The rapid purification of 3-hydroxybutyrate dehydrogenase and malate dehydrogenase on triazine dye affinity matrices

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3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) and malate dehydrogenase (EC 1.1.1.37) were purified to homogeneity on a large scale involving only two sequential affinity-chromatography steps on two triazine dye–Sephadex matrices. Recoveries of both enzymes were in excess of 60%. Malate dehydrogenase could also be purified by a combination of triazine dye affinity chromatography and gel filtration on Ultrogel AcA-44, but this offered no significant advantage over the purely affinity procedure.

3-Hydroxybutyrate dehydrogenase [D(–)-3-hydroxybutyrate:NAD⁺ oxidoreductase, EC 1.1.1.30] has been reported in several species of micro-organism (Shuster & Doudoroff, 1962; Bergmeyer *et al.*, 1967; Dhariwal & Venkatasubramanian, 1978). It has purified to homogeneity from *Rhodospirillum rubrum* by means of conventional techniques by an eight-step procedure that gives an overall recovery of about 9% (Bergmeyer *et al.*, 1967). This enzyme is of value as a reagent for the estimation of ketone bodies, namely 3-hydroxybutyrate and acetoacetate (Lowry & Passonneau, 1972; Williamson & Mellanby, 1974), in the blood of patients with diabetic ketoacidosis.

Malate dehydrogenase (L-malate:NAD⁺ oxidoreductase, EC 1.1.1.37) has been isolated from a large number of micro-organisms by means of conventional (Murphey *et al.*, 1967*a,b*; Kohn & Jakoby, 1968; You & Kaplan, 1975; Kristjansson & Ponnamperna, 1980) and affinity- (Wright & Sundaram, 1979) chromatographic procedures. The affinity-chromatographic procedures used employed AMP–Sephadex 4B and NAD⁺–hexyl-agarose. Malate dehydrogenase finds use as a reagent for the determination of malic acid in tissue and food samples (Lowry & Passonneau, 1972; Mellanby & Williamson, 1974).

The strain of *Rps. spheroides* employed in the present studies, N.C.I.B. 8253, which produces appreciable amounts of 3-hydroxybutyrate de-

hydrogenase, also produces large amounts of malate dehydrogenase (Williamson *et al.*, 1962). These two enzymes are difficult to separate by means of conventional chromatographic procedures, although for 3-hydroxybutyrate dehydrogenase to be of use as a reagent the extent of contaminating malate dehydrogenase must be kept below about 4% in activity units ($\mu\text{mol/min}$). It was therefore desirable to develop a procedure capable of separating and purifying both enzymes in high yield.

Affinity chromatography can provide an elegant and efficient solution to the problem of purifying individual enzymes from a complex mixture. However, conventional nucleotide or substrate-based affinity matrices are frequently difficult and expensive to synthesize. In addition, such matrices often have disappointingly low binding capacities. These drawbacks are a serious limitation to the application of such techniques on all but a laboratory scale. Affinity chromatography with triazine dye matrices is a relatively new technique that has seen widespread acceptance in recent years as a means of purifying enzymes (Lowe *et al.*, 1981).

The dyes are easily coupled to agarose or other supports without the use of toxic chemicals, and form stable conjugates that are less prone to leaching of ligand than are nucleotide and other similar ligands. The dyes are relatively inexpensive and often have binding capacities that are 10–100 times greater than for immobilized nucleotide matrices (Lowe *et al.*, 1981; Atkinson *et al.*, 1981). These factors make triazine dye affinity chromatography an attractive method for the large-scale purification of enzymes.

The present paper describes the development and application of this technique to the purification of

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two enzymes, 3-hydroxybutyrate dehydrogenase and malate dehydrogenase, from *Rhodopseudomonas spheroides*. The purification of these enzymes has been briefly reported previously (Atkinson *et al.*, 1981).

