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Chemical Evidence in Favor of a Stabilized Oxocarbonium-Ion Intermediate in the NAD$^+$ Glycohydrolase-Catalyzed Reactions

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The mechanism of the spontaneous solvolysis (hydrolysis and methanolysis) of the nicotinamide–ribose bond of NAD$^+$, studied under unimolecular rate conditions, was compared to the mechanism of the reactions catalyzed by NAD$^+$ glycohydrolase. Experimental arguments—i.e., sensitivity of the observed solvolytic rates to the medium effects, stereochemical course of the methanolysis of α- and β-NAD$^+$, partitioning ratio of the ADP-ribosyl intermediate, and entropy of activation—are presented, suggesting that the nonenzymatic solvolysis of β-NAD$^+$ proceeds through an enforced preassociation mechanism in which the acceptor molecules, methanol or (and) water, stabilize the developing ADP-ribosyl oxocarbonium ion generated by the bond cleavage. In aqueous solution this intermediate seems too unstable to exist as a fully developed solvent-equilibrated species. In contrast to the spontaneous solvolysis, the ADP-ribosyl intermediate generated in the active site of NAD$^+$ glycohydrolase shows a high selectivity when reacting with competing acceptor nucleophiles. This suggests that the enzyme provides a substantial stabilization of the intermediary ADP-ribosyl oxocarbonium ion. We conclude that catalysis of the nicotinamide–ribose bond cleavage by NAD$^+$ glycohydrolase would be due, in part, to the ability of the enzyme to effectively stabilize an oxocarbonium ion-like structure in the transition state.

INTRODUCTION

NAD$^+$ glycohydrolase[2] (EC 3.2.2.6) catalyzes the hydrolytic cleavage of the nicotinamide–ribose bond in NAD(P)$^+$ by an ordered uni–bi kinetic mechanism (*1*). The intermediary E · ADP-ribosyl complex, which occurs after the departure of nicotinamide, can react with acceptors such as water, methanol (*2*), or pyridines (*3*) with retention of configuration. A basic question concerning the chemical mechanism of the reaction was the nature of the bond-rupture step and consequently the structure of the intermediate (*4*). Several experimental arguments strongly suggest that NAD$^+$ glycohydrolase catalyzes unimolecular decomposition of its substrates with the generation of an ADP-ribosyl oxocarbonium-ion-like species (*5*, *6*). Recently, by use of linear free-energy relationships we

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[2] Abbreviations used: NAD$^+$-ase or NAD$^+$ glycohydrolase, NAD(P)$^+$ nucleosidase; pH*, the apparent pH of aqueous methanol.

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have shown that the scissile bond is broken, in the enzyme-catalyzed reaction, according to a mechanism involving a late transition state (*7*). This result confirmed earlier data on kinetic α-secondary deuterium isotope effects which, because of their magnitude, also pointed to a significant C–N bond cleavage in the transition state and to a sp^2-character at the C-1′ atom at which the bond is broken (*5*). However, according to considerations developed by Jencks and co-workers, glycosyl-derived oxocarbonium ions are too unstable to exist as free-solvent-equilibrated intermediates in chemical reactions (*8, 9*); it is known that such reactive species react with acceptors with near diffusion-controlled rates and show very little discrimination vs nucleophiles (*10*). Since enzymes derive their catalytic power through transition state stabilization (*11*), it was of interest to find out if NAD^+ glycohydrolase stabilizes the ADP-ribosyl oxocarbonium intermediate. In this work, in order to address this point, we have compared the rates of NAD^+ hydrolysis and methanolysis catalyzed by the enzyme and determined the mechanism of the spontaneous solvolysis, under experimental conditions favoring the unimolecular decomposition of the substrate. We have shown that, as predicted from its instability, the ADP-ribosyl species generated in the nonenzymatic reaction reacts with water and methanol at similar rates. In sharp contrast, the intermediate formed during the NAD^+ glycohydrolase-catalyzed reaction reacts about 50-fold faster with methanol than with water; such a discrimination is expected from relatively stable oxocarbonium ions. From our data we conclude that catalysis of NAD^+ hydrolysis by NAD^+ glycohydrolase derives in part from the ability of the enzyme to stabilize an oxocarbonium-ion-like transition state.

EXPERIMENTAL

β-NAD^+ and α-NAD^+ were obtained from Sigma Chemical Co. NAD^+ glycohydrolase was solubilized with detergent from calf spleen microsomes as described previously (*12*); the purification of this form of the enzyme was performed similarly to (*13*) and will be reported in detail elsewhere (H. M. Muller *et al.*); its specific activity was about 20 units/mg protein.

