SEPARATION OF THE ISOENZYMES OF LACTATE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY USING AN IMMOBILIZED AMP-ANALOGUE

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1. Introduction

The use of affinity chromatography [1] or biospecific affinity chromatography [2] for the separation and purification of enzymes is by now a widely accepted technique. In order to test whether this technique will permit separation of the multiple molecular forms of an enzyme as lactate dehydrogenase, the present investigation was initiated with the object of separating this enzyme into its five different isozymes.

In previous studies the advantages involved, using a general ligand for affinity chromatography, have been demonstrated. Thus Sepharose-bound N^6-(6-aminohexyl)-AMP, was used for separation and purification of a number of NAD^+-dependent enzymes [3–5]. The general underlying principle is that adenosine-5'-monophosphate (AMP) as inhibitor for a great number of enzymes will eliminate the need of binding a different inhibitor ligand for each enzyme to be separated. Subsequent application of specific eluents will then permit elution of one enzyme at a time [5]. The technique used in the present study involves application of the above Sepharose-bound AMP-analogue, elution subsequently being carried out with a weak gradient of NADH.

2. Experimental

Crystalline beef heart L(+)-lactate dehydrogenase (LDH) (type III), crystalline beef muscle L(+)-LDH (type X), pyruvate (type II, sodium salt), DL-lactic acid (grade DL-V, sodium salt), β-NADH (grade III), β-NAD^+ (grade III), nitro-blue tetrazolium (grade III) and phenazine methosulfate were purchased from Sigma Chemical Co., St. Louis, Mo., USA. 'AMP-Sepharose' was prepared as described elsewhere and contained 200 μmoles of nucleotide per g of dry polymer [6].

All other chemicals were of analytical grade and were used without further purification.

LDH activity was measured by the Warburg's assay [7], modified as follows: In the assay were included sodium phosphate (300 μ moles); pyruvate (5 μ moles); NADH (0.5 μ moles) and test solution (50 μl) in a total volume of 3.0 ml, pH 7.5.

Preparation of the isoenzyme mixture from beef heart LDH (containing mainly H_4 and H_3M) and beef muscle LDH (containing mainly M_4) was based on the 'quick-freeze' and 'slow-thaw' method [8]. Heart LDH (0.45 mg) and muscle LDH (0.55 mg) were dissolved together in 0.1 M sodium phosphate buffer pH 7.0, 1 mM β-mercaptoethanol (0.4 ml) and dialysed against the same buffer (500 ml) for 5 hr. The dialysed solution was diluted with the same buffer containing 2 M NaCl to give a protein concentration of 1 mg/ml. This solution was rapidly frozen over a mixture of acetone and dry ice. It was then allowed to thaw slowly at room temperature. About 90% of the enzyme activity remained after hybridisation.

The sample applied to the affinity column was prepared by mixing the above hybridised LDH (0.3 mg) with heart LDH (0.035 mg) and muscle LDH (0.040 mg). This was done in order to obtain a solution containing roughly equal amounts of each of the five isoenzymes.

A concave gradient of NADH was obtained using two parallel-sided containers of a cross-sectional area

