and dialysed against the same buffer. This preparation was subjected to affinity chromatography on 5′-AMP–Sepharose 4B.

Purification of T. aquaticus YT-1 malate dehydrogenase

Preparation of cell-free extract and DEAE-cellulose chromatography. Cells suspended in 10mM-Tris/HCl buffer, pH8, were passed twice in succession through a French press. The homogenate was centrifuged at 100000*g* and 4°C for 30min and the intense yellow supernatant extract was collected. The cell-free extract was fractionated on a column (1.5cm × 20cm) of DEAE-cellulose equilibrated with 10mM-Tris/HCl buffer, pH8, containing 1mM-EDTA and 1mM-mercaptoethanol. A linear gradient of 0–0.3M-NaCl in 300ml of the same buffer mixture was applied to elute the enzyme and the active fractions were pooled.

Affinity chromatography on NAD$^+$–hexane–agarose. The active preparation from the previous step was applied at a slow rate to a column of the NAD$^+$–hexane–agarose (1cm × 5cm) equilibrated with 10mM-sodium/potassium phosphate buffer, pH7, containing 0.2M-NaCl. A very low activity appeared in the effluent. The column was then washed with the phosphate/NaCl buffer until the A_{280} of the effluent was at a constant, low value. A very low amount of enzyme activity appeared in the effluent at these stages. The column was eluted with 150ml of buffer containing a linear gradient of 0–1mM-NADH. The bulk of the malate dehydrogenase activity emerged in a sharp peak around 80μM-NADH (Fig. 2). The most active fractions were pooled.

Purification of P. indigofera malate dehydrogenase

The method was essentially similar to that used for the *T. aquaticus* enzyme. The cell homogenate was centrifuged at 40000*g* and 4°C for 30min and the cell-free extract was fractionated on a DEAE-cellulose column (3cm × 50cm). The protein in the active fractions was precipitated with $(NH_4)_2SO_4$ (70% saturation) and dissolved in a small volume of 10mM-phosphate buffer, pH7, and the solution was dialysed against the same buffer containing 1mM-EDTA and 1mM-mercaptoethanol. On slow passage of this preparation through the NAD$^+$–hexane–agarose column, less than 50% of the malate dehydrogenase activity bound to the column. Some enzyme activity also appeared in the phosphate/NaCl-buffer washings. After elution with buffer containing the NADH gradient the best fractions were pooled. The large amount of the unbound malate dehydrogenase was

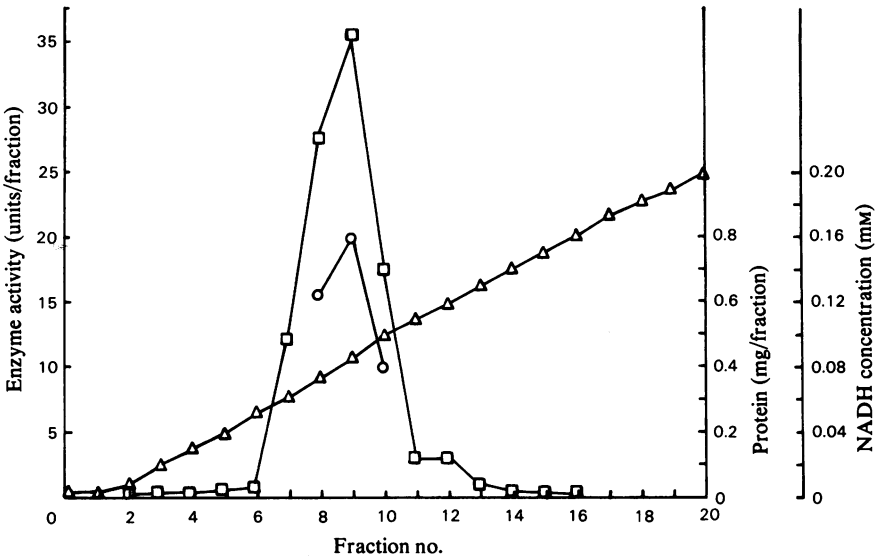

Fig. 2. *Fractionation of T. aquaticus malate dehydrogenase on NAD⁺–hexane–agarose column*
Details were as in Fig. 1. □, Enzyme activity; ○, protein; △, NADH concentration.

fractionated on a fresh NAD⁺–hexane–agarose column.

