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pH-Dependent Inactivation of Nicotinamide–Adenine Dinucleotide Glycohydrolase by Its Substrate, Oxidized Nicotinamide–Adenine Dinucleotide*

Saul Green and Areta Dobrjansky

ABSTRACT: Nuclear NAD glycohydrolase activity decreases rapidly during incubation with its substrate NAD^+ at pH 7.4 and 37°. The possibility that this enzyme, as well as its microsomal counterpart, might be inactivated during the incubation has been investigated. The enzymatic hydrolysis of NAD^+ at pH 6.0 by nuclear or microsomal NAD glycohydrolase preparations proceeded at a nearly linear rate for 1 hr with a 50% utilization of the substrate. At pH 8.0, the rate of NAD^+ hydrolysis rapidly declined to zero after 10% of the substrate was used up. The inactivation of NAD glycohydrolase by NAD^+ in 25 min at 4° was pH dependent, and was 0% at pH 7.0, 30% at pH 7.5, and 60% at pH 8.0. At pH 8.0, the inactivation was related to the NAD^+ concentration and was 0% at 10^{-7} M NAD^+, 50% at 10^{-5} M NAD^+, and 90% at 5×10^{-4} M NAD^+. The inactivation was stereochemically specific. Inactivation at equimolar concentrations was 95% for NAD^+, 71% for NADH, 63% for $NADP^+$, 58% for nicotinamide mononucleotide, and 61% for ribosylnicotinamide. 1-Methylnicotinamide, nicotinamide, adenosine diphosphoribose, AMP, ADP, and *d*-ribose 5-phosphate did not inactivate NAD glycohydrolase. Nicotinamide protected the enzyme from inactivation suggesting a competition by the nicotinamide for a specific NAD^+ binding site on the enzyme. The inactivation could not be reversed by temperature, pH, or dialysis, or by treatment with 1.0 M salt solutions, 2.0 or 4.0 M guanidine, SH binding, chelating, or reducing agents. These results show the presence of an uncommon form of pH-dependent enzyme inactivation by its substrate.

Nicotinamide–adenine dinucleotide glycohydrolase (EC 3.2.2.5) (NADase),[1] the enzyme that mediates the hydrolysis of NAD^+ to nicotinamide (Nam) and adenosine diphosphoribose (ADPR) (Handler and Klein, 1942) has been found in the microsomes and in the nuclei of normal and tumor cells (Waravdekar and Griffin, 1964). During a recent investigation in our laboratory into methods for the isolation and purification of NADase from nuclei of mouse Ehrlich ascites tumor cells, the NADase activity was found to decrease rapidly during incubation up to 10 min with substrate at 37° and pH 7.4, the conditions used to assess its activity (Mamaril *et al.*, 1970). These observations raised the possibility that the nuclear NADase might be inactivated during incubation with it susbstrate, NAD^+. Preliminary studies indicated the possibility of a similar phenomenon for microsomal NADase.

The present paper is concerned with establishing this similarity, investigating in some detail the conditions determining the inactivation of microsomal NADase by NAD^+ and proposing a mechanism for this inactivation.

Materials

Enzymes, substrates, and reagents were obtained from the following sources: twice-crystallized yeast alcohol dehydrogenase (EC 1.1.1.1), Worthington Biochemical Corp., Freehold, N. J.; β-NAD^+, β-$NADP^+$, β-NADH, Nam, EDTA, Tris, and *d*-ribose 5′-phosphate (d-R5P), the Sigma Chemical Co., St. Louis, Mo.; nicotinamide mononucleotide (NMN), adenosine diphosphoribose, sodium salt (ADPR), and adenosine 5′-phosphate (AMP), P-L Biochemicals, Inc., Milwaukee, Wis. Ribosylnicotinamide (NR) was prepared by incubating nicotinamide mononucleotide with purified bull seminal 5′-nucleotidase at pH 7.4 in 0.1 M Tris buffer at 37° until all the organic phosphate was split off. The 5′-nucleotidase was then inactivated by heating the reaction mixture in a boiling-water bath for 5 min. This solution was lyophilized, the dry material was dissolved in water, adjusted to a pH of 8.0 with 0.1 M sodium hydroxide, and the solution diluted withwater to yield a final concentration of 10^{-3} M NR.

