The Purification and Properties of Rat Liver Nicotinamide Adenine Dinucleotide Kinase

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SUMMARY

NAD kinase (ATP:NAD 2'-phosphotransferase, EC 2.7.1.23) has been purified from rat liver. Approximately a 1000-fold purification with a yield of 30% is routinely achieved. Preparations of up to 2000-fold purification have been achieved with a yield of 3 to 5%.

The apparent \( K_m \) values for NAD and ATP are 1.4 and 7.0 mM, respectively. The \( V_{max} \) was calculated to be 21 μmoles of NADP formed per mg of protein per hour.

The pH optimum is around 7.0. The enzyme is stable at pH 9 to 10. Rapid loss in activity occurs at lower pH values. Mg\(^{2+}\) is essential for enzymatic activity. Mn\(^{2+}\) can substitute for Mg\(^{2+}\), as can to a lesser extent, Zn\(^{2+}\) and Fe\(^{2+}\).

In 1938 von Euler and Adler (1) reported interconversion of NAD\(^{+}\) and NADP\(^{+}\) in yeast. Subsequently, Mehler et al. (2) reported the synthesis of NAD\(^{+}\) from NADP\(^{+}\) and ATP in pigeon liver extracts. By 1950, Kornberg (3) had partially purified the NADP\(^{+}\)-synthesizing protein from yeast and demonstrated that the enzyme catalyzed the following reaction:

\[
\text{NADP}^{+} + \text{ATP} \rightarrow \text{NAD}^{+} + \text{ADP} + \text{Mg}^{2+} \quad \text{and Mn}^{2+}
\]

Since then, enzymatic activity of this type has been detected in a wide variety of animal and plant tissues (4-7). NAD\(^{+}\) kinase has been partially purified from several sources: pigeon liver (5, 8), spinach (6), yeast (3), and Lactobacillus casei (7). In view of the practical considerations which make rats invaluable animals for experimentation \textit{in vivo}, and faced with a considerable body of literature concerned with the elucidation of NAD\(^{+}\) synthesis in rat liver, it seemed to us that the purification of the NAD\(^{+}\) kinase enzyme from rat liver would be of considerable interest.

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EXPERIMENTAL PROCEDURE

Purification of NAD\(^{+}\) Kinase—Fischer rats weighing 300 to 350 g were killed by decapitation. The livers were immediately removed, collected on crushed ice, and stored at \(-15\)°. The livers can be stored in the frozen state for several days without significant loss of enzymatic activity. Pooled frozen livers (40 to 80 g) were homogenized in a Teflon-glass homogenizer in 4 volumes of 0.05 M Tris-Cl\(^-\) buffer, pH 7.5. Rat liver, 200 g, was processed at one time. The crude homogenate (Fraction I) was centrifuged at 33,000 \( \times g \) for 60 min and the pellet was discarded. The addition of 35.3 g of solid ammonium sulfate per 100 ml of supernatant (Fraction II) precipitated the enzymatic activity which was collected by centrifugation at 33,000 \( \times g \) for 30 min. The pellet was then suspended in 200 ml of 0.01 M Tris-Cl\(^-\) buffer, pH 8.0, and this material was dialyzed overnight against 30 volumes of the same buffer. The dialysate (Fraction III) was heated for 5 min at 60°, cooled in an ice bath, and centrifuged at 35,000 \( \times g \) for 30 min. The pellet was discarded, and the supernatant material (Fraction IV) was treated with 0.1 ml of a calcium phosphate gel (90 mg, dry weight, per ml) per ml of supernatant material and allowed to stand for 15 min. After centrifugation at 2,000 \( \times g \) for 10 min, the pellet was washed once with 200 ml of 0.05 M Tris-Cl\(^-\) buffer, pH 8.0. The pellet was then eluted twice with 40-ml portions of 0.5 M potassium phosphate, pH 7.5, allowed to stand 15 min each time, and centrifuged at 2,000 \( \times g \) for 10 min. Both eluates were combined and solid ammonium sulfate (10.59 g/100 ml) was added. The material which sedimented after centrifugation at 35,000 \( \times g \) for 15 min was discarded, and 14.12 g of solid ammonium sulfate per 100 ml of supernatant were added. The material sedimented at 35,000 \( \times g \) for 15 min was dissolved in 10 ml of 0.05 M Tris-Cl\(^-\) buffer, pH 8.0, containing 0.005 M cysteine. This was then dialyzed for 2 hours against 1 liter of an identical buffer solution (Fraction V). Protamine sulfate (1%), in water, was then added slowly to the dialysate until no further precipitation was detected (about 3.5 ml/10 ml of dialysate). The pellet obtained after centrifugation at 35,000 \( \times g \) for 30 min was homogenized in 40 ml of 0.05 M Tris-Cl\(^-\) buffer, pH 8.0, containing 0.005 M cysteine and 5% ammonium sulfate. After standing for at least 2 hours, this material was centrifuged at 35,000 \( \times g \) for 30 min. The pellet was discarded and 35.3 g of ammonium sulfate/100 ml was added, the pellet obtained after centrifugation at 35,000 \( \times g \) for
Purification of NAD+ kinase from frozen rat liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude homogenate</td>
<td>1420</td>
<td>50.0</td>
<td>0.015</td>
<td>100</td>
</tr>
<tr>
<td>II. Supernatant of crude homogenate</td>
<td>1140</td>
<td>34.0</td>
<td>0.023</td>
<td>84</td>
</tr>
<tr>
<td>III. Dialyzed (NH₄)₂SO₄ fraction of the supernatant</td>
<td>330</td>
<td>76.0</td>
<td>0.047</td>
<td>111</td>
</tr>
<tr>
<td>IV. Heated dialysate</td>
<td>230</td>
<td>20.8</td>
<td>0.191</td>
<td>86</td>
</tr>
<tr>
<td>V. Dialyzed (NH₄)₂SO₄ fraction of extract from first calcium phosphate precipitation</td>
<td>15</td>
<td>25.0</td>
<td>1.49</td>
<td>52</td>
</tr>
<tr>
<td>VI. Dialyzed (NH₄)₂SO₄ fraction of extract from protamine sulfate precipitation</td>
<td>12</td>
<td>5.5</td>
<td>6.44</td>
<td>40</td>
</tr>
<tr>
<td>VII. (NH₄)₂SO₄ fraction of extract from second calcium phosphate precipitation</td>
<td>2</td>
<td>9.8</td>
<td>15.7</td>
<td>29</td>
</tr>
<tr>
<td>VIII. (NH₄)₂SO₄ fraction of Fraction VII after dilution with 4 M urea</td>
<td>0.5</td>
<td>0.027</td>
<td>30.0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Based on 100% recovery for Fraction I.