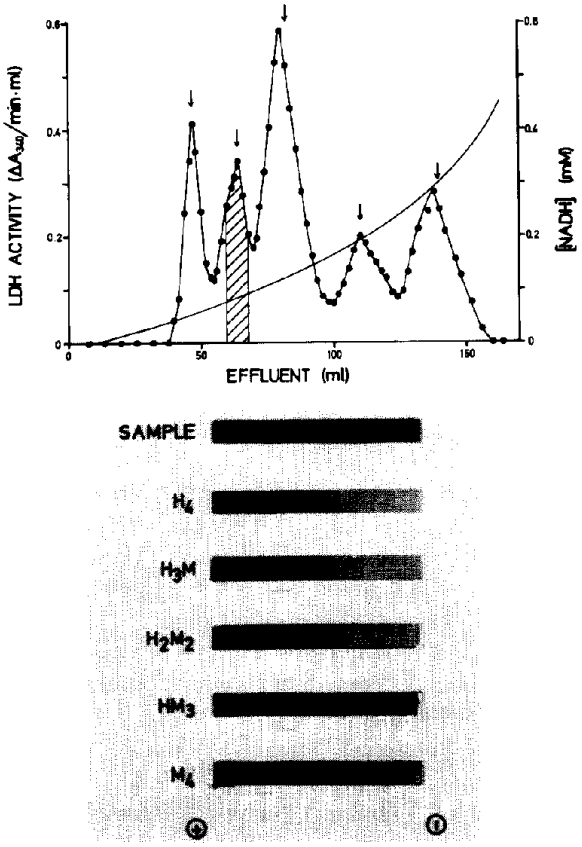

Fig. 1: a) Elution of LDH isoenzymes using a concave gradient of NADH. 0.2 mg of protein in 0.2 ml of 0.1 M sodium phosphate buffer pH 7.0, 1 mM β-mercaptoethanol and 1 M NaCl was applied to an AMP-Sepharose column (140 × 6 mm, containing 2.5 g of wet gel), equilibrated with 0.1 M sodium phosphate buffer pH 7.5. After washing of the column with this buffer (10 ml) the isoenzymes were eluted with a concave gradient of 0.0–0.5 mM NADH in the same buffer, containing 1 mM β-mercaptoethanol. 1.0 ml fractions were collected at a rate of 3.4 ml/hr. The arrows indicate the fractions taken for analysis by polyacrylamide gel electrophoresis. The hatched area indicates the pooled fractions, which were rechromatographed as described in the experimental section;
b) Polyacrylamide gel electrophoresis of applied sample and purified isoenzymes of LDH of the fractions indicated by arrows in fig. 1a. Electrophoresis and staining were carried out as described in the experimental section.

ratio of 2 (mixing chamber):1 (reservoir containing NADH). This concave gradient was applied in order to shorten the total time of elution, although roughly the same pattern was obtained with a linear gradient.

On rechromatography of the fractions containing H_3M, these were pooled and dialysed against 0.1 M sodium phosphate buffer pH 7.5 containing 1 mM β-mercaptoethanol for 5 hr to remove the nucleotide present. Although it is to be expected that even after dialysis the enzyme is present as a binary complex, it will bind to the AMP-analogue likely because of the relatively high AMP concentration in the column.

Gel electrophoresis was carried out as described by Dietz and Lubrano [9]. The gels contained 5.5% (w/w) of polyacrylamide with a relative concentration of cross-linking agent *N,N'*-methylene bis-acrylamide of 2.5% (w/w) and were prepared in 0.05 M Tris–HCl buffer, pH 8.9. The running buffer was 0.05 M Tris–glycine, pH 8.3. Samples (50 μl), prepared by mixing enzyme solution (50 μl), 40% sucrose (50 μl) and 0.05% bromphenolblue (10 μl) (tracking dye), were placed on the gels. The current flow applied was 2 mA per tube. The electrophoresis was stopped 30 min after disappearance of the tracking dye. The gels were stained for LDH-activity, utilising the nitro-blue tetrazolium reagent [9].

3. Results and discussion

Since the finding that lactate dehydrogenase exists in multimolecular forms as isoenzymes there has been a need for their efficient separation and isolation, including on a preparative scale. To this end, in particular ion-exchange matrices such as DEAE-Sephadex [10, 11] and DEAE-cellulose [12] have been used. In the present investigation the potentials of the technique of affinity chromatography for such a purpose were studied because of the general inherent advantages of the latter chromatography technique.

