Unusual Enzymatic Hydrolysis of NAD by Solubilized Form of NAD^+ Glycohydrolase

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Received November 20, 2001; accepted February 20, 2002

Using solubilized form (sNADase) of membrane-bound porcine brain NAD^+ glycohydrolase (pNADase), the NADase-catalyzed hydrolysis and transglycosidation reactions of NAD (1) were examined. Unexpectedly, products in the reactions were found to be nicotinamide (5′-*O*-diphosphono)-β-D-ribofuranoside (4) and adenosine (5). Adenosine 5′-diphosphate (ADP)-ribose (2) and nicotinamide (3) as well as a transglycosylated product, which are formed in a usual NAD/pNADase reaction system, were scarcely produced in the NAD/sNADase system. Setting aside the mechanical aspects of this unusual cleaving, it is quite interesting that the sNADase-catalyzed hydrolytic reaction of NAD resulted in the selective cleavage of the P–O bond of the adenosine side without the appreciable hydrolysis of the labile quaternary nicotinamide-ribose pyridinium linkage.

Key words membrane-bound particulate NADase; solubilized NADase; NAD; site-selective phosphatase-like activity

NAD^+ glycohydrolase (NADase) [EC 3.2.2.5] is widely known to have an ADP-ribosyltransferase-like activity (transglycosidation action) in addition to a hydrolase function for nicotinamide-ribose pyridinium linkage. There have been many reports[1—9] concerning substrates for the transferase-like activity of NADase, including synthetic low molecular compounds[2—5] and certain antibiotics components[6,7] as well as *in vivo* high molecular constituents.[8—10] NADase is a membrane-bound ecto-enzyme that is widely distributed in the organism, the richest source generally being the spleen, brain, and liver.[11] From the established viewpoint that the catalytic domain of the enzyme occurs in the extracellular region of the molecule,[12] it is of interest to examine whether enzyme activities of the parent particulate NADase (pNADase) are similarly observed with a solubilized extracellular portion (sNADase) of the pNADase.

Thus, we undertook the solubilization of the porcine brain pNADase according to the method for bovine spleen NADase of Augustin *et al.*,[13] since no procedure has not been established for porcine brain NADase. The crude sNADase thus obtained, however, showed unexpected catalytic properties of a phosphatase-like function rather than the usual hydrolase activity. It was a nicotinamide-bearing nucleotide compound that was isolated as a product of the sNADase-catalyzed cleavage reaction of NAD. This paper describes the formation of unusual products, nicotinamide 5′-diphosphorylribonucleotide (**4**) and adenosine (**5**), by the sNADase-catalyzed hydrolytic reaction of NAD (**1**). In this case, little ADP-ribose (**2**) or free nicotinamide (**3**) were produced, indicating there was no appreciable hydrolytic cleavage of the labile nicotinamide-ribose pyridinium linkage of **1**.

Results and Discussion

In order to obtain the sNADase, crude particulate NADase which was prepared from fresh porcine brain by the method of Zatman *et al.*,[14] was treated with a powder form of crude porcine pancreatic lipase (steapsin) which also contains protease activity.[13] After inactivation of the steapsin activity by its specific inhibitor phenylmethylsulfonylfluoride (PMSF), the reaction mixture was appropriately worked up according to the procedures of Augustin *et al.*,[13] giving a clear solution containing possible NADase-related activity. The clear solution thus obtained was used as sNADase.

The cleavage of NAD in the incubation system with sNADase was followed by high performance TLC. The system exhibited unexpected TLC behaviors distinct from those of the usual NAD/pNADase system that revealed the formation of adenosine 5′-diphosphate (ADP)-ribose (**2**) and nicotinamide (**3**): One of two main migratory spots was in a somewhat lower location than that of nicotinamide and the other was in a markedly lower location than that of ADP-ribose, suggesting the cleavage of another bond of the NAD molecule instead of the nicotinamide-ribose linkage. On the other hand, the treatment of **1** with steapsin for several hours resulted merely in the recovery of unchanged **1**, indicating that the unusual cleavage of **1** by sNADase can not be attributed to the steapsin activity that may possibly remain in the sNADase used.