15 min was dissolved in 10 ml of 0.05 M Tris-Cl- buffer, pH 8.0, containing 0.005 M cysteine and was dialyzed against 1 liter of an identical buffer solution for 3 hours (Fraction VI). Another calcium phosphate gel precipitation was then carried out with 0.3 ml of gel per ml of dialysate. The pellet was washed twice with 0.05 M Tris-Cl- buffer containing 0.005 M cysteine and 5% ammonium sulfate and was eluted twice with 10-ml portions of 0.5 M potassium phosphate, pH 7.5, containing 0.005 M cysteine; the supernatant fraction thus obtained was treated with 10.59 g of ammonium sulfate/100 ml and centrifuged for 15 min at 35,000 x g. The pellet was discarded and 14.12 g of ammonium sulfate precipitations, centrifugation was begun 15 min after complete solution of the salt. All operations were carried out at 4°C. This method is based on the measurement of the change in optical density at 340 nm obtained by reducing the NADP+ formed with isocitrate and NADP+-specific pig heart isocitrate dehydrogenase. The fractions obtained from the isolation procedure were assayed as follows. To a aliquot, 0.1 ml each, of the fractions or appropriate dilutions thereof, 0.4 ml of an incubation mixture containing the following was added: NAD+, 0.010 M, ATP, 0.013 M, nicotinamide, 0.010 M, sodium pyruvate, 0.010 M; MgCl₂, 0.010 M; and Tris, 0.350 M. This high level of Tris was employed to assure that the pH remained constant throughout the reaction. The sample tubes were incubated with shaking at 37°C and the reaction was stopped after 0, 15, and 30 min by heating in a boiling water bath for 1 min. The assay is linear with time under these conditions. The boiled samples were centrifuged 2 min in a Beckman microfuge, and 0.3 ml of the supernatant was removed and placed in a 1-ml quartz cuvette. Tris (0.05 M, 0.7 ml, pH 8.0) was added and the initial optical density reading at 340 nm was recorded. Then 0.05 ml of isocitrate (0.05 M) and 0.1 ml of pig heart isocitrate dehydrogenase (10 mg per ml) were added and mixed, and a final optical density reading was taken after 2 to 3 min when no further increase was noted. Zero time readings were subtracted from the 15- and 30-min readings to obtain the change in optical density per unit of time. Sodium pyruvate must be included in the reaction mixture for assay of fractions prior to Fraction V to prevent the high initial absorbance (prior to addition of isocitrate and isocitrate dehydrogenase) which increased linearly with time. It is thought that contaminating enzymes and substrates could be reducing NAD+ and the newly synthesized NADP+. Greenbaum, Clark, and McClean (9) have sidestepped this obstacle by developing a completely different assay procedure. Values for NAD+ kinase activity in homogenates and supernatants of rat liver obtained by either method are similar. In the experiments carried out with the purified enzyme, nicotinamide and sodium pyruvate were not added; cysteine, 0.005 M, was added unless otherwise specified.