Purification of T. fuscha malate dehydrogenase

Preparation of cell-free extract and DEAE-cellulose chromatography. Cells suspended in 10mM-Tris/HCl buffer, pH8, containing 1mM-mercaptoethanol and 1mM-EDTA were disrupted in the French press and the supernatant liquid after centrifugation of the homogenate at 40000*g* was collected. The cell-free extract was dialysed against 10mM-Tris/HCl buffer, pH8, containing 1mM-EDTA and fractionated on a DEAE-cellulose column (2.5cm × 25cm). Elution was with 600ml of the same buffer increasing linearly in NaCl concentration from 0 to 0.6M. The active fractions were pooled and dialysed against 10mM-sodium/potassium phosphate buffer, pH7.

Chromatography on hydroxyapatite. The dialysed preparation was applied to a column of hydroxyapatite (2cm × 20cm) equilibrated with 5mM-phosphate buffer, pH6.8, which was then eluted with 300ml of the buffer increasing linearly in phosphate concentration from 5 to 200mM. The active fractions, which appeared between 150 and 200mM-phosphate, were combined and the protein in them was precipitated with $(NH_4)_2SO_4$ (70% saturation), collected by centrifugation at 30000*g* for 15min, dissolved in a small volume of 10mM-phosphate buffer, pH7, and dialysed against the same buffer.

Affinity chromatography on NAD⁺–hexane–agarose and 5′-AMP–Sepharose 4B. The dialysed preparation was applied to a NAD⁺–hexane–agarose column (1cm × 3.5cm) equilibrated with 5mM-phosphate buffer, pH7, and after standing for 1h at room temperature (20°C) to facilitate binding of the enzyme, the column was exhaustively washed free of material absorbing at 280nm with buffer and eluted with a linear gradient of 0–1mM-NADH in 150ml of buffer. The most active fractions were pooled. Electrophoretic analysis of this preparation revealed a minor contaminant, which was removed by adsorption of the enzyme, after dialysis against 10mM-phosphate buffer, pH7, to a short 5′-AMP–Sepharose 4B column and elution with a gradient of 0–0.75mM-NADH.

Purification of B. caldotenax malate dehydrogenase

Preparation of the cell-free extract and its fractionation on DEAE-cellulose were essentially as described for the *T. fuscha* enzyme; the NaCl gradient for elution was from 0 to 0.5M. The protein from the active fractions was dissolved in and dialysed against 10mM-phosphate buffer, pH7, containing 1mM-EDTA.

Affinity chromatography on 5′-AMP–Sepharose 4B. The dialysed preparation was passed slowly through a column of AMP–Sepharose (1cm × 3.5cm) equilibrated with 20mM-phosphate buffer, pH7. The effluent contained slight malate dehydrogenase activity. After standing for 30min, the column was washed with buffer and then eluted with 150ml of buffer containing a linear gradient of NADH from 0 to 0.75mM.

Table 1. *Summary of purification of mesophilic and thermophilic bacterial malate dehydrogenase*

Some 20–50g (wet wt.) of cells and 2–3 vol. of buffer were used to prepare the cell-free extract. Data for *B. stearothermophilus* N.C.A. 2184 were similar to those for strain N.C.A. 1503. The specific activity of *B. licheniformis* malate dehydrogenase in cell-free extract was about 1.3 and that of the pure enzyme was 240; otherwise details of the purification were similar to those for the *B. subtilis* enzyme. In the calculation of the yield of *P. indigofera* malate dehydrogenase the large amount of enzyme that appeared in the effluent from the affinity column has not been taken into account. As stated in the text, this enzyme can be put through another affinity column. The recovery from the affinity step, based on the enzyme activity bound to the column and the activity eluted, was better than 80%. The overall yield, after allowing for the enzyme not bound to the NAD–agarose column, was better than 60%.

