AFFINITY CHROMATOGRAPHY OF ENZYMES ON AN AMP-ANALOGUE: SPECIFIC ELUTION OF DEHYDROGENASES FROM A GENERAL LIGAND

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Received 3 July 1972

1. Introduction

The use of general ligands such as NAD^+ for affinity chromatography has been the subject of many studies in this laboratory [1,2]. N^6-(6-aminohexyl)-AMP covalently bound through its terminal amino group to agarose has been shown to be an excellent biospecific adsorbent for NAD^+-dependent dehydrogenases. Although their affinity for this ligand is so strong that they are resistant to elution by salt gradients, satisfactory resolution of these enzymes can be achieved by eluting with pulses of oxidized or reduced cofactor at suitable concentrations. This method of elution can involve considerable effort before ideal eluent conditions are evolved; we now report on some other highly selective methods of elution of enzymes from this immobilized AMP analogue, some of which are more predictable in specificity and may be of rather wide application. These include the application of a linear gradient of NADH to the separation of GAPDH and LDH; the use of oxidized cofactor together with either oxidized substrate or specific inhibitor to separately elute, respectively, the corresponding enzymes LDH and YADH through ternary complex formation; similar specific elution of LDH by applying a preformed NAD^+-pyruvate adduct; and an extension of the model studies to the purification of LDH from a crude ox heart prepration using a mixture of NAD^+ and pyruvate to elute the enzyme by abortive ternary complex formation.

Abbreviations

GAPDH	: D-glyceraldehyde-3-phosphate dehydrogenase;
LDH	: L-lactate dehydrogenase;
YADH	: yeast alcohol dehydrogenase;
ICDH	: threo-D_S-isocitrate dehydrogenase;
GDH	: L-glutamate dehydrogenase;
PK	: pyruvate kinase;
MK	: myokinase;
MDH	: L-malate dehydrogenase.

2. Experimental

Crystalline YADH, ox heart L(+)-LDH (type III), DL-glyceraldehyde-3-phosphate (free acid), pyruvate (type II, sodium salt), L(+)-lactic acid (grade L-I), β-NADH (grade III) and β-NAD^+ (grade III) were purchased from Sigma Chemical Co., St.Louis, Mo., USA and crystalline rabbit muscle GAPDH from Boehringer Mannheim GmbH., Mannheim, Germany. The 'AMP-Sepharose' was prepared as described elsewhere [2] and contained 400 μmoles of nucleotide per g of dry polymer. All other chemicals were of analytical grade and were used without further purification except that acetaldehyde was predistilled.

YADH-activity was measured essentially according to a procedure described [3] except that it was found to be sufficient to use 0.5 μmole of NAD^+ in 3 ml of incubation mixture for the assays. LDH-activity was measured by the previously described modification [2] of the assay of Warburg [4]. The described assay of GAPDH-activity [2], based on the method of Warburg and Christian [5], was modified by the inclusion of 0.05 mmole of arsenate in 3 ml of incubation mixture. Protein was determined according to the procedure of Lowry et al. [6]. Corrections were made for coenzyme interference in the protein determinations. Completely reproducible chromatographic conditions were obtained by using an LKB-Beckman peristaltic pump and fraction collector at 4° for all column chromatography.

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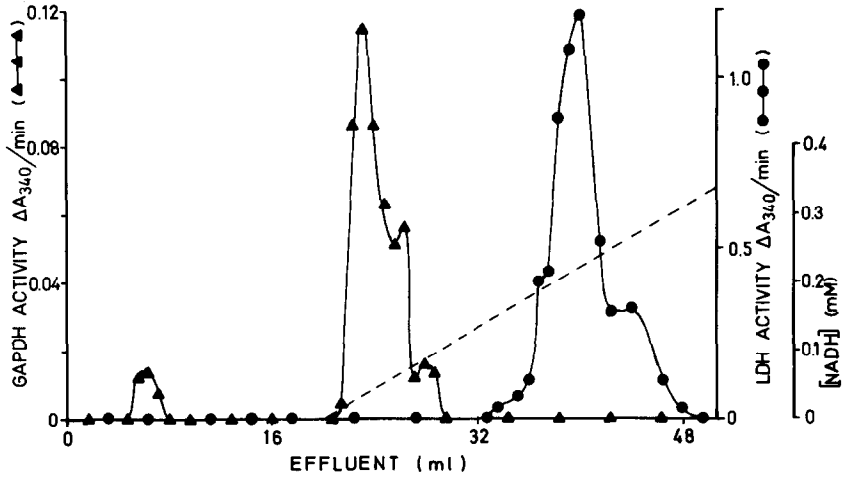

Fig. 1. Elution of GAPDH and LDH using an NADH-gradient. A mixture of 0.05 mg of each enzyme in 0.1 M phosphate buffer, pH 7.5, was applied to an AMP-Sepharose column (100 × 5 mm, containing 2.0 g of wet gel). The enzymes were eluted with a linear gradient of 0.0 – 0.5 mM NADH in the same buffer. 0.8 ml fractions were collected at a rate of 1.2 ml/hr. The somewhat uneven elution profiles were consistently observed.

