Some Properties of the Enzyme Nicotinamide Adenine Dinucleotide Glycohydrolase from Mouse Ehrlich Ascites Cells*

SAUL GREEN AND OSCAR BODANSKY

From the Division of Enzymology and Metabolism, Sloan-Kettering Institute for Cancer Research; Department of Biochemistry, Memorial Hospital, and James Ewing Hospital; and Sloan-Kettering Division of Cornell University Graduate School of Medical Sciences, New York 21, New York

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In the past decade several studies have been carried out on the physical, chemical, and kinetic properties of partially purified preparations of the enzyme nicotinamide adenine dinucleotide glycohydrolase (NADase) from the tissues of several mammalian and bacterial species (1-7). No information is available concerning the properties of this enzyme from Ehrlich ascites cells. Recently it was found in this laboratory that nitrogen mustard treatment of mice bearing an Ehrlich ascites tumor resulted in an increase in the activity of the nicotinamide adenine dinucleotide glycohydrolase in the tumor cells (8). This increase, which was directly related to a decrease in the rate of glycolysis of the ascites cells (9), made it desirable to study various characteristics of the activity of this enzyme.

EXPERIMENTAL PROCEDURE

Materials—The reagents used were obtained from the following sources: NAD+, NAD+P, and the NAD+ analogues from Pabst Laboratories, 3-acetylpipridine from Aldrich Chemical Company, Inc., isonicotinic acid hydrazide from Eastman Organic Chemicals Department, methyl bis(β-chloroethyl)amine from Merck Chemical Company, twice crystallized yeast alcohol dehydrogenase and Neurospora NADase from Worthington Biochemical Corporation, and yeast RNA from the Mann Chemical Company.

Methods.—The maintenance of the Ehrlich ascites cell tumor line and treatment of the tumor-bearing mice with HN2 have been previously described (9). Attempts to solubilize the NADase from tumor cell homogenates by methods described for other tissues (1, 5, 10) were unsuccessful. Consequently homogenates prepared by ultrasonic disintegration (9) were used for other tissues (1, 5, 10) were unsuccessful. Consequently homogenates prepared by ultrasonic disintegration (9) were used in the present study.

Assay of NADase—NADase was assayed by the method of Kaplan (11) with several modifications. The incubation mixture contained 0.30 μmole of NAD+ and 0.5 ml of a homogenate of ascites cells in a total volume of 1.0 ml. The pH of the incubation mixture was maintained at 6.5 with 0.1 M phosphate buffer. After incubation at 37° for 1 minute, the reaction was stopped by the addition of 0.5 ml of a solution of nicotinamide to give a final concentration of 0.04 M, followed immediately by heating in a boiling water bath for exactly 1 minute. The chilled mixture was adjusted to pH 9.5 with the addition of 2.5 ml of 0.2 M glycine-NaOH buffer containing 0.5 M ethanol, and was centrifuged at 3000 r.p.m. at 4° for 15 minutes. The NAD+ concentration of the clear supernatant solution was determined with alcohol dehydrogenase. The initial NAD+ concentration of each incubation mixture was also determined. A unit of NADase activity is defined as that amount of enzyme needed to cleave 1 μmole of NAD+ per hour.

Assay of RNase—Ribonuclease activity was determined according to the method of Siekevitz and Palade (12) with the following modifications. Ehrlich ascites cell homogenate, equivalent to 5.0 mg of nitrogen, was added to 1.0 ml of a 0.2 M Tris-HCl buffer solution, pH 7.4, containing 3.0 mg of yeast RNA, and the mixture was allowed to incubate for 10 minutes in a 23° water bath. The reaction was stopped by the addition of 2.0 ml of a cold 1.5 N perchloric acid solution containing 0.25% uranium acetate. After standing at 4° for 1 hour, the tubes were centrifuged at 2500 r.p.m. for 30 minutes. The absorbance of a 25-fold dilution of each of the clear supernatant solutions was determined at 260 μm. An appropriate reagent blank for each experimental tube was prepared, to which the RNA was added after the addition of the perchloric acid reagent. A unit of activity is defined as that amount of enzyme which results in an increase in optical density of 0.001 in 10 minutes under the conditions of the experiment described above.

The total nitrogen was determined by the micro-Kjeldahl method; for the extraction of NAD+ from tissues, the method of Lowry, Roberts, and Kapphahn (13) was employed.