Methods

Preparation of Microsomal NADase from Ehrlich Ascites Cells. Ehrlich ascites cells in 0.2 mM EDTA (pH 7.0) were allowed to swell for 10 min in 0.05 M potassium phosphate buffer (pH 7.0) and were disrupted in a 100-W, 20-kc MSE ultrasonic disintegrator (Green and Dobrjansky, 1970). Su-

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[1] Abbreviations used as follows: Nam, nicotinamide; ADPR, adenosine diphosphoribose; d-R5P, *d*-ribose 5′-phosphate; NR, ribosylnicotinamide. All other abbreviations are as listed in *Biochemistry 5*, 1455 (1966).

crose was added to the sonicate to yield a concentration of 0.25 M. The resulting suspension was centrifuged for 15 min at 30,000*g* in a Model L-2 Spinco ultracentrifuge to remove cellular, nuclear, and mitochondrial material. The opalescent supernatant solution was centrifuged for 1 hr at 105,000*g*, and the precipitate was washed once with 0.25 M sucrose and recentrifuged at 105,000*g*. The resulting precipitate, containing microsomal-bound NADase, was resuspended in five times its volume of 0.15 M KCl. This suspension could be stored at −20° and the NADase was stable for up to 1 year. When required, NADase was solubilized and further purified from this suspension (Green *et al.*, 1969). The NADase activity of these preparations was determined with alcohol dehydrogenase as described in a previous paper (Green and Bodansky, 1964), and the results were verified by assay with cyanide (Colowick *et al.*, 1951). A unit of NADase activity is the amount of enzyme needed to hydrolyze 1 μmole of NAD^+/hr under the specified conditions of the assay. Total protein was determined by the method of Folin as modified by Lowry *et al.* (1951).

Preparation of Nuclear NADase from Ehrlich Ascites Cells. Intact nuclei were isolated from Ehrlich ascites cells (Mamaril *et al.*, 1970). Yields of nuclei, free of cytoplasmic tags, were 60–80% based on nuclear counts and on DNA determinations. The isolated nuceli were suspended in cold 0.22 M sucrose solution containing 9.4 mM KH_2PO_4, 12.5 mM K_2HPO_4, 10.0 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM EDTA, and 0.3 mM $NaHCO_3$ (Wilbur and Anderson, 1951), and centrifuged for 15 min at 2500 rpm. The precipitate was washed once with 0.154 M NaCl containing 0.01 M EDTA and 0.01 M potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and sonicated for 1 min in a 100-W 20-kc/sec M. S. E. ultrasonic disintegrator. When this nuclear preparation was used immediately, it was suspended in five times its volume of 0.15 M KCl. If the nuclear material was to be used subsequently, it was suspended in five times its volume of Krebs–Ringer phosphate solution (Cohen, 1959) (pH 7.0) and stored at 4°. Under these conditions, this preparation lost 50% of its NADase activity by 72 hr.

Results

Hydrolysis of NAD^+ by NADase at pH 6.0 and at pH 8.0. The kinetics of the hydrolysis of NAD^+ during 1 hr at 37° by microsomal and by nuclear NADase at pH 6.0 and at pH 8.0 are shown in Figure 1. The NADase used in these experiments was present in the particulate material of microsomes or nuclei or was purified from the microsomes of Ehrlich ascites cells (see Methods).

At pH 6.0, the enzymatic hydrolysis of NAD^+ by all three NADase preparations proceeded at a nearly linear rate for 60 min with approximately 50% utilization of the substrate. At pH 8.0 the rate of hydrolysis of NAD^+ by all three enzyme preparations rapidly declined to zero after approximately 10% of the substrate had been used up. Figure 1 shows that for equivalent NADase activities, as standardized by assay at pH 6.5, the initial activity of the nuclear enzyme at pH 6.0 was lower than at pH 8.0; in contrast the activity of the microsomal preparations at pH 6.0 was higher than at pH 8.0. This observation is in accord with previous reports (Mamaril *et al.*, 1970).