Solvolytic experimental conditions. NAD^+ glycohydrolase-catalyzed hydrolysis and methanolysis of β-NAD^+ (final concentration 500 μM) were performed at 25°C in 50 mM sodium phosphate buffer, pH* 7.4 (apparent pH of aqueous methanol as measured with a glass electrode (*14*)), containing 0.05% (w/v) Emulphogene (final volume 2 ml) and various concentrations of methanol. Reaction progress was estimated by HPLC on aliquots (see below); β-NAD^+ was transformed into nicotinamide, ADP-ribose, and β-methyl ADP-ribose (*2*).

Nonenzymatic solvolysis reaction mixtures, i.e., NAD^+ (3 mM), maintained at constant sodium phosphate buffer (50 mM) and ionic strength (I = 500 mM, NaCl), pH* 6.0, containing adenine (internal standard) and various concentrations of methanol (final volume 1 ml), in screw cap septum vials were heated to 80 ± 0.5°C in hot blocks (Pierce Reacti-Therm). Aliquots were removed at time intervals, quenched in ice, and analyzed by HPLC. Alternatively, for rates of NAD^+

disappearance measurements, aliquots were injected into 1 M KCN and the absorbance read at 327 nm (*13*).

Product analysis by liquid chromatography and kinetic measurements. Reaction progress and product analysis were determined on aliquots (about 8–12 per kinetic run) of the reaction mixtures, by HPLC (*15*), by use of a Waters Co. modular system. Chromatography was carried out on a 3.9 × 300-mm reverse-phase μBondapak C_{18} column (Waters) operated at ambient temperature, pressures of 1000–1500 psi, and a flow rate of 1 ml/min. Separation of the compounds was performed by isocratic elution with 10 mM $(NH_4)H_2PO_4$ (pH 5.5) containing acetonitrile (1 to 5%, v/v); the compounds were detected by their uv absorbance at 259 nm. Peaks were identified by their retention times and their areas were integrated (Spectra Physics, Model SP 4270) for rate constants determination. To correct for small differences in the sample injection volumes, the areas of the peaks were normalized by using adenine as internal standard. These chromatography conditions allowed a good separation of β-NAD^+ from its solvolytic products: nicotinamide, β- and α-methyl ADP-ribose, and ADP-ribose. Under all the experimental conditions used in this study, hydrolysis and anomerization of methyl ADP-ribosides were found negligible.

Pseudo-first-order rate constants for NAD^+ disappearance, in the nonenzymatic solvolysis, were calculated from the slopes of conventional plots of ln (OD or normalized areas) vs time, using a least-squares computer program. The plots were linear to at least three to four half-lives ($r > 0.998$) and k_{obs} was reproducible to ca. 5%. In order to determine the pseudo-first-order rate constants for the appearance of the solvolytic products, we used a nonlinear regression program (KINFIT) which fitted the normalized areas to the curve of $A = A_\infty + (A_0 - A_\infty)e^{-kt}$ using A_∞ (end point), A_0, and k as variables (*16*). The best-fit of A_0 and A_∞ values, calculated by the program, agreed well with the experimentally observed values. Since the extinction coefficients of the solvolytic products, i.e., ADP-ribose and methyl ADP-ribosides, are identical, the product concentration ratio, e.g., hydrolysis vs methanolysis, could be obtained directly from the ratios of the peak areas. These ratios were found to be constant (±10%) over several half-lives.

For the NAD^+ glycohydrolase-catalyzed solvolysis, under our experimental conditions, reaction progress was linear with time up to ca. 40–50% of NAD^+ transformation. The rates of product appearance, i.e., ADP-ribose and β-methyl ADP-ribose, were estimated from initial rates determined with six time points.

Product identification. The methanolysis products of NAD^+ were identified as α- and β-isomers of methyl ADP-ribose. β-Methyl ADP-ribose was prepared from β-NAD^+ by action of calf spleen NAD^+ glycohydrolase as described previously (*2*); its structure was confirmed by NMR (*2, 17*). The peak of the chromatogram corresponding to α-methyl ADP-ribose was collected and its structure established by ^1H NMR (400 MHz; ^2H_2O and 3-trimethylsilyl-2,2,3,3-d_4-sodium propionate as internal standard). The methyl group resonance peaks of both α- and β-methyl ADP-ribosides are easily distinguishable by their chemical shifts (*2*): δ 3.389 ppm (s,α-O-CH_3) and 3.337 ppm (s,β-O-CH_3).

