Preparation of an Affinity Chromatographic System for the Separation of ADP Binding Proteins

Elke Bieber, Christoph Woenckhaus, and Harald Pauli

Klinikum der Johann Wolfgang Goethe Universität, Gustav Embden-Zentrum der Biologischen Chemie, Enzymologie, Theodor-Stern Kai 7, D-6000 Frankfurt 70, Bundesrepublik Deutschland

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[4-(3-Bromoacetylpyridinio)-butyl]adenosine pyrophosphate as a structural analog of NAD⁺ reacts covalently with the sulphydryl groups of thiopropyl agarose. 10–20 μmol can be bound to 1 ml gel. Stabilization of the insoluble coenzyme is attained by treatment with sodium borohydride (NaBH₄). This complex when applied to column chromatography, allows the separation of various dehydrogenases as a result of their different complex stability coefficients. Alcohol dehydrogenase from liver, lactate dehydrogenase, and adenylate kinase, which all bind to the ADP-analog residues of the gel matrix, can thus be separated by different salt gradients. Alcohol dehydrogenase from yeast, however, does not form a complex and can easily be eluted from the column with phosphate buffer. Glyceraldehyde-3-phosphate and aldehyde dehydrogenases can be eluted by the addition of NAD⁺ or NADH to the buffer. The uncharged 1,4-dihydropyridinio ring of the reduced coenzyme produces a more stable complex with the dehydrogenases than the oxidized form.

Introduction

The complex formation between substrate and enzyme allows the separation of the latter from crude extracts. If the substrate is bound to an insoluble matrix, the enzymes can be directly extracted from the solution. The quantitative separation of a specific enzyme depends on the characteristic complex stability coefficients which can be influenced by alteration of the salt gradient. Removal of the enzyme from the stationary phase can be affected by the addition of free ligands to the complex.

For the separation of dehydrogenases, certain nucleotides such as AMP, ADP and NAD⁺ are bound via spacer groups of different lengths to the agarose gel. The nucleotides are mainly linked by way of their adenine moieties at N6 or C8 [1–3]. Linkage of the ADP-residues as phosphate esters to the gel matrix has recently been described and a simple preparation of this type of affinity ligand is introduced in this paper.

Abbreviations: GAPDH, glyceraldehyde-3 phosphate dehydrogenase (EC 1.2.1.12); ADH-Y, alcohol dehydrogenase from yeast (EC 1.1.1.1); ADH-L, alcohol dehydrogenase from liver (EC 1.1.1.1). AldH, aldehyde dehydrogenase (EC 1.2.1.3); LDH, lactate dehydrogenase (EC 1.1.1.27); MK, myokinase, adenylate kinase (EC 2.7.4.3).

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Results

The structural analog of NAD⁺ i.e. [4-(3-acetylpyridinio)-butyl]adenosine pyrophosphate reacts with various NAD⁺ dependent dehydrogenases, indicating the formation of binary enzyme-coenzyme complexes with elevated dissociation constants when compared to the natural complexes. The dissociation constant $K_D = 0.014 \text{ M}$ for the yeast alcohol dehydrogenase-coenzyme analog complex is particularly high (Table I).

The coenzyme analog is prepared by condensation of adenosine-5 phosphomorphopholidate and 3-acetylpyridinio-n-butyl phosphoric acid ester in 2-chlorophenol. Bromination of the condensation product to [4-(3-bromoacetylpyridinio)butyl]adenosine pyrophosphate is attained by the irradiation of the acidic solution with white light in the presence of bromine [4]. The degree of halogenation is controlled by the cysteine reaction, in which the degree of halogenation is controlled by the cysteine reaction, in which the.

Table I. Dissociation constants of binary complexes formed with [4-(3-acetylpyridinio)-butyl]adenosine pyrophosphate and dehydrogenases.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_D \times 10^3 \text{ M}^{-1}$</th>
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<tbody>
<tr>
<td>ADH-Y</td>
<td>14</td>
</tr>
<tr>
<td>ADH-L</td>
<td>0.8</td>
</tr>
<tr>
<td>AldH</td>
<td>0.5</td>
</tr>
<tr>
<td>LDH</td>
<td>0.7</td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.2</td>
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</tbody>
</table>
sulfhydryl groups are converted to thiomethylene-carboxy residues leading to a shift in the uv absorption pattern of the derivatives (350 nm, ε 7.500).

Thiopropyl agarose consisting of up to 60 μmol SH-groups per 1 ml gel usually binds 19 μmol coenzyme analog. Treatment of the gel with sodium boro hydride at pH 4 stabilizes the gel by converting the thiomethylene-carboxy groups into the thiomethylene-carbinol residues [5]. This procedure renders the bond to the insoluble coenzyme analog more resistant and the gel more suitable for affinity chromatography. The extent of agarose-coenzyme binding can be determined by elementary analysis of phosphorus and nitrogen of the dried gel. The remaining sulfhydryl groups can be converted to carboxamidomethyl residues by treatment with iodoacetamide.

