Bull Semen Nicotinamide Adenine Dinucleotide Nucleosidase

III. PROPERTIES OF THE SUBSTRATE BINDING SITE*

JAMES H. YUAN AND BRUCE M. ANDERSON

From the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

SUMMARY

The properties of the substrate binding site of the bull semen nicotinamide adenine dinucleotide nucleosidase were studied with a titrimetric assay. The adenosine derivatives, adenosine, AMP, ADP, and ADP-ribose, were found to inhibit the NADase-catalyzed hydrolysis of NAD. Inhibition by these compounds was observed to be competitive with respect to NAD. The importance of an adenosine region and a pyrophosphate region at the substrate binding site of this enzyme was demonstrated by the relative $K_i$ values obtained for these compounds. N1-Alkylnicotinamide chlorides were also shown to be substrate-competitive inhibitors of the NADase-catalyzed hydrolysis of NAD. These compounds were suggested to be bound at a pyridinium ring region of the substrate binding site. The positive chain length effect observed in the binding of N1-alkylnicotinamide chlorides, N1-ethyl- to N1-dodecylnicotinamide chloride, inclusive, to the enzyme suggested the presence of a hydrophobic region close by the pyridinium ring region. Multiple inhibition studies further demonstrated that N1-alkylnicotinamide chlorides and adenosine derivatives can be simultaneously bound to the NAD binding sites of these enzymes. The specific binding of N1-alkylnicotinamide chlorides, facilitated by nonpolar interactions, is not a general feature of enzymes utilizing pyridine nucleotides as coenzymes. Yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49), beef heart and rabbit skeletal muscle lactate dehydrogenase (EC 1.1.1.27), and pig heart and rat liver malate dehydrogenase (EC 1.1.1.37) are not inhibited by low concentrations of the N1-alkylnicotinamide chlorides. Differences have also been recognized in the binding of adenosine derivatives to various dehydrogenases. The above observations prompted the investigation of the substrate binding properties of bull semen NADase (NAD glycohydrolase, EC 3.2.2.5). This report describes the interactions observed between these inhibitors and the bull semen NADase.

EXPERIMENTAL PROCEDURE

NAD, adenosine, adenylic acid, adenosine diphosphate, and adenosine diphosphoribose were obtained from Sigma. N1-Alkylnicotinamide chlorides were prepared as described previously (3). Bull semen was the gift of Curtiss Breeding Service, Cary, Illinois. Bull semen NADase was isolated and purified according to the procedure reported previously (8). NADase-catalyzed hydrolysis of NAD was studied with a titrimetric method. Multiple inhibition studies using two different competitive inhibitors were carried out as described previously (9). Measurements of pH were made at 25° with a Radiometer pH meter, type PHM 52, with a G-202 C glass electrode.

RESULTS

Titrimetric Assay for NADase Activity—The release of hydrogen ions in the NADase-catalyzed hydrolysis of NAD permitted the use of a titrimetric method for determining reaction rates. A titrimetric assay for the NADase-catalyzed hydrolysis of NAD

* The work was supported by Research Grant GB 25652 from the National Science Foundation. See preceding paper for Paper II of this series.
Effect of pH on Vmax and Km—The effect of pH on the NADase-catalyzed hydrolysis of NAD was studied between pH 6.0 and 9.0, and at each pH, five concentrations of the substrate were employed. The reactions were carried out at 25° by means of the assay conditions described above. Km and Vmax values were determined from the linear Lineweaver-Burk plots obtained at different pH values. The results of these studies are shown in Fig. 3. An optimal pH of 7.5 was observed for the maximum velocity, whereas only a slight change in the Km values was observed throughout the pH range studied.