The two major products (**4**, **5**) formed were then purified and isolated by column chromatographies on an anion exchanger (HCO_3^--form) to elucidate their entities. Compound **5** was sharply eluted with very dilute eluant (0.02 M ammonium hydrogencarbonate), whereas compound **4** was not eluted before the eluant attained a 0.2 M concentration. The FAB (+)- and (−)-MS spectra of **4** exhibited $[M+H]^+$ and $[M-H]^-$ ion peaks at *m*/*z* 415 and 413, respectively, indicative of the molecular weight 414. Based on a similar MS analysis, the molecular weight of **5** was estimated to be 268. These hydrolytic products of **1** were then subject to ^{1}H-NMR analyses. Surprisingly compound **4** was demonstrated to contain a nicotinamide riboside structure: The spectrum showed four separated one-proton signals due to four ring protons of the 3-substituted pyridinium system [δ 9.50 (s), 9.33 (d, J=6.3 Hz), 8.97 (d, J=8.1 Hz), and 8.32 (dd, J=6.3, 8.1 Hz)] together with the signals of an anomeric [δ 6.21 (d, J=5.0 Hz)]- and ribose-related five non-exchangeable protons. In contrast, compound **5**, which was the same as adenosine in spectrometirc and TLC properties, was identified as free adenosine. Based on these observations, it seemed most likely that the product **4** was a diphosphono compound. We accordingly treated **4** with phosphodiesterase-I (PDE-I) to

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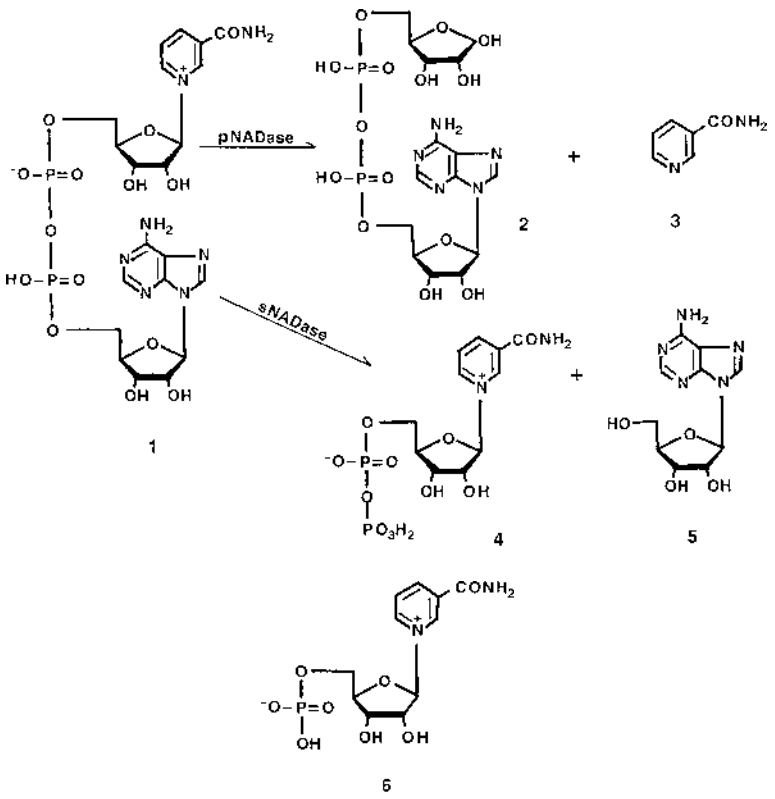

ascertain whether the monodephosphorylation of **4** occurs to give nicotinamide mononucleotide (NMN) (**6**),[15] which is produced as a counterpart by the treatment of **1** with PDE-I. The product obtained was identical with an authentic NMN as evidenced by MS- and NMR spectrometric data as well as TLC mobility and finally elemental analyses, demonstrating that compound **4** is an additional phosphoryl group-bearing NMN.

In this study, it was shown that sNADase-catalyzed reaction of NAD resulted, unexpectedly, in the selective cleavage of the P–O bond of the adenosine side without an appreciable hydrolysis of the labile quaternary nicotinamide–ribose linkage of NAD. Similar results were also obtained with the hydrolytic reaction of NAD using commercially available porcine brain pNADase-derived sNADase. Such an observation cannot immediately be interpreted as implying a new generation of a certain catalytic site in the sNADase molecule, but it is surprising that a relatively stable diphoshoryl P–O bond of the adenosine side underwent a selective hydrolytic cleavage, whereas much more hydrolizable pyridinium linkage remained unchanged.

