Purification and Separation of Pyridine Nucleotide-Linked Dehydrogenases by Affinity Chromatography Techniques

(general ligand affinity/enzyme purification/isoenzymes/mutant enzymes)

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ABSTRACT A number of different dehydrogenases have been shown to bind to Sepharose-bound N4-(6-aminohexyl)-AMP. These dehydrogenases can be specifically eluted by binary adducts of NAD+ or with cofactor gradients. In such manner pure enzymes can be obtained from crude extracts, as demonstrated in the purification on a preparative scale of lactate dehydrogenase from dogfish muscle. The data presented indicate the usefulness of general ligands as affinity agents. The techniques are particularly adaptable for the isolation of human mutant enzymes in blood or in the purification and concentration of enzymes present at low levels in fluids or tissues, as shown in the extensive purification of serum lactate dehydrogenase and glucose 6-phosphate dehydrogenase from hemolysate. Isoenzymes with different affinities for coenzymes can be separated by affinity techniques. Application of affinity techniques may lead to the separation of isoenzymes or mutant enzymes that are not separable by electrophoretic methods.

During the past few years, great interest has been focused on the development of affinity chromatography procedures for the purification of enzymes. The various approaches have recently been reviewed by Cuatrecasas (1), Porath and Kristiansen (2), and the techniques relevant to this paper by Mosbach (3). The advent of affinity techniques has marked a new era in enzymology by giving a rational approach for the purification of enzymes as well as in making possible the purification of enzymes that previously were not obtainable because of their low concentrations or stability characteristics.

In general, the purification of enzymes by affinity chromatography involves the coupling of a ligand to an insoluble matrix that can then selectively adsorb a given enzyme from a crude extract. In most cases the bound enzyme is subsequently eluted by a ligand that competes with the bound ligand, with high concentrations of salt, or with a shift in pH.

One of the advantages of affinity chromatography is that it may allow for the purification of a whole class of enzymes having common reagents on a single affinity column. For such a procedure to be successful, a "general" ligand must be used which will be able to interact with a group of enzymes. It is essential that the general ligand have a relatively weak affinity for the various enzymes so that they can be eluted with appropriately stronger binding ligands.

Abbreviations: LDH, lactate dehydrogenase; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase.

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This "general-ligand" approach (4) was first applied for pyridine nucleotide-linked dehydrogenases and related enzymes, using N4-(6-aminohexyl)-AMP; this analog of AMP, when covalently attached through its terminal amino group to Sepharose, has been found to bind several different dehydrogenases which could then be eluted with NAD+, NADH, or ternary complexes.

We report in the present communication how reduced NAD adducts (5), as well as agents that lead to abortive dead-end complexes, can be used to selectively separate dehydrogenases from the AMP-analog column. We demonstrate how this technique can be successfully applied for the rapid purification of dehydrogenases, including one example on a preparative scale. We will also discuss the potential of affinity chromatography techniques in detecting multiple forms of enzymes as well as enzyme variants.

MATERIALS AND METHODS

Chicken heart lactate dehydrogenase (EC 1.1.1.27; L-lactate:NAD+ oxidoreductase; LDH) and malate dehydrogenase (EC 1.1.1.37; L-malate:NAD+ oxidoreductase; MDH) from chicken heart mitochondria were prepared by the procedure outlined by Pese et al. (6) and Kitto and Kaplan (7), respectively. Alcohol dehydrogenase (EC 1.1.1.1; alcohol::NAD+ oxidoreductase; ADH) from equine liver was purchased from Boehringer Corp. Pyridine nucleotides were purchased from P.L. Biochemicals; hydroxylamine hydrochloride (Eastman) and oxaloacetate were obtained from Nutritional Biochemicals. All other reagents were purchased in the purest form obtainable from commercial sources.

Enzymatic assays, as well as the preparation of the various reduced binary pyridine nucleotide adducts used in this study, were performed as described by Everse et al. (5). Enzymatic assays were performed on a Perkin-Elmer model 46 Spectrophotometer equipped with a 165 recorder or a Gilford 240 Spectrophotometer with a 6040 recorder.

The N4-(6-aminohexyl)-AMP-Sepharose was prepared as described (8) and contained about 150 amoles of nucleotide per g of dry polymer. NADP+-Sepharose was synthesized by the method of Larsson and Mosbach (9).

RESULTS

Biospecific Elution of Dehydrogenases with Reduced Adducts. Reduced adducts of NAD+ are specific inhibitors of specific dehydrogenases (5). For example, the reduced NAD-pyruvate
adduct only binds to lactate dehydrogenase, whereas the reduced NAD-oxaloacetate adduct specifically inhibits malate dehydrogenases. The structures of these two reduced adducts are given in Fig. 1. A similar specificity for a reduced adduct was found for other dehydrogenases as well. The reduced adducts, therefore, appear to be ideally suited for the resolution of a mixture of pyridine nucleotide-linked dehydrogenases.