Inhibition of NADase by Adenosine Derivatives—Inhibition of the NADase-catalyzed hydrolysis of NAD was studied with adenosine, adenylic acid, adenosine diphosphate, and adenosine diphosphoribose. Two separate experiments were performed for the determination of inhibitor dissociation constants (Kf) from Lineweaver-Burk and Dixon plots. All four adenosine derivatives were found to inhibit the NADase-catalyzed hydrolysis of NAD competitively with respect to NAD. The competitive nature of the inhibition by these compounds is illustrated in Fig. 4 by the data obtained in studies using ADP-ribose as the inhibitor. The relationship shown in Fig. 4 is characteristic of those obtained with the other adenosine derivatives as well. The inhibition by adenosine derivatives was also measured, in a separate experiment, as a function of constant substrate concentration and varying inhibitor concentration. The inhibition obtained at two concentrations of adenylic acid, plotted according to Dixon (11), is shown in Fig. 5. Again, the relationship shown in Fig. 5 can serve to exemplify those obtained with the other adenosine derivatives studied. The inhibitor dissociation constants for the four adenosine derivatives, calculated from these two types of experiments, are listed in Table I. The average Kf values obtained from Lineweaver-Burk plots agree well with Kf values obtained from the Dixon plots.

The inhibition of NADase by adenosine derivatives was observed to be reversible upon dilution. Enzyme (12 μg) was incubated in 0.5 ml of a solution containing inhibitor at a concentration 10 times that required to produce 30% inhibition. The pH of this solution was maintained at 7.5 with the addition of NaOH. A 0.2-ml aliquot of this solution was then transferred to a 1.8-ml reaction mixture containing 5.0 × 10⁻² M NaCl and 1.31 × 10⁻⁴ M NAD for the titrimetric assay of enzyme activity. A second assay was performed by transferring 0.2 ml of a 24-μg per ml NADase solution into a 1.8-ml reaction mixture containing the same components described above plus a concentration of inhibitor equal to that obtained in the first assay mixture by dilution. Initial velocities of the hydrolysis of NAD were then measured in a titrimeter for these two reaction mixtures. With all adenosine derivatives, no additional inhibition was observed in those samples in which the enzyme had been incubated with a 10-fold higher concentration of inhibitor prior to assay.

Inhibition of NADase by N¹- Alkyl nicotinamide Chlorides—The NADase-catalyzed hydrolysis of NAD was studied in the presence of a homologous series of N¹-alkyl nicotinamide chlorides, N¹-methyl- to N¹-dodecyl nicotinamide chlorides, inclusive. Each inhibition was studied in two separate experiments, as described previously for adenosine derivatives. The inhibition...
of the enzyme, when studied as a function of varying substrate concentration, was observed to be competitive with respect to NAD. The inhibition of the enzyme at three concentrations of N'-methylnicotinamide chloride, plotted according to Line- weaver and Burk (10), is shown in Fig. 6. The inhibition caused by N'-methylnicotinamide chloride and N'-hexylnicotinamide chloride is representative of the relationships observed in the inhibition by all of the N'-alkylnicotinamide chlorides studied. Inhibitor dissociation constants for each of the 12 N'-alkylnicotinamide chlorides were calculated from the two independent experiments and are listed in Table II.

The inhibitor dissociation constants obtained in studies of the N'-ethyl- to N'-dodecylnicotinamide chlorides, inclusive, were observed to decrease with increasing chain length of the inhibitor, as shown in Fig. 8. The inhibitor dissociation constant obtained with N'-methylnicotinamide chloride is an obvious exception to this relationship.

**Table I**

<table>
<thead>
<tr>
<th>Adenosine derivatives</th>
<th>$K_I$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>$1.16 \times 10^{-2}$</td>
</tr>
<tr>
<td>Adenylate</td>
<td>$2.58 \times 10^{-3}$</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td>$1.64 \times 10^{-7}$</td>
</tr>
<tr>
<td>Adenosine diphosphoribose</td>
<td>$9.54 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**Fig. 4 (left).** Competitive inhibition of NADase by ADP-ribose. The reaction mixtures contained $5.85 \times 10^{-4}$ 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 ml. The reactions were measured at 25° and pH 7.5. Line 1, no inhibitor; Line 2, $5.23 \times 10^{-4}$ M ADP-ribose; Line 3, $5.74 \times 10^{-4}$ M ADP-ribose; Line 4, $1.79 \times 10^{-3}$ M ADP-ribose.

