Nicotinamide Adenine Dinucleotide Glycohydrolase from Rat Liver Nuclei

ISOLATION AND CHARACTERIZATION OF A NEW ENZYME*

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KUNIHIRO UEDA, MASANORI FUKUSHIMA, HIROTO OKAYAMA, AND OSAMU HAYAISHI

From The Laboratory of Molecular Biology, Institute for Chemical Research, Kyoto University, and The Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan

A new type of nicotinamide adenine dinucleotide glycohydrolase (NADase) has been isolated from rat liver nuclei. When partially purified chromatin is passed through a Sephadex G-200 column in the presence of 1 M NaCl, enzyme activities catalyzing the liberation of nicotinamide from NAD elute in two peaks. One, which appears in the void volume fraction, hydrolyzes the nicotinamide-ribose linkage of NAD to produce nicotinamide and ADP-ribose in stoichiometric amounts. This activity is not inhibited by 5 mM nicotinamide. The other, which elutes much later, catalyzes the formation of poly(ADP-ribose) from NAD and is completely inhibited by 5 mM nicotinamide. The former, NADase, is DNase-insensitive and thermostable, has a pH optimum of 6.5 to 7.0, a $K_m$ for NAD of 28 $\mu$M, and a $K_i$ for nicotinamide of 80 mM, and hydrolyzes NADP as well as NAD. The latter, poly(ADP-ribose) synthetase, is sensitive to DNase treatment and heat labile, has a pH optimum of 6 to 6.5, a $K_m$ for NAD of 250 $\mu$M and a $K_i$ for nicotinamide of 0.5 mM and is strictly specific for NAD. Further, the former NADase is shown to lack transglycosidase activity, which has been documented to be a general property of NADases derived from animal tissues. These results indicate that the NAD-hydrolyzing enzyme newly isolated from nuclei is a novel type of mammalian NADase which catalyzes the hydrolytic cleavage of the nicotinamide-ribose linkage of NAD.

NADase (NAD glycohydrolase or NAD nucleosidase) (EC 3.2.2.5) is a family of enzymes which catalyze the hydrolytic cleavage of the nicotinamide-ribose bond of NAD. These enzymes are widely distributed in various microorganisms (1-3) and animal tissues (4-6), and some of them have been partially purified and characterized (7, 8). Their enzymological properties appear to vary according to their origin. The enzymes found in microorganisms are soluble and usually accompanied by a heat-labile inhibitor protein (9). The microbial enzymes are further characterized by their high resistance to inactivation by nicotinamide, and by their inability to catalyze the transglycosidation or exchange reaction between the nicotinamide portion of NAD and exogenous nicotinamide or its analogs (10). The enzymes of animal origin, on the other hand, are bound to particulates, either microsomes or nuclei. The microsomal enzyme, which represents approximately 90% of the NADase activity in rat liver cells (5), has a prominent transglycosidase activity and exchanges the nicotinamide moiety of NAD with exogenous nicotinamide and its various analogs (11). This enzyme is fairly thermostable, is sensitive to nicotinamide inhibition, and cleaves NADP as well as NAD.

The enzyme found in the nucleus has been variously described as being similar to (6), identical with (12), or different from poly(ADP-ribose) synthetase (13). The latter enzyme, which has also been termed poly(ADP-ribose) polymerase (14) or ADP-ribose transferase (6, 15), catalyzes a transfer of the ADP-ribose moiety of NAD to nuclear protein and to the protein-attached ADP-ribose to yield a polymer composed of repeating ADP-ribose units. This enzyme may be considered as a kind of NADase, since it liberates nicotinamide concomitant with a transfer of ADP-ribose (6). Our previous investigation suggested that many properties, such as sensitivity to DNase, inhibition by nicotinamide, thermostability, and substrate specificity, are shared by both nuclear NADase and poly(ADP-ribose) synthetase (6). Kinetic studies by Römer et al. (12) also supported this view. However, we have observed, with the use of a newly developed assay method, that the activity catalyzing the liberation of nicotinamide from NAD in nuclei is much less sensitive to nicotinamide inhibition than that responsible for poly(ADP-ribose) synthesis. Furthermore, the former activity is more resistant to DNase treatment than the latter. These discrepancies led us to re-examine the NADase in rat liver chromatin. The results to be presented in this paper show that there exists a discrete NADase in addition to poly(ADP-ribose) synthetase in nuclei and that the nuclear NADase is of a type hitherto unknown in mammalian tissues.