Inactivation of NADase During Hydrolysis of NAD^+ at pH 8.0. The mechanism of the decreased rate of hydrolysis at pH 8.0 beyond the first 10% of substrate utilization was investigated. In view of the findings that the nuclear enzyme and the microsomal enzyme behaved in a similar way at pH 8.0 or at pH 6.0 and because of the greater ease and economy with which the microsomal enzyme could be obtained, subsequent studies were carried out with NADase from Ehrlich ascites cell microsomes. The possibility existed that this inactivation was due to a progressive inhibition of NADase activity by the products of the reaction. Nam and ADPR are produced when NAD^+ is hydrolyzed by NADase (Handler and Klein, 1942). ADPR may further decompose to adenosine 5′-monophosphate (AMP) and *d*-ribose 5′-phosphate (d-R5P) at alkaline pH (Colowick *et al.*, 1951).

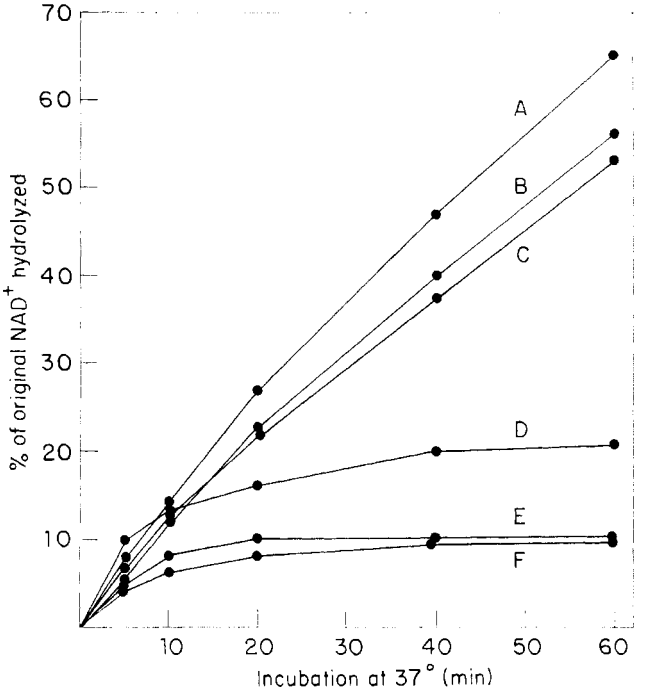

FIGURE 1: Hydrolysis of NAD^+ by Ehrlich ascites cell NADase at pH 6.0 and 8.0. Reaction mixtures contained 8.0 mM potassium phosphate buffer (pH 6.0) or glycylglycine buffer (pH 8.0), 2.5×10^{-4} M NAD^+, and 0.6 unit of NADase in a final volume of 6.0 ml. (All NADase preparations were standardized after assay by the method described in the text.) At pH 6.0 and 8.0, respectively, purified soluble microsomal NADase (curves A and E), microsome-bound NADase (curves B and F), and nuclei-bound NADase (curves C and D). The NAD^+ hydrolyzed at each time interval was calculated by difference from the amount of NAD^+ present at zero time.

The effects of the end products of NAD^+ breakdown on NADase activity at alkaline pH were studied. Reaction mixtures (2 ml) contained 8.0 mM glycylglycine buffer (pH 8.0), 2.0 units of purified NADase, and 0.5 μmole of either NAD^+, Nam, ADPR, AMP, or d-R5P. NADase activity was determined immediately and after incubation for 60 min at 37°. For this assay, an aliquot of each mixture was adjusted to pH 6.0.

Table I shows that neither ADPR nor any of its breakdown products at a concentration of 2.5×10^{-4} M exert any inhibitory or inactivating effect on NADase at pH 8.0. Nam, at 2.5×10^{-4} M, exerts only a small inhibitory effect, 26–32% on NADase initially or by the end of 1 hr at pH 8.0. It is, therefore, highly unlikely that the much smaller concentrations of these reaction products which would be produced at the end of 10 min at pH 8.0 (*i.e.*, about 0.25×10^{-4} M (see Figure 1), would play a role in the 94% inactivation that occurs when NAD^+ is present in the incubation mixture. These results