When applied to affinity chromatography, the coenzyme analog revealed different binding characteristics to the enzymes. ADH-Y does not form a stable complex with the free coenzyme analog and consequently cannot be retained by the gel. It can be washed out from the gel bed with albumine directly after preparing the column. For the separation of the various enzymes (0.01 m phosphate buffer, pH 7), a maximum salt gradient of 0.23 m for ADH-L, 0.35 m for LDH, and 0.38 m for adenylate kinase is required. GAPDH and A1DH can be eluted with buffer containing NAD⁺ or NADH (Fig. 1).

The simple synthesis and the excellent yield of the agarose bound dinucleotide allow the preparation of large columns to purify enzymes from crude protein extracts. In particular 5 mg aldehyde dehydrogenase were separated from 100 mg protein mixture of partly purified tissue extracts. The isolated enzyme did not show impurities in the SDS gel electrophoresis. The specific activity increased from 0.08 to 0.8 U/mg. Caused by the simple preparation this affinity chromatographic system offers an alternative as compared to others [1].

The reduction of the insoluble coenzyme analog with sodium boro hydride in alkaline solution leads to complete hydrogenation of the heterocyclic nucleus, which can be detected by the disappearance of the absorption band at 390 nm. Treatment of the gel with sodium dithionite at pH 8 yields the 1,4-dihydro derivative. The degree of reduction can be controlled spectroscopically and chemically. In the presence of cyanide ions an addition product is formed from the oxidized form of the coenzyme analog that shows identical absorption characteristics as the original free form. If, in spite of further cyanide addition to the solution, the extinction of the absorption at 390 nm does not increase, complete reduction can be assumed. The dihydro coenzyme analog is slightly stable in Tris/HCl buffer at pH 8. Decomposition starts after a few days storage at 4 °C which leads to products showing an uv absorption at 310 nm. In the reduced state, however, the affinity chromatographic system provides a stronger bonding power for the enzymes LDH and ADH-L. GAPDH can only be separated from the column by addition of NAD⁺ to the buffer (Fig. 2).

**Discussion**

The formation of NAD⁺-dehydrogenase complexes is initiated by the interaction of the pyrophosphate residues of the coenzyme with the positively charged groups of the protein *i.e.* the arginine side chains [6]. The two nucleotide moieties are attached to the different binding areas [7]. Fixation
of the coenzyme analog to thiopropylagarose should lead to complex formation that resembles the binding characteristics of the free form with various dehydrogenases. Considering the AMP and NMN nucleotides residues of the coenzyme NAD+, however, only the adenosine nucleotides such as AMP and ADP are able to form stable complexes with the dehydrogenases. NMN is not bound to the catalytic center and the adenine free fragment NMP only forms very weak complexes with a high dissociation constant. This indicates, that the positively charged pyridinium ring is repelled by the positive amino acid residues within the nicotinamid binding site [6]. The 1,4 dihydropyridine ring of the partially reduced coenzyme is better suited in forming stronger complex bonds.

The replacement of ribose in NAD+ by a hydrophobic hydrocarbon chain also diminishes complex stability.

Treatment of the covalently bound coenzyme analog with sodium borohydride in acid solution leads to the reduction of the carbonyl group. The absence of the carbonyl group renders the linkage between coenzyme and the gel matrix more resistant although the complex stability between coenzyme and dehydrogenase suffers somewhat as a result of this procedure. In the natural NAD+-enzyme complexes, the pyridine ring is attached via hydrogen bonding to the carbonyl function and the side chain of the protein. It is apparent, then, that most enzymes can be liberated from the column by alterations in the salt gradient. The complexes with
GAPDH and ADH and the coenzyme analog can only be solved by prior addition of free nucleotide to the eluant. On the other hand it is the different affinity of the ligand to different dehydrogenases that allows a selective elution of aldehyde dehydrogenase from the column.

Nearly 1% of the covalently bound coenzymes are involved in the complexation process with the dehydrogenases which indicates that many of the bound molecules are not in very favorable positions to guarantee complex formation.

The lack of positively charged dihydropyridine rings allows a tighter bonding of the enzymes to the insoluble coenzyme analog. But dihydropyridine can be easily destroyed by low pH values and certain anions.

