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SEPARATION OF ISOZYMES OF HORSE LIVER ALCOHOL DEHYDROGENASE AND PURIFICATION OF THE ENZYME BY AFFINITY CHROMATOGRAPHY ON AN IMMOBILIZED AMP-ANALOGUE

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SUMMARY

A mixture of the two homogeneous isozymes EE and SS of horse liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) was separated on a column of N^6-(6-aminohexyl)-AMP substituted Sepharose with a linear gradient of NAD^+ at pH 7.5 in the presence of 1.5 mM cholic acid. SS was eluted first at 0.4 mM NAD^+, and EE later at 3.4 mM NAD^+. This separation is due to true biospecific adsorption since both isozymes showed no affinity to a gel to which *n*-hexylamine had been covalently bound.

From a horse liver crude extract alcohol dehydrogenase could be purified in a one-step procedure using a pulse of 0.1 mM NAD^+ plus 1.1 mM pyrazole resulting in 22 times purification with a yield of 36%. The enzyme obtained was homogeneous on dodecylsulphate-gel electrophoresis and showed a specific activity of about 3.1 units/mg when measured at pH 10.0.

INTRODUCTION

Horse liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) is a dimeric enzyme for which three isozymes EE, ES and SS [1], or AA, AB and BB [2], are formed by the combination of two different types of protein chains. In addition, a number of subfractions have been found [1, 2]. The primary structures of the two subunits E and S are very similar and a difference of only six amino acid residues has been found by peptide mapping experiments [3]. The isozymes differ in substrate specificity towards 3β-hydroxysteroids [4–6] and ω-hydroxylated steroids [7]. Only isozymes with the S-subunit are active towards the former substrate, while with the latter substrate the S-subunit yields higher activity than the E-subunit.

The isozymes have been separated by conventional methods [6, 8, 9], but there is a need for their efficient separation in high yield, preferentially in a single-step procedure. To achieve this, biospecific affinity chromatography on the general ligand N^6-(6-aminohexyl)-AMP attached to Sepharose was used in the present investigation. While this work was in progress, the separation of the isozymes of beef lactate de-

hydrogenase (EC 1.1.1.27) using this ligand was reported [10]. Separation of the SS and EE isozymes of horse liver alcohol dehydrogenase is now demonstrated. Elution is effected by ternary complex formation of the enzyme with NAD^+ and a 3α-hydroxy-steroid, cholic acid. Alcohol dehydrogenase was also purified from a crude liver extract in a one-step procedure utilizing the ternary complex between the enzyme, NAD^+ and the inhibitor pyrazole [11] for specific elution [12].

MATERIALS AND METHODS

Materials. Nitro blue tetrazolium, phenazine methosulphate, β-NAD^+ (grade III), β-NADH (grade III), glutathione (reduced form, crystalline), cholic acid (sodium salt) and bovine serum albumin (crystalline) were obtained from Sigma (St. Louis, Mo., U.S.A.). Pyrazole was bought from Schuchardt (München, G.F.R.), Sepharose 4B from Pharmacia (Uppsala, Sweden) and *n*-hexylamine from Merck (Darmstadt, G.F.R.). Acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethyl-ethylenediamine were obtained from Eastman (Rochester, N.Y., U.S.A.) and coomassie brilliant blue and sodium dodecylsulphate from Schwarz/Mann (Orangeburg, N.Y., U.S.A.). All other chemicals were of analytical grade and were used without further purification.

Sepharose derivatives. "AMP-Sepharose" was prepared by coupling N^6-(6-aminohexyl)-AMP to Sepharose utilizing BrCN [13, 14]. It contained about 140 μmoles of nucleotide per g of dry polymer.

Sepharose 4B substituted with hexylamine was prepared analogously with the exception that coupling was performed at pH 10–10.5 in 50% (v/v) dimethylformamide in 0.1 M $NaHCO_3$. The hexylaminesubstituted Sepharose gel was exhaustively washed with 50% (v/v) dimethylform-amide in 1 M NaCl, with 1 M NaCl and finally with water. Bovine serum albumin, which is known to bind to alkylamine-substituted Sepharose [15], was used to test the substitution of the gel.