TABLE I: Microsomal NADase Activity after Incubation at pH 8.0 with NAD^+ or End Products of NAD^+ Hydrolysis.[a]

Addns to Reaction Mix.	Rel NADase Act.[b] (%)	
	0 min	60 min
None	100	96
NAD^+	96	6
Nam	74	68
ADPR	100	96
AMP	94	102
ADP	96	96
d-R5P	98	100

[a] Reaction mixtures and incubation conditions were as described in the legend of Figure 1. The final concentration of each of the added compounds was 2.5×10^{-4} M. [b] NADase activity was determined in an aliquot of each reaction mixture after incubation at 37° for the time shown.

suggested the possibility that the pH-dependent inactivation of NADase was related to NAD^+ per se.

Relationship of pH, NAD^+ Concentration, and Time of Inactivation. The inactivation of NADase by NAD^+ was studied. The NADase used, as noted previously, was a microsomal preparation in which the NADase was bound; the enzyme could, therefore, be quantitatively recovered by centrifugation for 10 min at 105,000*g* at 4°. The total time which elapsed between the mixing of NAD^+ add NADase in pH 8.0 buffer and the removal of the supernatant solution containing the NAD^+ was 25 min. The precipitate containing the NADase was further freed of the components of the mixture by washing twice with 0.15 M KCl at 4° and its NADase activity was

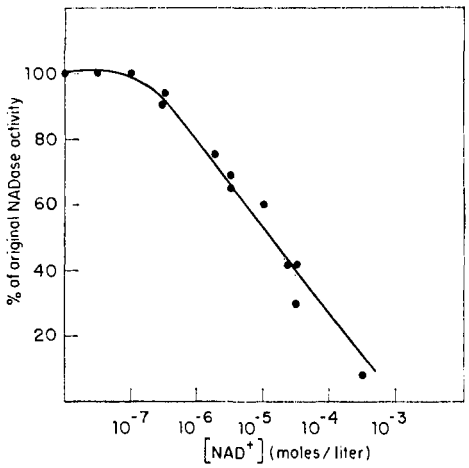

FIGURE 2: Effect of NAD^+ on microsomal NADase activity at pH 8.0 and 4°. The reaction mixture (1 ml) contained 1.0 unit of microsome-bound NADase suspended in 0.15 M KCl, 8.0 mM glycylglycine buffer (pH 8.0), and NAD^+ which ranged in concentration from 0 to 5×10^{-4} M. Immediately upon mixing, all tubes were centrifuged for 10 min at 105,000*g* in a Model L-2 Spinco ultracentrifuge. Total NAD^+–NADase contact time was 25 min at 4°. The precipitated material was washed twice by homogenization with ten volumes of ice-cold 0.15 M KCl centrifuged, and suspended in ice-cold 0.15 M KCl. The results of duplicate determinations of the NADase activity in each sample are presented relative to the activity of the control, *i.e.*, incubated with 8.0 mM glycylglycine buffer (pH 8.0), containing no NAD^+.

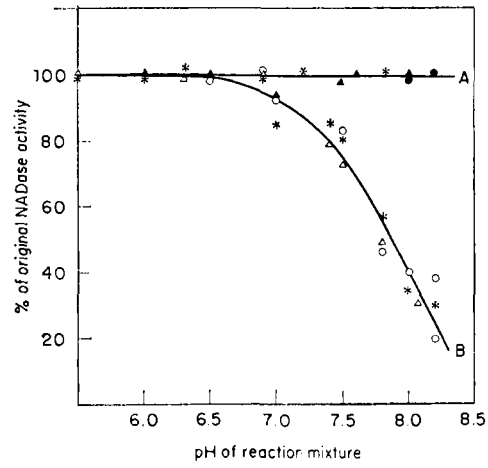

FIGURE 3: Effect of pH on NADase inactivation by NAD^+. Each milliliter of reaction mixture contained 1.0 unit of microsome-bound NADase and either 10^{-5} M NAD^+ (curve B) or no NAD^+ (curve A). The final concentration of each buffer was 8.0 mM and the buffers used were: glycylglycine (○), potassium phosphate (*), and Tris-HCl (△). Experimental conditions and assays were carried out as described. (See legend of Figure 2.) Each point represents a single determination.

determined by the standard technique. Figure 2 shows the effects on NADase activity of contact with various concentrations of NAD^+ at pH 8.0 and at 4° during the period of 25 min. The control value was obtained with NADase which was similarly handled in the absence of NAD^+.