In order to test this hypothesis, purified chicken H\textsubscript{2} LDH was applied to the AMP-analog Sepharose column, which was equilibrated in 0.1 M phosphate buffer, pH 7.5. The enzyme could be completely eluted from the column with 0.12 mM reduced NAD-pyruvate adduct, whereas the reduced NAD-oxaloacetate adduct was ineffective in eluting this enzyme.

Alcohol dehydrogenase from horse liver is successfully and specifically eluted from the AMP-analog Sepharose column with 0.12 mM reduced NAD-acetaldehyde adduct. This adduct is, however, somewhat unstable, and it is therefore difficult to prepare it in a pure form. This fact makes the reduced NAD-acetaldehyde adduct somewhat less attractive to use as an eluent.

Various dehydrogenases are capable of forming tightly bound dead-end ternary complexes with NAD\textsuperscript{+} and their oxidized substrates or substrate analogs (10). Thus, LDH forms a dead-end complex with NAD\textsuperscript{+} and pyruvate, and ADH reacts with NAD\textsuperscript{+} and hydroxylamine in a similar manner (11). The formation of dead-end complexes has previously been used for the elution of ox heart LDH and yeast ADH from the AMP-analog Sepharose column (12). We found that liver ADH may be rapidly and quantitatively eluted from the AMP-analog Sepharose column with a mixture of 0.5 mM NAD\textsuperscript{+} and 3 mM hydroxylamine in 0.1 M phosphate buffer, pH 7.5.

Resolution of LDH, MDH, and ADH from the N\textsuperscript{4}-(6-amino-hexyl)-AMP-Sepharose Affinity Column. Samples of the three enzymes, LDH, MDH, and ADH, were mixed and diluted with 0.1 M phosphate buffer, pH 7.5. In a 5-\textmu{l} sample, the activity of each of the three enzymes in the mixture was found to be equivalent to a change in absorbance of 0.150, 0.050, and 0.050 unit/min, respectively, under standard assay conditions. A 0.5-\textmu{l} sample of the mixture of the three enzymes was applied to the AMP-analog Sepharose column that had been previously equilibrated with 0.1 M phosphate buffer, pH 7.5, at 4\textdegree, and the remainder of the enzyme mixture was stored at 4\textdegree. The column was about 4.0 \times 1.2 cm in size. Phosphate buffer (0.1 M), pH 7.5, was then pumped through the column at a rate of 15-20 ml/hr. Fractions were collected every 10 min and assayed for the presence of any of the three enzymes. As is illustrated in Fig. 2, small quantities of the MDH, totaling about 0.5\%, appeared in several of the early fractions. The nature of the leakage is not understood at this time. In general, MDH has a lower affinity for the AMP-analog Sepharose than ADH or LDH.

After sufficient buffer was pumped through the column to remove any nonadsorbed protein, the column was eluted with a freshly prepared solution of 0.12 mM reduced NAD-oxaloacetate adduct at a rate of 15-20 ml/hr. The collected fractions were assayed for the presence of all three enzymes. MDH was found to be eluted as a sharp peak with almost all of the activity being present in three fractions. No LDH or ADH activity could be detected in these fractions.

The alcohol dehydrogenase was subsequently eluted from the column with a mixture of 3 mM hydroxylamine and 0.5 mM NAD\textsuperscript{+} in phosphate buffer. The enzyme was eluted as a sharp peak (Fig. 2) with no apparent contamination by either MDH or LDH.

The lactate dehydrogenase was eluted with a 0.12 mM solution or reduced NAD-pyruvate adduct in phosphate buffer, as shown in Fig. 2. No contamination by MDH or ADH was found in the LDH fractions.

Since the fractions that contained the separated enzymes also contained the inhibiting compounds that were used for the elution of the enzymes, it was necessary to dialyze these fractions before an estimate of the total recovery could be made. In our experiment, all three enzymes were recovered in almost quantitative yields (>90%).

