Bull Semen Nicotinamidc Adenine Dinucleotide Nucleosidase

V. KINETIC STUDIES*

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SUMMARY

The substrate specificity of bull semen NAD nucleosidase for various NAD analogs was studied. It was found that NAD, NADP, and nicotinamide hypoxanthine dinucleotide served well as substrates for the enzyme. The enzyme also catalyzed the hydrolysis of nicotinamide mononucleotide, 3-acetylypyridine adenine dinucleotide, 3-acetylypyridine hypoxanthine dinucleotide, 3-pyridinealdehyde adenine dinucleotide, thionicotinamide adenine dinucleotide, and 3-pyridinealdehyde hypoxanthine dinucleotide but at much slower rates. The hydrolysis of NADH, NADPH, and α-NAD was found not to be catalyzed by the enzyme. The enzyme was observed to be inhibited noncompetitively in the presence of nicotinamide; however, it was observed not to catalyze a pyridine base exchange reaction. Product inhibition patterns with nicotinamide and adenosine diphosphoribose were consistent with an “Ordered Uni Bi” reaction in which nicotinamide is the first product released by the enzyme.

NADases (NAD glycohydrolase, EC 3.2.2.5), capable of catalyzing the hydrolysis of the nicotinamide N-ribosidic linkage of NAD, have been isolated from a variety of sources (1-5). The enzymes from mammalian tissues, in most cases, have been isolated as lipoprotein complexes (6-8). In contrast to this, NADase from bull semen was found to exist in an extracellular soluble form (9). The enzyme from bull semen has been purified (10), and the chemical and physical properties of the enzyme were reported (11). Studies of the properties of the substrate binding site of the enzyme with several groups of substrate competitive inhibitors suggested the presence of an adenosine region, a pyrophosphate region, a hydrophobic region, and a pyridinium ring region at the substrate binding site of the enzyme (12, 13). It was reported previously (14) that NADase isolated from bull semen not only catalyzed the hydrolysis of NAD and NADP, but also catalyzed the hydrolysis of NMN and nicotinamide riboside. The rate of hydrolysis of NMN was approximately 10% of that of NAD (14). It was reported (15) that NADase from rabbit erythrocytes catalyzed the hydrolysis of NMN but at a rate 200 times less than that of NAD. NADase from human erythrocytes was also reported to catalyze the hydrolysis of NMN (16). Very little information is available concerning the ability of NADases to catalyze the hydrolysis of riboside bonds to nitrogen bases other than nicotinamide since previous studies of bull semen NADase (14) have been restricted exclusively to nicotinamide riboside derivatives. It was of interest to extend these studies to include a greater variety of ribosides. It was reported (17, 18) that NADases isolated from pig brain and other mammalian tissues were sensitive to inhibition by nicotinamide free base and functioned as transglycosidases, capable of transferring the adenosine diphosphoribose moiety of NAD from one molecule of nicotinamide to another, or to other substituted pyridine bases. The ability of nicotinamide to inhibit the hydrolysis of NAD was proposed to involve a competition between nicotinamide and water for an adenosine diphosphoribose-enzyme intermediate. Since the ribosyl transfer reaction was not restricted to nicotinamide as the only acceptor, it was used successfully to prepare a large series of NAD analogs containing pyridine bases other than nicotinamide (19-22). Nicotinamide protection of the hydrolysis of NAD catalyzed by bull semen NADase was also observed (23). However, the nature of the nicotinamide protection of the hydrolysis of NAD was not clear. The present study, therefore, concerns the investigation on the substrate specificity of this enzyme and the nature of the nicotinamide protection of the hydrolysis of NAD by this enzyme.

EXPERIMENTAL PROCEDURE

Materials—NAD, NADH, NADP, NADPH, NHD, 3-AcPyAD, 3-AcPyHID, 3-PyAlAD, 3-PyAlHID, NMN, α-NAD, and ADP-ribose were obtained from Sigma. TNAD was prepared according to the procedure previously reported (22). Nicotinamide was purchased from Eastman. 3-AcPy was the product of Aldrich. l-Ergothionine hydrochloride was obtained from Mann. Yeast alcohol dehydrogenase was purchased from Worthington. Pig brain NADase was partially purified

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according to Windmueller and Kaplan (6) to a specific activity of 80 units per mg. The bull semen sample was a gift of the Curtiss Breeding Service, Cary, Ill. Bull semen NADase was isolated and purified according to the procedure reported previously (10). The purified NADase with a specific activity of 7630 units per mg was stored at -15°C.

