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On the Mechanism of Action of Calf Spleen NAD$^+$ Glycohydrolase

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The hydrolysis of NAD$^+$ analogs bearing different substituents in the pyridinium moiety, catalyzed by solubilized calf spleen NAD$^+$ glycohydrolase, was studied. The enzyme was specific for analogs possessing a carbonyl function at position C-3. The observed maximal rates showed no simple dependence either on the leaving ability of the parent pyridines or on the observed binding energies. The analogs without carbonyl substituents were not found to be hydrolyzed under our experimental conditions; and, compared to the substrates, they all presented much higher affinities for the active site, which could not be related to specific interactions. These results indicate that the rate-limiting step of the NAD$^+$ hydrolysis, which is the formation of an enzyme–ADP–ribosyl intermediate, is probably more complex than a simple chemical step, i.e., pyridinium–ribose bond breakage. At a molecular level we favor a catalytic mechanism which, through nonbonded interactions between the substrate and the active site of the enzyme, results in the destabilization of the pyridinium–ribose bond, i.e., unimolecular decomposition involving an oxocarbonium ion intermediate.

INTRODUCTION

NAD$^+$ glycohydrolases (EC 3.2.2.6) catalyze the hydrolysis of NAD$^+$ at the nicotinamide–ribose bond. The minimum kinetic mechanism of calf spleen NAD$^+$ase[1] was found to be ordered Uni–Bi, the formation of an ADP–ribosyl intermediary complex (E–ADP–Rib) being rate limiting (*1*). The reactivity of E–ADP–Rib toward nucleophiles (*1*, *2*) and the properties of ADP–ribonolactone as an inhibitor of the NAD$^+$ase (*3*) are in favor of an oxocarbonium ion intermediate in NAD$^+$ hydrolysis. In order to obtain better insight into the molecular mechanism of nicotinamide–ribose bond breaking, we have now examined the action of calf spleen NAD$^+$ glycohydrolase on NAD$^+$ analogs bearing different substituents in the pyridinium ring.

Examples of application of linear free-energy relationships to the mechanistic probing of reactions catalyzed by enzymes are numerous (*4*). In principle, alteration of the electronic nature of the C-3 pyridinium ring substituent could be expected to modify the reactivity of the analogs in the NAD$^+$ase catalyzed hydrolysis, thus providing information on changes in charge density at the reaction center.

[1] Abbreviations used: NAD$^+$ase, NAD(P)$^+$ nucleosidase; PdAD$^+$, pyridine adenine dinucleotide; ac^{3}PdAD$^+$, 3-acetylpyridine adenine dinucleotide; cn^{3}PdAD$^+$, 3-cyanopyridine adenine dinucleotide; f^{3}PdAD$^+$, 3-formylpyridine adenine dinucleotide; hy^{3}PdAD$^+$ and hy^{4}PdAD$^+$, 3 (and 4)-carboxyhydrazidepyridine adenine dinucleotide; io^{3}PdAD$^+$, 3-iodopyridine adenine dinucleotide; n^{3}PdAD$^+$, 3-aminopyridine adenine dinucleotide; sNAD$^+$, thionicotinamide adenine dinucleotide; NaAD$^+$, nicotinic acid adenine dinucleotide; meNaAD$^+$, methylnicotinate adenine dinucleotide; X-Pd, substituted pyridine; INH, isonicotinic acid hydrazide; and E–ADP–Rib, enzyme–adenosine diphosphoribosyl complex.

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No correlation was found between the rate of hydrolysis of the NAD^+ analogs studied and, e.g., the pK_a of the leaving pyridine, suggesting that the rate-limiting step leading to the formation of the intermediate E–ADP–Rib is probably not simple pyridinium–ribose bond breakage. The implications of this result for the mechanism of action of calf spleen NAD^+ase are discussed.