**Chemicals**—NAD+, ATP, Tris (Sigma 121), sodium pyruvate, nicotinamide, calcium phosphate gel, and pig heart isocitrate dehydrogenase, Type I, were obtained from Sigma Chemical Company. L-Cysteine and protamine sulfate were purchased from Nutritional Biochemicals Corporation and trisodium salt (allo-free), from Calbiochem.

**Protein Determination**—The protein content of the fractions was determined according to the modification by Lowry et al. (10) of the Folin-Ciocalteu procedure. L-Cysteine gives a positive color reaction in this protein assay. Protein solutions which contain 0.005 M cysteine must be diluted at least 1:50 to eliminate interference.

**RESULTS**

**Purification of NAD+ Kinase**—The results of a typical purification procedure are shown in Table I. The enzyme has undergone up to a 2000-fold purification with a 4% yield. The enzyme at this stage, however, still contains contaminating proteins as measured by disc gel electrophoresis. Fraction VII, which was employed in most of the studies reported, is free of detectable amounts of NAD glycohydrolase, adenylate kinase, ADPase, and the various pyrophosphatases dealing with pyridine nucleotide metabolism.

**Intracellular Location of Enzymatic Activity**—With the use of the procedure of Schneider and Hogeboom (11), 98% of NAD+ kinase activity in rat liver was found associated with the supernatant fraction, i.e. that material not sedimented by centrifuga-
tion at 105,000 \times g for 2 hours. This is also true for pigeon liver (5), yeast (3), and spinach (6). NAD\(^+\) kinase in thyroid gland tissue is associated with the nuclear fraction (12).

**Determination of \(K_m\) Values for NAD\(^+\) and ATP**—The apparent \(K_m\) values for NAD\(^+\) and ATP under the experimental conditions described were 1.4 \times 10^{-3} M and 7.0 \times 10^{-3} M, respectively (Fig. 1). The \(V_{\text{max}}\) was calculated to be 21 umoles of NADP\(^+\) formed per mg of protein per hour. The kinetic parameters were calculated from \(V \text{ versus } V/\text{substrate concentration}\) plots as described by Dowd and Riggs (13).

**Substrate Specificity**—The following nucleotides were tested with regard to their ability to replace ATP: GTP, CTP, TTP, dATP, ADP, UTP, and dGTP. dATP was as active as ATP and was the only compound to have any activity.

**Various NAD analogues** were qualitatively tested in order to determine whether NAD kinase could convert them to their respective NADP analogues. Thionicotinamide adenine dinucleotide, 3-acetyl pyridine adenine dinucleotide, and 6-aminonicotinamide adenine dinucleotide were so utilized. Pyridine aldehyde adenine dinucleotide, \(\alpha\)-NAD, and hypoxanthine adenine dinucleotide did not serve as substrate for NAD kinase.

**Table II**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg(^{++})</td>
<td>100</td>
</tr>
<tr>
<td>Mn(^{++})</td>
<td>80</td>
</tr>
<tr>
<td>Co(^{++})</td>
<td>6</td>
</tr>
<tr>
<td>Fe(^{++})</td>
<td>19</td>
</tr>
<tr>
<td>Fe(^{+++})</td>
<td>0</td>
</tr>
<tr>
<td>Zn(^{++})</td>
<td>41</td>
</tr>
<tr>
<td>Cu(^{++})</td>
<td>0</td>
</tr>
<tr>
<td>Ni(^{++})</td>
<td>0</td>
</tr>
<tr>
<td>Sn(^{++})</td>
<td>0</td>
</tr>
<tr>
<td>Ba(^{++})</td>
<td>10</td>
</tr>
<tr>
<td>Ca(^{++})</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Based on 100% activity in the presence of Mg\(^{++}\).

**Fig. 1.** Determination of the kinetic parameters of rat liver NAD\(^+\) kinase. The concentration of NAD\(^+\) was varied in the presence of 0.013 M ATP, 0.010 M MgCl\(_2\), 0.350 M Tris-chloride buffer, pH 8.0, 0.005 M L-cysteine, and enzyme of specific activity 16.0. In the experiment where the concentration of ATP was varied, 0.010 M NAD\(^+\) was employed, concentration of enzyme and reactants otherwise being unchanged. \(K_m\) values and \(V_{\text{max}}\) were calculated according to the procedure of Dowd and Riggs (13). ○, activity at various levels of ATP; ●, activity at various levels of NAD.

**Fig. 2.** Effect of varying concentrations of Mg\(^{++}\) and Mn\(^{++}\) on the activity of rat liver NAD\(^+\) kinase. The concentrations of the metals were varied in the presence of 0.010 M NAD\(^+\), 0.013 M ATP, 0.350 M Tris buffer, pH 8.0, and 0.005 M L-cysteine. A dilution of enzyme specific activity 10.0 was used.