It may be seen that at concentrations of NAD^+ higher than 10^{-7} M the degree of inactivation of the NADase was directly proportional to the concentration of NAD^+. To assure that the pH itself was not affecting NAD^+ to produce an inactivating substance, NAD^+ solutions were kept at pH levels of 6.0, 7.0, and 8.0 for 25 min at 4°, then adjusted to pH 6.0. Each of these solutions was then mixed with NADase in 0.15 M KCl at 4°, centrifuged, and the precipitates were washed as previously described. Assay of the NADase activity in the precipitate showed no inactivation in any instance.

The relationship between the pH of the reaction mixture and the inactivation of NADase activity by NAD^+ during the 25-min period is shown in Figure 3. The concentration 10^{-5} M NAD^+, which produced about a 50% decrease in NADase activity at pH 8.0 (see Figure 2), was used in the reaction mixtures. The pH ranged from 5.5 to 8.2 and the final concentration of buffer was 8.0 mM. Glycylglycine, potassium phosphate, and Tris-HCl were used as buffer and gave essentially the same results at any pH. The degree of inactivation of NADase in the presence of NAD^+ increased as the medium became more alkaline. No loss in enzyme activity resulted over this pH range in the absence of NAD^+.

The time course of the inactivation of NADase by NAD^+ is shown in Figure 4. In the absence of NAD^+, NADase activity was not decreased by incubation at 4° for up to 75 min at pH 6.0 or at pH 8.0. In the presence of NAD^+ (5×10^{-4} M), no inactivation took place at pH 6.0; however, at pH 8.0, a time-dependent inactivation occurred which was directly related to the concentration of NAD^+.

The question whether the continuous presence of NAD^+ was required for the inactivation of NADase at pH 8.0 was considered. A 4-ml reaction mixture containing 10^{-5} M NAD^+, 4.0 units of NADase, and 8 mM glycylglycine buffer (pH 8.0), was prepared and divided immediately into two equal parts.

TABLE II: NADase Activity after Contact with NAD+ or Related Compounds.[a]

Addns to Mix.	Rel NADase Act. (%)
None	100
NAD+	5
NADH	29
NADP+	37
Nicotinamide mononucleotide	42
Nicotinamide riboside	39
1-Methylnicotinamide	104
Nicotinamide	100

[a] Incubation was for 50 min at 4° in a final volume of 4.0 ml. Each mixture contained 4.0 units of NADase (0.43 unit/mg of protein) and 2.0 μmoles (0.5 mM) of one of the compounds shown. After incubation, the NADase was collected by centrifugation, washed, suspended in 0.15 M KCl, and assayed.