EXPERIMENTAL

NAD^+ and f^3PdAD^+ were obtained from Sigma Chemical Company and ac^3PdAD^+ from Serva. The pyridines used in NAD^+ analog syntheses were Fluka products. [*Adenine*-^{14}C]NAD^+ was purchased from the Radiochemical Centre (Amersham). The following NAD^+ analogs were prepared according to published procedures: cn^3PdAD^+ (*5*, *6*), io^3PdAD^+ (*7*), n^3PdAD^+ (*8*), $NaAD^+$ (*9*), $sNAD^+$ (*10*), hy^4PdAD^+ (*11*), hy^3PdAD^+ (*12*), $meNaAD^+$ (*13*), and $PdAD^+$ (*14*). The analogs prepared by transglycosidation were cochromatographed several times with ^{14}C-labeled NAD^+ on Dowex 1-X2. Purified analogs were free of radioactivity and revealed a single spot on paper electrophoresis (0.05 *M* sodium acetate, pH 5.0 and 75 V/cm).

Enzymes

Neurospora crassa, pig brain and calf spleen NAD^+ glycohydrolases were purchased from Sigma Chemical Company. The solubilized calf spleen NAD^+ glycohydrolase used for the kinetic studies was prepared as reported previously (*15*). Its specific activity was about 7 units/mg protein.

Kinetic Measurements

Initial rates of hydrolysis were determined using a titrimetric method according to the conditions described previously (*15*). The measurements were carried out at 37°C, pH 7.4 in the presence of 0.1 *M* NaCl; the reaction mixture had a final volume of 1.9 ml. The kinetic constants were calculated from Lineweaver–Burk plots. The inhibition constants were determined at fixed inhibitor concentrations with varying NAD^+ concentrations (Lineweaver–Burk plots).

The pK_a values of the pyridines were measured at 37°C and $I = 0.1$ *M* using a Radiometer titration instrument. The constant of nicotinic acid was taken from the literature (*16*).

RESULTS

Several NAD^+ analogs were studied as substrates for solubilized calf spleen NAD^+ glycohydrolase. Only those analogs bearing a carbonyl function at C-3 of the pyridinium moiety were found to be substrates. The kinetic constants obtained are shown in Table 1. Since the maximal hydrolysis rates of f^3PdAD^+ and $NaAD^+$ are much lower than the rate observed with NAD^+, these analogs were studied as inhibitors of NAD^+ase. Both analogs were found to be competitive inhibitors, and K_i were determined. In contrast, all other NAD^+ analogs tested were not hydrolyzed at a measurable velocity, i.e., under

TABLE 1

KINETIC PARAMETERS OF THE NAD ANALOGS WHICH ARE SUBSTRATES[a]

Substrate	Substituent	K_m (μM)	Maximal rate (%)	pK_a[c]
NAD^+	$-CONH_2$	56	100	3.33
s NAD^+	$-CSNH_2$	57	53	3.35
hy^3PdAD^+	$-CONHNH_2$	400	93	3.58
$meNaAD^+$	$-COOCH_3$	500	372	3.13
ac^3PdAD^+	$-COCH_3$	150	25	3.13
f^3PdAD^+	$-CHO$	390[b]	3	3.80
$NaAD^+$	$-COO^-$	300[b]	3	4.77

[a] Determined at pH 7.4, 37°C, and $I = 0.1$ M.
[b] K_i values.
[c] pK_a of the parent pyridinium ion.

our experimental conditions, their maximal rates being less than 0.5% relative to NAD^+. However, these analogs revealed themselves to be very potent competitive inhibitors; the inhibition constants are listed in Table 2. It appears that, contrary to the microbial NAD^+ glycohydrolases, e.g., *Neurospora crassa*, the calf spleen enzyme is not particularly specific. Similar conclusions were reached with other mammalian NAD^+ases (*17*, *18*).