RESULTS

Effect of HN2 Treatment on NADase Activity of Several Tissues in Normal and Tumor-bearing Mice—The NADase activity of several tissues in each of the four groups of mice was measured in order to determine the specificity of the HN2 effect. The groups consisted of HN2-treated and untreated normal mice as well as HN2-treated and untreated mice bearing an Ehrlich ascites tumor. HN2 administration was started in the normal as well as the tumor-bearing mice 24 hours after implantation of the tumor. Table I shows that at the dosage of HN2 used,
**TABLE I**

NADase activity in several mouse tissues after treatment of animals with HN2

HN2 treatment of mice bearing an ascites cell tumor has been described (8). Animals were killed 8 days after implantation of the tumor. After the tissues were removed and rinsed several times in ice-cold 0.9% NaCl, a 20% homogenate in cold 0.25 M sucrose was assayed for NADase activity with alcohol dehydrogenase (see "Experimental Procedure"). In other experiments it was found that at this dose of HN2, there was no change from control values with respect to the nitrogen content per g of liver or kidney. Each value is the mean of values from tissues of 10 mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Liver</th>
<th>Kidney</th>
<th>Erythrocyte stroma</th>
<th>Ehrlich ascites cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice ...........</td>
<td>271 ± 35</td>
<td>160 ± 18</td>
<td>0.59 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>Normal mice + HN2 ..........</td>
<td>260 ± 31</td>
<td>177 ± 18</td>
<td>0.50 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Ehrlich ascites tumor-bearing mice</td>
<td>302 ± 22</td>
<td>216 ± 19</td>
<td>0.58 ± 0.07</td>
<td>0.98 ± 0.32</td>
</tr>
<tr>
<td>Ehrlich ascites tumor-bearing mice + HN2</td>
<td>272 ± 22</td>
<td>210 ± 19</td>
<td>0.59 ± 0.14</td>
<td>1.32*</td>
</tr>
</tbody>
</table>

* p < 0.01.

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**Fig. 1.** Effect of pH on activity of ascites cell NADase from HN2-treated (X) and from untreated mice (O). Incubation mixtures (1 ml) were 0.1 M with respect to potassium phosphate buffers (pH 5.5 to 8.0). Initial NAD+ concentration in the reaction mixture was 0.23 mM. A 20% Ehrlich ascites cell homogenate (0.5 ml) was the enzyme source. Appropriate controls from which the enzyme was omitted were run for each experiment. Each point is the average of four determinations.

**Fig. 2.** Effect of substrate concentration on NADase activity. Each incubation mixture contained, in a final volume of 1.0 ml, 2.0 units of NADase activity as ascites cell homogenate, and any one of several concentrations of NAD+ ranging from 0.03 mM to 0.40 mM, in 0.1 M potassium phosphate buffer, pH 6.5. The initial NAD+ concentration of each mixture was determined before incubation. Assay of NADase activity was carried out as described in "Experimental Procedure." O, control; X, HN2-treated.

**Fig. 3.** Effect of pyridine derivatives on Ehrlich ascites cell NADase. The reaction mixtures were the same as described in "Experimental Procedure" for NADase assay, with the exception that each contained the final concentration of pyridine bases shown. Each point is the average of three determinations and is expressed as the extent of inhibition relative to the control rate. O, NADase controls; X, HN2-treated.
only the NADase activity of the Ehrlich ascites tumor cells was increased significantly.

**Effect of pH**—Fig. 1 shows the effect of pH on the NADase activity of homogenates of ascites cells from HN2-treated and untreated mice. The preparation from each source was active over a broad pH range and showed the same pH optimum, 6.5.

**Effect of Substrate Concentration**—Homogenates of ascites cells lost about 10% of their NADase activity on standing for 1 hour at 4°. Accordingly, velocity determinations at several substrate concentrations, usually five or six, were performed within 20 minutes of the preparation of the homogenate. Fig. 2 shows the results obtained with the homogenates of the ascites cells from two control mice and two mice treated with HN2. Maximal velocity was attained at a concentration of about 0.20 mM NAD+ for either type of homogenate. For the calculation of $K_m$ values (14), velocities were determined at five different concentrations below this maximum with the enzyme source as ascites cell homogenate from each of 10 control and 10 HN2-treated mice. There was no significant difference between the mean $K_m$ values for the control and HN2-treated groups, 6.6 $\pm$ 1.5 $\times$ $10^{-4}$ M and 7.3 $\pm$ 0.9 $\times$ $10^{-4}$ M, respectively. The mean NADase activities for the control and HN2-treated groups were 1.20 $\pm$ 0.23 units per mg of nitrogen and 7.24 $\pm$ 2.92 units per mg of nitrogen, respectively.