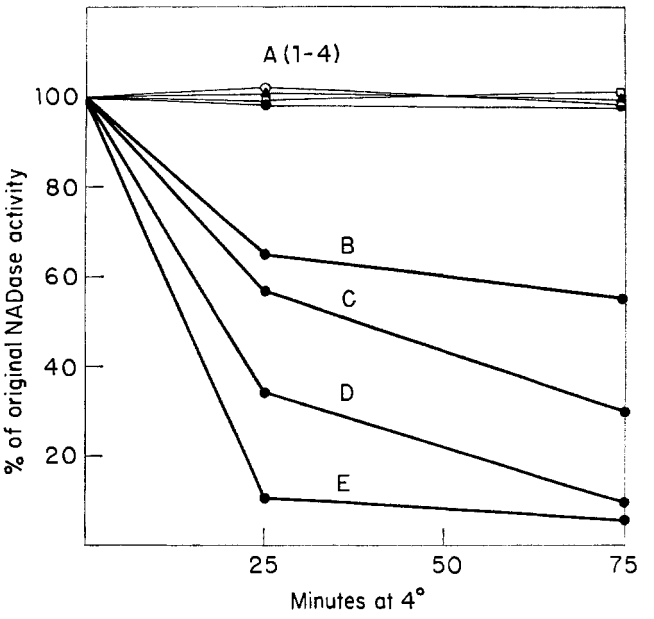

FIGURE 4: The kinetics of NADase inhibition by NAD+. Each 4.0-ml reaction mixture contained 4.0 units of NADase and the following: 8.0 mM potassium phosphate buffer (pH 6.0) (A_1); 8.0 mM potassium phosphate buffer (pH 6.0) and 5×10^{-4} M NAD+ (A_2); 8.0 mM glycylglycine buffer (pH 8.0) (A_3); 8.0 mM glycylglycine buffer (pH 8.0) and 5×10^{-7}, 5×10^{-6}, 10^{-5}, 5×10^{-5}, or 5×10^{-4} M NAD+, respectively (A_4, B, C, D, E). The reaction was started by adding the enzyme to the mixture at 4°. Experimental and assay conditions were as described in the legend of Figure 2.

The first of these was kept at 4° for a total of 75 min. The second was further divided into 0.5- and 1.5-ml aliquots. The enzyme in each was precipitated by centrifugation and the precipitate washed, a procedure which took 25 min, as previously noted. The precipitate from the 0.5-ml aliquot, suspended in 0.15 M KCl, was assayed and showed a 40% decrease from the original NADase activity. The precipitate from the 1.5-ml aliquot was resuspended in 1.5 ml of glycylglycine buffer (pH 8.0) containing no NAD+, and samples assayed were at two successive 25-min intervals. No further inactivation occurred. However, in the 2-ml portion of the original mixture which had been allowed to incubate in the presence of NAD+, inactivation continued to the extend already noted in Figure 4.

Relationship of the Structure of NAD+ to the Inactivation of NADase. Several NAD+ congeners were mixed with the enzyme at 4° for a total of 75 min which included the procedure for precipitating and washing the enzyme. Table II shows that NAD+ was the most effective inactivator of NADase, and that considerable inactivating activity was still present when NAD+ was altered by reduction to NADH or a phosphate-transfer reaction to NADP+. The possibility that the 71% inhibition in NADase activity, found when NADH was used, was due to the partial oxidation of NADH to NAD+ by components of the crude enzyme preparation was considered. Alcohol dehydrogenase and ethanol, in amounts found to be able to maintain essentially all of the NAD+ present (0.5 mM) in the reduced state, were added to the incubation mixture containing NADH. After incubation and centrifugation at 4°, as has been described, the NADase activity was 31% relative to the control, and was not significantly different from the value for the mixture containing NADH alone. Essentially, an inactivating effect of the same order of magnitude occurred with compounds where the Nam moiety was combined with ribose (NMN and NR). However, substitution of a methyl group for the ribose moiety produced a compound with no inactivating effect. No inactivation was obtained with Nam, or with cogeners (AMP, ADPR, ADP, d-R5P) which did not contain Nam (see also Table I).

The possibility that the formation of an NADase:NAD+ complex was involved in the inactivation of NADase at pH 8.0 was explored. Table III shows that several concentrations of ADPR did not prevent NADase inactivation by NAD+. In contrast, amounts as low as 0.5 μmole of Nam in the incubation mixture exerted a measureable protective effect, and this effect increased with increasing concentrations of Nam. This finding indicates the possibility that for inactivation to

TABLE III: Effect of Nicotinamide and Adenosine Diphosphoribose on the Inactivation of NADase by NAD+.[a]

Addns to Reaction Mix. (mM)	Rel NADase Act. (%)
1. None	100
2. NAD+ [b]	8
3. NAD+ + ADPR (0.25)	8
4. NAD+ + ADPR (0.50)	6
5. NAD+ + ADPR (1.00)	10
6. NAD+ + Nam (0.13)	16
7. NAD+ + Nam (0.25)	28
8. NAD+ + Nam (0.50)	37
9. NAD+ + Nam (0.75)	40
10. NAD+ + Nam (1.00)	42

[a] Each reaction mixture contained 8.0 mM glycylglycine buffer (pH 8.0) and 4.0 units of NADase (0.43 unit/mg of protein) in a total volume of 4.0 ml. After incubation for 50 min at 4°, NADase was collected by centrifugation, washed, suspended in 0.15 M KCl, and assayed as has been described in the text. [b] The final concentration of added NAD+ was 0.5 mM.

occur NAD^+ must combine with NADase at a site for which Nam can compete.