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MATERIALS AND METHODS

Chemicals—[7-14C]Nicotinamide was obtained from Daiichi Pure Chemical Co., Tokyo, and [adenine-14C]NAD and d-([1-14C]glucose from the Radiochemical Centre, Amersham. NAD, its analogs, cyclic AMP, and other nucleotides are the products of Sigma. Crystalline yeast alcohol dehydrogenase was obtained from Sigma, and bovine pancreatic DNase from Schwarz/Mann. Sephadex G-200 was purchased from Pharmacia, and Dowex 1 (X2, 200 to 400 mesh) from Dow Chemical.


Thin-[adenine-14C]NAD and AdPy-[adenine-14C]AdP were prepared by the method of Horecker and Wood (24).

Preparation of Yeast Alcohol Dehydrogenase—Yeast alcohol dehydrogenase was obtained from Sigma, and bovine spleen as described by Kaplan and Stolzenbach (18). (100/60/1.6, v/v/v) as the solvent. After being air-dried at room temperature, the paper was cut into 1-cm strips. Each strip was immersed in a toluene-based scintillator, and its radioactivity was determined with a liquid scintillation spectrometer.

Preparation of Microsomes—The microsomal fraction was prepared from rat liver by the method of Hogeboom (21).

Enzyme Assays—NADase activity was assayed by measuring nicotinamide released from NAD. The standard reaction mixture contained 100 mM Tris-HCl buffer (pH 7.5), 0.1 mM [nicotinamide-14C]NAD, 100 mM MgCl2, 1 mM dithiothreitol, 100 mM [adenine-14C]NAD (2,500 cpm per nmol), and enzyme in a total volume of 0.2 ml. Incubation was carried out for 10 min at 37°C and terminated by the addition of 4 ml of ice-cold 20% Cl2COOH. The acid-insoluble radioactivity was determined with the use of a Millipore filter as previously described (19). In Assay 2, which was applied to samples containing a high concentration of salt, 1.6 mM (NH4)2SO4 (pH 8.0) and heat-inactivated chromatin (23) (80 mg of protein) were added to the reaction mixture (0.3 ml) of the same composition as in Assay 1, and incubation was carried out for 60 min at 37°C. The acid-insoluble radioactivity was determined as described above.

Glucose-6-phosphatase activity was assayed according to the method of Morey and Wood (24).

Paper Chromatography—Descending paper chromatography was carried out with isobutyric acid/1 M NH4OH/0.1 M EDTA Na2 (100/60/16, v/v/v) as the solvent. After being air-dried at room temperature, the paper was cut into 1-cm strips. Each strip was immersed in a toluene-based scintillator, and its radioactivity was determined using a liquid scintillation spectrometer.

Sephadex G-200 Gel Filtration—Purified chromatin (6 to 10 mg of protein) was stirred for 1 hour at 0°C in a solution (4 ml) containing 1 M NaCl, 1 mM Tris-HCl (pH 7.5), and 0.5 mM dithiothreitol. The mixture was applied to a Sephadex G-200 column (2.5 x 90 cm) which had been equilibrated with a solution identical to that used to dissolve the chromatin. The column was eluted with the equilibrating salt-buffer solution at a flow rate of 10 ml per hour. Each fraction (4.5 ml) was assayed for protein and DNA by measurement of the absorbance at 230 and 260 nm, respectively, and for NADase and poly(ADP-ribose) synthetase activities.

Protein Determination—Protein was estimated according to the method of Lowry et al. (35) with bovine serum albumin as the standard.