Step	Protein (mg)	Enzyme activity (units)	Specific activity (units/mg of protein)	Yield (%)
B. subtilis				
Cell-free extract	2040	13200	6.5	100
DEAE-cellulose chromatography	1500	12000	8.0	91
5′-AMP–Sepharose affinity chromatography	27.3	10100	370	77
P. indigofera				
Cell-free extract	1700	10000	5.9	100
DEAE-cellulose chromatography	133	8000	61.5	80
NAD^+–hexane–agarose affinity chromatography	1.2	3020	2610	30
B. stearothermophilus N.C.A. 1518 Ra2				
Cell-free extract	1500	1300	0.87	100
DEAE-cellulose chromatography	222	1200	5.4	92
5′-AMP–Sepharose affinity chromatography	3.6	1058	294	81
B. stearothermophilus N.C.A. 1503				
Cell-free extract	1323	315	0.24	100
DEAE-cellulose chromatography	199	270	1.35	85
5′-AMP–Sepharose affinity chromatography	0.9	258	287	82
BI strain				
Cell-free extract	880	2340	2.7	100
DEAE-cellulose chromatography	151.5	2170	14.3	92
5′-AMP–Sepharose affinity chromatography	6.3	1830	289	78
T. fuscha				
Cell-free extract	2197	94	0.043	100
DEAE-cellulose chromatography	147.2	88	0.6	94
Hydroxyapatite chromatography	100	80	0.8	85
NAD^+–hexane–agarose affinity chromatography	7.4	60	8.1	64
5′-AMP–Sepharose affinity chromatography	6.6	57	8.6	61
T. sacchari				
Cell-free extract	1998	196	0.098	100
DEAE-cellulose chromatography	840	120	0.145	61
Hydroxyapatite chromatography	192.4	108	0.56	55
5′-AMP–Sepharose affinity chromatography	3.7	85	23.3	43
T. aquaticus				
Cell-free extract	450	150	0.3	100
DEAE-cellulose chromatography	180	130	0.72	87
NAD^+–hexane–agarose affinity chromatography	2.7	119	44.3	80
B. caldotenax				
Cell-free extract	2394	575	0.25	100
DEAE-cellulose chromatography	400	440	1.1	77
5′-AMP–Sepharose affinity chromatography	26.2	330	12.6	57

Purification of T. sacchari malate dehydrogenase

Preparation of cell-free extract and DEAE-cellulose chromatography. Cells suspended in 10mM-Tris/HCl, pH8, containing 1mM-EDTA and 1mM-mercaptoethanol were treated with glass beads in a Braun homogenizer and after centrifugation at 40000*g* and 4°C for 30min the supernatant cell-free extract was collected. It was fractionated on a DEAE-cellulose column (2cm × 20cm) equilibrated with the same buffer mixture by using a gradient of 0–0.6M-NaCl in 600ml of buffer for stripping the enzyme. The active fractions were combined and the protein was precipitated with $(NH_4)_2SO_4$ (65% saturation). The precipitate was dissolved in 5mM-sodium/potassium phosphate buffer, pH8, containing 1mM-mercaptoethanol and the solution was dialysed against the same buffer mixture.