The capability of temperature, pH, dialysis, and of various compounds to reactivate NADase was investigated. NADase was inactivated by incubation with NAD^+ at pH 8.0 for 75 min at 4°, precipitated, washed, and resuspended. Aliquots which were kept for 1 hr at 37° either at pH 8.0 in 8 mM glycylglycine buffer or at pH 6.0 in 8.0 mM potassium phosphate buffer showed no reactivation. At a concentration of 5×10^{-4} M in the incubation mixture, the following compounds did not reactivate NADase either at pH 6.0 or 8.0: *n*-ethylmaleimide, *p*-chloromercuribenzoate, reduced glutathione, EDTA, histidine, Nam, ADPR. In addition, reactivation was not effected by incubation with 1.0 M concentrations of $(NH_4)_2SO_4$, $(NH_4)Cl$, K_2SO_4, NaCl, and KCl or by 2.0 or 4.0 M guanidine.

On the assumption that the formation of specific chemical bonds might be the cause of the enzyme inactivation, active NADase was pretreated to effect various functional groups. The compounds used and conditions employed were those described for the reactivation experiments. After preincubation with each of these compounds, the enzyme preparation was washed and incubated with NAD^+ at pH 8.0 for 1 hr at 4°. Determination of the NADase activity in these preparations showed that none of the compounds tested were effective in preventing the inactivation of NADase by NAD^+.

Discussion

Although the reversible inhibition of an enzyme by excess concentrations of substrate is well known and its kinetics well formulated (Dixon and Webb, 1964), the apparently irreversible inactivation of an enzyme by its substrate is an uncommon phenomenon. There are two indications of its existence in the literature. Lieberman (1957) reported that NADase in mouse fibroblasts grown in tissue culture was inactivated when the cells were grown in the presence of NAD^+, NMN, or NR, and Smith and Lands (1970) have recently reported that lipoxygenase from soybean catalyzes its own destruction in the presence of its substrates, oxygen, and fatty acids.

Our results show the degree to which the inactivation of NADase by its substrate NAD^+ is dependent on the pH, the concentration of NAD^+, and the time and temperature of interaction. It was not possible to reverse this inactivation by altering pH or temperature, by dialysis or by treatment with SH binding, reducing or chelating agents, high salt concentrations, or by agents which break hydrogen bonds. Our results also demonstrate that the structural configuration of NAD^+ and more specifically its ribosylnicotinamide moiety is closely related to the pH-dependent inactivation of NADase, for the modification of this molecule to NADH, $NADP^+$, NMN, or NR still allows the inactivation of NADase to proceed, though not to the same extent. In contrast, adenine nucleotides, as in ADPR, AMP, and ADP do not cause inactivation, even at 37°. Since Nam but not ADPR influenced the ability of NAD^+ to inactivate NADase, it would appear that the inactivation involves a site on the enzyme to which the nicotinamide moiety of NAD^+ can bind.

Chambon *et al.* (1966) reported the existence in animal cell nuclei of an enzymatic system which catalyzed the incorporation of the adenylic moiety of NAD^+ into an acid-insoluble product. The pH optimum for this reaction was found to be 8.0 (Fujimura *et al.*, 1967). Other workers have suggested that the enzyme responsible for this activity was nuclear NADase (Roemer *et al.*, 1968; Nakazawa *et al.*, 1968). These conclusions were based on data obtained in experiments involving incubations of the nuclear homogenates in the presence of NAD^+ at 37° to 45° for periods as long as 2 hr and at pH levels as high as 8.5. In view of the rapid inactivation of both nuclear and microsomal NADase by NAD^+ at alkaline pH as reported in the present paper, it is advisable to reevaluate the conclusion by Roemer *et al.* (1968) that there is identity between NADase and the ADPR-polymerizing enzyme in the cell nucleus.

Studies are currently underway into the mechanism of the specific effect of NAD^+ on NADase at alkaline pH and into the possibility that NADase from other species may be similarly inactivated by NAD^+.

Acknowledgments

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