Enzyme assay. Alcohol dehydrogenase activity of both the isozymes EE and SS was measured at room temperature (22–25 °C) with ethanol as substrate according to the method of Dalziel [16].

Protein determination. Protein was determined according to Lowry et al. [17]. Bovine serum albumin was used as standard and corrections were made for interference from coenzyme and glutathione in the protein determinations.

Electrophoresis. Isozymes were identified by electrophoresis in 1% agarose gel in 0.05 M Tris–HCl, pH 8.5, followed by staining for activity with NAD^+ (30 mg), nitro blue tetrazolium (7 mg), phenazine methosulphate (1.5 mg) and ethanol (75 μl) in 0.1 M carbonate buffer, pH 10 (50 ml). Dodecylsulphate-gel electrophoresis at pH 7.3 was performed according to Weber and Osborn [18].

Enzyme preparations. Crystalline horse liver alcohol dehydrogenase from Sigma (St. Louis, Mo., U.S.A.), principally made up of EE, and pure SS isozyme, isolated from horse liver [9] were used for the separation experiments. The crude extract of alcohol dehydrogenase was prepared from frozen horse liver, which was thawed, ground in a meat grinder and extracted at 4 °C overnight with two volumes of distilled water. After centrifugation at 23 000 $\times$ *g* for 4 h and removal of the lipid layer the crude extract was subjected to affinity chromatography.

Alcohol dehydrogenase thus purified was dialyzed for 6 h against 0.05 M

sodium phosphate buffer, pH 7.0, 5 mM in glutathione and then for 6 h against the same buffer made 1 mM in glutathione.

Affinity chromatography. All chromatographic procedures were performed at 4 °C. The columns (145 mm·5 mm), packed with about 2 g of wet gel, were washed prior to each experiment with a few volumes of eluting buffer (0.1 M sodium phosphate, pH 7.5, supplemented with 1 mM glutathione). Samples containing around 3 units of alcohol dehydrogenase activity (1 unit converts 1 μmole of ethanol per min under the assay conditions described) were applied in the separation experiments and samples containing about 10 units in the purification experiments. The sample volumes applied were 0.5–2 ml and gradient volumes 100 ml.

RESULTS

Separation of isozymes

Mixtures of the EE and SS isozymes of horse liver alcohol dehydrogenase were prepared, such that each isozyme contributed an equal number of units with ethanol as the substrate. Samples were applied to columns containing N^6-(6-aminohexyl)-AMP ("AMP-Sepharose"). In the initial studies elution was attempted with