RESULTS

Nonenzymatic Hydrolysis of NAD$^+$

It was shown previously that the rate of hydrolysis of the cationic *N*-ribose bond of β-NAD$^+$ is pH-independent in the range of ca. pH 2 to 7 (*18*). At higher pH values this unimolecular decomposition is superseded by a specific base-catalyzed hydrolysis which also yields nicotinamide and ADP-ribose (*18*). We have now studied the solvolysis of β-NAD$^+$ in the presence of methanol; this solvent was selected because we have found that it is a good nucleophilic acceptor in the NAD$^+$ glycohydrolase-catalyzed reaction (*2*). Our aim was to compare the rates of hydrolysis and methanolysis in the spontaneous solvolysis of NAD$^+$ and to determine some other parameters, such as the stereochemical course of the methanolysis and the influence of the solvent on the observed rates of solvolysis.

The solvolysis of β-NAD$^+$ was studied at pH* 6.0 in a sodium phosphate buffer maintained at constant ionic strength (I = 0.5 M; NaCl). This buffer does not participate per se in the solvolysis reaction (*18*) and conveniently its pH changes minimally with temperature. Under these experimental conditions solvolysis of β-NAD$^+$ followed pseudo-first-order kinetics and we have confirmed that at pH 6.0 hydrolysis is in the pH-independent region (not shown). Moreover a similar rate vs pH* profile was observed when solvolysis was studied in the presence of methanol. With 20% (v/v) methanol the observed solvolysis rates fitted the equation

$$k_{obs} = k_{solv} + k_{OH^-}[OH^-],$$

with $k_{solv} = 9.12\ 10^{-3}$ min^{-1} and $k_{OH^-} = 8.55\ 10^4$ liters · mol^{-1} · min^{-1} at 80°C and $pK_w = 14$ ($r = 0.998$). We have also determined the effect of various concentrations of methanol (0–30%, v/v) on the solvolysis rates of β-NAD$^+$. Increased observed rates were obtained with increasing alcohol concentrations (Fig. 1). The fact that the solvent components can act as nucleophiles introduces some

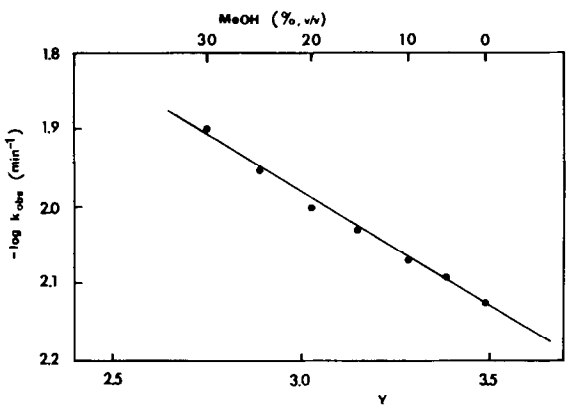


FIG. 1. Pseudo-first-order rate constants for the solvolysis of β-NAD$^+$, in methanol (0–30%, v/v)/water, as a function of Y, at pH* 6.0 and 80°C. Y-values are based on the solvolysis of *tert*-butyl chloride (*20*).

TABLE 1

Solvolysis of β-NAD^+ in Aqueous Methanol

Methanol				
Percentage (v/v)	M	α/β (±SD)	Methanolysis/ hydrolysis (±SD)	K
10	2.47	1.5 (0.4)	0.106 (0.014)	2.16
20	4.94	2.4 (0.4)	0.161 (0.018)	1.44
30	7.41	2.9 (0.3)	0.238 (0.011)	1.27

Note. The solvolysis of β-NAD^+ was performed at 80.0°C and pH* 6.0 in the presence of various concentrations of methanol as indicated under Experimental. About 8–10 aliquots per run, up to three half-lives, were analyzed by HPLC. The methanolysis/hydrolysis ratio corresponds to the peak area ratio [methyl ADP-ribosides]/[ADP-ribose]. The partitioning ratio, *K*, is defined in the text. The stereochemical ratio α/β (i.e., inversion vs retention) corresponds to the peak area ratio [α-methyl ADP-ribose]/[β-methyl ADP-ribose].

difficulties in interpreting such results. A priori, the changes in k_{obs} due to variations in the binary solvent composition can originate from changes in the ionizing power of the solvent and (or) by an acceleration due to a second-order reaction between β-NAD^+ and methanol as nucleophile. A unimolecular heterolysis of the *N*-glycosidic bond of NAD^+ would not create a charge and therefore would not be expected to be much affected by solvent polarity changes; this was documented for the solvolysis of, e.g., *N-tert*-alkylpyridinium salts (*19*). When the solvolysis rates of NAD^+ were correlated with the Grunwald–Winstein *Y*-scale, i.e., according to the $\log(k/k_0) = mY$ equation (Fig. 1), a negative slope $m = -0.31$ ($r = 0.994$) was found, with *Y*-values based on the solvolysis of *tert*-butyl chloride (*20*). This result can be rationalized if one assumes that the passage to the transition state of the solvolytic reaction involves a dispersion of the localized charge of the initial bond. Similar values were found for ethanol/water mixtures in the solvolysis of *N*-(methoxymethyl)-*N*,*N*-dimethylanilinium ion (*21*). On the other hand, if we expect that water and methanol can act as competing nucleophiles in a bimolecular reaction with β-NAD^+, i.e., SN-2 rate component with nucleophilic assistance by the solvent molecules, the observed rates of solvolysis could be analyzed in a first approximation by the rate equation