**Fig. 5 (center).** Inhibition of NADase as a function of AMP concentration. The reaction mixtures contained $5.85 \times 10^{-4}$ M NaCl, NAD and inhibitor concentrations 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. Line 1, $1.43 \times 10^{-4}$ M NAD; Line 2, $7.95 \times 10^{-4}$ M NAD.

**Fig. 6 (right).** Competitive inhibition of NADase by N'-hexylnicotinamide chloride. The reaction mixtures contained $5.85 \times 10^{-4}$ M NaCl, NAD concentrations varying from $6.13 \times 10^{-5}$ M to $1.51 \times 10^{-4}$ M, N'-hexylnicotinamide chloride 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. Line 1, no inhibitor; Line 2, $1.17 \times 10^{-3}$ M N'-hexylnicotinamide chloride; Line 3, $2.34 \times 10^{-3}$ M N'-hexylnicotinamide chloride; Line 4, $3.42 \times 10^{-3}$ M N'-hexylnicotinamide chloride.

**Fig. 7 (left).** Inhibition of NADase as a function of N'-hexylnicotinamide chloride concentration. The reaction mixtures contained $5.85 \times 10^{-4}$ M NaCl, NAD and inhibitor concentrations 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. Line 1, $1.43 \times 10^{-4}$ M NAD; Line 2, $7.95 \times 10^{-4}$ M NAD.

**Fig. 8 (center).** The relationship of the logarithm of the reciprocals of the inhibitor constants to the chain length of the alkyl substituents of the inhibitors.

**Fig. 9 (right).** Multiple inhibition of NADase by N'-methylnicotinamide chloride and AMP. The reaction mixtures contained $5.85 \times 10^{-4}$ M NaCl, $1.23 \times 10^{-4}$ M NAD, inhibitor concentrations 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 concentration of N'-methylnicotinamide chloride was varied from zero to $2.81 \times 10^{-3}$ M and the concentrations of AMP used were as follows: Line 1, zero; Line 2, $1.26 \times 10^{-3}$ M; Line 3, $2.52 \times 10^{-3}$ M; Line 4, $3.78 \times 10^{-3}$ M.
Adenosine derivatives and N1-methylnicotinamide chloride were observed to inhibit the bull semen NADase catalyzed hydrolysis of NAD competitively with respect to NAD. Since both adenosine derivatives and N1-methyl nicotinamide chloride are structurally analogous to different portions of the NAD molecule and the over-all inhibition of the enzyme by these inhibitors observed in each case was competitive, the binding of the different inhibitors could occur at different parts of the bull semen NADase substrate binding site. The possibility of interactions occurring between any given pair of inhibitors in binding to the enzyme was studied by the multiple inhibition analysis described previously by Yonetani and Theorell (9). Inhibition of an enzyme system in the presence of two competitive inhibitors, I1 and I2, in the enzyme-I1-I2 complex. A plot of 1/V against vi at the different fixed concentrations of I2 will result in a series of straight lines that intersect at an abscissa value of -αK11. The interaction constant, α, can be calculated from this term by means of the values of K11 determined from inhibitor studies of I1 alone. The same relationship exists if one plots the ratio of the initial velocities in the absence of inhibitor (v0) to the initial velocities in the presence of inhibitors (v1) versus the concentration of I1. Multiple inhibition by inhibitor pairs composed of a combination of inhibitors from the two different types of inhibitors results, in many cases, in a converging line relationship. Such a converging line relationship was observed in the multiple inhibition of NADase by the inhibitor pair, N1-methyl nicotinamide chloride and AMP, as shown in Fig. 9. The lines converge at a point equal to -αK11 and the interaction constant, α, obtained by dividing the value of αK11 by the known K11 value for N1-methyl nicotinamide chloride, was 0.966. Inhibitor interaction constants calculated from multiple inhibition studies of various combinations of adenosine derivatives with N1-methyl nicotinamide chloride are listed in Table III.

