Separation of rat lactate dehydrogenase isoenzyme C₄ from other isoenzymes by affinity and ion-exchange chromatography

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Lactate dehydrogenase C, an isoenzyme composed of C polypeptide subunits and found only in mature testes and spermatozoa, differs kinetically, chemically and immunologically from the five common isoenzymes of lactate dehydrogenase, each of which is a tetramer of A and/or B subunits. In the rat lactate dehydrogenase C exists in two molecular forms, isoenzymes C₄ and A₁C₃. In addition to these two forms of lactate dehydrogenase C, rat testicular homogenate contains all the five isoenzymes of A and B type. Purification of isoenzyme C₄ requires its separation from the other six isoenzymes, of which isoenzymes A₁C₃ and A₃B₁ are the most difficult ones to separate. In the present study isoenzyme A₃B₁, along with other isoenzymes, was separated from isoenzyme C₄ by AMP-Sepharose chromatography by using a gradient of increasing concentration of NAD⁺–pyruvate adduct. In the next step, isoenzyme A₁C₃ was separated from isoenzyme C₄ by DEAE-cellulose chromatography, resulting in a pure lactate dehydrogenase isoenzyme C₄ preparation.

Lactate dehydrogenase in animals occurs in five common isoenzyme forms, each being one of the possible random tetrameric combinations of two different polypeptide chains designated A (or M) and B (or H) (Markert, 1963). A special form of lactate dehydrogenase, namely lactate dehydrogenase C (lactate dehydrogenase X; EC 1.1.1.27) has been found in mature testes and spermatozoa of mammalian and avian species (Goldberg, 1963, 1974, 1977; Blanco & Zinkham, 1963; Zinkham *et al.*, 1964*b*; Wheat & Goldberg, 1975). Whereas the isoenzymes containing A and B subunits are found in all tissues including testes and spermatozoa, lactate dehydrogenase C is found only in mature testes and spermatozoa. Lactate dehydrogenase C differs from the five major isoenzymes in its kinetic (Goldberg, 1972), chemical (Goldberg, 1972) and immunological properties (Goldberg, 1971). These properties make lactate dehydrogenase C attractive for biochemical, genetic and anti-fertility studies, as well as in the studies related to mutation monitoring (Ansari *et al.*, 1980; Ansari & Mallin, 1981).

Lactate dehydrogenase C has been purified from testes of several species, including rat (Schatz & Segal, 1969; Ansari *et al.*, 1980; Chang *et al.*, 1980), bull (Kolb *et al.*, 1970), mouse (Wong *et al.*, 1971; Goldberg, 1972; Lee *et al.*, 1977; Wheat & Goldberg, 1977), rabbit (Wheat & Goldberg, 1977) and human (Svasti & Viriyachai, 1975; Wheat & Goldberg, 1977; Kolk *et al.*, 1978; Toowicharanont

& Svasti, 1980). In spermatozoa and/or testicular extracts from mouse, rabbit, human, ram and dog, only a single molecular form of lactate dehydrogenase C has been detected, i.e. isoenzyme C₄ (Goldberg, 1963; 1965; Zinkham *et al.*, 1964*a,b*; Blackshaw & Samisoni, 1967). However, in rat, bull, pig, guinea pig, pigeon and bat, multiple forms of lactate dehydrogenase C have been observed (Zinkham *et al.*, 1964*a,b*; Valenta *et al.*, 1967; Goldberg, 1971; Gutierrez *et al.*, 1972).

In the rat, lactate dehydrogenase C exists in two forms, isoenzymes C₄ and A₁C₃ (Goldberg, 1977). Thus rat testicular homogenate contains a total of seven lactate dehydrogenase isoenzymes. These seven isoenzymes exhibit anodic electrophoretic mobilities, in basic buffer, in the order: A₄ < A₁C₃ < C₄ = A₃B₁ < A₂B₂ < A₁B₃ < B₄ (see the electrophoretic pattern of the homogenate in Fig. 1). Purification of isoenzyme C₄ requires its separation from the other six isoenzymes, of which isoenzymes A₁C₃ and A₃B₁ are the most difficult ones to separate. Isoenzyme A₃B₁ is identical with isoenzyme C₄ in electrophoretic mobility, and therefore cannot be separated on the basis of charge. Isoenzyme A₁C₃ gives a band very close to that of isoenzyme C₄ in electrophoresis, and the separation requires fine control of ion-exchange chromatography conditions.