**Effect of Pyridine Derivatives on Ehrlich Ascites Cell NADase**—Differences in the effect of various pyridine compounds on NADase activity have been reported by Zatman et al. (4) and by Kaplan et al. (3) to depend on the species and tissue of origin. Differences in the effect of various pyridine compounds on NADase activity from two control mice and two mice treated with HN2. Maximal velocity was attained at a concentration of about 0.20 mM NAD+ for either type of homogenate. For the calculation of $K_m$ values (14), velocities were determined at five different concentrations below this maximum with the enzyme source as ascites cell homogenate from each of 10 control and 10 HN2-treated mice. There was no significant difference between the mean $K_m$ values for the control and HN2-treated groups, 6.6 $\pm$ 1.5 $\times$ $10^{-4}$ M and 7.3 $\pm$ 0.9 $\times$ $10^{-4}$ M, respectively. The mean NADase activities for the control and HN2-treated groups were 1.20 $\pm$ 0.23 units per mg of nitrogen and 7.24 $\pm$ 2.92 units per mg of nitrogen, respectively.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wavelength (nm)</th>
<th>$\times 10^{-4}$</th>
<th>Initial substrate concentration</th>
<th>Relative rates of hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD+</td>
<td>340</td>
<td>6.22</td>
<td>0.23</td>
<td>100</td>
</tr>
<tr>
<td>TNAD+</td>
<td>395</td>
<td>11.30</td>
<td>0.15</td>
<td>60</td>
</tr>
<tr>
<td>NADP+</td>
<td>340</td>
<td>6.22</td>
<td>0.23</td>
<td>43</td>
</tr>
<tr>
<td>NID+</td>
<td>338</td>
<td>6.20</td>
<td>0.20</td>
<td>25</td>
</tr>
<tr>
<td>3-AcPyAD+</td>
<td>303</td>
<td>9.10</td>
<td>0.20</td>
<td>4</td>
</tr>
<tr>
<td>3-PyAlAD+</td>
<td>358</td>
<td>9.30</td>
<td>0.20</td>
<td>$&lt;2$</td>
</tr>
</tbody>
</table>

* NID+ nicotinamide inosine dinucleotide; TNAD+, thionicotinamide adenine dinucleotide; 3-PyAlAD+, 3-pyridinealdehyde adenine dinucleotide.

Thus, for example, whereas all animal NADases show essentially the same degree of inhibition by nicotinamide, marked differences may exist in the effect of isonicotinic acid hydrazide on the NADases of the different tissues of the same species, and of the same tissues from different species (2). Fig. 3 shows that different pyridine derivatives inhibited NADase from Ehrlich ascites cells to markedly different degrees. The effect of a given pyridine base on ascites cell NADase from control and from HN2-treated mice was the same.

**Hydrolysis of NAD+ Analogues by Ehrlich Ascites Cell NADase**—Table II shows that, except for 3-AcPyAD+, the NADase from ascites cells of HN2-treated mice hydrolyzed the analogues of NAD+ at the same relative rates as the NADase from untreated ascites cells. The basis for the single difference is being investigated.
Transglycosidase Activity of NADase from Ehrlich Ascites Cells—Pig brain enzyme has been used extensively to synthesize analogues of NAD+ (17, 18). The transglycosidase activity of Ehrlich ascites cell NADase was demonstrated by measuring the ability of the ascites cell homogenates to synthesize 3-AcPyAD+ and isonicotinic acid hydrazide adenine dimucleotide. Fig. 4 shows that the analogue-synthesizing capacity of NADase was the same in ascites cells from HN2-treated mice and from untreated mice.

Possible Mechanisms of Increased NADase Activity—In the course of the present studies, it was observed that treatment of mice with HN2 also resulted in an increase in the activity of Ehrlich ascites cell RNase. Thus, the average RNase activity of ascites cells from five HN2-treated mice was 168 ± 54 units per mg of nitrogen, and that from five untreated mice was 20 ± 6 units per mg of nitrogen. The possibility was investigated that the increases in these enzyme activities might be due to activation. The results of a typical experiment (Table III) show that the RNase activity of homogenates of Ehrlich ascites cells was increased after treatment in vitro with HN2. This is in agreement with the findings of Hilz and Klempien (20). Incubation with HN2 was also found to increase the activity of RNase in ascites cells from mice treated with HN2. These findings on RNase were in sharp contrast to the absence of any activation in vitro of the NADase by HN2 in ascites cell homogenates from either HN2-treated mice or control mice. Indeed, inhibition rather than activation occurred (Table III).