Experimental

Materials

NADH, NAD⁺, oxaloacetic acid and sodium DL-3-hydroxybutyrate were obtained from the Boehringer Corporation. Other chemicals were of the highest purity available. Potassium phosphate buffers were prepared by mixing stock solutions of K₂HPO₄ and KH₂PO₄ and diluting to the required concentration. Triazine (Procion) dyes were obtained from Imperial Chemical Industries.

Growth of organism

Rhodopseudomonas spheroides (N.C.I.B. 8253) was grown aerobically at 30°C in a culture medium adapted from that described by Williamson *et al.* (1962) in a 400-litre stainless-steel fermenter operated as described previously (Elsworth & Stockwell, 1968; Melling & Scott, 1972). One litre of the culture medium contained: KH₂PO₄, 5 g; K₂HPO₄, 5 g; (NH₄)₂HPO₄, 0.8 g; monosodium glutamate, 2 g; DL-malic acid, 2.7 g; MgSO₄·7H₂O, 0.2 g; CaCl₂, 40 mg; MnSO₄·4H₂O, 4 mg; ferric citrate, 6 mg; nicotinic acid, 1 mg; thiamin, 1 mg; biotin, 0.1 mg. The pH was adjusted to 7.0 with 5 M-NaOH. During the growth of the organism the pH was maintained at 7.0 ± 0.2 by the automatic addition of 5 M-NaOH or 3.2 M-H₃PO₄ as necessary. The cells were harvested by centrifugation, washed with 50 mM-potassium phosphate buffer, pH 7.0, and stored at -20°C until required.

Chromatography media

Sephacrose 4B and Ultrogel AcA-44 and AcA-34 were obtained from Pharmacia and LKB Produkter respectively and prepared for use as described by the manufacturers. For the estimation of molecular weights a column (1.6 cm × 85 cm) of Ultrogel AcA-34 was calibrated with rabbit fructose bisphosphate aldolase (*M_r* 156 000), bovine serum albumin (*M_r* 67 000), hen ovalbumin (*M_r* 45 000), bovine chymotrypsinogen (*M_r* 25 000) and bovine ribonuclease (*M_r* 13 700) (Pharmacia calibration proteins) and rabbit phosphorylase *b* (*M_r* 94 000) (Sigma Chemical Co.). The flow rate was 6 ml/h.

Preparation of dye-Sephacrose conjugates

Procion dye-Sephacrose conjugates were prepared as described by Baird *et al.* (1976), but with the use of NaOH in place of Na₂CO₃ to provide the alkaline conditions necessary for coupling (Atkin-

son & Harvey, 1979). Differing degrees of substitution by Procion Blue MX-4GD on Sepharose 4B were obtained by withdrawing samples from the reaction mixture over a period of 5 min to 3 h. The degree of substitution was determined by hydrolysing samples of gel in 7.5 M-acetic acid for 15 min at 85°C followed by spectrophotometric determination of the dye. The usual degree of substitution was in the range 2–4 μmol/g of Sepharose.

Enzyme assays

L-Malate dehydrogenase activity was assayed largely as described by Bergmeyer (1974*b*). The reaction mixture contained (final concentrations) neutralized 0.5 mM-oxaloacetic acid in buffer and 0.25 mM-NADH, with sufficient 0.1 M-potassium phosphate, pH 7.5, to make 1 ml of solution. The reaction was performed in semi-micro cuvettes, and was started by adding 0.02 ml of enzyme solution and followed by recording the decrease in absorbance at 340 nm at 30°C.

3-Hydroxybutyrate dehydrogenase activity was assayed by a modification of the method described by Bergmeyer (1974*a*). The reaction mixture contained (final concentrations) 2 mM-NAD⁺ and 25 mM-sodium DL-3-hydroxybutyrate, with sufficient 0.2 M-Tris/HCl buffer, pH 8.0, to make 1 ml of solution. The reaction was performed in semi-micro cuvettes, and was started by adding 0.02 ml of enzyme solution and followed by recording the increase in absorbance at 340 nm at 30°C.

In each case 1 unit of enzyme is defined as the amount of enzyme that catalyses the oxidation or reduction of 1 μmol of substrate/min at 30°C.