Even in the presence of ethanol, one of the most efficient substrates for the transglycosylational action of NADase,[5] no substantial transglycosylated ethanol which was available in good yield in the parent NAD/pNADase system, was produced. This unexpected catalytic alteration of sNADase could be caused by its possible stereoconformational change which was attributed to dissociation from a membrane anchor. On the other hand, it can be viewed that pNADase originally has a site-selective phosphatase-like activity, weak though it may be. The marked decrease of inherent NADase activity during the solubilization of pNADase may possibly link to the relative occurrence of the phosphatase-like activity in sNADase. However, the activity of the sNADase used here (0.01 U/ml, see Experimental) did not seem low enough to be regarded as that of a concomitant contaminated with the parent pNADase.

Setting aside such controversial points, it is of significance from the chemical and catalytic points of view that this enzymatic cleavage has been permitted to yield compound **4** effectively in only one step from **1**. In any case, it seems quite interesting that sNADase shows a site-selective phosphatase-like activity, even though its biological significance remains unclear, instead of a hydrolyzing activity for the quaternary pyridinium linkage of NAD. Further purification and subsequent molecular analyses of sNADase will be required to elucidate the formation of such unexpected products.

Experimental

FAB-MS spectra were determined with a JEOL JMX-AX500 spectrometer using glycerol or triethanolamine as a matrix. ^{1}H-NMR spectra were measured in D_2O with a Bruker HSL-400 spectrometer with sodium [2,2,3,3-d_4]3-(trimethylsilyl)propionate as an internal standard. The abbreviations "s, d, dd, t, dt, br, and bs" denote "singlet, doublet, doubledoublet, triplet, doubletriplet, broad, and broad singlet", respectively. Analytical TLCs were run on precoated silica gel $60F_{254}$ HPTLC plates (Merck, 10 cm×10 cm) using 2-propanol/0.2% aqueous ammonia (7 : 3, v/v) as the developing system. β-NAD (N 7004), NADase (N 9879, purified acetone-dried powder derived from porcine brain), and crude porcine pancreatic lipase (steapsin) (L 3126) were obtained from Sigma Chemical Co.

Particulate NADase (pNADase) Prepared from fresh porcine brain by the method of Zatman *et al.*[14] and used without further purification when employed as pNADase.

Solubilized NADase (sNADase) Prepared from pNADase by the modified method of Augustin *et al.*[13] Briefly, to the crude pNADase or the Sigma acetone-dried powder NADase suspended in 10 mM of Tris–HCl (pH 7.7), crude steapsin powder was added so that the ratio of steapsin powder to net pNADase was *ca.* 3 : 1 (w/w). The suspension mixture was left under mild stirring for 120 min at 30 °C in the presence of 1 mM $CaCl_2$. The reaction mixture was then treated with 1 mM PMSF to inactivate the steapsin activity and then the mixture was centrifuged at 50000 *g* for 60 min. The resulting clear supernatant (adjusted to pH 7.2) containing sNADase was concentrated

to one-third of its original volume under lyophilization conditions. Debris formation was removed by additional centrifugation. The activity of the concentrated supernatant thus obtained was estimated to be *ca.* 0.01 U/ml. In this case, U is the activity of sNADase that disrupts 1 μmol of NAD per min.