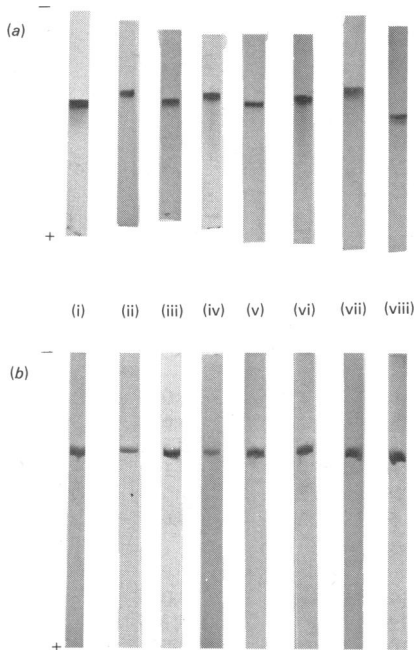

Fig. 3. *Gel-electrophoretic profiles of purified malate dehydrogenases*

(*a*) shows gels for the native enzymes and (*b*) shows sodium dodecyl sulphate/polyacrylamide gels for the denatured enzymes. All gels were stained for protein as described in the text. Sources and amounts of enzyme applied: (i) *B. subtilis*, *a* (20μg) and *b* (5μg); (ii) *B. licheniformis*, *a* (10μg) and *b* (5μg); (iii) BI, *a* (15μg) and *b* (5μg); (iv) *B. stearothermophilus* N.C.A. 1503, *a* (10μg) and *b* (5μg); (v) *T. sacchari* (both 10μg); (vi) *T. fuscha* (both 10μg); (vii) *B. caldotenax*, *a* (10μg) and *b* (5μg); (viii) *T. aquaticus*, *a* (15μg) and *b* (5μg).

Chromatography on hydroxyapatite. Details of this step were similar to those described for *T. fuscha* malate dehydrogenase. A 0–0.3M-phosphate gradient at pH6.8 was set up for elution.

Affinity chromatography on 5′-AMP–Sepharose. The preparation dialysed into 10mM-phosphate buffer, pH7, containing 1mM-EDTA was fractionated on an AMP–Sepharose column (1cm × 3.5cm), which was eluted with 150ml of buffer containing a gradient of 0–0.4mM-NADH.

Results

The purification schedules for the various malate dehydrogenases are summarized in Table 1. In most cases a combination of DEAE-cellulose chromatography and affinity chromatography on AMP–Sepharose and/or on NAD^+–hexane–agarose constitutes an efficient purification procedure. In two instances an additional step, hydroxyapatite chromatography, has been incorporated. This step eliminates some contaminants which otherwise appear in the final preparations. The final preparation of each

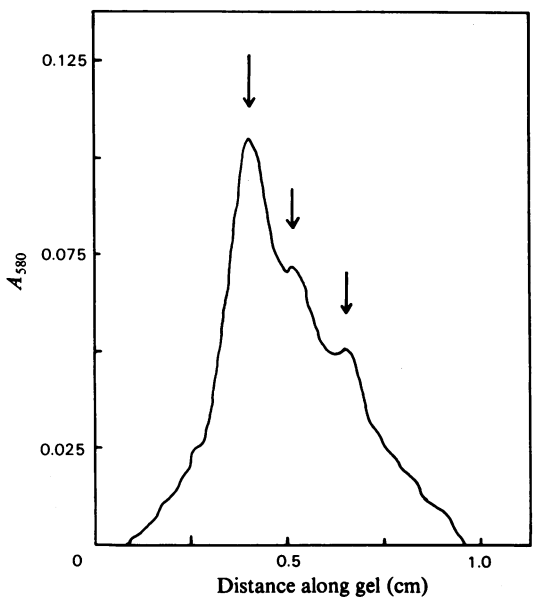

Fig. 4. *Gel-electrophoretic profile of T. aquaticus YT-1 malate dehydrogenase*

Purified *T. aquaticus* malate dehydrogenase was electrophoresed in polyacrylamide and the gel after being stained for enzyme activity was scanned at 580nm by using a Beckman model 25 spectrophotometer fitted with a scanning carriage, at a slit width of 0.1mm, scanning speed of 0.5cm/min and chart speed of 2in/min. Peaks indicated by arrows represent the three malate dehydrogenase species.

malate dehydrogenase was apparently homogeneous as judged by the finding that on electrophoresis of the native enzyme in polyacrylamide or of the denatured enzyme in sodium dodecyl sulphate/polyacrylamide gels a single major protein band resulted. Representative electrophoretic profiles are presented in Fig. 3. The band of native protein could also be detected by the enzyme-activity stain. *T. aquaticus* malate dehydrogenase was exceptional in that, when electrophoresed in the native state, it yielded more than one protein band. Staining of the gel for enzyme activity and a scan of the gel revealed that the malate dehydrogenase consisted of one major and two minor active forms (Fig. 4). This pattern of multiple bands was seen even when the purification was carried out in the presence of the serine-proteinase inhibitor, phenylmethanesulphonyl fluoride, at a concentration of 10 or 50μg/ml. Homogeneity of several of the malate dehydrogenase preparations was also indicated by the results of sedimentation-equilibrium-centrifugation experiments (I. P. Wright, T. K. Sundaram & A. E. Wilkinson, unpublished work). A noteworthy feature of the purification methods developed in this study, apart from their rapidity and ease, is the high recovery of enzyme generally achieved, which varies from 43% to over 80% (Table 1).

