Nicotinamid Mononucleotide Pyrophosphorylase Activity in Animal Tissues*

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L. S. Dietrich, Laphalle Fuller, I. L. Yero, and Laura Martinez

From the Department of Biochemistry, University of Miami, School of Medicine, Miami 36, Florida

SUMMARY

An enzyme which catalyzes the formation of nicotinamide mononucleotide in the presence of nicotinamide, 5-phosphoribosylpyrophosphate, adenosine triphosphate, and Mg++, has been purified from rat liver. The inhibitor is removed by protamine sulfate. A broad activity peak is observed between pH 7.5 and 9.5 with maximal activity around pH 8. The enzymatic activity is stable at these pH values, but is acid labile. The apparent K, values for nicotinamide, PP-ribose-P, and ATP are 2.62 μM, 3.57 × 10⁻⁴ M, and 3.83 × 10⁻⁴ M, respectively. The V_max was calculated to be 5.4 mmol per mg of protein per hour.

Recently, the observation was made in our laboratory (2) that when ascites cell extracts were incubated with 14C-nicotinamide, 5-phosphoribosylpyrophosphate, adenosine triphosphate, and Mg++, 14C-nicotinamide mononucleotide was formed. The K_a for nicotinamide was around 10⁻⁴ M, several magnitudes lower than previously reported for NMN pyrophosphorylase activity (3). Attempts to find NMN pyrophosphorylase activity with a similar high affinity for nicotinamide were unsuccessful until it was observed that the supernatant material remaining after protamine sulfate precipitation consistently exhibited NMN pyrophosphorylase activity. The further addition of 0.05 M Tris-chloride buffer, pH 7.3. The crude homogenate was centrifuged at 22,000 × g for 30 min, and the pellet was discarded. Protamine sulfate (1%) was then added dropwise to the supernatant material (Supernatant I) to a final concentration of 0.7 ml of protamine sulfate solution per 10 ml of supernatant material. After standing in the cold for 30 min, the material was centrifuged at 22,000 × g for 15 min, the pellet discarded, and the supernatant material (Supernatant II) fractionated with ammonium sulfate. The ammonium sulfate precipitates formed, upon standing for 10 min after complete solution of the salt, were collected by centrifugation at 22,000 × g for 15 min. The addition of 31.77 g of solid ammonium sulfate per 100 ml of Supernatant II resulted in the precipitation of a protein fraction containing all of the nicotinamide acid mononucleotide pyrophosphorylase activity. The further addition of 7.06 g of ammonium sulfate per 100 ml of Supernatant II resulted in the precipitation of a protein fraction containing all of the NMN pyrophosphorylase activity. All of the isolation steps were carried out at 4°.

Assay of NMN Pyrophosphorylase Activity—The reaction mixture contained 0.7 μmole of PP-ribose-P, 2.0 μmoles of ATP, 10 μmoles of MgCl₂, 0.1 μmole of 14C-nicotinamide (8.0 μC per μmole), 50 μmoles of Tris, pH 7.3, and enzyme in a total volume of 1 ml. Incubation was carried out at 37° for 30 min or 1 hour. The reaction was stopped by heating in a boiling water bath for 15 min. The samples were then centrifuged, and the supernatant material was chromatographed with Solvent C of Preiss and Handler (4). The areas corresponding to NMN were cut out, and the radioactivity was quantitated with a liquid scintillation spectrometer as previously described (5). Under those conditions, enzymatic activity is proportional to the enzyme concentration and the time of incubation.

Chemicals—14C-Nicotinamide was purchased from New England Nuclear Corporation. Immediately upon receipt the compound was dissolved in water and stored frozen. Periodical analysis of this compound by comparison of its Rf value in three different chromatographic solvents indicates that under these conditions no detectable deterioration occurs. PP-ribose-P was purchased from Pabst Laboratories as the magnesium salt. The Mg++ was removed by shaking with Dowex 50 cation exchange resin in the sodium form.