In the present study isoenzyme A₃B₁ was successfully separated from isoenzyme C₄ on an

AMP-Sepharose column by using a gradient of increasing concentration of NAD^+ -pyruvate adduct. Other lactate dehydrogenase isoenzymes, including isoenzyme A_1C_3 , were removed from isoenzyme C_4 by gradient elution from a DEAE-cellulose column, resulting in a pure preparation of lactate dehydrogenase isoenzyme C_4 .

Experimental

Materials

Rat testicles were obtained frozen from Pel Freeze, Rogers, AR, U.S.A. N^6 -(6-Aminohexyl)-5'-AMP-Sepharose was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Sodium pyruvate, NAD^+ and NADH were from Calbiochem, San Diego, CA, U.S.A. Lactic acid, 2-oxoglutaric acid and 2-hydroxyvaleric acid were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Determination of protein

In the crude extract, protein was determined by the Coomassie Blue method of Bradford (1976), with bovine serum albumin as a standard. Bio-Rad Laboratories (Richmond, CA, U.S.A.) Protein Assay Kit (cat. no. 500-0002) was used for this purpose. Protein in all other fractions was determined by measurement of A_{280} , an absorption coefficient of $1 \text{ (mg/ml)}^{-1} \cdot \text{cm}^{-1}$ being assumed.

Assay of enzyme activity

Lactate dehydrogenase activity was assayed by a spectrophotometric method (Pesce *et al.*, 1967) with pyruvate as substrate for all lactate dehydrogenase isoenzymes and 2-oxoglutarate as substrate specific for lactate dehydrogenase C. The reaction mixture, of volume 1 ml, contained 100 mM-potassium phosphate buffer, pH 7.6, 1 mM-pyruvate or 2-oxoglutarate, 0.15 mM-NADH and 10–25 μl of the enzyme solution. A Gilford model 250 spectrophotometer was employed to measure the decrease in A_{340} at room temperature (20°C).

One unit of lactate dehydrogenase is defined as the amount reducing 1 μmol of 2-oxoglutarate (or of pyruvate for other isoenzymes)/min under the above conditions.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed with 6% polyacrylamide gels (5 mm $\times$ 120 mm) in 30 mM-Tris/5 mM-glycine buffer, pH 9.3, with a recirculating buffer system maintained at 5°C. At 5 mA/tube, the electrophoresis was usually complete in approx. 50 min. The gels were stained for enzyme activity by the tetrazolium method (Dietz & Lubrano, 1967), with lactate for all isoenzymes or 2-hydroxyvalerate for specific lactate dehydrogenase C staining.

Purification of enzyme

A 100 g batch of frozen testicles was homogenized in 200 ml of 50 mM-potassium phosphate buffer, pH 6.5, containing 1 mM-dithiothreitol (buffer A). The homogenate was centrifuged at 14 000 g for 30 min at 2°C and the sediment was discarded.

To the supernatant (crude extract), 250 mg of enzyme-grade $(\text{NH}_4)_2\text{SO}_4$ /ml was slowly added with stirring in an ice bath. After being stirred for an additional 20 min, the solution was centrifuged at 14 000 g for 30 min at 2°C. The precipitate was discarded. To the supernatant an additional 300 mg of $(\text{NH}_4)_2\text{SO}_4$ /ml of the solution was added as before. After centrifugation, the supernatant was discarded. The precipitate was suspended in a small volume of buffer A and dialysed extensively against the same buffer. The dialysed solution was centrifuged at 50 000 g for 1 h at 2°C to remove any aggregated protein.

The $(\text{NH}_4)_2\text{SO}_4$ fraction was subjected to affinity chromatography on an AMP-Sepharose column (2.5 cm $\times$ 25 cm) equilibrated with buffer A (Chang *et al.*, 1980). The AMP-Sepharose column was mounted at room temperature close to a cold-box (LKB CombiCold Lab, model 5201B) maintained at 2–4°C. The effluent tube from the column was connected to a fraction collector in the cold-box. The top of the column was connected, with tubing through a peristaltic pump, to the $(\text{NH}_4)_2\text{SO}_4$ fraction, which was kept on ice. This arrangement allowed me to maintain the protein solution and effluent fractions at a low temperature while maintaining the column at room temperature to utilize its high affinity. The cold $(\text{NH}_4)_2\text{SO}_4$ fraction was passed through the column at a rate of 30 ml/h. The column was washed free of unbound protein with the buffer at room temperature at a flow rate of 100 ml/h until the A_{280} of the effluent was less than 0.005. The bound proteins were eluted with NAD^+ -pyruvate adduct by using a gradient elution technique. The adduct was freshly prepared as described by Lee *et al.* (1977): 200 mg of NAD^+ and 200 mg of pyruvate were dissolved in 5 ml of distilled water. The pH of the mixture was raised to and maintained at 11.5 by dropwise addition of 1 M-NaOH at room temperature for 20 min. The solution was diluted to 2 $A_{340}^{1\text{cm}}$ /ml in buffer A. The A_{260}/A_{340} ratio was close to 2.8.