Protein determination

Protein concentrations were determined by using the Folin reagent of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co.) as standard.

Electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed with the discontinuous buffer system of Laemmli (1970). For the determination of subunit molecular weights a calibration mixture was used containing rabbit phosphorylase *b* (*M_r* 94 000), bovine serum albumin (*M_r* 67 000), bovine carbonic anhydrase (*M_r* 30 000), soya-bean trypsin inhibitor (*M_r* 21 000) and hen lysozyme (*M_r* 14 300) (standard mixture supplied by Bio-Rad Laboratories) and pig deoxyribonuclease II (*M_r* 38 000) (Sigma Chemical Co.). Gels were stained with Coomassie Blue R250. Densitometer traces were obtained at 595 nm by using a Gilford 2600 spectrophotometer with a linear transport attachment.

Preparation of crude cell extracts for column screening

(a) A 150g batch of *Saccharomyces cerevisiae* (baker's yeast) was suspended in 600ml of 0.1M-potassium phosphate buffer, pH 7.5, and passed four times through a Manton-Gaulin homogenizer (APV Ltd., Crawley, Sussex, U.K.) at 600 kg/cm². The homogenate was cooled on ice after each passage.

(b) A 10g portion of *Rps. spheroides* cell paste was suspended in 35ml of 0.1M-potassium phosphate buffer, pH 7.5, and sonicated for eight 30s bursts with a Dawe Soniprobe (Dawe Instruments), with cooling between each burst. Both cell extracts were clarified by centrifugation at 27000g for 30min. They were then exhaustively dialysed against 10mM-potassium phosphate buffer, pH 7.5, at 4°C.

Screening of Procion dyes for enzyme binding

A total of 38 Procion dye-Sepharose conjugates were prepared and packed into 1ml (1.6cm × 0.9cm) columns and equilibrated in 10mM-potassium phosphate buffer, pH 7.5. Crude extract containing either 28 units of yeast malate dehydrogenase or 18 units of malate dehydrogenase and 0.35 unit of 3-hydroxybutyrate dehydrogenase from *Rps. spheroides* was loaded on each column.

The columns were washed with 5ml of equilibration buffer and eluted sequentially with 5ml of 1M-KCl followed by 5ml of 1M-KCl containing 5mM-NADH, both in equilibration buffer. The columns were run at a flow rate of about 0.2ml/min. Samples of each eluate were examined for malate dehydrogenase and 3-hydroxybutyrate dehydrogenase activities.

The preliminary experiments were performed at room temperature.

Results

Screening of Procion dye-Sepharose matrices

The comparison of the behaviour of yeast and *Rps. spheroides* malate dehydrogenases is a good illustration of the way in which the same enzyme from different sources can behave differently on triazine dye affinity chromatography. The yeast enzyme does not bind to five dyes, Procion Yellow MX-8G, Procion Yellow H-A, Procion Turquoise H-A, Procion Turquoise H-7G and Procion Blue MX-7RX, to which the *Rps. spheroides* enzyme bound. The yeast enzyme could be eluted by 1M-KCl from eight dyes, Procion Red MX-2B, Procion Red MX-5B, Procion Red P-3BN, Procion Red H-8BN, Procion Red H-3B, Procion Red HE-3B, Procion Blue HE-RD and Cibachron Blue F-3GA, whereas the *Rps. spheroides* enzyme required the presence of 5mM-NADH. There was only

one dye, Procion Rubine MX-B, for which the yeast enzyme exhibited a stronger affinity, and that required the presence of NADH for elution. The differences in behaviour between the two enzymes were particularly marked for the eight red dyes examined, six of which required NADH for the elution of the *Rps. spheroides* enzyme, although the yeast enzyme could be eluted by KCl in each case.