Statistical Analysis—In order to avoid unconscious bias in the plotting of Lineweaver-Burk lines, the kinetic data were analyzed by a computer program developed and kindly furnished to us by Dr. W. W. Cleland (6). The data were processed by an IBM 7040 computer. The program gives the values for K_a, V_max, and V₅₀.
TABLE I
Purification of nicotinamide mononucleotide pyrophosphorylase from rat liver
See “Experimental Procedure” for details of assay medium.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Activity (cpm/hr/mg protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>32.2</td>
<td>333</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant I</td>
<td>19.2</td>
<td>2,405</td>
<td>442</td>
</tr>
<tr>
<td>Supernatant II</td>
<td>14.0</td>
<td>13,229</td>
<td>1,731</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate (45 to 55%)</td>
<td>23.8</td>
<td>42,442</td>
<td>942</td>
</tr>
</tbody>
</table>

*14 C-NMN formed at 37°C.

TABLE II
Requirements for MNM pyrophosphorylase purified from rat liver
See “Experimental Procedure” for details of assay medium.
The enzyme source was 45 to 55% ammonium sulfate precipitate of Supernatant II.

<table>
<thead>
<tr>
<th>Flask additions</th>
<th>NMN formed at 37°C (cpm/0.1 ml reaction mixture/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete medium</td>
<td>9295</td>
</tr>
<tr>
<td>-PP-ribose-P</td>
<td>275</td>
</tr>
<tr>
<td>-ATP</td>
<td>106</td>
</tr>
<tr>
<td>-Mg++</td>
<td>127</td>
</tr>
</tbody>
</table>

RESULTS
Identification of Enzymic Product as NMN—The reaction product has been characterized by four different paper chromatographic systems, Solvents B and C of Preiss and Handler (4), isobutyrate-ammonia (7), 1-butanol saturated with water in a NH₄HCO₃ atmosphere (8), and paper electrophoresis. Mild alkaline hydrolysis with NH₄OH at pH 10 overnight at 37°C (5) resulted in the release of a radioactive compound that behaved, with the above mentioned chromatographic and electrophoretic procedures, in an identical manner to nicotinamide. Incubation with purified NAD pyrophosphorylase (9) resulted in the formation of a radioactive compound which behaves similarly to NAD.

Data showing the increase in specific activity during purification are presented in Table I. It is of interest to note the very marked increase in specific activity and recovery obtained after protamine sulfate precipitation (Supernatant II). Protamine has no enzymic activity nor does the sulfate ion stimulate enzymic activity. It is assumed that protamine sulfate removed an inhibitor of NMN pyrophosphorylase.

The requirements of NMN pyrophosphorylase purified from rat liver are presented in Table II. The elimination of PP-ribose-P, ATP, or Mg++ resulted in the loss of enzymic activity. The enzyme is not as yet in a pure enough state to permit a quantitative stoichiometric relationship. Changes in the ATP concentration of the reaction mixture produced by the enzymic preparation under the assay condition, with the luciferin-luciferase reaction (10), are independent of nicotinamide concentration. Although the decrease in ATP levels (4 to 8%, depending on the enzymic preparation) is several times the amount of NMN formed, the sensitivity of the ATP assay is such as to permit the tentative conclusion that the role of ATP in the NMN pyrophosphorylase reaction is primarily catalytic. Nicotinamide lost during the reaction can be quantitatively accounted for by the NMN formed.

ATP Requirements—ATP appears to be an essential requirement for enzymic activity. Increasing the levels of PP-ribose-P in the absence of ATP did not stimulate enzymic activity, indicating that the function of ATP is not that of simply sparing PP-ribose-P. Specificity studies (Table III) indicate...
was 45 to 55\% ammonium sulfate precipitate of Supernatant II. ATP
phosphorylase isolated from rat liver. O-O, enzymic activity
PP.ribose-P . Nicotinamide... (2.96 ± 0.3) X 10^{-6}

45\% ammonium sulfate precipitate of Supernatant II. Nicotinic
acid mononucleotide pyrophosphorylase was assayed according
to the procedure of Imsande and Handler (11). Each value is the
average of at least three experiments run in duplicate.

mononucleotide pyrophosphorylase was determined on the 25 to
monium sulfate precipitate of Supernatant II. Nicotinic acid
pyrophosphorylase activity was determined on the

The enzymic activity is destroyed by boiling and exposure to
cold acetone. It is stable to storage at \(4^\circ\) and \(-20^\circ\) and can
withstand 55\% for 1 min.