$$k_{obs} = k_{hydr}\,[H_2O] + k_{met}\,[CH_3OH].$$

The experimental data, when analyzed by a nonlinear regression program (*22*), fitted this equation relatively well with $k_{hydr} = 1.26\ 10^{-4}$ liters · mol^{-1} · min^{-1} and $k_{met} = 9.83\ 10^{-4}$ liters · mol^{-1} · min^{-1} ($\sigma = 0.374$), at 80°C and pH* 6.0. Importantly, the ratio of the apparent second-order rate constants k_{met}/k_{hydr} was about 7.8. As we shall see below (Table 1), this ratio is much higher than the partitioning ratio obtained by HPLC analysis of the solvolytic products. This indicates that the increased solvolysis rates observed in the presence of methanol

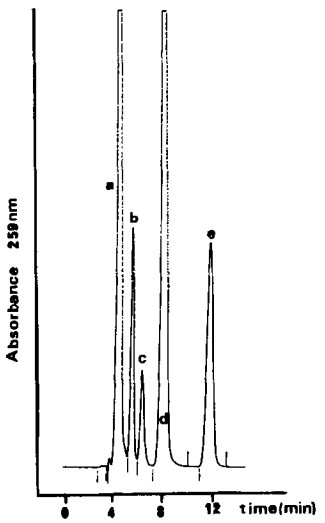


FIG. 2. Elution profile obtained by HPLC, on a reverse-phase μBondapak C_{18} column, of the solvolytic products of β-NAD^+ in methanol/water (25 : 75, v/v). ADP-ribose (a), α-methyl ADP-ribose (b), β-methyl ADP-ribose (c), β-NAD^+ (d), and nicotinamide (e).

cannot be explained only on the basis of a first-order reaction in this alcohol and must also take into account medium effects.[3]

The important data of this study came from the analysis of the solvolysis of β-NAD^+ by HPLC; this technique allowed us to follow the reaction progress and to analyze the products formed. In addition to nicotinamide and ADP-ribose, the solvolysis conducted in the presence of methanol gave two additional product peaks (Fig. 2) which were assigned as α- and β-anomers of methyl ADP-ribose. This remarkable separation on a reverse-phase column permitted determination of, in addition to the solvolysis rate constants, the selectivity toward the solvent molecules, and the stereochemical course of the methanolysis.

Analysis of the progress curves, as indicated under Experimental, allowed the determination of the rate constants of β-NAD^+ disappearance and of the solvolytic products appearance. The reaction followed pseudo-first-order kinetics and, as expected, the $t_{1/2}$ values obtained were identical within the experimental precision (±10%). During the time course of the solvolysis, up to at least three half-lives, the ratio of methanolysis to hydrolysis, i.e., peak areas ratio [methyl ADP-ribosides]/[ADP-ribose], was constant (±10%) (Table 1). The same was found for the stereochemistry of the methanolysis, i.e., peak areas ratio [α-methyl ADP-ribose]/[β-methyl ADP-ribose], although the scatter was higher at low methanol concentration due to experimental imprecision (Table 1). For each

[3] A better fit of the solvolysis data was obtained with the equation

$$k_{obs} = k_{hydr}[H_2O] + k_{met}[CH_3OH] + k'_{met}[CH_3OH]^2,$$

with $k_{hydr} = 1.34$, 10^{-4} liters · mol^{-1} · min^{-1}; $k_{met} = 5.38$, 10^{-4} liters · mol^{-1} · min^{-1} *and* $k'_{met} = 0.62$ (liters · $mol^{-1})^2$ · min^{-1} ($\sigma = 0.173$).

methanol concentration we have also determined the product partitioning ratio, defined as

$$K = \frac{[\text{methyl ADP-ribosides}]}{[\text{ADP-ribose}]} \times \frac{[H_2O]}{[CH_3OH]}.$$

This ratio assumes that each of the solvent components of the binary water/methanol mixture reacts according to a second-order rate in the product-determining step. This may be justified since both solvents compete for a position in the immediate solvation shell around the reactive intermediate. The data are summarized in Table 1. It follows that if the solvolysis of β-NAD^+ occurs through a unimolecular process, the intermediate which is formed by the *N*-ribosidic bond cleavage shows only a small selectivity when reacting with methanol and water as competing nucleophiles; this is indicative of a highly unstable carbocationic intermediate. Methanol reacts slightly faster; this might be due to the higher polarizability and nucleophilicity of this molecule relative to water (*10*). Interestingly the partitioning factor is dependent on the solvent composition. Such cases, where substrates exhibit higher selectivity in solvents of higher polarity, have been described before (*23*).