Methods—The bull semen NADase-catalyzed hydrolytic reaction was studied with a titrimetric method (12). A Radiometer type TTT11 titrator, type SBR 2c titrigraph, PHM 26e pH meter, type ABU 12 automatic burette unit with a 0.25-ml burette, and type TTA 31 micititrination assembly equipped with a G-2222 glass electrode and a K-4112 calomel electrode were used for this purpose. Initial velocity measurements were made at five or more substrate concentrations. Michaelis constants (K_m) and maximum initial velocities (V_max) were calculated from plots according to Lineweaver and Burk (24). In studies of the inhibition of NADase-catalyzed hydrolysis of NAD, two different types of experiments were performed for the determination of the inhibitor dissociation constant (K_i) for each inhibitor. In the first set of experiments, the inhibition was studied as a function of varying substrate concentration at a constant inhibitor concentration. The data obtained from these studies were plotted according to Lineweaver and Burk (24). In the second set of experiments, the inhibition was studied as a function of varying inhibitor concentration at a constant substrate concentration and the data obtained were plotted according to Dixon (25).

The measurements of the pyridine base exchange reaction catalyzed by NADase from pig brain and bull semen were carried out in reaction mixtures containing 0.1 m sodium phosphate buffer, pH 7.5, 3.93 × 10^{-3} m NAD, 0.1 m 3-AcPy, and NADase, in a total volume of 2.5 ml. The reactions were initiated with the addition of the enzyme, and, at the time intervals indicated, 0.2-ml aliquots from the reaction mixture were pipetted into test tubes and the reactions were stopped with the addition of 0.2 ml of 10% trichloroacetic acid. Any precipitate in the solutions was removed by centrifugation, and then 0.2 ml of this solution was pipetted into a cuvette containing 9.0 × 10^{-5} m Tris and 0.5 ml ethanol in a total volume of 2.8 ml. The optical densities of the samples before the addition of yeast alcohol dehydrogenase were read at 365 nm and 340 nm against a blank containing the same concentration of Tris and ethanol. Then 0.2 ml of a 10 mg per ml yeast alcohol dehydrogenase solution was added to the sample cuvette to reduce all the pyridine dinucleotides in the sample. Again, optical densities at 365 nm and 340 nm were read against the same blank. The optical densities obtained before the addition of yeast alcohol dehydrogenase for each sample at both wave lengths were subtracted from those obtained in the presence of yeast alcohol dehydrogenase. The ratios of the optical density values obtained at 365 nm and 340 nm were plotted against the reaction time.

RESULTS

Substrate Specificity—Various NAD analogs were studied as substrates for bull semen NADase. The data obtained from studies of NADase-catalyzed hydrolysis of each compound, plotted according to Lineweaver and Burk (24), were used to calculate the Michaelis constants (K_m) and maximum velocity (V_max) values, which are listed in Table I. Of the compounds tested, NAD, NHD, and NADP showed the highest activities as substrates. The enzyme also catalyzed the hydrolysis of 3-AcPyAD, 3-AcPyHD, 3-PyAlAD, 3-PyAlHD, TNAD, and NMN, but at much slower rates. The hydrolysis of α-NAD, NADH, and NADPH was found not to be catalyzed by the enzyme under the conditions employed.

Inhibition of NADase—Bull semen NADase-catalyzed hydrolysis of NAD was found to be inhibited by nicotinamide. The inhibition obtained with nicotinamide was observed to be noncompetitive with respect to NAD. The noncompetitive nature of the inhibition by nicotinamide was determined using double reciprocal plots where the initial velocities were measured at five concentrations of NAD and a constant nicotinamide concentration. When this inhibition was studied under the conditions of varying nicotinamide concentrations and a constant substrate concentration, a noncompetitive type of inhibition was also observed in plots of reciprocal velocity versus inhibitor concentration. The inhibition of the enzyme by nicotinamide was observed to be a linear noncompetitive inhibition as demonstrated from the replot of the slopes and the intercepts of these relationships against the concentration of nicotinamide (Fig. 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_m (M)</th>
<th>V_max (mole/min/mg)</th>
<th>Relative activity %</th>
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<tr>
<td>NAD</td>
<td>1.02 × 10^{-4}</td>
<td>5.46 × 10^{-4}</td>
<td>100</td>
</tr>
<tr>
<td>NHD</td>
<td>7.14 × 10^{-4}</td>
<td>4.91 × 10^{-4}</td>
<td>89.9</td>
</tr>
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<td>NADP</td>
<td>1.05 × 10^{-4}</td>
<td>4.57 × 10^{-4}</td>
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<tr>
<td>3-PyAlHD</td>
<td>1.96 × 10^{-4}</td>
<td>5.29 × 10^{-4}</td>
<td>9.7</td>
</tr>
<tr>
<td>3-AcPyHD</td>
<td>2.50 × 10^{-4}</td>
<td>6.01 × 10^{-4}</td>
<td>11.0</td>
</tr>
<tr>
<td>3-PyAlAD</td>
<td>1.43 × 10^{-4}</td>
<td>3.18 × 10^{-4}</td>
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</tr>
<tr>
<td>TNAD</td>
<td>7.25 × 10^{-6}</td>
<td>1.38 × 10^{-4}</td>
<td>2.5</td>
</tr>
<tr>
<td>3-AcPyAD</td>
<td>6.76 × 10^{-6}</td>
<td>5.00 × 10^{-4}</td>
<td>9.2</td>
</tr>
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<td>NMN</td>
<td>1.56 × 10^{-6}</td>
<td>4.39 × 10^{-4}</td>
<td>8.4</td>
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</table>