Studies comparing the effect of various compounds on the
activity of NMN pyrophosphorylase isolated from rat liver (Table
V) indicates that compounds such as heparin, dextran sulfate,
and probably RNA, inhibit enzymic activity while at the same
concentration (100 \(\mu\)g per reaction vessel), sialic acid, hyaluronic
acid, chondroitin sulfate, and DNA have no effect. Nicotinoid
acid mononucleotide pyrophosphorylase is, on the other hand,
unaffected by heparin and dextran sulfate.

Distribution of Enzymic Activity — The NMN pyrophosphorylase
activity of rat liver appears to be restricted to the cytoplasmatic
components of the cell. i.e. it is not sedimented at 105,000 \(\times g
for 3 hours. No activity was detected in the nuclear, mitochondrial,
or microsomal fractions. It is found in all of the cell
types thus far studied (Table VI). Normal rat tissues fall in the
following order of decreasing activity: liver, kidney, heart, brain,
skeletal muscle, and lung. \(K_m\) determinations with nicotinamidc
as the substrate have been carried out on the supernatant
material obtained after protamine sulfate precipitation in rat brain,
kidney, heart, and lung. The \(K_m\) values were \(1.0 \times 10^{-4} \text{ M}\) in
all of the cases.\(^1\)

### Table IV

<table>
<thead>
<tr>
<th></th>
<th>Apparent (K_m) ± s.e.</th>
<th>(V_{max}) ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide</td>
<td>((2.96 ± 0.33) \times 10^{-4})</td>
<td>(4.8 ± 0.1)</td>
</tr>
<tr>
<td>ATP</td>
<td>((3.82 ± 0.61) \times 10^{-4})</td>
<td>(6.4 ± 0.7)</td>
</tr>
<tr>
<td>PP-ribose-P</td>
<td>((3.57 ± 0.89) \times 10^{-5})</td>
<td>(5.5 ± 0.4)</td>
</tr>
</tbody>
</table>

Fig. 2, pH optimum and pH stability studies of NMN pyrophosphorylase isolated from rat liver. ●—●, enzymic activity at given pH values; ○—○, samples were incubated at pH indicated for 1 hour. pH was then adjusted to 7.3 in all of the cases, and enzymic activity was determined.

### Table V

<table>
<thead>
<tr>
<th>Flask addition</th>
<th>NMN</th>
<th>Nicotinamide acid mononucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/0.02 ml enzyme/hr</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5946</td>
<td>4098</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>6289</td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>6585</td>
<td></td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>5002</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>1015</td>
<td>4818</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>1228</td>
<td>4363</td>
</tr>
<tr>
<td>DNA</td>
<td>6554</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>4200</td>
<td>3829</td>
</tr>
</tbody>
</table>

Table VI

<table>
<thead>
<tr>
<th>Tissue</th>
<th>NMN formed ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>163 ± 12 (10)</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>80 ± 1 (6)</td>
</tr>
<tr>
<td>Rat heart</td>
<td>60 ± 2 (6)</td>
</tr>
<tr>
<td>Rat brain</td>
<td>24 ± 1 (6)</td>
</tr>
<tr>
<td>Rat skeletal muscle</td>
<td>21 ± 2 (6)</td>
</tr>
<tr>
<td>Rat lung</td>
<td>18 ± 3 (6)</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>146 ± 4 (6)</td>
</tr>
<tr>
<td>Ehrlich leue ascites cells</td>
<td>90 ± 7 (12)</td>
</tr>
<tr>
<td>755 Adenocarcinoma</td>
<td>130 ± 9 (7)</td>
</tr>
<tr>
<td>Chick embryo liver (12 days)</td>
<td>128 ± 6 (6)</td>
</tr>
</tbody>
</table>

Assay was carried out on the supernatant fluid obtained upon
protamine sulfate precipitation. Figures in parentheses represent
number of animals used in calculating the mean.

that the ATP requirement cannot be replaced by GTP, CTP,
TTP, dAMP, dATP, dGTP, AMP, or ADP. The slight stimulation
observed in the case of ADP is probably due to the presence
of adenylate kinase in the enzyme preparation.