**DISCUSSION**

The titrimetric assay developed for assaying bull semen NADase activity provides a convenient method for studying directly the kinetics and various other properties of the enzyme. In a previous study of this enzyme, a spectrophotometric method using 3-acetylpyridine adenine dinucleotide as substrate was reported (8). This method, although more sensitive than the titrimetric method described in the present study, would not permit a study of the enzyme in the presence of ultraviolet-absorbing compounds. For this reason, the titrimetric method was developed and various properties of the enzyme were studied so that a comparison of these two methods could be made. For example, the effect of pH on Km and Vmax values and the linear relationship between enzyme activity and enzyme concentration agree well with similar relationships obtained previously by means of the spectrophotometric method (8). Also, the Km value for NAD at 38° (3.23 × 10^-4 M) is essentially equal to that reported earlier (3.2 × 10^-4 M) by Abdel-Latif and Alvisatos (12).

The substrate binding properties of the enzyme were studied through the inhibition of the enzyme by two different groups of structural analogs. The first group of inhibitors, the compounds structurally related to the adenosine moiety of the NAD molecule, was adenosine, AMP, ADP, and ADP-ribose. Inhibition by these four adenosine derivatives was demonstrated to be competitive with respect to NAD. From the inhibitor dissociation constants listed in Table I, it can be seen that adenosine was least effectively bound to the enzyme compared to the other adenosine derivatives studied. There is no increase in the binding of AMP, compared to adenosine, suggesting a favorable interaction of the additional negatively-charged group. However, no further increase in the binding of ADP was observed. ADP-ribose, which represents a greater portion of the NAD molecule, was more efficiently bound to the enzyme than the other three adenosine derivatives.

The second group of inhibitors studied, N1-alkyl nicotinamide derivatives and 3'-O-alkyladenosine diphosphoribose.
chlorides, are structurally analogous to the nicotinamide moiety of NAD. The type of inhibition observed with N'-alkylnicotinamide chlorides was shown to be competitive with respect to NAD in each case. As shown in Table II, the inhibitor dissociation constants decreased with increasing chain length of the alkyl group of these inhibitors, with the exception of N'-methylnicotinamide chloride. The effect of chain length on the change in free energy of binding of these compounds to the enzyme was calculated from a linear relationship obtained from the plotting of the logarithm of 1/K, versus the number of alkyl chain carbons of these inhibitors. Using the change in the logarithm of 1/K, per methyl group (ΔpK1), free energy changes were calculated from the following equation:

$$\Delta G = 2.3 RT \Delta pK_1$$

A free energy change per methyl group of 0.10 kcal per mole was obtained. The interactions of nonpolar groups on substrates or inhibitors with nonpolar residues on an enzyme can contribute a significant portion of the total binding energy of the binding process. Taking into account dispersion forces only, Webb (13) suggested a free energy range of 0.36 to 0.59 kcal per mole per methylene group of a hydrocarbon chain. The value obtained for the change in free energy per methylene group in the studies of inhibition of the NADase-catalyzed hydrolysis of NAD by the N'-alkylnicotinamide chlorides was below this range. These results indicated a more complicated nature of the interaction of alkyl groups of N'-alkylnicotinamide chlorides with this enzyme.

The free energy changes per methylene group observed in the binding of N'-alkylnicotinamide chlorides to yeast alcohol dehydrogenase and rabbit muscle L-a-glycerophosphate dehydrogenase were 0.37 kcal per mole (2) and 0.471 kcal per mole (6), respectively.