RESULTS

NAD-degrading Activities of Chromatin—Partially purified chromatin of rat liver actively degrades NAD, and at least part of the ADP-ribose released is incorporated into acid-insoluble material (20). This incorporation, which is ascribed to poly(ADP-ribose) synthetase, is known to be very sensitive to nicotinamide inhibition (19, 26). We examined whether the NAD-degrading activity associated with chromatin could be completely inhibited by nicotinamide. Table I shows the effect of 5 mM nicotinamide on the NADase and poly(ADP-ribose) synthetase activities under identical conditions. While poly(ADP-ribose) synthetase was almost completely inhibited by the nicotinamide, the NADase was only partially inhibited and retained a quarter of its original activity. Furthermore, the decrease in total NADase activity in the presence of nicotinamide.

| Table I
<p>| Effect of nicotinamide on NADase and poly(ADP-ribose) synthetase activities of chromatin |</p>
<table>
<thead>
<tr>
<th>Nicotinamide (5 mM)</th>
<th>NADase</th>
<th>Poly(ADP-ribose) synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>2.48 ± 0.05</td>
<td>1.89 ± 0.05</td>
</tr>
<tr>
<td>+</td>
<td>0.65 ± 0.05</td>
<td>1.6 ± 0.05</td>
</tr>
</tbody>
</table>

1 The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; deamido-NAD, nicotinic acid adenine dinucleotide; deamido-NMN, nicotinic acid mononucleotide; deamido-NAD, nicotinamide hypoxygenine dinucleotide; thioNAD, thionicotinamide adenine dinucleotide; AcPyAD, 3-acetylpyrindine adenine dinucleotide.
mide may be entirely attributable to inhibition of poly(ADP-ribose) synthetase, since the decrease in poly(ADP-ribose) synthetase activity corresponded almost exactly to the decrease in total NADase activity.

The enzyme activity catalyzing the cleavage of the pyrophosphate bond of NAD (i.e. NAD pyrophosphatase or phosphodiesterase (27)) was present in chromatin at a level corresponding to less than 5% of that of the total NADase activity, as estimated from the cleavage of [NMN-ribose-14C]NAD under the same conditions. 2

These results suggest that at least two types of enzymes capable of splitting the nicotinamide-ribose linkage are associated with chromatin: one (poly(ADP-ribose) synthetase) is highly sensitive to nicotinamide while the other (NADase) is relatively insensitive.

Separation of NADase and Poly(ADP-ribose) Synthetase by Gel Filtration—In order to separate the nicotinamide-insensitive NADase from poly(ADP-ribose) synthetase, chromatin was treated with a buffered solution containing 1 M NaCl ("Materials and Methods") and passed through a Sephadex G-200 column in the presence of the same buffered salt solution. This procedure has been reported to be effective in dissociating the various proteins of chromatin from DNA (28). Fig. 1 shows the elution profile of DNA, protein, and the enzyme activities from the Sephadex column.

Deamido-NAD, deamido-NMN, and nicotinamide ribonucleotide were not cleaved by the enzyme.

The peak fractions of nicotinamide-insensitive NADase appeared in the void volume along with DNA, but poly(ADP-ribose) synthetase was retarded and eluted apart from the NADase. When 5 mM nicotinamide was included in the assay mixture, the peak of synthetase activity observed in Fig. 1 was almost completely abolished.

These results clearly indicate that there are two distinct NAD-cleaving enzymes in nuclei: one is a NADase which is unaffected by nicotinamide, and the other is poly(ADP-ribose) synthetase, which is highly sensitive to nicotinamide.

The peak fractions of nicotinamide-insensitive NADase were combined, dialyzed against a large volume of 1 mM Tris-HCl buffer (pH 7.5), and concentrated about 10-fold by the use of a Diaflo apparatus equipped with an XM-100A membrane. The concentrated material was used in the following experiments without further purification. Attempts to solubilize or purify the enzyme in the presence of deoxycholate, butanol, etc. have so far been unsuccessful.

Kinetic Properties of Nuclear NADase—Under the assay conditions used, the reaction proceeded linearly for at least 2 hours. The amount of nicotinamide released increased linearly with addition of enzyme preparation, up to at least 70 μg of protein.