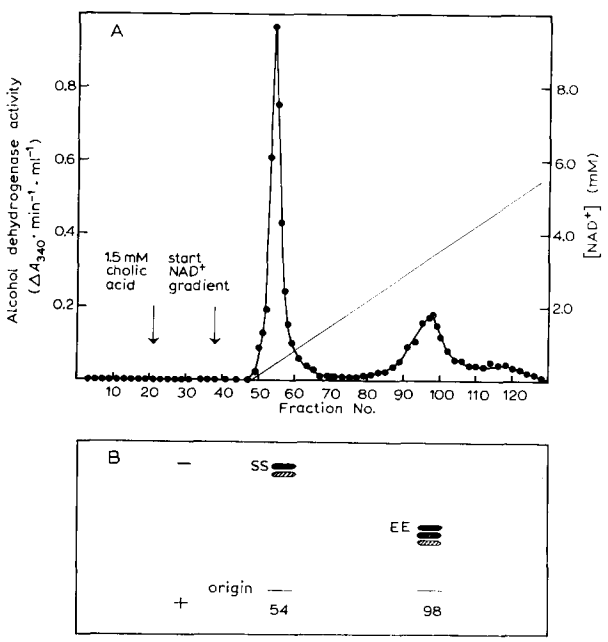

Fig. 1. Separation of a mixture of the EE and SS isozymes on an "AMP-Sepharose" column by elution with NAD^+ and cholic acid. A. Elution curve from the column (145 mm·5 mm, containing 2.0 g of wet gel) loaded with a mixture of 1.5 units of EE in 0.07 ml of 0.1 M sodium phosphate, pH 7.5, made 1 mM in glutathione and 1.5 units of SS in 0.48 ml of 0.05 M Tris–HCl, pH 8.5. The arrows indicate starting of a pulse of 1.5 mM cholic acid and a linear gradient of 0–10 mM NAD^+ (total volume 100 ml) in the presence of 1.5 mM cholic acid. Fractions of 0.6 ml were collected at a flow rate of 2.3 ml/h. ●—●, Horse liver alcohol dehydrogenase activity (corrected for inhibition by cholic acid). B. Agarose gel electrophoresis in 0.05 M Tris–HCl, pH 8.5, followed by staining for enzymatic activity. Hatched areas indicate less intense bands. Forms just below main isozymes are subfractions. Numbers refer to the fractions in A.

various gradients of NADH in different buffers. At pH 7.5 (0.1 M sodium phosphate buffer) both isozymes were eluted together in one peak. At pH 9.0 (0.05 M Tris–HCl, 0.05 M KCl), however, separation was observed with the EE form eluting first. When the ionic strength was raised (0.05 M Tris–HCl, 0.1 M KCl) a poor separation was obtained. In all cases where only NADH gradients were used, recoveries were low. Ternary complex formation between the enzyme, NAD^+ and an inhibitor was therefore tried as an alternative elution method. Since the difference in substrate specificity between the isozymes involves steroids [4–7], steroid derivatives such as 3α-hydroxysteroids known to be inhibitors of the enzyme [4], were tried in the elution by ternary complex formation. Cholic acid (3α, 7α, 12α-trihydroxy-5β-cholanoic acid), which is comparatively soluble in water, was therefore included in the elution buffer.

The elution profile obtained, using a linear gradient of NAD^+ in the presence of 1.5 mM cholic acid, is shown in Fig. 1. SS and EE are well separated and elute at NAD^+ concentrations of 0.4 mM and 3.4 mM, respectively. The fact that SS elutes before EE is in accordance with the reported inhibitory effect of lithocholic acid (3α-hydroxy-5β-cholanoic acid) on the oxidation of ethanol where K_i-values of 0.54 mM and 1.34 mM were obtained for the reactions catalyzed by SS and EE, respectively [6]. The recovery of total enzyme activity, SS plus EE, was 60–70% after correction for inhibition by cholic acid. The yield was, however, dependent on the presence of thiols in the elution buffers, and could be increased by performing the separations with 1 mM glutathione in the buffer, indicating that the losses are partly due to oxidations. It may also be added that the electrophoretic analyses of the peak fractions indicated formation of subfractions, especially in those samples, which were separated in the absence of added thiol and at a more alkaline pH or in samples not directly analyzed after chromatographic separations.

In order to ascertain that the separation found was due to ternary complex formation involving cholic acid, similar chromatographic procedures were performed but with the omission of cholic acid. The elution picture obtained was quite different, as shown in Fig. 2. Only 7% of SS is eluted within the range of 0–1 mM NAD^+, whereas about 75% (corrected for inhibition by cholic acid) of SS is eluted in the presence of cholic acid. EE is eluted at 3.9 mM NAD^+ in the absence of cholic acid compared to 3.4 mM in its presence. These results, together with the fact that cholic acid alone does not elute any enzyme (see Fig. 1) demonstrate that elution is in fact the result of ternary complex formation.

Finally, in order to ascertain that the binding of alcohol dehydrogenase is due to the AMP moiety and not to unspecific interaction with the hexamethylene arm of the analogue, EE and SS were each applied to hexylamine-substituted Sepharose. About 90% of the applied activities appeared in the void volume.