TABLE 2

INHIBITION CONSTANTS OF THE NAD ANALOGS WHICH ARE COMPETITIVE INHIBITORS[a]

Analogs	Substituent	K_i (μM)	pK_a
cn^3PdAD^+	$-CN$	3.0	1.39
io^3PdAD^+	$-I$	9.0	3.25
hy^4PdAD^+	$-CONHNH_2$	1.7	3.79
$PdAD^+$	$-H$	6.0	5.23
n^3PdAD^+	$-NH_2$	4.7	5.98

[a] Determined at pH 7.4, 37°C, and $I = 0.1$ M.

DISCUSSION

In the hydrolysis of NAD^+ catalyzed by calf spleen NAD^+ glycohydrolase, the rate-determining step is the formation of the intermediary complex E–ADP–Rib. Therefore substituent changes at C-3 of the pyridinium ring were believed to exert a major influence on the observed hydrolysis rates of the NAD^+ analogs. Such a rate dependence would be in agreement with the known properties of the pyridines as leaving groups. Demethylation of *N*-methyl-pyridinium salts by PPh_3, an SN_2 process, was shown to give linear

Hammett and Brønsted plots (*19*). Under experimental conditions where an A-1 mechanism prevails, i.e., intermediary formation of an oxocarbonium ion, Jones, Sinnott, and Souchard (*20*) demonstrated that the rates of hydrolysis of β-D-galactopyranosyl pyridinium salts are correlated with the pK_a of the leaving pyridines. In both cases, as expected, the leaving ability of the pyridines increases with electron-withdrawing substituents. Preliminary results obtained in this laboratory indicate that a linear relationship exists also between the nonenzymatic hydrolysis rates of NAD^+ analogs (base-catalyzed) and the pK_a of the leaving pyridines. The slope of the log k vs pK_a plot is −0.7 (Muller and Schuber). It was previously demonstrated that rate constants for quaternization of substituted pyridines, e.g., alkylation and acylation reactions, are also well correlated by substituent constants (*21–24*). In this case, for which pyridines act as nucleophiles, the reactions are accelerated by electron-donating substituents.

Our results (Tables 1 and 2) clearly indicate that the leaving ability of the parent pyridine, which is a function of its pK_a,[2] is not correlated with the maximal hydrolysis rate of the analog studied. Interestingly, a linear relationship between log k_{cat} and the pK_a of the leaving pyridine was found in the hydrolysis of β-D-galactopyranosyl pyridinium salts, catalyzed by *Escherichia coli* β-galactosidase (*26*). Considering the substrates (Table 1), it appears that compared to the amide and thioamide, substitution of the pyridinium ring by other carbonyl groups results in apparent decreased affinities[3] of the analogs for the active site, which are not correlated with changes in maximal hydrolysis rates. In terms of substrate specificity, however, as expressed by V/K_m, NAD^+ remains the best substrate for NAD^+ glycohydrolase. The observation that the analogs, which all yield a common enzyme–ADP–ribosyl intermediate, are hydrolyzed at different rates is in agreement with the fact that the formation of E–ADP–Rib is rate limiting (*1*). In relationship to the low hydrolysis rate of f^3PdAD^+ and $NaAD^+$, one should note that these analogs have modified carbonyl groups, i.e., the existence of a hydrated form for f^3PdAD^+ and the presence of a delocalized negative charge for $NaAD^+$.

The analogs of this study which do not have a carbonyl substituent at position C-3 of the pyridinium moiety are not hydrolyzed, or at best very slowly, by calf spleen NAD^+ glycohydrolase. All these compounds compared to the substrates present much greater affinities for the enzyme (up to two orders of magnitude; Table 2). Such binding energies cannot be accounted for by increased specific interactions between the pyridine ring and residues of the active site, since the different C-3 substituents studied are characterized by widely different electronic, steric, and hydrophobic constants.