For the gradient elution an Ultrograd Gradient Mixer (LKB model 11300) was used with a linear-gradient chart. The conditions for gradient elution were: initial eluent, buffer A; final eluent, adduct with $A_{340}^{1\text{cm}}$ 2.0; flow rate, 60 ml/h; gradient setting was such that the concentration of the adduct in the eluent would increase linearly, reaching its maximum strength at 300 ml, after which pure adduct ($A_{340}^{1\text{cm}}$ 2.0) would keep running through the

column (see the theoretical gradient plot in Fig. 1). Fractions of volume 10ml were collected and monitored for enzyme activity with pyruvate as well as with 2-oxoglutarate; dilution in the assay procedure was sufficient to overcome inhibition of the enzyme by the NAD⁺-pyruvate adduct; A₂₈₀ for protein could not be monitored because of high absorbance of the adduct at 280nm. The fractions containing enzyme activity towards 2-oxoglutarate (peak I in Fig. 1) were pooled, concentrated to 10ml through an Amicon PM-10 membrane, and dialysed against 10mM-potassium phosphate buffer, pH8.2, containing 1mM-dithiothreitol (buffer B). The solution was centrifuged at 3000g for 20min to remove any precipitate. The AMP-Sepharose column was regenerated by passing 200ml of the adduct in 1M-NaCl followed by equilibration with buffer A; the column could be used several times without any noticeable loss of its efficiency. It should be stored in the cold with 0.02% NaN₃.

The enzyme solution from the AMP-Sepharose column was further chromatographed on a DEAE-cellulose (Whatman DE-52) column (2.5cm × 25cm), the entire procedure being performed in the cold. The enzyme solution was applied to the column and eluted with the equilibrating buffer (buffer B) at a flow rate of 60ml/h, 10ml fractions being collected and the effluent being monitored continuously by a u.v. detector. The first protein peak (Ia) appeared within 100ml of elution (Fig. 2), after which a linear gradient was set as follows: initial eluent, buffer B; final eluent, 0.1M-NaCl in buffer B; flow rate, 60ml/h; fraction size, 10ml; the Ultrograde Gradient Mixer was set so that the gradient would be complete at 100ml. This resulted in the elution of the second protein peak (Ib). A second gradient was set at this point: initial eluent, 0.1M-NaCl in buffer B; final eluent, 0.5M-NaCl in buffer A; flow rate, 60ml/h; fraction size, 10ml; the gradient mixer was set to complete the

Table 1. Purification of rat lactate dehydrogenase C from 100g of rat testicles
Experimental details are given in the text. The activity given here is the one measured with 2-oxoglutarate, a specific substrate from lactate dehydrogenase C.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Crude extract	31000	400	0.01
(NH ₄) ₂ SO ₄ fraction	2200	308	0.14
AMP-Sepharose chromatography	17.2	198	11.5
DEAE-cellulose chromatography	4.3	135	31.4

Fig. 1. Affinity chromatography of the (NH₄)₂SO₄ fraction of 100g of rat testes on a AMP-Sepharose column (2.5cm × 25cm)

Gradient elution conditions are described in the text. The gradient plot (— · —) shown in the chromatogram is theoretical. Activity was measured with 2-oxoglutarate (●) and pyruvate (○) as substrate. On the right-hand side of the Figure are shown electrophoretograms of peak I, peak II and crude extract. The gels were stained by the tetrazolium method with HV (2-hydroxyvalerate) and LA (lactate).

gradient at 200 ml. This resulted in the elution of the third protein peak (Ic). The fractions were monitored for A_{280} activity with pyruvate and activity with 2-oxoglutarate. Fractions were pooled, concentrated and analysed by polyacrylamide-gel electrophoresis. Pool Ib was found to be pure isoenzyme C_4 . This fraction was either stored at 4°C as a 55%-saturated- $(\text{NH}_4)_2\text{SO}_4$ suspension or concentrated by pressure filtration [Amicon PM-10; 140 kPa (20 lbf/in²) of N_2] and stored at 4°C with 0.02% NaN_3 .