When equal volumes of ascites cell homogenates from control and from HN2-treated mice were mixed and allowed to incubate at 37° for 30 minutes, the NADase activity of the mixture was 4.60 units per ml, essentially the average of the activities, 1.08 and 7.76 units per ml, of the two homogenates. These findings indicate that there was no enzyme-like activation or no other activator present in excess in the ascites cells of the HN2-treated mice.

DISCUSSION

The present study has shown that administration of HN2 to mice bearing an Ehrlich ascites tumor resulted in an increased activity of NADase in the tumor cells, but not in other tissues. Consideration of our results on the properties of Ehrlich ascites cell NADase shows that this enzyme does not bear a consistent pattern of resemblance to the NADases from other sources. The pH-activity curve of ascites cell NADase showed a sharp peak at pH 6.5, and thus resembled the peak obtained with the NADase from pig spleen (pH 6.0), but differed from the enzyme from bull semen, which has a maximum at pH 8.0, and from pig brain, which has a broad range of optimal activity of between pH 5.0 and 8.5 (1, 5, 10). The value for $K_m$ of ascites cell NADase, 6.5 x $10^{-5}$ M (Table II), was similar to the value of 2.3 x $10^{-5}$ M reported by Dickerman, San Pietro, and Kaplan (10) for pig spleen, but different from that of bull semen NADase, 3.9 x $10^{-4}$ M (5). The inhibition of ascites cell NADase by 2.0 mM nicotinamide was 53%, essentially the same as that found for brain and spleen NADase of beef, lamb, rat, mouse, horse, and human (4). Inhibition by isonicotinic acid hydrazide was of the same order as that of the NADase from rat spleen, rabbit spleen, rabbit brain, and pig brain, but different from the inhibition of NADase from beef brain and spleen, lamb brain and spleen, mouse brain and lymphosarcoma, and human spleen (4). Comparison of the relative rates of hydrolysis of the analogues of NAD+ by ascites cell NADase also revealed similarities and differences between this enzyme and those from other tissues. The ratio of hydrolysis of NADP to that of NAD+ was about 0.5 (Table II) and is comparable to the ratio reported by Abdel-Latif and Alivisatos (5) for the bull semen enzyme, but contrasts with the ratio of 1.2 for the pig spleen enzyme obtained by Dickerman et al. (10).

As indicated above, the properties of NADase from a given tissue may vary widely with the species and, within a species, may vary from tissue to tissue. The properties of the enzyme NADase from mouse ascites tumor cells reported in this paper differ in several respects from those reported by Zatman et al. (4) for mouse brain, spleen, and lymphosarcoma.

The question arises whether the increases in NADase and RNase activity of the ascites cells after treatment of the mice with HN2 represents activation of existing enzyme or an increase in the number of enzyme molecules. The increased RNase activity of Ehrlich ascites cells after incubation in vitro with HN2 has been attributed to the removal of an RNase inhibitor (20, 21). Although it has not been investigated, the possibility exists that the increase of RNase activity in vivo after treatment of the mice with HN2 may be similarly explained. However, the absence of any increase in NADase activity in vivo makes it difficult to ascribe an activating mechanism for the increase in vivo. The possibility that the increased NADase activity represents formation of new enzyme is under investigation.

**SUMMARY**

Several properties of the enzyme nicotinamide adenine dinucleotide glycohydrolase (NADase) from mouse Ehrlich ascites cells have been measured. The pH optimum, $K_m$ value, transglycosidase activity, ability to hydrolyze various NAD+ analogues, and sensitivity to inhibition by some pyridine derivatives were the same for the enzyme from ascites cells of mice treated with methyl bis(β-chloroethyl)amine (HN2) and from untreated mice.

HN2 treatment of mice with Ehrlich ascites tumors also resulted in a marked increase in the ribonuclease activity of the
ascites cells. RNase but not NADase was activated in vitro by HN2. Experiments to explore the possibility that the increased ascites cell NADase from HN2-treated animals represented activation of the enzyme proved to be negative.

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Some Properties of the Enzyme Nicotinamide Adenine Dinucleotide Glycohydrolase from Mouse Ehrlich Ascites Cells
Saul Green and Oscar Bodansky


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