The results of this study are of interest in elucidating the mechanism of action of calf spleen NAD^+ glycohydrolase. The hydrolytic pyridinium–ribose bond breaking seems to involve a specific interaction between the substituent at position C-3 of the pyridinium ring and the active site of the enzyme. Such a catalytically productive interaction appears, so far, to be restricted to carbonyl groups. Comparing NAD^+ to a poor substrate $PdAD^+$ one observes that the added carboxamide, which could in principle

[2] The leaving ability of pyridines can be characterized indiscriminately by their basicities or by the substituent constants (σ); these parameters are linearly related (*25*).

[3] It was previously shown that according to the minimum kinetic mechanism for calf spleen NAD^+ase the Michaelis constant K_m should reduce to the dissociation constant of the Michaelis complex (*1*).

provide an additional binding, interacts with the enzyme in such a way as to decrease the free energy of the catalyzed reaction but unexpectantly also by decreasing the observed binding energy. Such a behavior is typical for an induced substrate destabilization, in which a part of the intrinsic binding energy between the enzyme and a specific substrate may be utilized to provide the driving force for catalysis (*27*). As a consequence a lower apparent affinity is observed (*27*). Such a destablization mechanism, which might arise in the present case from nonbonded interactions between the carbonyl group at C-3 of the pyridinium moiety and the active site of the enzyme, could provide substrate specificity and contribution to catalysis. Interestingly it was recently shown by X-ray crystallography that, compared to the normal coenzyme, PdAD$^+$ and io^{3}PdAD$^+$ are bound quite differently by horse liver alcohol dehydrogenase. The adenosine moiety of the analogs remains in the adenine nucleotide pocket; however, the pyridine moieties are bound at a remote place from the active site. Such a result indicates that the carboxamide group on the nicotinamide moiety plays an important role for proper positioning of the coenzyme molecule in the active site of this enzyme (*28*).

The lack of relationship between log V and the pK_a of the parent pyridines in the hydrolysis of NAD$^+$ analogs by calf spleen NAD$^+$ase, which cannot be explained a priori by steric or nonpolar partitioning effects, seems to indicate that the rate-limiting step leading to the E–ADP–Rib intermediate cannot be exclusively the chemical step, i.e., pyridinium–ribose bond breakage. A similar conclusion was reached by Bull, Romero, Stein, Ferraz, and Cordes (*29*) from studies of the α-deuterium isotope effect on the rates of hydrolysis of NAD$^+$ and NMN$^+$ catalyzed by pig brain NAD$^+$ glycohydrolase. Thus, the rate-determining step is rather more complex and might involve an additional step between the Michaelis complex and the catalytic step.

$$\mathrm{E + S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{(E \cdot S)} \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} \mathrm{(E \cdot S)^*} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{E\text{–}ADP\text{–}Rib + X\text{-}Pd}$$

$$\mathrm{E\text{–}ADP\text{–}Rib} \xrightarrow[\mathrm{H_2O}]{k_5} \mathrm{E + ADP\text{–}ribose + H^+}$$

SCHEME 1

One can speculate that such a step could be an unimolecular transformation of the Michaelis complex into a catalytically active complex, (E · S)* (Scheme 1), involving a specific conformational response of the enzyme on substrate binding and/or substrate distortion. Indeed enzyme-catalyzed reactions where a specific conformational change can be rate limiting are now recognized to be rather common (*30*, *31*). A kinetic analysis of such a scheme [see, e.g., (*32–34*)] predicts that poor substrates ($k_2'/k_1' > 1$) have much-decreased maximal rates and can be potent inhibitors of the enzyme. In nonequilibrium situations ($k_3 \gg k_2'$) V reduces to k_1' if this step is rate limiting; it follows that the specificity of the enzyme depends on the ability of a substrate to induce the transformation (E · S) $\longrightarrow$ (E · S)* or on the lifetime of (E · S)*, i.e., k_2'. As another consequence, the observed K_m has a more complex meaning than the Michaelis-complex dissociation constant. There are now numerous cases where the specificity of an enzyme is reflected by the step(s) which follow(s) the Michaelis complex (*27*, *35*).