An examination of the behaviour of malate dehydrogenase and 3-hydroxybutyrate dehydrogenase from *Rps. spheroides* reveals that in general 3-hydroxybutyrate dehydrogenase shows a much weaker degree of interaction with the dyes than does malate dehydrogenase. It could be eluted by 1M-KCl from all the dyes, except for Procion Rubine MX-B, Procion Yellow MX-R and Procion Blue MX-4GD. In these cases the enzyme could be eluted by 5mM-NADH in 1M-KCl. In comparison, malate dehydrogenase showed a much greater affinity for the dyes and required 5mM-NADH in 1M-KCl for elution from eight dyes, to which 3-hydroxybutyrate dehydrogenase either did not bind or from which it could be eluted by 1M-KCl.

For the large-scale purification of malate dehydrogenase and 3-hydroxybutyrate dehydrogenase, two dyes were selected, Procion Red H-3B and Procion Blue MX-4GD. These two dyes were chosen because they gave the highest recovery of the enzymes in the small-scale screening procedure. The behaviour of the two enzymes on these dyes was complementary. With the Procion Red H-3B-Sepharose column 3-hydroxybutyrate dehydrogenase was eluted by KCl, whereas malate dehydrogenase required NADH in the presence of KCl; on the Procion Blue MX-4GD-Sepharose column malate dehydrogenase could be eluted by KCl, but 3-hydroxybutyrate dehydrogenase required NADH in the presence of KCl. By using these dye columns a two-step procedure was devised that initially separated the two enzymes and then purified them individually to homogeneity.

Both adsorption and desorption of 3-hydroxybutyrate dehydrogenase were affected by ligand concentration, as shown in Fig. 1. The decreased capacity of the affinity matrix at high degrees of substitution may be due to the steric effect of a large number of dye molecules. However, the enzyme that is bound under these conditions is not readily eluted, perhaps because the enzyme is bound at several non-specific sites. From these results a degree of substitution of 3.5–4.0 μmol of Procion Blue MX-4GD/g of Sepharose was adopted for the purification of 3-hydroxybutyrate dehydrogenase.

The maximum capacity for the Procion Red H-3B-Sepharose column for 3-hydroxybutyrate dehydrogenase was about 6 units/ml of column. The degree of substitution was about 3 μmol of dye/g of Sepharose. However, in practice a greater degree of

Fig. 1. Effect of ligand concentration on adsorption and elution of 3-hydroxybutyrate dehydrogenase on Procion Blue MX-4GD-Sepharose

Columns (1 ml, 0.9 cm × 1.6 cm high) were prepared containing Sepharose substituted with various amounts of Procion Blue MX-4GD. The columns were equilibrated in 10 mM-potassium phosphate buffer, pH 7.5, at a flow rate of about 0.2 ml/min and loaded with 1 ml of a 10-unit/ml solution of 3-hydroxybutyrate dehydrogenase in 10 mM-potassium phosphate buffer, pH 7.5. The enzyme used had been partially purified by chromatography on Procion Red H-3B-Sepharose. The columns were washed with 5 ml of equilibration buffer containing 1 M-KCl and eluted with 5 mM-NADH in equilibration buffer containing 1 M-KCl. The washings and eluates were examined for 3-hydroxybutyrate dehydrogenase activity. The experiments were performed at room temperature. ●, Enzyme bound, % of that applied; ○, enzyme eluted, % of that bound.

purification and improved recovery were obtained by loading about 4 units of enzyme/ml of column.

The capacity of Procion Blue MX-4GD-Sepharose for 3-hydroxybutyrate dehydrogenase was 7–10 units/ml of column (0.4–0.5 mg of enzyme), whereas the capacity for malate dehydrogenase was much greater, at 600–800 units/ml of column (2–2.5 mg of enzyme).

Purification of malate dehydrogenase and 3-hydroxybutyrate dehydrogenase

All operations were performed at 4°C where possible.

Preparation of cell extract. A 1 kg batch of *Rps. spheroides* cell paste was suspended in 3 litres of 0.1 M-potassium phosphate buffer, pH 7.5, and passed twice through a Manton-Gaulin homogenizer at 500 kg/cm². Deoxyribonuclease (4 mg) was added to

decrease the viscosity of the homogenate. The homogenate was centrifuged at 13 000 g for 1 h, and the supernatant was dialysed against 20 litres of 10 mM-potassium phosphate buffer, pH 7.5.