**Materials and Methods**

*Enzymes and coenzymes:* Alcohol dehydrogenase from yeast and liver (EC 1.1.1.1), glyceraldehyde-3 phosphate dehydrogenase (EC 2.7.4.3), lactate dehydrogenase (EC 1.1.1.27), adenylate kinase (EC 2.7.4.3), NAD* and NADH were products of Boehringer, Mannheim. Aldehyde dehydrogenase (EC 1.2.1.3) was prepared to have a maximum specific activity of 0.1 U/mg [8]. Bovine serum albumine was obtained from Behringwerke, Marburg. [4-(3-Acetylpyridinio)-butyl]adenosine pyrophosphate was prepared as described [4]. Agarose was purchased as Sepharose 6B from Pharmacia, Uppsala, Sweden. Enzymic activities were controlled at 340 nm by the formation of NADH or NAD* [9].
Protein concentrations were determined at 280 nm [10] or by the Biuret- or Bradford-method [11, 12].

\[4-(3\text{-Bromoacetylpyridinio})\text{butyl} \text{adenosine pyrophosphate}\]

350 mg (0.5 mmol) of \[4-(3\text{-acetylpyridinio})\text{-n-buty 1}\text{ adenosine pyrophosphate}\] were dissolved in 15 ml 3\% aqueous HBr. 160 mg bromine were added and the solution irradiated with a Shandon lamp, white light 3 x 6 W. After 48 h, an excess of bromine and hydrogen bromide was removed by extraction with 12 x 50 ml ether. The coenzyme analog was precipitated from the aqueous phase by addition of cold acetone. At \(-20^\circ\)C the compound is stable for some days. To determine the degree of bromination a small 10 mg amount of the coenzyme was dissolved in 0.1 ml 1 M acetate buffer, pH 4 and 5 - 20 \(\mu\)l given to 1 ml of the same buffer containing 0.05 M cysteine. After 2 h standing at ambient temperature, 0.1 ml from the solution were given to 2 ml 2 M glycine/NaOH buffer, pH 10. The absorbance at 350 nm was measured. The cysteine coenzyme compound shows an absorption maximum at 350 nm, \(\varepsilon = 7500\).

For the preparation of the affinity gel 400 mg of the bromo-compound were dissolved in 50 ml 1 M acetate buffer, pH 4 and added to the corresponding amount of freshly prepared thiopropyl agarose [13]. To determine the rate of incorporation into the agarose 1 ml of the gel was washed with water and dried in vacuo. The dry material was analyzed for nitrogen and phosphorus. The content of SH groups was measured photometrically according to Ellman [14]. A 500 ml suspension of the affinity gel was treated with 1 g sodium borohydride and slowly stirred at pH 4. After 15 min the gel was collected by vacuum filtration and resuspended in 0.5 M acetic acid. According to the content of free SH groups a twofold excess of iodoacetamide was added to the mixture and the suspension stirred at room temperature for 24 h. The gel was filtered off and washed with 1 M acetate buffer until the filtrate showed no trace of iodoacetamide. As a final procedure the gel was washed with the elution buffer.

For the preparation of the 1,4-dihydroform of the insoluble coenzyme 50 ml of the suspension were added to 200 ml 5\% aqueous sodium hydrogen carbonate. In this solution 5 g sodium dithionite were dissolved and the mixture left in a boiling water bath for 5 min. The gel was then filtered off and washed with 5 L 10 mM Tris/HCl buffer, pH 8. The formation of the dihydroform could be optically determined at 390 nm. The content of remaining oxidized coenzyme could be detected by addition of potassium cyanide. The cyanide adduct shows an absorption at 380 nm.

For affinity chromatography, a column (1 x 30 cm) was filled with the gel containing the oxidized coenzyme analog and washed with 0.01 M phosphate buffer, pH 7.0, in 2 mM EDTA. The protein mixtures were applied to the gel and the column washed with the buffer until no absorption at 280 nm could be detected. Then the gel was washed with a linear salt gradient in the same buffer. The gradient was prepared by mixing of 100 ml 1 M NaCl in 0.01 M phosphate buffer, pH 7, in 2 mM EDTA and 100 ml saltfree buffer. When the concentration of 1 M NaCl was reached, the column was washed with the elution buffer and a linear NAD\(^+\) gradient dissolved in elution buffer. The latter was prepared by mixing of 15 ml 10 mM NAD\(^+\) to 15 ml NAD\(^+\) free elution buffer. GAPDH was completely eluated with 10 mM NADH in 0.01 M phosphate buffer, in 2 mM EDTA. For the separation of aldehyde dehydrogenase we used 5 mM phosphate buffer, pH 6 with 1 mM EDTA and 0.1% mercaptoethanol.

For affinity chromatography with the reduced dihydroform of the insoluble coenzyme 10 mM Tris/HCl buffer, pH 8, was used. The dissociation constants of the binary complexes of coenzyme analog and enzymes were determined by equilibrium dialysis using carbonyl \([^{14}\text{C}]\text{labeled }[4-(3\text{-acetylpyridinio})\text{butyl}]}\text{adenosine pyrophosphate (0.118} \mu\text{Ci}/\mu\text{mol)} [10] or by fluorescence measurements [15].