The fact that the rate-limiting step in the formation of the E–ADP–Rib intermediate is probably not a simple chemical step could in principle help to explain the following observations in the hydrolysis of NAD^+ by calf spleen NADase: (a) the lack of sensitivity of the kinetic parameters to pH and ionic strength (*15*) and (b) the relatively high activation energy of the reaction; Ea = 16.2 kcal/mole (67.8 kJ/mole). (This activation energy could also include the enthalpy of a transconformation.) Circumstantial evidence, based on inactivation of NAD^+ glycohydrolases, exist in the literature which supports the idea that the binding of NAD^+ is followed by a conformational change of the enzyme (*36–39*).

At a molecular level, the mechanism of NAD^+ hydrolysis represents a special case among enzyme-catalyzed reactions. Because the pyridine bears a full positive charge, its departure is not subject to specific or general acid catalysis. Owing to the leaving ability of the pyridines bearing electron-withdrawing substituents (*19*, *20*), nucleophilic catalysis is not indispensable; and it is likely that the pyridinium–ribose undergoes a dissociative reaction, i.e., unimolecular decomposition resulting in the formation of an oxocarbonium intermediate (see Introduction). Such a reaction pathway would be in accordance with the mechanism of the uncatalyzed NAD^+ decomposition. The spontaneous nicotinamide–ribose bond hydrolysis is characterized by a biphasic pH-rate profile (*40*). At basic pH (above 7.0 to 7.5 depending on the buffer) the observed rate is proportional to the hydroxyl ion concentration, i.e., specific base catalysis (*41*). At lower pH however, the rate is essentially independent of proton concentration, which is consistent with a unimolecular decomposition. This latter mechanism has been demonstrated for the hydrolysis of β-D-galactopyranosyl pyridinium salts (*24*), and according to Bull *et al.*, seems also to occur for the nonenzymatic and enzymatic (pig brain NAD^+ase) hydrolysis of NAD^+ (*29*).

Different mechanisms in keeping with the above discussion can be invoked so as to provide the driving force for such a decomposition reaction; they all involve a pyridinium–ribose bond destabilization. A medium effect can be invoked: a specific conformational change which sets up the catalytic step could alter, in the $(E \cdot S)^*$ complex, the environment of the pyridinium–ribose to bring about a charge destabilization via ground-state destabilization (e.g., hydrophobic microenvironment, water expulsion, charge or dipolar repulsion) or transition-state stabilization (e.g., stabilization of the developing oxocarbonium).

Finally we wish to present a hypothesis on the contribution to a destabilization of the pyridinium–ribose bond by a conformational effect. With molecular models, one can show that the ribose ring linked to the pyridine can be forced into conformations in which the scissile bond becomes approximatively transantiperiplanar to an electron lone-pair orbital of the ring oxygen, e.g., O(1′)-exo or C(1′)-endo. These are high-energy conformations, the former in which the four coplanar carbon atoms have their substituents eclipsed being particularly unfavorable. If the ribose is locked in such a conformation in $(E \cdot S)^*$, the enzyme providing the energy for this ring distortion,[4] one would expect a ground-state destabilization due to dipole–dipole interactions. Furthermore ab initio calculations have indicated that such conformations would introduce a C_1–N^+

[4] According to energy calculations of ribofuranose ring puckering, this distortion requires less than 2.5 kcal/mole^{-1} (*44*).

bond lengthening by 0.05 Å [Lehn and Wipff, personal communication; see also (*42*)], resulting in a bond weakening and an electronic delocalization closer to that of the transition state. Such a transantiperiplanar arrangment is also highly favorable to the expulsion of the pyridine by the assistance of the electron lone-pair yielding the oxocarbonium ion in which these described effects are released. Such stereoelectronic control in hydrolytic reactions has been well studied by Deslongchamps and co-workers (*43*).

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