The stereochemistry of the methanolysis proved very informative. At pH* 6.0, β-NAD^+ reacts with methanol preferentially by inversion of configuration; however, the extent of inversion is too small to account for a strict bimolecular reaction, i.e., a SN-2 mechanism. The ratio inversion/retention of methanolysis is also sensitive to the polarity of the medium, i.e., more inversion is found at higher methanol concentrations (Table 1). The effect of solvent-ionizing power on both the stereochemical course and the selectivity of the solvolysis reaction can be tentatively explained if one assumes that in a more polar solvent (i) that the intermediate formed is more stabilized, and therefore shows a greater selectivity; and (ii) that the transition state is more "open," leaving an increased probability to react also by retention. If inversion of configuration was the major route for methanolysis at pH* < 7.0, under unimolecular rate conditions, paradoxically at pH* > 7.0, when the solvolysis rates become dependent on pH, retention becomes the preferred route (Table 2). Interestingly, the stereochemistry of the methanolysis of α-NAD^+ proved somewhat different; e.g., under experimental conditions where β-NAD^+ showed a 3 : 1 preference in favor of inversion, α-NAD^+ yielded a 1 : 1 mixture of α- and β-methyl ADP-ribose.

Finally, in order to complete our data on the reaction mechanism of β-NAD^+ hydrolysis, we have also determined the activation parameters. The Arrhenius plot of the k_{obs} obtained in the range of 70–100°C is shown in Fig. 3; it gave ($r = 0.998$) $\Delta H^{\neq} = 26.88$ kcal $\cdot$ mol^{-1} and $\Delta S^{\neq} = -0.54$ cal K^{-1} $\cdot$ mol^{-1} (if the rate constants were considered second order with respect to water, the entropy of activation was -8.60 cal K^{-1} $\cdot$ mol^{-1}).

NAD^+ Glycohydrolase-Catalyzed Solvolysis of NAD^+

Solvolysis of β-NAD^+ catalyzed by NAD^+ glycohydrolase was studied in the presence of varying concentrations (0.5–2.5 M) of methanol. This solvent mole-

TABLE 2

Influence of pH on the Stereochemical Course of the Methanolysis of β-NAD^+

	pH*					
	5.5	6.0	6.5	7.0	7.5	8.0
α/β	3.83 (0.19)	2.80 (0.15)	2.52 (0.12)	1.28 (0.03)	0.78 (0.05)	0.54 (0.06)

Note. The solvolysis of β-NAD^+ was studied at 80.0°C in aqueous methanol (30% methanol, v/v). The stereochemistry of the methanolysis was estimated from the ratio of the α- and β-methyl ADP-ribose peak areas obtained by HPLC (see Experimental). The data represent the average (±SD in parentheses) of eight time points obtained over three to four half-lives.

cule was shown previously to be an excellent alternate acceptor for the ADP-ribosyl intermediate formed during the enzyme-catalyzed reaction (*1, 2*). The minimum kinetic Scheme I describes the partitioning of the common intermediate between water and methanol (*1*). Methanolysis occurs exclusively with retention of configuration (*2*); moreover, in the concentration range used, methanol was not found to have an adverse effect on the enzyme activity (*1*).

β-NAD^+ disappearance and products (i.e., nicotinamide, ADP-ribose, and β-methyl ADP-ribose) appearance were now conveniently monitored by HPLC; this technique allows a much better quantification of the reaction products than our earlier methods (*1, 2*). The effect of methanol on the rate of β-NAD^+ breakdown was found to be relatively minor; e.g., at 2.5 M, when methanolysis accounts for more than 70% of product formation, the rate was reduced by only about 20%. These data are in agreement with our previous conclusions that the E · ADP-ribosyl intermediary complex is formed subsequent to the rate-limiting step(s) (*1, 7*). If according to Scheme I the transfer reactions are first order in the acceptor concentrations; i.e., the transfer proceeds through a simple nonbinding