These results show that a mixture of LDH, MDH, and ADH may be effectively resolved by biospecific elution from an affinity column that contains a general ligand. The specific elution was achieved with either reduced NAD adducts or with the compounds necessary to form a dead-end complex with the enzyme.
Preparative Purification of Dogfish Muscle Lactate Dehydrogenase. To demonstrate that the AMP-analog Sepharose column could also be used in a preparative purification of large quantities of enzyme, we undertook the following purification of dogfish lactate dehydrogenase. One hundred grams of dogfish (Squalus acanthias) muscle was minced with scissors and added to 100 ml of cold distilled water. The mixture was homogenized for 60 sec in a Waring Blender. All subsequent procedures were carried out at 4°C. The suspension was stirred for 30 min and centrifuged for 30 min at 29,000 × g. The clear supernatant solution (115 ml) was applied to the AMP-analog Sepharose column (6.0 × 2.5 cm) that had been previously equilibrated with 0.1 M potassium phosphate buffer at pH 7.0. After the sample was applied, the column was washed with phosphate buffer until the absorbance at 280 nm of the eluant approached zero. The enzyme was then eluted with 0.1 M phosphate buffer at pH 7.0, containing 0.12 mM reduced NAD-pyruvate adduct (Fig. 3). Those fractions containing enzymatic activity were pooled and concentrated in a Diaflow cell, with a PM-50 filter. Removal of the reduced pyridine nucleotide adduct by chromatography on a Sephadex G-200 column (21.0 × 2.5 cm) yields an enzyme that has a specific activity of 696 units/mg (Fig. 4). This enzyme crystallizes readily. The specific activity is equivalent to the specific activity of crystalline dogfish LDH obtained by the previously used more lengthy purification procedure (13), which involves ammonium sulfate fractionation, DEAE-cellulose chromatography, and multiple recrystallizations after hydroxyethyl-cellulose chromatography. The yield varies from 20 to 40%, with the longer procedure, whereas the yield was better than 90% with affinity chromatography. The amino-acid composition of the dogfish LDH eluted from the AMP-analog Sepharose column corresponds to that of homogeneous crystalline dogfish M₄ LDH. An analysis of the capacity of the column, as well as the yield obtained during the purification and changes in specific activity, are presented in Table 1.

![Fig. 3. AMP-Sepharose chromatography of dogfish muscle extract.](image)

![Fig. 4. Removal of the reduced NAD-pyruvate adduct from the LDH obtained by AMP-Sepharose chromatography using Sephadex G-200 chromatography. The column was eluted with 0.1 M phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol.](image)

**Table 1. Purification of dogfish M₄ LDH by affinity chromatography on AMP-analog Sepharose and elution with reduced NAD-pyruvate adduct**

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification (×-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>115</td>
<td>874</td>
<td>20,470</td>
<td>23.4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>AMP-analog Sepharose</td>
<td>29</td>
<td>27</td>
<td>18,560</td>
<td>687</td>
<td>91</td>
<td>30</td>
</tr>
</tbody>
</table>

**Purification of Transhydrogenase from Pseudomonas aeruginosa.** Two grams of *Pseudomonas* cells were sonicated in 5 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.1 M mercaptoethanol and 1 mM EDTA. The specific activity of the crude sonicated extract was 0.18 units/mg. The extract was applied to an AMP-analog Sepharose column, the column was extensively washed with buffer, and the transhydrogenase was eluted from the column with 5 mM NADH in buffer. The enzyme was recovered with a 90% yield (see Table 2) and a 314-fold purification. Since the enzyme has a very high molecular weight, a subsequent chromatography of the enzyme over Sephadex G-200 yielded the pure enzyme with an overall yield of 85% and a 1680-fold purification. The conventional purification procedure for this enzyme involves eight steps, and only 15% of the original amount of enzyme is recovered (14).

Other Uses of Affinity Columns. One of the striking features of affinity chromatography is allowing for the purification of an enzyme (protein) that is present in very small concentrations in a tissue or a body fluid. This is illustrated in Table 3. Serum LDH can be adsorbed on the AMP-analog Sepharose column, and after extraneous protein is washed off with buffer, the enzyme can be eluted from the column using the pyruvate adduct with almost full recovery. The purification of the LDH by this procedure is about 17,000-fold and can be as high as 100,000-fold. This type of experiment suggests that through the use of affinity techniques, enzymes (proteins) present in very low concentrations can be purified from tissues as well as from body fluids.

Another example of the effectiveness of affinity techniques is given in Table 4, which demonstrates that glucose-6-phosphate dehydrogenase can be effectively purified from human erythrocyte hemolysates. The enzyme is bound to a NADP⁺-Sepharose column and eluted with a gradient of NADP⁺ (0–0.2 mM) in 0.1 M phosphate buffer, pH 7.4. This results in a purification of 4500-fold with about a 50% yield. The enzyme can be further purified about seven times with the use...
TABLE 2. Purification of Pseudomonas aeruginosa transhydrogenase by affinity chromatography on AMP-analog Sepharose and elution with NADH

<table>
<thead>
<tr>
<th></th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude sonicate</td>
<td>0.18</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>AMP-analog Sepharose</td>
<td>55</td>
<td>90</td>
<td>306</td>
</tr>
<tr>
<td>Sephadex G-200 (void volume)</td>
<td>294</td>
<td>65</td>
<td>1633</td>
</tr>
</tbody>
</table>

of a DEAE-Sephadex column. The enzyme is homogeneous on gels and appears to be in the area of purity described by Yoshida (15). Some of the activity is lost during the concentration steps.