Purification of alcohol dehydrogenase from a crude extract

A crude extract of horse liver was applied to an AMP-analogue column. The object of this part of the study was to achieve purification of alcohol dehydrogenase itself and not its separation into isozymes. To this end the strong competitive inhibitor pyrazole [11] was used together with NAD^+ in the elution buffer. After application of the crude extract, before application of pyrazole, the column was washed with 0.3 M NaCl and 0.1 mM NAD^+ to remove non-biospecifically adsorbed proteins or weakly bound dehydrogenases. Alcohol dehydrogenase could then be eluted with a

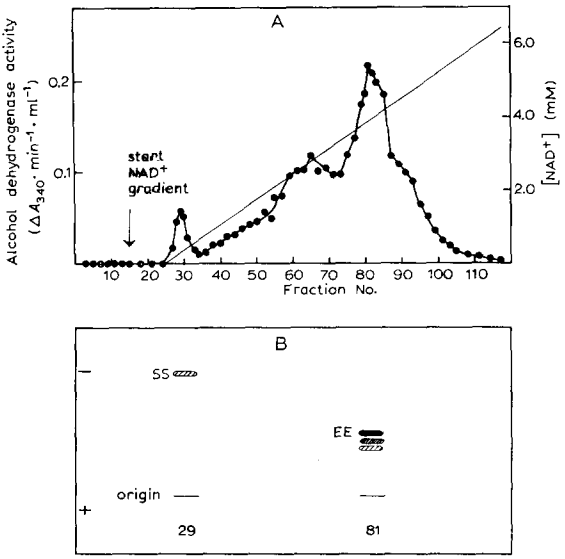

Fig. 2. Separation of a mixture of the EE and SS isozymes on an "AMP–Sepharose" column by elution with NAD^+. A. Elution curve from the column (145 mm·5 mm, containing 2.0 g of wet gel) loaded with a mixture of 1.3 units of EE in 0.09 ml of 0.1 M sodium phosphate buffer, pH 7.5, made 1 mM in glutathione and 1.3 units of SS in 0.45 ml of 0.05 M Tris–HCl, pH 8.5. The arrow indicates starting of a linear gradient of 0–10 mM NAD^+ (total volume 100 ml). Fractions of 0.6 ml were collected at a flow rate of 2.3 ml/h. ●—●, Horse liver alcohol dehydrogenase activity. B. Agarose gel electrophoresis in 0.05 M Tris–HCl, pH 8.5, followed by staining for enzymatic activity. Hatched areas indicate less intense bands. Forms just below main isozymes are subfractions. Numbers refer to the fractions in A.

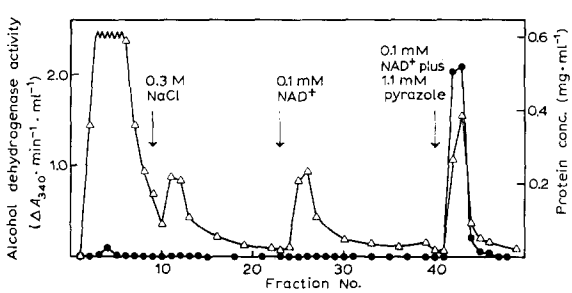

Fig. 3. Affinity Chromatography of alcohol dehydrogenase from a horse liver crude extract on an "AMP-Sepharose" column. The "AMP–Sepharose" column (145 mm·5 mm, containing 2.2 g of wet gel) was loaded with 2 ml of a crude extract containing 11 units of alcohol dehydrogenase. Solutions of 0.3 M NaCl, 0.1 mM NAD^+ and 0.1 mM NAD^+ plus 1.1 mM pyrazole in 0.1 M sodium phosphate buffer, pH 7.5, made 1 mM in glutathione were applied as indicated by the arrows. Fractions of 2 ml were collected at a flow rate of 2.7 ml/h. ●—●, Horse liver alcohol dehydrogenase activity; △—△, protein concentration.