The Michaelis constant for NAD, as determined from a Lineweaver-Burk plot, was 28.5 μM. This value is considerably lower than those of either poly(ADP-ribose) synthetase (250 μM) or the microsomal NADase (100 μM) examined under identical conditions.

The nuclear NADase had a fairly sharp pH optimum between pH 6.5 and 7.0. This range is lower than that of poly(ADP-ribose) synthetase, which is most active between pH 8.0 and 8.5. 3

4K. Ueda and M. Fukushima, unpublished results.

5H. Okayama and K. Ueda, unpublished results.

Stoichiometry of reaction catalyzed by nuclear NADase

<table>
<thead>
<tr>
<th>Substrate or products</th>
<th>Reaction</th>
<th>Radioactivity from</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>Degraded</td>
<td>[Nicotinamide-14C]NAD</td>
<td>4902</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>Produced</td>
<td>[NMN-ribose-14C]NAD</td>
<td>4866</td>
</tr>
<tr>
<td>MNM</td>
<td>Produced</td>
<td>[Adenine-14C]NAD</td>
<td>159*</td>
</tr>
<tr>
<td>AMP</td>
<td>Produced</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>Poly(ADP-ribose)</td>
<td>Produced</td>
<td>149*</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from a parallel analysis on a Dowex 1-formate column.  
+ Incorporation into acid-insoluble material, as determined by the Millipore method.

Substrate Specificity—The substrate specificity of the nuclear NADase was studied using variously labeled NADs. As shown in Table II, nicotinamide and ADP-ribose were produced in approximately stoichiometric amounts from NAD. Although about 3% of the degraded NAD was recovered as NMN (and AMP), probably due to the action of a trace of phosphodiesterase (27), no other by-products and no poly(ADP-ribose) were detected among the reaction products.
narrower than that of microsomal NADase; the latter enzyme hydrolyzed not only NADP, thioNAD, and NMN, but also the deamido-analogs of NAD and NMN at rates of 53, 71, 12, 6, and 6% respectively, of the rate of NAD hydrolysis under identical conditions.

**Inhibitors**—Various NAD analogs and related compounds inhibited the nuclear NADase to different degrees. Among them, NADP, thioNAD, and deamido-NAD were the most potent inhibitors, producing almost complete inhibition at a concentration of 5 mM with 100 μM NAD. Addition of NMN or α-NAD to 5 mM inhibited the activity approximately 70% relative to the uninhibited activity. The inhibition by this group of compounds was probably produced by competition for the substrate site on the enzyme. Nicotinamide activated the enzyme slightly at a concentration of 5 mM, but promoted 33% inhibition at 50 mM. ADP-ribose and cyclic AMP, which are potent inhibitors of poly(ADP-ribose) glycohydrolase (29), AMP, which is an inhibitor of rat liver phosphodiesterase (27), and thymidine and dTTP, which are inhibitors of poly-(ADP-ribose) synthetase (26, 30), all yielded approximately 20 to 40% inhibition of the nuclear NADase at 5 mM.

The pattern of inhibition of nuclear NADase by various compounds was very similar to that of microsomal NADase, except that cyclic AMP gave a slightly activating effect on the microsomal enzyme. However, when the NADase activity was assayed photometrically by measuring the decrease of NAD, rather than by release of [14C]nicotinamide from [14C]NAD, nicotinamide was found to be a potent inhibitor of microsomal, but not nuclear, NADase at 5 mM. This difference in behavior may derive from the inhibition of the microsomal enzyme by nicotinamide due to the exchange reaction (see below).

**Mode of Inhibition by Nicotinamide**—The inhibition of the nuclear NADase by high concentrations (>30 mM) of nicotinamide was noncompetitive. The Kₐ value for nicotinamide, estimated by Dixon’s plot (31), was 80 mM.