Incubation of NAD (1) with sNADase Leading to an Unusually Cleaved Product (4) and Its Counterpart (5) NAD (500 mg, 0.75 mmol) was incubated with sNADase (20 ml) in 0.1 M Tris–HCl (20 ml/pH 7.2) with mild stirring at 37 °C for 48 h. During the incubation, the cleavage of NAD was checked by TLC, and the pH of the incubation mixture was occasionally adjusted because its gradual lowering occurred with the progress of the reaction. After TLC had indicated the complete disappearance of NAD, the reaction mixture was treated with 80% TCA (1.5 ml) at 4 °C to inactivate the sNADase activity and resultant white precipitates were removed by centrifugation. To supernatant thus obtained cold acetone (200 ml) was added to give a white suspension. The suspension was allowed to stand overnight at 4 °C precipitating a solid white mass. The supernatant liquid layer was removed by decantation and was once laid aside. The residual white mass (285 mg) was collected and dissolved in water (30 ml), and the aqueous solution was applied to a column (2.5 cm×15 cm) of DEAE-Sephadex A-25 (HCO_3^--form). The column was washed with 0.8% (w/w) aqueous NH_4HCO_3 (500 μmho, 150 ml) to remove any non-adsorbing component and then eluted with a 4% solution (2500 μmho) of the same hydrogen carbonate. Major fractions exhibiting a strong UV-absorption peak at around 260 nm and a single spot on TLC, were collected and evaporated to dryness *in vacuo* giving a white solid mass. The solid mass was repeatedly lyophilized to provide **4** (234 mg) as its monoammonium salt. An analytical sample was obtained by further drying over P_2O_5 *in vacuo* for 12 h. **4**: FAB-MS (negative) *m/z*: 413 $[M-H]^-$; (positive) *m/z* 415 $[M+H]^+$. ^{1}H-NMR (D_2O) δ: 9.50 (1H, s, py-2), 9.33 (1H, d, J=6.3 Hz, py-6), 8.97 (1H, d, J=8.1 Hz, py-4), 8.32 (1H, dd, J=6.3, 8.1 Hz, py-5), 6.20 (1H, d, J=5.0 Hz, anomeric), 4.65 (1H, t, J=4.9 Hz, rib-4′), 4.60 (1H, m, rib-3′ or -2′), 4.56 (1H, m, rib-2′ or -3′), 4.40 (1H, dd, J=12.8, 4.0 Hz, one of rib-5′-methylene protons), 4.31 (1H, dd, J=12.8, 12.8, 3.0 Hz, another one of rib-5′-protons). *Anal.* Calcd for $C_{11}H_{15}N_2O_{11}P_2\cdot NH_3$: C, 30.70; H, 4.19; N, 9.77. Found: C, 30.54; H, 4.26; N, 10.02. The above supernatant solution laid aside was neutralized with 1 M tris (hydroxymethyl) aminomethane and was evaporated to dryness *in vacuo*. The resulting residue was treated with an appropriate amount of ethanol and an ethanol-insoluble part was dissolved in a small volume of hot water. The aqueous solution was centrifuged to remove any turbidity. The clear supernatant thus obtained was allowed to stand at 4 °C giving a white crystalline mass **5**. The melting point (233—235 °C) and the MS and ^{1}H-NMR spectral data of **5** were compatible with those of authentic adenosine. *Anal.* Calcd for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.87; N, 26.22. Found: C, 44.82; H, 4.86; N, 26.09.

Dephosphorylative Conversion of Compound 4 into Nicotinamide Mononucleotide (NMN) (6) with Phosphodiesterase I (PDE-I) Compound **4** (20 mg) was incubated with appropriate units of PDE-I in 0.1 M Tris–HCl (pH 8.8, 1.2 ml) at 37 °C. The incubation reaction was continued until a TLC spot due to **4** was found to disappear. After the heat-inactivation (70—80 °C, 1 min) of the PDE-I activity and the removal of any resulting turbidity by centrifugation, the reaction mixture was diluted with ten volumes of water (12 ml). The solution was then applied to a column (2.5 cm×10 cm) of DEAE-Sephadex A-25 and eluted with a 4% aqueous NH_4HCO_3 solution. Strong UV-absorbing fractions which showed the same TLC mobility were collected and evaporated to dryness *in vacuo* to give **6** (12 mg). Compound **6** agreed well with an authentic NMN in its TLC mobility as well as various spectral properties: MS {335 $[M+H]^+$} and ^{1}H-NMR [δ: 9.47 (s), 9.29 (d), 8.98 (d), 8.30 (dd), 6.21 (d), 4.65 (t), 4.57 (m), 4.45 (m), 4.31 (dd), 4.15 (dd)]. *Anal.* Calcd for $C_{11}H_{14}N_2O_8P\cdot NH_3$: C, 37.22; H, 4.86; N, 12.00. Found: C, 37.96; H, 4.82; N, 11.75.

Acknowledgments We thank Mr. E. Yamada (Laboratory of NMR, Faculty of Engineering, Hokkaido University) and Dr. E. Fukushi (GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University) for measuring ^{1}H-NMR- and MS spectra, respectively.

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