FIG. 1. Replot of the slopes and the intercepts obtained in double reciprocal plots against the concentration of nicotinamide. Open circles, slopes; closed circles, intercepts.
ADP-ribose was found to inhibit the NADase-catalyzed hydrolysis of NAD, and the inhibition by this compound was found to be competitive with respect to NAD. The competitive nature of the inhibition by ADP-ribose is illustrated in Fig. 2. The inhibition of the enzyme by ADP-ribose was found to be a linear competitive type, and this is exemplified in Fig. 3, which is the replot of the apparent $K_m$ values and slopes obtained in Fig. 2 as a function of the concentration of ADP-ribose. The kinetic data obtained in the studies of inhibition of bull semen NADase by nicotinamide and ADP-ribose are summarized and presented in Table II.

In the spectrophotometric assay method reported previously (10), 3-AcPyAD served as a substrate for the NADase-catalyzed hydrolytic reaction. Since the maximum velocity obtainable with 3-AcPyAD as substrate is considerably lower than that observed with NAD, 3-AcPyAD could be studied as an inhibitor of NAD hydrolysis. When studying the inhibition of the enzyme at a constant 3-AcPyAD concentration and varying the substrate concentration, a competitive inhibition of the enzyme with respect to NAD was observed. This is illustrated in Fig. 4, in which an average inhibitor dissociation constant ($K_i$) of $6.57 \times 10^{-6}$ M was obtained from this study. The $K_i$ value obtained essentially equals the $K_m$ value obtained when 3-AcPyAD was studied as the substrate for the enzyme in the spectrophotometric assay method (10).

Studies of Pyridine Base Exchange Reaction—It was reported that nicotinamide inhibited the pig brain NADase-catalyzed hydrolysis of NAD noncompetitively (18), and the enzyme was also shown to catalyze a pyridine base exchange reaction. The pyridine base exchange reaction between the nicotinamide moiety of the NAD molecule and the free base 3-AcPy catalyzed by pig brain NADase is illustrated in Fig. 5 (Line 1). Although the bull semen NADase was also found to be inhibited by nicotinamide noncompetitively, no pyridine base exchange reaction was observed, as shown in Line 8 of Fig. 5. NADases from Neurospora crassa and bacteria were shown not to catalyze pyridine base exchange reactions (2, 3, 5, 26-28). However, Grossman and Kaplan (29) demonstrated that ergothionine induced a nicotinamide sensitivity in the NADase from N. crassa, resulting in the ability of this enzyme to catalyze the pyridine base exchange reaction. In contrast to this, no pyridine base exchange reaction was observed with bull semen NADase in the presence of 5.85 mm ergothionine in the reaction mixture, and this is illustrated in Line 2, Fig. 5. The pyridine base exchange reaction between the 3-AcPy moiety of the 3-AcPyAD and the free base nicotinamide catalyzed by bull semen NADase has also been studied (10).