TABLE I

PURIFICATION OF ALCOHOL DEHYDROGENASE FROM A HORSE LIVER CRUDE EXTRACT BY AFFINITY CHROMATOGRAPHY ON A SEPHAROSE-BOUND AMP-ANALOGUE

Step	Enzyme activity		Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
	(units/ml)	(total units)				
Crude extract	5.5	11	77	0.14	1	100
Affinity chromatography (fraction Nos 42 and 43 from Fig. 3)	1.98	4.0	1.31	3.1	22	36

pulse of 1.1 mM pyrazole and 0.1 mM NAD^+. The results are shown in Fig. 3 and the purification summarized in Table I. The fractions obtained were dialyzed prior to enzyme assay. Some denaturation may have taken place at this stage, which in part can account for the moderate yield. The specific activity obtained, about 3.1 units/mg, is roughly equivalent to that of commercially available crystalline horse liver alcohol dehydrogenase preparations. As shown in Fig. 4, gel electrophoresis of the purified enzyme revealed only one band. The mobilities in the gel of this and commercially available alcohol dehydrogenase were identical.

Fig. 4. Dodecylsulphate-gel electrophoresis at pH 7.3. A. Horse liver crude extract. B. Horse liver alcohol dehydrogenase after affinity chromatography.

DISCUSSION

The present results show conclusively that separation of the isozymes of horse liver alcohol dehydrogenase, as well as the purification of the enzyme from a crude liver extract, is possible by biospecific affinity chromatography using the Sepharose-bound AMP-analogue, N^6-(6-aminohexyl)-AMP. The best isozyme separation is obtained when elution is effected by ternary complex formation utilizing a steroid inhibitor, that is preferentially bound together with NAD^+ to the isozyme with the greatest activity towards steroid substrates. The SS isozyme is then eluted first and EE later (Fig. 1) and complete separation is thus obtained. This order of biospecific elution is in agreement with the catalytic properties of the isozymes and with their sensitivity to inhibition by 3α-steroids.

In recent years doubts have been expressed about some reports claiming separations based on so called biospecific affinity chromatography but rather involving, at least in part, hydrophobic interactions between protein and the often hydrophobic spacers and not to the biospecific ligand moiety itself [19]. A further complication may arise from the use of cyanogen bromide activated gels. During the activation step positively charged NH groups may be introduced on the matrix leading to electrostatic interactions between protein and matrix, unless a medium of rather high ionic strength (0.15 M NaCl) is applied [20]. The risk of non-specific binding is most pronounced when applying the usual procedure of preparing affinity adsorbents where the ligand is coupled to a matrix already provided with arms. Here the risk of leaving a large number of unsubstituted arms on the matrix is acute. In the alternative approach a ligand analogue is synthesized with a spacer and then coupled. The latter procedure has been applied in this and other studies reported involving general ligands such as coenzymes [21, 22]. To assure that binding of alcohol dehydrogenase in fact is due to the AMP moiety and not to unspecific interaction with the hexamethylene arm of the analogue, EE and SS each were applied to hexylamine-substituted Sepharose. About 90% of the applied isozyme came off in the void volume showing that the isozyme is not bound non-specifically to arm or matrix.

The formation of subfractions of EE and SS is also of interest. The structural explanation for their occurrence in the horse enzyme is unknown but conformational changes [2,8] or desamidations [23] have been suggested. In the case of the rat enzyme [24] preferential oxidation of certain cysteine residues has been demonstrated. In the present work it was found that formation of subfractions was most extensive in those samples that were separated in low yield by affinity chromatography in the absence of added thiols and at a more alkaline pH, or in those that were not directly analyzed by electrophoresis. It seems possible therefore, that oxidation of SH groups may be associated with formation of this type of subfractions in the horse enzyme, too, and that further oxidation may lead to inactivation of the enzyme.

The affinity columns used in this study, were repeatedly used for several months in the separation studies without any detectable loss in binding capacity. Even the columns to which crude extracts had been applied could be used several times. Generally, after three runs the columns were regenerated by washing with 8 M urea.

Based on the results of the present investigation, demonstrating both the separation of the isozymes of alcohol dehydrogenase using affinity chromatography as well as purification of the enzyme from crude extracts, it is concluded that isolation of the individual isozymes on a preparative scale can be performed.

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