Lineweaver-Burk plots of NMN pyrophosphorylase activity
with nicotinamide, ATP, and PP-ribose-P are presented in Fig. 1.
The kinetic parameters are presented in Table IV. In these
determinations, the components whose concentrations were not
varied were kept at the concentrations used in the standard
assay.

pH optimum studies are presented in Fig. 2. The enzymic
activity exhibits a broad peak between pH 7.5 and 9.5 with
maximal activity around pH 8.0. Stability studies (Fig. 2)
indicate that the enzyme is acid labile.

The enzymic activity is destroyed by boiling and exposure to
cold acetone. It is stable to storage at \(4^\circ\) and \(-20^\circ\) and can
withstand 55\% for 1 min.

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unaffected by heparin and dextran sulfate.

### Discussion

The presence of an enzyme, in all of the tissues studied so far,
that converts nicotinamide to NMN and which has a very high
affinity for nicotinamide, places new importance on the role of nicotinamide in pyridine nucleotide metabolism. Preiss and Handler (3) purified a NMN pyrophosphorylase from acetone powder of erythrocytes which exhibited a \( K_m \) for nicotinamide in the order of 0.1 M. Dold, Mielsch, and Holzer (12) showed that extremely high concentrations of nicotinamide (0.1 M) were needed before fortified acetone powder extracts of ascites cells would synthesize NAD from nicotinamide. This is in contrast to the enzymes giving rise to nicotinic acid mononucleotide either from quinolinic acid (13) or nicotinic acid (11) where the \( K_m \) values for their respective pyridine derivatives are 7 \( \mu \)M or approximately 1 \( \mu \)M, respectively. Thus the role of nicotinamide as a direct precursor of NAD was open to question. The discovery of a NMN pyrophosphorylase with a high affinity for nicotinamide reopens the possibility of the direct utilization of nicotinamide in the biosynthesis of NAD.

Evidence that NMN pyrophosphorylase is not the same enzyme that catalyzes the conversion of nicotinic acid to nicotinic acid mononucleotide is demonstrated by the fact that (a) the two enzymes can be separated by (NH\(_4\))\(_2\) SO\(_4\) fractionation (see “Experimental Procedure”) and (b) the NMN pyrophosphorylase is acid labile while the nicotinic acid mononucleotide pyrophosphorylase is acid stable (11).

The observation that the enzyme is apparently in an inhibited form after homogenization gives rise to the question as to the nature of the inhibitor, and whether the inhibition occurs in vivo or is an artifact of homogenization. The fact that enzymic activity is very sensitive to polyanionic polymers such as heparin and dextran sulfate (Table V) indicates that the latter may be the case.

The requirement for ATP as well as PP-ribose-P is not a usual requirement for pyrophosphorylase, but it is similar to that observed for nicotinic acid mononucleotide pyrophosphorylase activity (11). Whether the function of ATP is to control the direction of the reaction as has been recently demonstrated by Nakamura, Nishizuka, and Hayashi (14) for nicotinic acid mononucleotide pyrophosphorylase remains to be exhaustively studied. Preliminary studies indicate, however, that ATP does not effect the degradation of \(^{14}C\)-NMN by this enzyme preparation. Since the ATP affect appears to be catalytic, this may be an example of another allosteric system (15).

Polyanionic polymers may act as chelating agents and bind Mg\(^{++}\) needed for the NMN pyrophosphorylase reaction. If this were the case, then these same anionic polymers should inhibit a nicotinic acid mononucleotide pyrophosphorylase which has a Mg\(^{++}\) requirement similar to NMN pyrophosphorylase (5). This, as can be seen in Table V, is not the case, and it appears that the effects of heparin and dextran sulfate may be specific for NMN pyrophosphorylase.

REFERENCES
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