Chromatography on Procion Red H-3B-Sepharose. The dialysed supernatant was applied to the column as described in Fig. 2. The fractions containing 3-hydroxybutyrate dehydrogenase and malate dehydrogenase activities were pooled separately, and each was concentrated to about 200 ml by ultrafiltration with Amicon PM10 membranes. The concentrated pools were dialysed against 10 mM-potassium phosphate buffer, pH 7.5.

Final purification of 3-hydroxybutyrate dehydrogenase. The dialysed enzyme from the Procion Red H-3B-Sepharose column was loaded on to a column of Procion Blue MX-4GD-Sepharose as described in Fig. 3.

The fractions containing the homogeneous enzyme were pooled. The enzyme was concentrated by precipitation with 70%-saturation (NH₄)₂SO₄ and was finally stored at 4°C as a suspension in 3.2 M-(NH₄)₂SO₄, pH 6–7.

Final purification of malate dehydrogenase. This enzyme could be purified to homogeneity by two methods: chromatography on Procion Blue MX-4GD-Sepharose or on Ultrogel AcA-44.

(a) Chromatography on Procion Blue MX-4GD-Sepharose. The dialysed enzyme from the Procion Red H-3B-Sepharose column was loaded on to a column of Procion Blue MX-4GD-Sepharose as described in Fig. 4. The fractions containing the homogeneous enzyme were pooled.

(b) Chromatography on Ultrogel AcA-44. The dialysed enzyme from the Procion Red H-3B-Sepharose column was further concentrated to a volume of 40 ml. It was applied to a 1.9-litre column (5 cm × 95 cm high) of Ultrogel AcA-44 equilibrated in 10 mM-potassium phosphate buffer, pH 7.5, containing 50 mM-KCl at a flow rate of 72 ml/h. Fractions of volume 10 ml were collected. The fractions containing the homogeneous enzyme were pooled.

The purified enzyme from both purification procedures was concentrated by precipitation with 70%-saturation (NH₄)₂SO₄ and was stored at 4°C as a suspension in 3.2 M-(NH₄)₂SO₄, pH 6–7.

The results of a large-scale purification procedure for both enzymes are summarized in Table 1.

Properties of the purified enzymes

Both enzymes were homogeneous when examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 5). From these gels the subunit molecular weights of the two enzymes were estimated to be 23 000 for 3-hydroxybutyrate dehydrogenase and 36 000 for malate dehydrogenase.

Fig. 2. Chromatography of 3-hydroxybutyrate dehydrogenase and malate dehydrogenase on Procion Red H-3B-Sephacrose

The column was 1.8 litres (12.4 cm $\times$ 15 cm high) and equilibrated in 10 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 1.0 litre/h. After loading of the sample, the column was eluted with (A) 5 litres of 10 mM-potassium phosphate buffer, pH 7.5, (B) 5 litres of 10 mM-potassium phosphate buffer, pH 7.5, containing 1 M-KCl, and (C) 3 litres of 10 mM-potassium phosphate buffer, pH 7.5, containing 1 M-KCl and 2 mM-NADH. O, 3-Hydroxybutyrate dehydrogenase activity; □, malate dehydrogenase activity; Δ , A_{280} .

Fig. 3. Chromatography of 3-hydroxybutyrate dehydrogenase on Procion Blue MX-4GD-Sephacrose

The column was 800 ml (9 cm $\times$ 12.5 cm high) and equilibrated in 10 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 400 ml/h. After loading of the sample, the column was eluted with (A) 1.5 litres of 10 mM-potassium phosphate buffer, pH 7.5, (B) 0.5 litre of 10 mM-potassium phosphate buffer, pH 7.5, containing 1 M-KCl, and (C) 1.5 litres of 10 mM-potassium phosphate buffer, pH 7.5, containing 1 M-KCl and 2 mM-NADH. O, 3-Hydroxybutyrate dehydrogenase activity; Δ , A_{280} .