Results and discussion

The results on purification of lactate dehydrogenase C from 100 g of rat testicles are summarized in Table 1. The amount of the purified enzyme obtained was 4.3 mg with a specific activity of 31.4 units/mg, representing a 34% yield and about 2400-fold purification.

As stated in the Experimental section, the crude extract was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and chromatographed on an AMP-Sepharose column with a gradient of increasing concentration of NAD^{+} —

pyruvate adduct (Fig. 1). The use of the gradient resulted in the separation of two peaks; the first peak contained mostly lactate dehydrogenase C, whereas the second peak contained the other isoenzymes. The gel-electrophoresis patterns of the two peaks are diagrammatically represented in Fig. 1. The electrophoresis patterns for the crude homogenate, also depicted in Fig. 1, show a common band for isoenzymes C_4 and A_3B_1 . This means that separation of isoenzymes C_4 and A_3B_1 on the basis of charge is either impossible or difficult. The electrophoresis patterns for peak II (Fig. 1) show a band at the position of isoenzymes $C_4 + A_3B_1$ in the gel stained with lactate, but the corresponding band in the gel stained with 2-hydroxyvalerate is missing. Thus the separation of isoenzymes C_4 and A_3B_1 has been achieved on the basis of their differential affinities towards AMP, isoenzyme C_4 being eluted in the first peak and isoenzyme A_3B_1 being eluted in the second peak along with the other isoenzymes. One batch of N^6 -(6-aminohexyl)-5'-AMP-Sepharose did not effect separation of the two peaks. The electrophoresis patterns for peak I (Fig. 1) show the presence of isoenzymes C_4 and A_1C_3

Fig. 2. Chromatography of peak I of Fig. 1 on a DEAE-cellulose column (2.5 cm × 25 cm)

Experimental conditions are described in the text. The gradient plot (-----) showing the NaCl concentration is theoretical. Activity was measured with 2-oxoglutarate (●) and pyruvate (○) as substrate. A_{280} of the fractions is also shown (—). On the right-hand side are shown electrophoretograms of the three peaks. The gels were stained by the tetrazolium method with HV (2-hydroxyvalerate) and LA (lactate).

along with small amounts of isoenzymes A₄, A₂B₂, A₁B₃ and B₄.

Peak I from the AMP-Sepharose chromatography was subjected to DEAE-cellulose chromatography (Fig. 2). Under the conditions of the chromatography, three protein peaks were obtained. By polyacrylamide-gel electrophoresis, the first peak (Ia) was found to contain isoenzymes A₄, A₁C₃ and a trace of C₄. The second peak (Ib) contained only isoenzyme C₄. The third protein peak (Ic) had enzyme activity towards pyruvate but very little activity with 2-oxoglutarate, and hence contained very little lactate dehydrogenase C. Peak Ib was found to be pure isoenzyme C₄ by polyacrylamide-gel electrophoresis.

In this fractionation procedure I have avoided the heating step used by some investigators (Goldberg, 1972; Schatz & Segal, 1969; Lee *et al.*, 1977) to get rid of other lactate dehydrogenase isoenzymes. This decision was made because in the rat the B-type isoenzyme has a thermostability comparable with that of lactate dehydrogenase C.

Purification of isoenzyme C₄ from rat testicles requires its separation not only from other proteins and enzymes but also from other lactate dehydrogenase isoenzymes, of which the most difficult ones to separate are isoenzymes A₁C₃ and A₃B₁. The latter was separated in the present study by AMP-Sepharose chromatography, and the former was separated by ion-exchange chromatography.

The yield of the pure isoenzyme C₄ in the present study was 4.3 mg/100 g of testes, which is 67% higher than in a previous study on rat lactate dehydrogenase isoenzyme C₄ (Schatz & Segal, 1969). However, rat testicles have a considerably lower concentration of lactate dehydrogenase C than do mouse testicles; Lee *et al.* (1977) and Wheat & Goldberg (1977) purified 62 mg of lactate dehydrogenase C/100 g of mouse testes. On the other hand only 3 mg of lactate dehydrogenase C could be purified from 100 g of rabbit testes (Wheat & Goldberg, 1977).

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