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor Type of inhibition</th>
<th>Michaelis constant ($K_m$)</th>
<th>Inhibitor constant ($K_i$)</th>
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</thead>
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<tr>
<td>NAD</td>
<td>Nicotinamide</td>
<td>Linear non-competitive</td>
<td>$1.02 \times 10^{-4}$ M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$2.13 \times 10^{-2}$ M  (intercept)</td>
</tr>
<tr>
<td>NAD</td>
<td>ADP-ribose</td>
<td>Linear competitive</td>
<td>$1.02 \times 10^{-4}$ M</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>$2.08 \times 10^{-2}$ M  (slope)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$9.54 \times 10^{-4}$ M  (intercept)</td>
</tr>
</tbody>
</table>

Fig. 2. Competitive inhibition of NADase by ADP-ribose. The reaction mixture contained $5.85 \times 10^{-5}$ M NaCl, NAD concentrations varying from $6.13 \times 10^{-5}$ M to $1.51 \times 10^{-4}$ M, ADP-ribose as indicated, and 5.2 mg of NADase, in a total volume of 1.71 m1. The reactions were measured at 25° and pH 7.5. The concentrations of ADP-ribose used were as follows: Line 1, 0; Line 2, $5.25 \times 10^{-4}$ M; Line 3, $8.74 \times 10^{-4}$ M; Line 4, $1.79 \times 10^{-3}$ M.

Fig. 3. Replot of the slopes and apparent $K_m$ values obtained in Fig. 2 against the concentration of ADP-ribose. Open circles, slopes; closed circles, apparent $K_m$ values.

Fig. 4. Competitive inhibition of NADase-catalyzed hydrolysis of NAD by 3-AcPyAD. The reaction mixtures contained $5.85 \times 10^{-2}$ M NaCl, NAD concentrations varying from $6.13 \times 10^{-3}$ M to $1.51 \times 10^{-4}$ M, 3-AcPyAD concentration as indicated, and 5.2 mg of NADase, in a total volume of 1.71 ml. The reactions were measured at 25° and pH 7.5. The concentrations of 3-AcPyAD used were as follows: Line 1, 0; Line 2, $7.01 \times 10^{-4}$ M; Line 3, $1.40 \times 10^{-4}$ M; Line 4, $2.11 \times 10^{-3}$ M.
of the pyridine ring, lead to larger changes in the functioning of dinucleotide, such as those involving the grouping in position 3 of adenosine-3'-(diphosphate-4'-ribose). The relatively low rates of hydrolysis obtained with 3-acetylpyridine, pyridine-3-aldehyde, and thionicotinamide derivatives. This is exemplified by the relatively slow turnover observed with 3-AcPyAD as substrate. The adenosine region was shown to differ from the pyridinium ring region of the substrate site on the basis of multiple inhibition studies. The effective binding of 3-AcPyAD to bull semen NADase is of particular interest since it represents a major difference in binding associated with a rather minor change in chemical structure. Further studies on the binding of pyridine nucleotide-derivatives substituted in position 3 with a variety of functional groups are currently underway.

**Discussion**

Using a previously described (12) titrimetric method, we have studied 12 nucleotide derivatives as possible substrates for bull semen NADase. Nine of the 12 were observed to be used as substrates (Table I). Thus, a requirement for the oxidized pyridinium ring system is indicated with a stereochemical preference for the β-ribosidic linkage. This stereospecificity is consistent with that observed with other pyridine nucleotide-requiring enzymes, such as dehydrogenases.

The better substrates, NAD, NADP, and NHD, showed only slight differences in utilization by the bull semen enzyme. The additional phosphate of NADP and the hydroxyl substitution at position 6 of the purine ring of NHD are both tolerated well by the enzyme. In fact, complete removal of the adenylate portion of the molecule, as in NMN, does not abolish substrate activity but does lower the maximum velocity to less than one-twentieth of that observed with NAD. That the adenylate moiety does play a role in the enzyme reaction is supported by the finding that adenylate acid functions as a substrate-competitive inhibitor of the enzyme (12) and is bound at an adenosine region of the substrate binding site. The adenosine region was shown to differ from the pyridinium ring region of the substrate site on the basis of multiple inhibition studies (12).

On the other hand, structural changes in the other half of the dinucleotide, such as those involving the grouping in position 3 of the pyridine ring, lead to larger changes in the functioning of compounds as substrates. This is exemplified by the relatively low rates of hydrolysis obtained with 3-acetylpyridine, pyridine-3-aldehyde, and thionicotinamide derivatives.

The $K_m$ for the NADase-catalyzed hydrolysis of 3-AcPyAD was $6.4 \times 10^{-4}$ M in the spectrophotometric assay reported previously (10). The $K_m$ value for 3-AcPyAD is smaller than that for NAD; however, the rate of the hydrolysis of this compound is considerably slower than that observed with NAD. These data suggest that NADase bind 3-AcPyAD better but the turnover number for 3-AcPyAD is smaller than that for NAD. This was further confirmed by the observation that 3-AcPyAD also inhibited the NADase-catalyzed hydrolysis of NAD. The inhibitor dissociation constant ($K_i$) obtained for 3-AcPyAD was $6.57 \times 10^{-4}$ M, which was essentially the same as the $K_m$ value obtained for 3-AcPyAD. Thus, with 3-AcPyAD as substrate, the rate constant that governs the rate-limiting catalytic process is considerably smaller than the rate constant for the dissociation of 3-AcPyAD-enzyme complex.