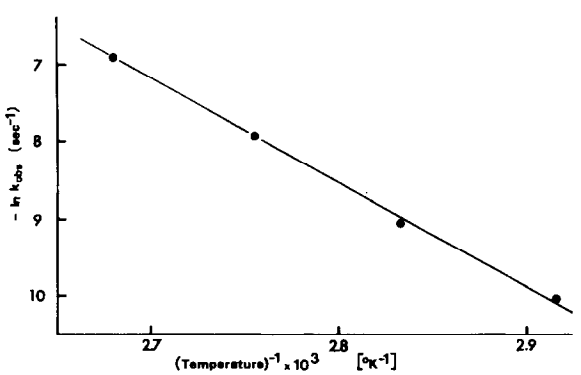
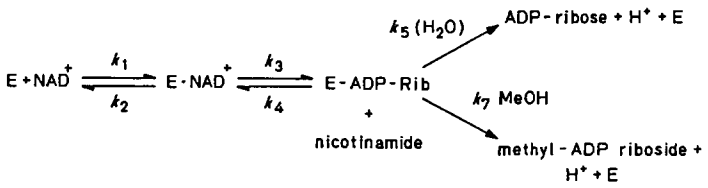


FIG. 3. Arrhenius plot of k_{obs} for the hydrolysis of β-NAD^+ at pH 5.6.



SCHEME 1

bimolecular attack by water and methanol on the E · ADP-ribosyl intermediate, then the product ratio is given by the equation

$$[\beta\text{-methyl ADP-ribose}]/[\text{ADP-ribose}] = k_7\,[CH_3OH]/k_5\,[H_2O].$$

This ratio, which remained constant throughout the time course of the reaction, was indeed found to increase linearly with respect to increasing methanol concentrations (Table 3). Accordingly, the partitioning ratio of methanolysis vs hydrolysis ($K = k_7/k_5$) was constant with an average value of 50.2 ± 3.7 ($n = 5$) (Table 3). Two important conclusions can be drawn from these data: (i) there is no evidence for saturation kinetics in the methanol concentration range studied (0.5–2.5 M), i.e., there seems to be no distinct binding and reaction steps for methanol and water in their attack of the E · ADP-ribosyl intermediary complex; and (ii) the ADP-ribosyl intermediate generated in the active site of NAD^+ glycohydrolase shows a high selectivity vs nucleophiles. This selectivity reflects

TABLE 3

Solvolysis of β-NAD^+ Catalyzed by NAD^+ Glycohydrolase

Methanol (M)	Methanolysis/ hydrolysis	K
0.5	0.435	46.9
1.0	1.024	54.1
1.5	1.515	52.2
2.0	1.818	46.0
2.5	2.564	50.8

Note. The solvolysis of β-NAD^+ catalyzed by NAD^+ glycohydrolase was performed at 25.0°C and pH* 7.4 in the presence of various concentrations of methanol. About 10–12 aliquots per run were analyzed by HPLC. Normalized peak areas of β-methyl ADP-ribose and ADP-ribose were plotted as a function of time. The methanolysis/hydrolysis ratio corresponds to the ratio of the initial rates obtained from such plots. The partitioning ratio K is defined in the text.

the intrinsic reactivity of the enzyme-stabilized ADP-ribosyl oxocarbonium ion intermediate.

DISCUSSION

The energy of activation of β-NAD^+ hydrolysis, under unimolecular rate conditions, is 27 kcal · mol^{-1} (at 37°C); under optimal conditions calf spleen NAD^+ glycohydrolase lowers this energy barrier by about -12 kcal · mol^{-1} (*24*). We ignore the exact origin of this catalytic effect. In the present work we took advantage of the fact that methanol can compete with water as a nucleophile in the spontaneous solvolysis of β-NAD^+ and can act as an alternate acceptor in the NAD^+ glycohydrolase-catalyzed reactions. Both enzymatic and spontaneous NAD^+ methanolysis reactions involve the initial cleavage of the nicotinamide–ribose bond; the comparison of a similar reaction in solution or occurring in the active site of the enzyme yielded insights on the nature of the catalytic efficiency of NAD^+ glycohydrolase.

Although the purpose of our study was not to elucidate the precise mechanism of the nonenzymatic solvolysis of NAD^+, the results obtained here and elsewhere (*5, 7*) give some interesting information. In the region where the spontaneous hydrolysis of β-NAD^+ is pH-independent, a kinetic α-secondary deuterium isotope effect of 1.10 was measured (*5*). Similarly, the hydrolysis of pyridinium analogs of β-NAD^+ showed a high sensitivity to the basicity of the leaving pyridine ($\beta_{lg} = -1.11$) (*7*). It was concluded that the pH-independent pathway consists of a unimolecular dissociative process, with the formation of an oxocarbonium-ion-like intermediate, which is essentially fully developed in the transition state. Jencks and Young (*8, 9, 25*) have presented arguments suggesting that an oxocarbonium ion, such as the one generated from NAD^+ by the departure of nicotinamide, would be too unstable to exist as a free solvent-equilibrated intermediate. Therefore the solvolysis of β-NAD^+ is more likely to proceed through, e.g., an enforced preassociation mechanism. The results of our study are in favor of such a hypothesis.