This result contrasts with the marked inhibitory effects of nicotinamide on poly(ADP-ribose) synthetase and the microsomal NADase. The inhibition of the former enzyme is competitive with respect to the substrate, NAD (12); and the inhibition of the latter, which is noncompetitive, has a Kₐ value of 6 mM.

**Exchange Reaction**—The transglycosidation, or exchange reaction between the nicotinamide moiety of NAD and exogenous nicotinamide, has previously been thought to be one of the characteristics which distinguish mammalian NADase (a transglycosidase type) from the microbial enzyme (a glycohydrolase type). We examined the nuclear NADase and, for reference, the microsomal NADase for this activity by incubating nonradioactive NAD with a large excess of [14C]nicotinamide and analyzing the incorporation of radioactivity into NAD. As shown in Table III, the incorporation of [14C]nicotinamide into NAD was negligible with the nuclear NADase (3% of the NAD cleaved), while it was very marked in the case of the microsomal enzyme (44%). The value for the microsomal enzyme is similar to that reported for rat liver NADase by Swislocki et al. (8). The small amount of exchange observed with the nuclear NADase might be intrinsic to this enzyme, or the preparation might be contaminated by microsomal components; this problem is currently under investigation.

From these results, it is concluded that the nuclear NADase predominantly hydrolyzes the nicotinamide-ribose linkage of NAD and transfers ADP-ribose from NAD to only a slight extent, if at all.

**Thermal Stability at Various pH Levels**—Another property which clearly distinguishes the nuclear NADase from the microsomal enzyme is the pH range in which it is relatively thermostable. When the preparations were treated at 60° for 5 min, decreases in enzyme activities were observed to varying extents at various pH levels (Fig. 2). The nuclear enzyme was most thermostable at acidic pH levels, while the microsomal enzyme was most stable at neutral pH levels.

**Insensitivity to DNase**—In contrast to poly(ADP-ribose) synthetase, which is well known to be very sensitive to DNase (19, 39), the nuclear NADase was not affected at all by pretreatment (37°, 15 min) with bovine pancreatic DNase (50 μg/ml). Furthermore, the elution profile of the nicotinamide-insensitive NADase of chromatin upon Sephadex G-200 gel filtration (Fig. 1) did not change when the filtration was carried out after DNase treatment. These results suggest that, although this enzyme coelutes with DNA during Sephadex G-200 chromatography, there is no structural correlation between the two.

**Localization in Nucleus**—When either chromatin or the nuclear NADase preparation was treated with 1 M NaCl and centrifuged at 105,000 x g for 60 min, more than 80% of the enzymatic activity was retained with the microsomal enzyme (open marks) were treated at various pH levels, as indicated, for 5 min at 60°, and assayed under the standard conditions.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Nuclear NADase</th>
<th>Microsomal NADase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol %</td>
<td>nmol %</td>
</tr>
<tr>
<td>Nicotinamide released</td>
<td>13.3</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinamide incorporated</td>
<td>0.45</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Release of nicotinamide from NAD in the absence of nicotinamide.

![Graph showing thermal stability of nuclear and microsomal NADases at various pH levels.]

Fig. 2. Thermal stability of nuclear and microsomal NADases at various pH levels. The nuclear NADase (closed marks) and microsomal NADase (open marks) were treated at various pH levels as indicated, for 5 min at 60°, and assayed under the standard conditions. Activity is expressed relative to the original (untreated) activity. The following buffers (5 mM each) were employed for the pretreatment: O, potassium phosphate buffer; Δ and △, sodium citrate buffer; ●, Tris-HCl buffer.
total activity of nicotinamide-insensitive NADase was recovered in the precipitate, suggesting that the nuclear NADase is insoluble under these conditions. As described above, this NADase is excluded by Sephadex G 200, is not affected by DNase, and is insoluble in 1 M NaCl. Taken together, these facts suggest that the enzyme might be bound to some nonchromosomal nuclear components, i.e. nucleonemata or nucleolus. More precise localization of this enzyme in the nucleus is currently under investigation.