The effective binding of 3-AcPyAD to bull semen NADase is of particular interest since it represents a major difference in binding associated with a rather minor change in chemical structure. Further studies on the binding of pyridine nucleotide-derivatives substituted in position 3 with a variety of functional groups are currently underway.

NADase-catalyzed hydrolysis of NAD was found to be inhibited noncompetitively by nicotinamide. It was reported earlier (17, 18) that NADases from pig brain and beef spleen were inhibited by nicotinamide noncompetitively, and further studies with isotope experiments showed that labeled NAD was formed from labeled nicotinamide and unlabeled NAD in a reaction catalyzed by these enzymes. These findings suggested that nicotinamide acted by competing with water molecules for an intermediate adenosine diphosphoribose-enzyme complex. The reaction of nicotinamide with the enzyme intermediate results in the formation of NAD which is viewed as an inhibition of the hydrolytic reaction. In contrast to this, bull semen NADase was found not to catalyze the pyridine base exchange reaction. The bull semen NADase catalyzed the hydrolysis of NAD to form nicotinamide and ADP-ribose, and these two products were shown to inhibit the enzyme.

The inhibition of the enzyme by nicotinamide was found to be a linear noncompetitive type (Fig. 1), and a linear competitive inhibition was observed with ADP-ribose (Fig. 3). The data obtained in the inhibition of the NADase-catalyzed hydrolysis of NAD by nicotinamide and ADP-ribose are summarized and presented in Table II. The linearity of the Lineweaver-Burk plots of the inhibition studies with nicotinamide and ADP-ribose indicates that the NADase reaction follows the simple rate law:

$$v = \frac{VA}{K_a + A}$$

where $v$ is the initial velocity of the forward reaction, $V$ is the maximum velocity, $A$ is the concentration of substrate, and $K_a$ is the Michaelis constant. Based on the kinetic treatment as described by Cleland (30, 31), one can first assume a simple mechanism as follows:

$$A \xrightarrow{k_1} P \xrightarrow{k_2} EQ \xrightarrow{k_3} E$$

where $P$ is the first product and $Q$ the second product from the substrate $A$, the rate equation for this mechanism can be written as described by Hsu et al. (32):

$$v = \frac{VA}{K_a + A + \frac{K_{iA}P}{K_{iA}} + \frac{K_iQ}{K_i} + \frac{K_pQ}{K_p} + \frac{A}{K_p}}$$

![FIG. 5. The NADase-catalyzed pyridine base exchange reaction. The reaction mixtures contained 0.1 M sodium phosphate buffer, pH 7.5, 3.3 x 10^{-3} M NAD, 0.1 M 3-AcPy, and the enzyme, in a total volume of 2.5 ml. Line 1, 20 µg of pig brain NADase; Line 2, 323 µg of bull semen NADase with 5.85 x 10^{-3} M ergothionine; Line 3, 325 µg of bull semen NADase.](image-url)
where $K_a$ is the dissociation constant of $A$, $K_{PQ}$ and $K_{Q}$ are the inhibitor constants for $P$ and $Q$, and $K_P$ is the Michaelis constant for $P$ in the reverse reaction. If the reaction is allowed to proceed in the presence of $P$ but not $Q$, the above equation in a reciprocal form becomes:

$$\frac{1}{v} = \frac{K_a}{V} \left[ 1 + \frac{K_{a}P}{K_{PQ}} \right] \left[ 1 + \frac{A}{A} \right] + \frac{1}{V} \left[ 1 + \frac{P}{K_P} \right]$$

When the reaction proceeds with the presence of $Q$ but not $P$, the rate equation is:

$$\frac{1}{v} = \frac{K_a}{V} \left[ 1 + \frac{Q}{K_{Q}} \right] \left[ 1 + \frac{A}{A} \right] + \frac{1}{V}$$

Therefore, if the reaction of the enzyme proceeds according to the above scheme, the first product $P$ should exhibit a linear noncompetitive inhibition and the second product $Q$ should exhibit a linear competitive inhibition.

The kinetic features observed for the NADase-catalyzed hydrolysis of NAD are consistent with the Ordered Uni Bi mechanism as described in the above mechanism. These facts suggest that nicotinamide is the first product released and ADP-ribose the last.

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REFERENCES