Discussion

Affinity chromatography on AMP–agarose or on NAD^+–hexane–agarose is a key step in our methods for the isolation of malate dehydrogenase. The versatility of these methods may be appreciated by comparison with the only two published reports on the purification of thermophile malate dehydrogenase. In one of these, by Murphey *et al.* (1967*b*), malate dehydrogenase from *B. stearothermophilus* N.C.A. 2184 was isolated by a laborious sequence of steps not involving affinity chromatography with a recovery of less than 5%. Presumably because of the difficulties inherent in that procedure, the purification was attempted only once. By the present method malate dehydrogenase from the same strain of *B. stearothermophilus* can be purified in two easy steps with a yield of over 80%. The second report, by Biffen & Williams (1976), describes the isolation of *T. aquaticus* YT-1 malate dehydrogenase by a procedure consisting of nine steps with a recovery of 3%. Our method, comprising just two fractionation steps, affords a yield of 80%. Multiple enzymically active forms were also seen by J. H. F. Biffen & R. A. D. Williams (personal communication) in their purified preparations, and this was attributed to a possible proteolytic cleavage of a single parent form of the enzyme. In the present study multiple forms were observed even when the enzyme was purified in the presence of the serine-proteinase inhibitor, phenylmethanesulphonyl fluoride. This finding, however, does not preclude the possibility of proteolysis by a proteinase not sensitive to this inhibitor. Thus it remains an open question whether the multiple forms are genuine isoenzymes or arise through proteinase action on one parent form of malate dehydrogenase. Three electrophoretically distinctive forms of malate dehydrogenase were also detected by us in a partially purified preparation isolated in the presence of phenylmethanesulphonyl fluoride (10μg/ml) from another strain, *T. aquaticus* B. For the *B. subtilis* malate dehydrogenase the method developed here is simpler and provides a better recovery than that described by Yoshida (1965) without the affinity-chromatography step. Our preparation from the affinity step had a specific activity at least as high as that obtained by the more conventional method (Yoshida, 1965), but for some reason not clear yielded a somewhat diffuse band when a relatively large amount was electrophoresed in polyacrylamide gel at pH9.2 (Fig. 3). A sharp protein band was, however, obtained when the electrophoresis was carried out at pH7 or 8. In general, it appears that the 5′-AMP–Sepharose affinity step works better with the tetrameric malate dehydrogenases than with the dimers, since, of the 11 enzymes examined here, it has recently been found that those from *P. indigofera*, *T. fuscha* and *T. aquaticus* are dimers and those from the other bacteria are tetramers (I. P. Wright & T. K. Sundaram, unpublished work). It is likely that the general principles of the procedures developed in this study for malate dehydrogenase from bacteria from several genera will prove applicable to the purification of malate dehydrogenase from other organisms, including eukaryotes.

The catalytic efficiency varies a great deal among the different malate dehydrogenases. Although the specific activities of the enzymes from the *B. stearothermophilus* strains and strain BI, which are nearly identical, are only a little lower than that of the mesophilic *B. subtilis* malate dehydrogenase, the enzymes from the moderately thermophilic *T. fuscha* and *T. sacchari* and from the extremely thermophilic *B. caldotenax* and *T. aquaticus* possess considerably less catalytic activity (Table 1). The *P. indigofera* enzyme is by far the most active malate dehydrogenase and its activity is comparable to that of the malate dehydrogenase from *P. testosteroni* (You & Kaplan, 1975). It is not clearly established, however, whether low catalytic activity is a universal feature of thermophile proteins correlating with their thermostability. Although the *P. indigofera* enzyme is the most active of all the malate dehydrogenases examined here, there is no consistent indication that the dimeric enzymes are more efficient catalysts than their tetrameric counterparts.

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