Methanolysis of β-NAD^+, in the pH-independent region, occurs predominantly with inversion of configuration at the central atom; this preference, however, is too small to account for a bimolecular reaction mechanism with a nucleophilic participation of methanol in the rate-limiting step. Conversely, comparison with the data of ribose-5-phosphate anomerization (*26*) indicates that inversion at C-1′ is opposite the configuration expected from a reaction under strict thermodynamic control. Intriguingly the ratio $\beta/\alpha = 1.95$, corresponding to the equilibrium composition of ribose-5-phosphate (at pH 4.5), is very close to the anomeric ratio observed in the methanolysis of β-NAD^+ under more basic conditions, i.e., when NAD^+ is hydrolyzed according to a specific base-catalyzed reaction. Also important is the observation that, although the rates of solvolysis of β- and α-NAD^+ are not very different (*7*), the stereochemical courses of their methanolyses are not identical. This suggests that the solvolysis of both NAD^+ isomers does not proceed through a common, solvent-equilibrated, oxocarbonium ion

intermediate and that methanol reacts before complete separation of the leaving nicotinamide.

The entropy of activation for the hydrolysis of β-NAD^+, in the pH-independent region, is small and negative, i.e., -0.54 cal $K^{-1} \cdot mol^{-1}$ ($\Delta S^{\neq} = -8.6$ cal $K^{-1} \cdot mol^{-1}$ when converetd into a second-order rate constant, which takes into account water concentration). Such values are consistent with a transition state which includes weak interactions of the central atom with the leaving nicotinamide and the entering solvent molecules. Similar $\Delta S^{\neq}$ values were found by Knier and Jencks (*21*) for the solvolysis, in water, of *N*-(methoxymethyl)-*N*,*N*-dimethyl-*m*-nitroanilinium ion; this molecule is an example where the predicted lifetime of the intermediary oxocarbonium ion is too short to exist and consequently where the reaction with nucleophiles proceeds through a concerted mechanism. In contrast to β-NAD^+, other glycosyl pyridinium-containing molecules such as β-D-galactosyl pyridinium salts, when hydrolyzed under similar conditions, exhibit large positive $\Delta S^{\neq}$ (*27, 28*). Recently it was shown by Bennet and Sinnott that, in water, the solvolysis of these substrates is truly unimolecular, without preassociation reactions (*29*). Interestingly, such a difference in $\Delta S^{\neq}$ for the hydrolysis of glycosides in the furanoside or pyranoside form has been noted long ago (*30*). Solvolysis of *N-tert*-alkylpyridinium salts in water, which follow SN-1-type reaction mechanisms, also show large positive entropies of activation (*19*).

The lifetime of the ADP-ribosyl oxocarbonium ion in solvolysis can also be deduced from its selectivity when reacted with competing nucleophiles such as water and methanol. It is well documented that the selectivity of solvolytic reaction intermediates depends markedly on their stability (*8–10, 31*). When the intermediate is too unstable to diffuse in the solvent, it will react with acceptors at near diffusion-controlled rates and consequently shows very little discrimination vs nucleophiles. Conversely, in solvolytic reactions which involve relatively stable carbocationic intermediates, water and methanol exhibit distinct nucleophilicities; e.g., in cases where the approach of the central atom is sterically unhindered a partitioning ratio $k_{methanol}/k_{water}$ up to 70 was measured (*10, 32*). Since the solvolysis of β-NAD^+ in the presence of methanol shows very little selectivity it follows, in agreement with the other observations, that the oxocarbonium ion generated by the departure of nicotinamide is too reactive to exist as a fully developed solvent-equilibrated intermediate. In conclusion, the different experimental results obtained in the study of the spontaneous solvolysis of β-NAD^+ can be rationalized by an enforced-preassociation mechanism where water, the final nucleophilic acceptor, reacts in a solvent cage in the rate-limiting step. The transition state of the reaction can be considered as an open structure, with considerable oxocarbonium ion character, which is weakly stabilized both by the leaving nicotinamide moiety and incipient solvent molecules.