A crude extract, obtained by 2 min homogenisation of 100 g of ox heart in a Sorvall omnimixer in 120 ml of 0.03 M phosphate buffer, pH 7.3, 1 mM in cysteine, was filtered first through a double layer of cheese-cloth then through glass wool. The filtrate was centrifuged for 2 hr at 20,000 *g* and the precipitate was discarded. Gel electrophoresis was carried out essentially as described [7] regarding gel composition and staining procedure but using 5 mM Tris-glycine (0.039 M) buffer, pH 8.3, and omitting sodium dodecyl sulphate. Ultracentrifugation of LDH was carried out in a Spinco analytical ultracentrifuge model E at a rotor speed of 59,780 rpm.

3. Results and discussion

The effectiveness of Sepharose-bound N^6-(6-aminohexyl)-AMP as a general ligand for affinity chromatography is a reflection of the fact that AMP is an inhibitor for a great number of both NAD^+-dependent and other enzymes. When 'groups' of enzymes are adsorbed by such a ligand, a successful separation depends on the degree of specificity that can be achieved during subsequent elution. The technique of pulse elution has been applied to the separation of GAPDH and LDH using respectively NAD^+ and NADH as eluents [2]. Potassium chloride gradients have been successfully used with other affinity systems [8]. However, in cases involving in particular strong affinity, a mild and more specific method of elution seems preferable to one involving extreme conditions, especially if enzymic activity is to be completely retained. To this end the desirable aspects of both approaches have been combined and the use of an NADH gradient has been examined. As shown in fig.1 at low concentrations the reduced cofactor elutes first GAPDH and then LDH efficiently. The naturally occurring binary complex formation between enzyme and coenzyme appears to be operative. These complexes are generally stronger for NAD^+-dependent dehydrogenases than for those involving the corresponding enzymes and the inhibitor AMP. In addition, in each case there are differences in the binding strength not only of the enzyme–coenzyme complex but also of that between enzyme and AMP. The dual operation of these affinity properties should ensure a high specificity in gradient elution. This technique may also avoid problems involving non-specific ionic interactions in cases where, for instance, charged matrices are used. It is possible that even more subtle separations e.g. of isoenzymes might be accomplished by this method.

Another method of gaining specificity utilises

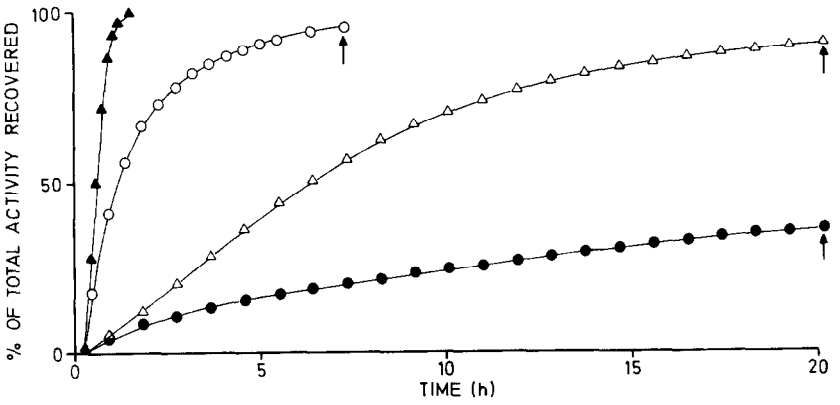

Fig. 2. Efficiency of different eluent systems on ox heart LDH bound to a AMP-Sepharose column (40 × 15 mm, containing 1.0 g of wet gel). 0.1 mg of LDH in 0.5 ml 0.1 M phosphate buffer, pH 7.5, was applied. The following systems in the same buffer were used: 0.5 mM NAD^+ + 0.5 mM L-lactate (●–●–●), 0.5 mM NAD^+ + 0.5 mM pyruvate (△–^–△), 0.5 mM oxidized NAD-pyruvate adduct (○–○–○) and 0.5 mM NADH (▲–▲–▲). The arrows indicate a pulse of 2.0 ml of 10 mM NADH to permit elution of the remaining bound enzyme. Corrections are made for the inhibition effects in enzyme assays. 5.5 ml fractions were collected at a rate of 6 ml/hr.

ternary complex formation. A comparative model study was first carried out on biospecifically-adsorbed LDH to establish the elution efficiency of different complexes, based on the findings described for ternary complex formation between enzyme, coenzyme and substrate or substrate analogues ([9,10] and references cited therein).