Other Properties—Divalent cations such as Mg²⁺, Mn²⁺, Ca²⁺, Cd²⁺, Cu²⁺, Hg²⁺, and Zn²⁺ had no significant effect on the nuclear NADase activity at a concentration of 5 mM. High concentrations of salt (e.g. 0.5 M NaCl or KCl), which are very inhibitory to poly(ADP-ribose) synthetase (20), had no influence on the NADase.

The nuclear NADase did not require —SH-protecting reagents such as dithiothreitol, which are stimulatory to poly- (ADP-ribose) synthetase, for the enzyme activity (13). Rather, the presence of these reagents in the dialyzing medium at concentrations higher than 1 mM slightly inactivated the enzyme.

**DISCUSSION**

Since the discovery of poly(ADP-ribose) synthetase in mammalian cell nuclei, it has been known that there is a discrepancy between the amount of NAD cleaved (nicotinamide liberated) and the amount of poly(ADP-ribose) synthesized (ADP-ribose incorporated into acid-insoluble material) (19). This discrepancy has been explained partly by the discovery of protein-bound ADP-ribose and the instability of the protein-ADP-ribose linkage (33, 34), and partly by the existence of an enzyme called poly(ADP-ribose) glycohydrolase, which degrades the polymer (30, 35). On the other hand, evidence has been presented from a number of laboratories for the existence of a separate NADase in nuclear preparations. For example, the report by Green and Dobrjansky has suggested that there are two forms of NADase in tumor cell nuclei, one bound in a complex with DNA and the other associated with the nuclear membrane (36). The DNA-associated enzyme appears to be identical with poly(ADP-ribose) synthetase, as judged by its ability to incorporate ADP-ribose into acid-insoluble material, while the membrane-bound enzyme resembles the microsomal NADase in various properties. Yamada et al. (13) also suggested the multiplicity of nuclear NADases; these workers have isolated poly(ADP-ribose) synthetase free of NADase activity.

Recently, we re-examined the NADase activity of chromatin with a sensitive, newly developed assay method and found that there is a residual activity of NADase which is neither inactivated by DNase nor inhibited by high concentrations of nicotinamide. The nuclear NADase has various properties which distinguish it from poly(ADP-ribose) synthetase and also from the microsomal NADase. The fact that it has a very weak transglycosidase activity makes it unique among animal tissue NADases. Rather, it resembles the microbial enzymes. In order to obtain a preparation practically free from transglycosidase activity, special caution must be taken to minimize microsomal contamination, since the microsomal fraction contains a potent NADase activity of the transglycosidase type. In this study, the initial homogenate of rat liver was prepared in 0.25 M sucrose, the centrifugation in 2.2 M sucrose was performed twice, and finally, the isolated nuclei were further washed with 0.25 M sucrose. This procedure yields nuclei which are essentially free of microsomal contamination as judged by the glucose-6-phosphatase activity.

Although our NADase has been isolated from partially purified chromatin, it resembles the membrane-bound enzyme from Ehrlich ascites tumor cell nuclei described by Green and Dobrjansky (36), in its pH activity profile, its insensitivity to DNase, its substrate specificity, and its inability to catalyze poly(ADP-ribose) synthesis. However, our preparation is almost two orders of magnitude less sensitive to nicotinamide inhibition than theirs. This might be due to the difference of species, tissues, or methods of preparation. The difference is under investigation as is the possibility that an NADase originally of the transglycosidase type is modified during preparation such that the transglycosidase activity is lost.

The physiological importance of the nuclear NADase is unknown. However, judging from its relatively low Kₘ value for NAD (28 μM), it seems plausible that this enzyme is active *in situ* and contributes significantly to the cellular NAD metabolism.

Finally, in the interest of a clearer nomenclature, we would like to propose that the trivial name “NAD glycohydrolase” be applied to the microbial and the newly described nuclear enzymes and that the term “NAD transglycosylase” be used for other mammalian NADases possessing a transglycosidase activity.

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Nicotinamide adenine dinucleotide glycohydrolase from rat liver nuclei. Isolation and characterization of a new enzyme.
K Ueda, M Fukushima, H Okayama and O Hayaishi


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