Since the bull semen NADase catalyzes the hydrolysis of the nicotinamide-ribose linkage of NAD, one would expect that certain catalytically functional groups would be located in the area of the pyridinium ring region of the substrate binding site. Furthermore, one might also expect that the presence of these functional groups in this area could interfere with the binding of N'-alkylnicotinamide chlorides to the enzyme. The deviation from the linearity observed in the study of N'-methylnicotinamide chloride (Fig. 8), which has a smaller inhibitor dissociation constant than most of the other N'-alkylnicotinamide chlorides studied, would indicate that the smaller N'-methyl derivative can be accommodated at the binding site, whereas an increase in size of the alkyl group to the N'-ethyl derivative creates steric problems resulting in poorer binding. It is felt that the relatively low change in free energy per methylene group observed in the binding of N'-alkylnicotinamide chlorides reflects the negative influence of steric effects on a normal process of nonpolar interactions.

The results of the studies of inhibition of NADase by the substrate-competitive inhibitors, nicotinamide and adenosine derivatives, revealed that there was an apparent recognition by the enzyme of the various compounds studied, and suggested the binding of the substrate to the enzyme to involve interactions with several parts of the substrate molecule. From these studies, the adenosine moiety, the pyrophosphate group, and the positively-charged nicotinamide ring moiety would be expected to contribute to the over-all binding of the substrate.

Although binding of these inhibitors can in each case exclude the binding of substrate, the individual binding process of different types of inhibitors can involve interactions at different regions of the substrate binding site. In this respect, substrate-competitive inhibitors interacting with the enzyme at different regions of the substrate binding site would be expected to be bound simultaneously to the enzyme. Mutual exclusion was observed in the multiple inhibition analysis with inhibitor pairs composed of various combinations of adenosine derivatives. These results indicated that any two of these compounds could not be bound to the enzyme at the same time. This was demonstrated by an interaction constant of infinity observed with the inhibitor pair ADP and ADP-ribose (Table III). Such a mutual exclusion with an interaction constant of infinity would be expected if the inhibitors interact with or overlap the same regions of the substrate binding site. The combinations of N'-methylnicotinamide chloride and adenosine derivatives were studied in the multiple inhibition analysis, and simultaneous binding of these inhibitors was observed (Table III). An interaction constant of unity signifies the absence of interaction between inhibitors in the binding process and an interaction constant greater than unity indicates an unfavorable interaction between inhibitors in binding to the enzyme. In the multiple inhibition study of the inhibitor pair AMP and N'-methylnicotinamide chloride, an interaction constant of 0.966 was obtained (Fig. 9). Therefore, little or no interaction between these inhibitors in their binding to the enzyme was observed. An unfavorable interaction between the inhibitor pair ADP-ribose and N'-methylnicotinamide chloride was observed, as shown by the interaction constant greater than unity (Table III). This again suggests steric problems in the binding of compounds that interact with the enzyme near the region of the substrate binding site where the catalytic process occurs.

According to the present studies, the properties of the substrate binding site of NADase, with respect to interactions with nicotinamide and adenosine derivatives, appear very similar to those of the NAD binding site of the L-a-glycerophosphate dehydrogenase from rabbit muscle (6). In the present studies of NADase, the hydrophobic region was not as clearly demonstrated as in the case of L-a-glycerophosphate dehydrogenase; however, the positive chain length effect observed in the binding of N'-alkylnicotinamide chlorides, although a relatively minor effect, does suggest nonpolar interactions at the substrate binding site. Preliminary studies of the inhibition of NADase-catalyzed hydrolysis of 3-acetylpyridine adenine dinucleotide by alkylphosphates have suggested the existence of a hydrophobic region in the substrate binding site of the enzyme. Further investigation of nonpolar interactions in the binding of the NAD molecule to bull semen NADase is currently underway.

Acknowledgment—We would like to acknowledge the generosity of the Curtiss Breeding Service, Cary, Illinois, in providing bull semen samples for this study.

REFERENCES

Bull Semen Nicotinamide Adenine Dinucleotide Nucleosidase: III. PROPERTIES OF THE SUBSTRATE BINDING SITE
James H. Yuan and Bruce M. Anderson


Access the most updated version of this article at http://www.jbc.org/content/247/2/515

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/2/515.full.html#ref-list-1