Chromatography on Ultrogel AcA-34 showed that 3-hydroxybutyrate dehydrogenase was a tetramer of molecular weight 89 000, which is similar to the value of 85 000 found by sedimentation equi-

brium (Bergmeyer *et al.*, 1967). Malate dehydrogenase was a dimer of native molecular weight 70 000. The *Rps. spheroides* enzyme is typical of the low-molecular-weight dimeric malate dehydrogen-

Fig. 4. *Chromatography of malate dehydrogenase on Procion Blue MX-4GD-Sepharose*

The column was 400 ml (6.4 cm $\times$ 12.5 cm high) and equilibrated in 10 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 160 ml/h. After loading of the sample the column was eluted with 800 ml of 10 mM-potassium phosphate buffer, pH 7.5, followed by a 3-litre linear gradient (—) of 0–700 mM-KCl in 10 mM-potassium phosphate buffer, pH 7.5, $\square$, Malate dehydrogenase activity; Δ , A_{280} .

Table 1. *Purification of 3-hydroxybutyrate dehydrogenase and malate dehydrogenase from Rps. spheroides*

The Table shows a summary of a typical purification starting from 1 kg of frozen cell paste. Experimental details are described in the text.

3-Hydroxybutyrate dehydrogenase

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification per step (fold)	Yield (%)	Malate dehydrogenase (units)
Cell extract	6860	76 200	0.09		100	414 000
Procion Red H-3B-Sepharose chromatography	6800	2200	3.1	34	99	273
Procion Blue MX-4GD-Sepharose chromatography	5380	278	19.2	6	78	112

Malate dehydrogenase

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification per step (fold)	Yield (%)
Cell extract	414 000	76 200	5.4		100
Procion Red H-3B-Sepharose chromatography	327 000	3100	105	19	79
Procion Blue MX-4GD-Sepharose chromatography	265 000*	906	292	2.8	64
Ultrogel AcA-44 chromatography	267 000*	924	289	2.8	64

* Preparation divided into two and each half treated as shown. The results are multiplied by 2 to indicate what would happen in a normal purification.

ases that have been isolated from both prokaryotes and eukaryotes (Murphey *et al.*, 1967a,b; Sundaram *et al.*, 1980).

Discussion

The purification of these two enzymes represents a good example of the application of triazine dye affinity chromatography to the purification of enzymes, particularly as in this case both enzymes

could be purified to homogeneity solely by means of affinity chromatography on two separate dye columns. Malate dehydrogenase could also be purified by chromatography on Ultrogel AcA-44, but this offered no significant advantage over the affinity-chromatography procedure. Indeed, for large-scale operations the affinity procedure would be the preferred method.

By virtue of the small number of steps involved the recoveries of both enzymes were in excess of

Fig. 5. Scanning densitometer traces of gel electrophoretograms of (a) 3-hydroxybutyrate dehydrogenase and (b) malate dehydrogenase

Experimental details are described in the text. The second peak in each case is the dye front.

60%, in strong contrast with the conventional purification procedure for 3-hydroxybutyrate dehydrogenase, for which a recovery of 9% was achieved (Bergmeyer *et al.*, 1967).

The capacities of both dye columns for both enzymes were very high, an important factor in considering the applicability of a purification protocol to large-scale working. With 3-hydroxybutyrate dehydrogenase, by the use of 7-litre and 3-litre columns it would be possible to prepare 1g of homogeneous enzyme from 3 kg of cell paste, a scale of operation that is readily achievable. No comparable data exist detailing the behaviour of this enzyme on conventional affinity matrices, but for bacterial malate dehydrogenases the capacities were as much as 20 times lower: 60 units/ml of column for AMP-Sepharose and 30 units/ml of column for NAD⁺-hexyl-agarose (Wright & Sundaram, 1979).

Assuming a similarly decreased capacity for 3-hydroxybutyrate dehydrogenase, the large-scale purification of these two enzymes by means of conventional nucleotide affinity adsorbents would be prohibitively expensive and require the use of impractically large columns of 50–150 litres volume.

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