As seen from fig. 2, NAD^+ plus pyruvate elutes the enzyme quite efficiently (neither substance alone effects elution). An abortive ternary complex between LDH, NAD^+ and pyruvate is easily formed, although its formation is time dependent [10] as can also be seen from the figure. A mixture of NAD^+ and lactate is also effective, albeit less so. This is consistent with our previous observations with batchwise elution [2] and is probably due to non-abortive ternary complex formation. That elution by NAD^+ plus lactate is caused by formation of NADH *in situ* seems unlikely since under the conditions chosen the amount of NADH present should be negligible. In any event utilisation of abortive ternary complex formation is more efficient. A closely related eluent with efficiency approaching that of NADH is the oxidized form of the adduct synthesised by base-catalysed condensation of NAD^+ and pyruvate [9] *.

Ternary complex formation was subsequently utilised for enzyme separation studies. In fig.3 a complete separation of YADH and LDH is depicted. A solution of NAD^+ and hydroxylamine eluted YADH only, then NAD^+ together with pyruvate yielded LDH. Neither enzyme was obtained by applying these substances separately. We ascribe the elution of YADH to the formation of a ternary complex between the enzyme, NAD^+ and the competitive inhibitor hydroxylamine [11]. It is interesting that no YADH could be eluted by an NAD^+-acetaldehyde mixture. This observation seems to be consistent with the results of recent fluorescence experiments [10]. LDH is eluted on forming an abortive ternary complex as outlined above.

The experiments so far described have all involved simple enzyme mixtures. The practical possibilities of the general ligand system in combination with one of these specific elution techniques is demonstrated by the purification of LDH from a crude ox heart extract. The results are summarised in fig.4. Much protein with no apparent affinity for the AMP-analogue appears in the void volume. On application of a pulse of NAD^+ more protein was eluted but also essentially lacking LDH-activity. NAD^+ and pyruvate

* As part of a joint project with this laboratory, Drs. N.O. Kaplan and J.Everse generously donated this adduct. Their fruitful discussions with one of us (K.M.) is gratefully acknowledged. That part of this paper dealing with this adduct constitutes a preliminary report of this collaboration.

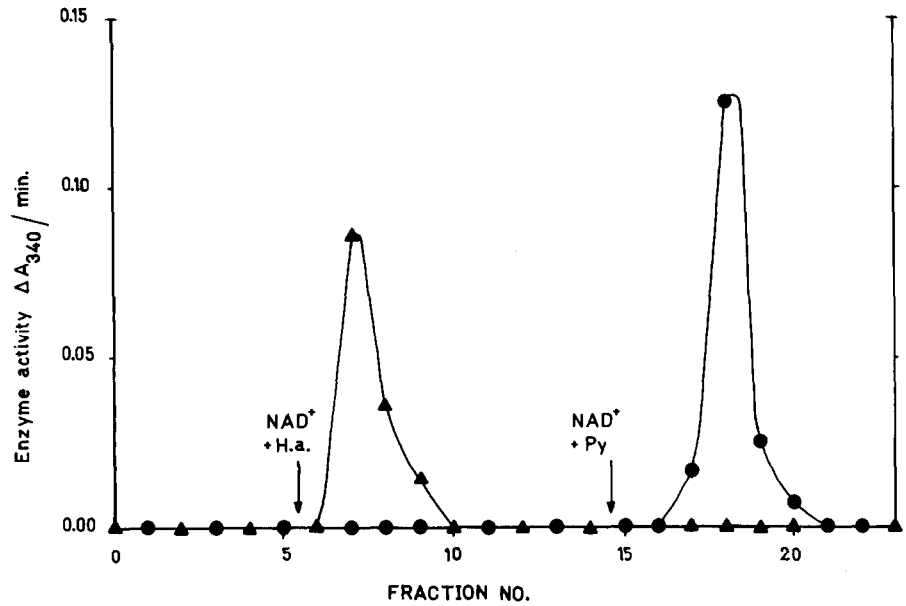

Fig. 3. Chromatography of a mixture of YADH (▲–▲–▲) and LDH (●–●–●) using different eluent systems. A mixture of 0.1 mg LDH and 0.025 mg YADH, dissolved in 0.5 ml of 0.1 M phosphate buffer, pH 7.5, was applied to an AMP-Sepharose column (40 × 15 mm, containing 1.0 g of wet gel). Applications were made, as indicated above by the arrows, of 0.5 mM NAD^+ plus 3 mM hydroxylamine (H.a.) and 0.5 mM NAD^+ plus 5 mM pyruvate (Py) all dissolved in the above buffer. Activities were determined for 100 μl of effluent. 2.2 ml fractions were collected at a rate of 3.3 ml/hr.