It is of interest that the peak tube showed, after elution from the Sepharose column, an activity corresponding to a 50,000-fold purification from the original hemolysate.

DISCUSSION

The data presented above demonstrate the utility of general affinity chromatography techniques. With a general ligand and a whole family of enzymes can be adsorbed. Knowledge of the inhibitors or substrates of the various enzymes may allow for their selective elution. As illustrated in the present communication, the application of specific binary adducts of NAD" allows for the elution of specific dehydrogenases.

The affinity chromatography techniques have not only made enzyme purification a more rational activity, but have also shortened and simplified the procuring of pure enzymes. In the case of the dogfish Mi LDH, the enzyme can be purified to such an extent that it can be crystallized in a day's time. In the method that we previously used, a number of steps were necessary and several weeks of work were required. With a liter of the N"-(6-aminohexyl)-AMP-Sepharose, we believe we can obtain about 2 g of the LDH in a day.

What is quite striking about the affinity columns is their effectiveness in the purification and concentration of enzymes that are present in low levels in a fluid or tissue. The results of our purification of serum LDH and erythrocyte glucose-6-phosphate dehydrogenase suggest that the procedure can be used to purify small amounts of enzymes. This, for example, can become significant in obtaining human mutant enzymes present in blood in a highly purified form. With the advent of new micro-procedures for fingerprinting and sequencing, it may then be possible to detect the modification in the mutant protein.

Through the use of general affinity columns, several enzymes may be obtained quickly in pure form by either using different substrates or adducts or eluting with a gradient of the same ligand (i.e., NAD" or NADH). For example, the LDH Mi and H4 isoenzymes have different affinities for both the reduced NAD-pyruvate adduct and NADH; this property was used in the separation of the two forms of the enzyme as well as the three intermediate hybrid types by elution from an AMP-analog Sepharose column with a gradient of NADH (16).

It is possible that eventually affinity techniques will be used in the same way as electrophoretic methods are now applied in detecting multiple forms of a given enzyme. It has been a general practice to detect isoenzymes largely by electrophoretic techniques. Electrophoresis will distinguish multiple forms only when they possess differences in charge. There are a number of mutant hemoglobins that have the same charge as the normal form, but that were later found to have differences that do not involve changes in charge. Application of the affinity technique could thus possibly separate the various isoenzymes of a dehydrogenase that have the same charge but are different in their relative Kd values or K... of NAD" or NADH or in their affinities to various coenzyme analogs.

TABLE 3. Purification of LDH from human serum by affinity chromatography on AMP-analog Sepharose and elution with reduced NAD-pyruvate adduct

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>42</td>
<td>4 X 10^4</td>
<td>5.4</td>
<td>0.00135</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>AMP-analog Sepharose</td>
<td>10.6</td>
<td>0.2</td>
<td>4.7</td>
<td>23</td>
<td>87</td>
<td>17,000*</td>
</tr>
</tbody>
</table>

* This is a minimal value, since peak tubes have shown an increase in purification well over 100,000-fold.

TABLE 4. Purification of glucose-6-phosphate dehydrogenase from human erythrocyte by affinity chromatography on NADP"-Sepharose and elution with a gradient of NADP"

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate</td>
<td>1200</td>
<td>43 X 10^4</td>
<td>121</td>
<td>0.0028</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>NADP&quot;-Sepharose*</td>
<td>148</td>
<td>4.2</td>
<td>54</td>
<td>12.8</td>
<td>45</td>
<td>4.571</td>
</tr>
<tr>
<td>DEAE-Sephadex†</td>
<td>53</td>
<td>0.24</td>
<td>22.6</td>
<td>94</td>
<td>19</td>
<td>33,214</td>
</tr>
</tbody>
</table>

* The exact structure of this product is not established. It is prepared by linking e-aminocaproic acid to Sepharose, followed by coupling of NADP" with carbodiimide in 80% pyridine to this matrix (9). It is likely that in the above affinity binding nonspecific binding, i.e., ionic and/or hydrophobic, by the spacer inserted between Sepharose and NADP"; i.e., e-aminocaproic acid, also is involved. Elution was carried out with a gradient of NADP", 0–0.2 mM.
† Elution with 0.05–0.25 mM NaCl in 0.02 M phosphate buffer (pH 6.4).
The studies reported in this paper have been done with the 6-substituted hexylamine AMP derivative. This ligand is an effective one, but recent work in one of our laboratories (17) with 8-substituted AMP derivatives has indicated that the substitution at the 8 position has been effective in "catching" certain pyridine nucleotide-linked enzymes for which the 6-substituted column was ineffective. Further, an 8-substituted NAD\(^+\) derivative (17) as well as a 6-substituted NAD\(^+\) (18) have recently been synthesized and appear to be promising ligands. Studies involved in the modifications of the pyridine nucleotides or their derivatives may give special and more effective affinity characteristics for dehydrogenases.

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