On applying a mixture of all five LDH isoenzymes to a column containing Sepharose-bound N^6-(6-aminohexyl)-AMP, the enzymes remained bound to the ligands. Such binding was to be expected in view of the fact that the K_i of free N^6-(6-aminohexyl)-AMP for LDH lies in the order of 1×10^{-4} M. As seen from fig. 1a, on subsequent application of a gradient of NADH, five distinct peaks were obtained representing the five different isoenzymes as shown by gel electrophoresis (fig. 1b). Whereas both H_4 and M_4 appear pure, the remaining isoenzymes are somewhat contaminated

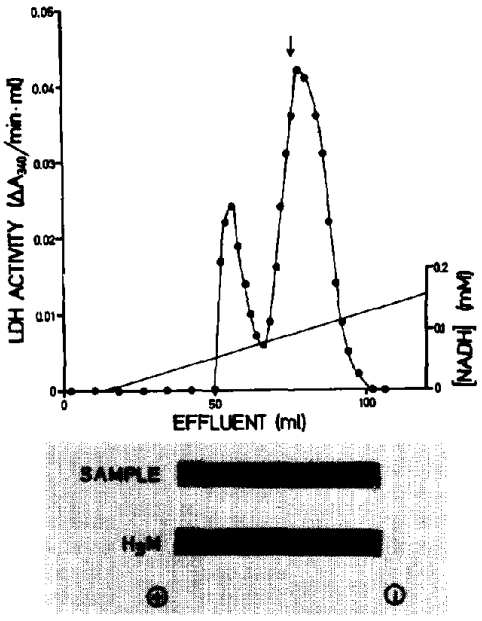

Fig. 2: a) Elution of the rechromatographed H_3M isoenzyme peak using a linear gradient of NADH. 4.0 ml of dialysed enzyme solution obtained from the preceding chromatography run was applied to an AMP-Sepharose column (140 × 6 mm, containing 2.5 g of wet gel), equilibrated with 0.1 M sodium phosphate buffer pH 7.5. The isoenzyme was eluted with a linear gradient of 0.0–0.2 mM NADH in the same buffer, containing 1 mM β-mercaptoethanol. 1.0 ml fractions were collected at a rate of 6 ml/hr. The arrow indicates the fraction taken for analysis by polyacrylamide gel electrophoresis; b) Polyacrylamide gel electrophoresis of applied and purified H_3M isoenzyme. Electrophoresis and staining were carried out as described in the experimental section.

with isoenzyme from the preceding peak. To prove that complete separation can be obtained by this technique, the fractions containing H_3M were pooled and rechromatographed. As is seen from fig. 2 a and b, pure H_3M could thus be obtained. The separation achieved on gradient elution using NADH can be ascribed to the different affinities for NADH of each isoenzyme. In line with such interpretation are reported K_{diss} constants for the binary complexes H_4–NADH = 0.39×10^{-6} M [13] and M_4–NADH = $1.4–2 \times 10^{-6}$ M [14]. The fact that two different parameters come into play in this technique, i.e. besides varying affinities of the isoenzymes for the eluting ligand NADH, also likely different affinities for the bound ligand, may account for the excellent separation obtained using a relatively weak gradient of 0–0.4 mM NADH (= a total of 20 mg). Analogous with the separation described are preliminary data obtained on the separation of the isoenzymes of liver alcohol dehydrogenase [15].

The bound AMP-analogue utilised in this investigation was used repeatedly over a period of several months. Excellent reproducibility was obtained as to the molarity of NADH at which each isoenzyme was eluted (e.g. H_3M being eluted at 0.095, 0.098 and 0.096 mM of NADH respectively in three different runs). The recovery of enzyme activity was about 60%, the activity loss likely to be caused by partial denaturation taking place on standing of the very diluted enzyme solutions.

It should be possible to apply the described affinity chromatography of isoenzymes of LDH in clinical diagnosis. One advantage in this context is the fact that isoenzyme mixtures containing inactive isoenzyme will easily be resolved even if the latter are only denatured in their active sites since these will not adsorb to the column and therefore thus not interfere with the elution pattern. Scaling-up of the procedure for a preparative purpose should also be relatively easy to accomplish.

Acknowledgements

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