**Fig. 3.** Determination of the pH optimum and pH stability of NAD\(^+\) kinase. A dilution of the enzyme, specific activity 10.0, was incubated in 0.010 M MgCl\(_2\), 0.010 M NAD\(^+\), 0.013 M ATP, 0.0050 M L-cysteine, and 0.030 M Tris-maleate buffer at the pH indicated (○—○). In the pH stability studies, 0.02 ml of a dilution of enzyme, specific activity 16.0, was incubated in 0.2 ml of 0.05 M Tris-maleate buffer at the pH indicated for 1 hour at 37°C in the presence (Δ—Δ) and absence (○—○) of 0.005 M cysteine. Aliquots were then adjusted to pH 8.0 and assayed for enzymatic activity.

\(\text{dATP, ADP, UTP, and dGTP. dATP was as active as ATP and was the only compound to have any activity.}

Various NAD analogues were qualitatively tested in order to determine whether NAD kinase could convert them to their respective NADP analogues. Thionicotinamide adenine dinucleotide, 3-acetyl pyridine adenine dinucleotide, and 6-aminonicotinamide adenine dinucleotide were so utilized. Pyridine aldehyde adenine dinucleotide, \(\alpha\)-NAD, and hypoxanthine adenine dinucleotide did not serve as substrate for NAD kinase.
**Metal Requirements**—The results of substituting Mg\(^{2+}\) for other cations are shown in Table II. The optimal Mg\(^{2+}\) concentration is obtained in the presence of a 1:1 ratio of Mg\(^{2+}\):ATP. Mn\(^{2+}\) was found to be a slightly more powerful activator in concentrations one-fourth to one-fifth that of Mg\(^{2+}\) (Fig. 2). Both Mg\(^{2+}\) and Mn\(^{2+}\) inhibited the reaction if present in excess amounts.

**pH Optimum and pH Stability**—The pH optimum of the NAD\(^{+}\) kinase reaction is around 7.0 (Fig. 3). At lower pH values there is a rapid decrease in enzymatic activity, although this may be a reflection of the high instability of the enzyme at neutral and acid pH values. On the basic side of the plateau the values decrease very slowly. The enzyme is most stable at pH 9 to 10. Cysteine has a marked protective action and thus was included in all stages of purification after Fraction IV.

**Discussion**

NAD\(^{+}\) kinase has been purified previously from several sources: yeast (3), pigeon liver (5, 8), spinach (6), and more recently from A. vinelandii (7). NAD\(^{+}\) kinase from pigeon liver, spinach, and rat liver is susceptible to precipitation with protamine sulfate and shows positive adsorption to calcium phosphate gel. In the earlier stages of purification the enzyme from the various sources survives heating to 60\(^\circ\)C. DEAE-cellulose columns have been employed successfully by Nemchinskaya et al. (8) and Chung (7) in the isolation of the pigeon liver and A. vinelandii NAD\(^{+}\) kinase enzymes, respectively. Acetate buffer, pH 5.0, has been used to extract the protamine sulfate pellet in the purification of both pigeon liver and spinach enzymes. This procedure, however, will completely inactivate rat and bovine liver NAD\(^{+}\) kinase.

Since the apparent \(K_m\) values for NAD\(^{+}\) and ATP are quite high, it is possible that this enzymatic activity is a secondary manifestation of some enzyme, the main function of which is different from the catalytic process observed here. Attempts to lower the apparent \(K_m\) values by altering the concentration of cations and anions, pH, etc., have failed to affect these kinetic parameters.

Slater and Sawyer (14, 15) have calculated that in rat liver, NADP\(^{+}\) turns over in an interval of 5 hours. If an optimal rate of synthesis (at saturation levels of the required substrates) is calculated for the total amount of NADP\(^{+}\) kinase estimated to be present per g of liver, approximately 2000 \(\mu\)g of NADP\(^{+}\) could be synthesized per g of liver per hour. Based on the turnover rates reported by Slater and Sawyer (14, 15), approximately 71 \(\mu\)g of NADP could be synthesized per g of liver per hour. Thus, the enzyme is present in excess. The concentration of NADP\(^{+}\) and ATP in rat liver is 1 \(\times\) \(10^{-4}\) M and 0.5 to 1.0 \(\times\) \(10^{-3}\) M (16), respectively, the same magnitude as the apparent \(K_m\) values of the enzyme. Although more detailed studies are required in order to answer this question conclusively, it appears that this enzyme could well supply the NADP\(^{+}\) required by the cell.

**References**

7. **Chung, A. E., J. Biol. Chem., 244, 1182 (1967).**
11. **Schneider, W. C., and Hogueoom, G. H., J. Biol. Chem., 183, 123 (1950).**