The picture emerging from the data on the NAD^+ glycohydrolase-catalyzed reactions is somewhat different. We know that this enzyme catalyzes a stepwise process which involves, after the departure of nicotinamide, the formation of an E · ADP-ribosyl intermediary complex that reacts subsequently, in a fast step, with acceptors such as water, methanol (solvolysis), or pyridines (transglycosidation) (*1*). The mechanism of the reaction leading to this intermediate has been

probed by isotope effects and by use of linear free-energy relationships. Under conditions where the *N*-ribose bond cleavage is rate limiting, a kinetic α-secondary deuterium isotope effect of 1.13 was measured (*5*) and a $\beta_{lg} = -0.90$ was found when the pK_a of the departing pyridines was varied (*7*). Both results are indicative of a late transition state, i.e., a large degree of bond breaking to the leaving pyridine, and are in favor of the generation of an ADP-ribosyl oxocarbonium ion intermediate. The present study on the solvolysis of β-NAD^+ catalyzed by NAD^+ glycohydrolase enabled us to evaluate the reactivity of this intermediate with nucleophiles. We have found that, in sharp contrast to the spontaneous solvolysis of β-NAD^+, in the enzyme-catalyzed reaction the ADP-ribosyl intermediate displays a high selectivity, favoring methanolysis over hydrolysis by a factor of 50. Since the product ratio β-methyl ADP-ribose/ADP-ribose increases linearly with respect to increasing methanol concentrations, this partitioning ratio cannot be explained by a preferential binding of methanol. As discussed above, this implies that the intermediary ADP-ribosyl oxocarbonium ion must be sufficiently stable within the active site of the enzyme in order to react with acceptors in an activation-limited step. In their study on the solvolysis of substituted 1-phenethyl derivatives Richards and Jencks (*10*) have reported selectivities of such magnitude for methanol vs water in the case of their most stable carbocations, e.g., 1-(4-(dimethylamino)phenyl)ethyl cation. Similar important selectivities in enzyme-catalyzed solvolysis of glycosidic bonds have been reported for *B. fasciatus* venom NAD^+ glycohydrolase (*33*) and β-galactosidase (*34*). In this context, it is interesting to mention the study of Yost and Anderson on the partitioning of a series of pyridines in a NAD^+ glycohydrolase (*B. fasciatus*)-catalyzed reaction (*33*). A linear Brönsted plot was obtained with $\beta_{nuc} = 0.43$. This large sensitivity of the ADP-ribosyl intermediate to the pK_a of the incoming pyridines in the transglycosidation reaction, is consistent with (i) a transition state in which a great amount of *N*-ribosyl bond is already formed and (ii) a stabilized intermediate which reacts with the nucleophilic pyridines in an activation-limited step. The obligation for NAD^+ glycohydrolase to stabilize the ADP-ribosyl intermediate can also be inferred intuitively from the stereochemical course of the reactions catalyzed by the enzyme. Both methanolysis and transglycosidation occur with complete retention of configuration at the C-1′ of the ribose moiety (*2*). The intermediate must therefore exist in the active site with a lifetime that is sufficient to allow the diffusion of the departing nicotinamide prior to the nucleophilic attack by the incoming molecules. In this respect calf spleen NAD^+ glycohydrolase is different from the toxins and enzymes which catalyze, e.g., mono-ADP-ribosylation reactions (*35*). These enzymes transfer ADP-ribose to acceptors such as proteins with inversion of configuration at the C-1′ of the ribose moiety (*36, 37*) in a central ternary complex. Therefore such enzymes, which do not catalyze methanolysis of β-NAD^+, would not be expected to stabilize an intermediary oxocarbonium ion to an extent similar to that of NAD^+ glycohydrolase.

To summarize, since the ADP-ribosyl oxocarbonium ion derived from β-NAD^+ in the spontaneous solvolysis is too reactive, it must necessitate a substantial stabilization by the active site of NAD^+ glycohydrolase for existing as a reaction

intermediate. According to the Hammond postulate, we are left with the conclusion that the decrease in the energy barrier of the nicotinamide–ribose bond breaking would be due, in part, to the ability of the enzyme to effectively stabilize an oxocarbonium-ion-like species in the transition state. This stabilization could be electrostatic, e.g., by the formation of an ion pair with more or less bond order, between the oxocarbonium ion and a carboxylate from the enzyme. Such a residue, which would also shield the α-face of the ribosyl moiety of the intermediate, was shown to exist in the active site of the NAD^+ glycohydrolase (*38*). According to the ideas developed by Warshel, the active site of the enzyme could also be viewed as a "super-solvent" (*39* and references therein); i.e., the enzyme would act by solvating, more efficiently than water, the ionic resonance form of the ADP-ribosyl oxocarbonium ion intermediate relative to the substrate in its ground state.

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