together then yielded 95–100% of the total amount of LDH-activity applied. Finally further protein could be eluted by NADH, but practically no LDH-activity was found. The enzyme was purified by a factor of about thirty-five, the whole procedure starting from fresh heart being completed within 24 hr. The enzyme was shown to be pure by the following criteria: a single, homogeneous peak was obtained by analytical ultracentrifugation: on polyacrylamide gel electrophoresis [7] two peaks only were obtained corresponding to the two isoenzymes LDH_1 and LDH_2, in contrast to the extra minor bands observed with commerically available crystalline LDH; finally no activities of enzymes normally present in the extract, e.g. ICDH ($NADP^+$–dependent), GDH, MDH, PK, MK or diaphorase could be detected in the LDH prepared.

These results clearly illustrate that the problem of specific elution is not a limitation on the usefulness of general ligands in affinity chromatography, in spite of recently-expressed scepticism [12]. Rapid and efficient purification of a great number of enzymes using just one ligand is now feasible. Furthermore, valuable information about multi-substrate mechanisms is to be expected to emerge from future studies, for instance the establishing of the occurrence of abortive ternary complexes hitherto undetected.

Acknowledgements

The authors wish to thank Dr.Hugh Guilford for stimulating discussions and linguistic advice, and Margaretha Scott for the work on gradient elution. The investigation has been supported in part by the Swedish National Science Foundation.

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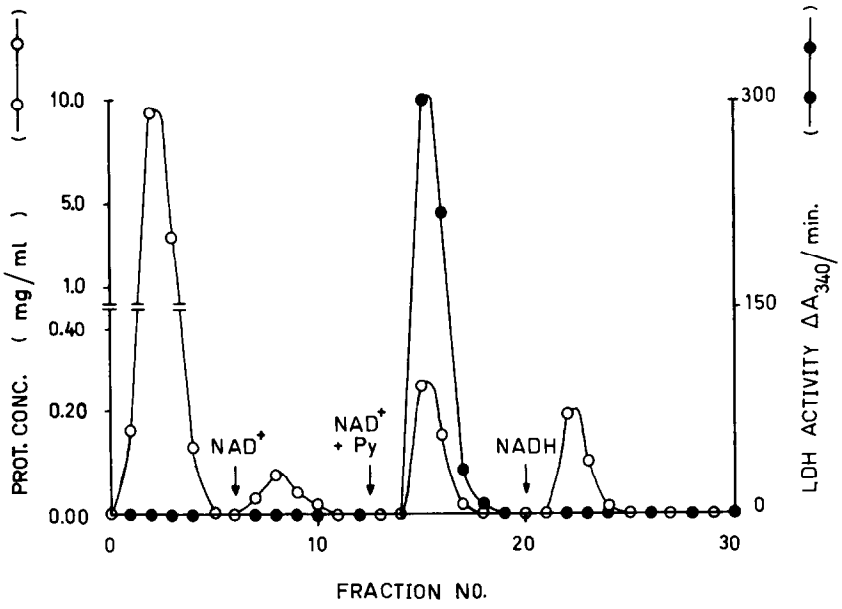

Fig. 4. Purification of LDH from ox heart by affinity chromatography. Crude extract (1.0 ml) was applied to an AMP-Sepharose column (40 × 15 mm, containing 1.6 g of wet gel). The following applications were made with the different substances dissolved in 0.03 M phosphate buffer, pH 7.3, 1 mM in cysteine: at arrow 1, 0.5 mM NAD^+; at arrow 2, 0.5 mM NAD^+ plus 5 mM pyruvate; and at arrow 3, 0.5 mM NADH. To ensure adequate ternary complex formation, the flow was stopped for 12 hr after applications of the NAD^+–pyruvate mixture at fraction 14 corresponding to one void volume. The total LDH activity found per fraction of 2.3 ml and collected at a flow rate of 3.4 ml/hr is given in the figure.

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