Synthesis of Pyridine Nucleotide Analogs Consisting of Nicotinoylaminoo Acids by Means of Transglycosidation Reactions Catalyzed by Mammalian Pyridine Nucleotide Transglycosidases

Tadayoshi IMAI* and Makoto HATORI

Graduate Course of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

(Received September 26, 2001)

Summary Besides isonicotinic acid analogs of pyridine nucleotides, 24 novel pyridine nucleotide cofactors that have an amino acid residue at the carbonyl carbon of the nicotinamide moiety have been prepared by means of transglycosidation reactions catalyzed by rabbit spleen and guinea pig spleen pyridine nucleotide transglycosidases. Their chemical properties were characterized by means of proton NMR, Fab-mass, and UV spectral measurement and phosphodiesterase digestion. Except for the isonicotinic acid ones, these nicotinoylaminoo acid analogs were shown to function as substrates for both the hydrolysis and the transglycosidation reactions catalyzed by the mammalian NAD glycohydrolases, though their substrate activities were lower than those with the original pyridine nucleotides (NMN, NAD, and NADP). They were inactive in regard to yeast alcohol dehydrogenase- and Thermoaerobium brockii alcohol dehydrogenase (NADP dependent)-oxidation.

Key Words isonicotinic acid, nicotinoylaminoo acids, pyridine nucleotide analogs, pyridine nucleotide transglycosidase, transglycosidation reaction

One of the characteristic properties of a mammalian tissue-associated NAD glycohydrolase [EC 3.2.2.6] is its catalytic function not only as a transglycosidase, but also as a hydrolase. Until now, several kinds of pyridine nucleotide analogs have been synthesized with the transglycosidase activity of such enzymes and used for many biochemical studies (1).

In a previous paper (2), Imai, a present author showed that mammalian pyridine nucleotide transglycosidase activities are distributed ubiquitously, but they differ in the substrate specificity toward pyridine nucleotide structures: Rabbit spleen pyridine nucleotide transglycosidase is more active toward NMN than toward NAD, and, in contrast, guinea pig spleen pyridine nucleotide transglycosidase is more active toward NAD than toward NMN. Moreover, the two enzymes showed very broad substrate specificities toward nicotinamide analogs that have an amino acid residue at carbonyl carbon.

Based on the enzymatic properties of the enzymes, we have tried to prepare several kinds of novel pyridine nucleotide cofactors. In this report, the preparation and the chemical and enzymatic properties of the analogs will be described.

MATERIALS AND METHODS

A purification of the pyridine nucleotide transglycosidases, identical with NAD glycohydrolase, from rabbit spleen and guinea pig spleen and a preparation of nicotinoyl-amino acids, i.e., nicotinoyl-Ala, -β-Ala, -γ-aminobutyric acid (GABA), -6-aminohexanoic acid (6AH), -Val, -Met, -Ser, -Thr, -Asp, -Glu, -Asn, -Gln, and -GlyGly were performed as described in the previous paper (2). Nicotinoylglycine (nicotinoyl-Gly) was purchased from Tokyo Kasei. To determine the nicotinoyl amino acid concentrations, the molecular absorption coefficient for nicotinoyl-Gly at pH 2 (5.7×10³ M⁻¹ cm⁻¹) was used with the assumption that the spectrophotometrical properties of all the nicotinoyl amino acids are identical. Nicotinic acid mononucleotide (NaMN) was prepared as described (3). Yeast alcohol dehydrogenase was purchased from Oriental, and Thermoaerobium brockii alcohol dehydrogenase (NADP-dependent) and Crotalus adamanteus phosphodiesterase were from Sigma.

The velocity of hydrolysis at the C-N glycoside linkage of the pyridine nucleotides was assayed as described (2). The velocity of the transglycosidation reactions was monitored by both a spectrophotometrical method (Method A) and assaying the transglycosidation product spectrophotometrically by use of the molar absorption coefficients described below after separation by paper chromatography with a solvent system of n-BtOH/ethanol/water=13/8/4 (solvent A), followed by paper electrophoresis at pH 5 (Method B) as described.
in the previous paper (2). The digestion of the NAD- and NADP-analogs with venom phosphodiesterase was carried out as described previously (4).

The preparation of pyridine nucleotide cofactor analogs was carried out as follows. For the synthesis of NMN analogs, mixtures consisting of 0.1 m nicotinoyl-amino acid, 0.05 m Tris-maleate (pH 6.5), 16 mM NMN, and 1.0 unit of rabbit spleen NAD glycohydrolase in a total volume of 3.0 mL were incubated at 39°C for 3 h. After the reaction was terminated by the addition of 1 m HCl (0.1 mL/mL), each mixture was spotted onto Toyo #50 paper and chromatographed with solvent A. The transglycosidation products retained at the origin were eluted with water and adsorbed on a DEAE-cellulose column that had been equilibrated with 0.05 m ammonium acetate (pH 5). After the nonreactive NMN had been washed out with 0.01 m NaCl/0.05 m ammonium acetate (pH 5), the NMN analogs, except for NMN-Glu and NMN-Asp, were eluted with 0.04 m NaCl/0.05 m ammonium acetate (pH 5). NMN-Asp and NMN-Glu were eluted by the addition of 0.15 m NaCl to the same buffer. The fractions containing the analogs were pooled and desalted by means of charcoal treatment (Norit extra: 2 mg/density unit at 266 nm) and applied on a Sephadex G-10 column (elution volume/gel volume=0.38).

The reaction systems for the preparation of NAD- and NADP analogs were the same as those described for NMN analogs, except that 1.0 unit of guinea pig spleen NAD glycohydrolase and 16 mM NAD or NADP were used. The following NaCl concentrations were used for elution: 0.1 m for NAD-Gly, -Ala, and -Ser; 0.2 m for NAD-Asp and -Glu; 0.16 m NaCl for NADP-Gly, -Asp, -GABA, and -GlyGly; and 0.2 m for NADP-Asp. Under these conditions, NMN, NAD, and NADP were eluted with 0.01 m+, 0.02 m+, and 0.1 m NaCl/0.05 m ammonium acetate (pH 5), respectively.

The isonicotinic acid analogs, i.e., isonicotinic acid mononucleotide (IsoNaMN), isonicotinic acid-adenine dinucleotide (IsoNaAD), and isonicotinic acid-adenine dinucleotide phosphate (IsoNaADP), were prepared from NMN, NAD, and NADP by essentially the same methods described previously for the preparation of nicotinic acid analogs (3). The yields were 38, 33, and 31%, respectively.

The purity of the analogs thus obtained was examined in several different ways and shown to be more than 95%. A single ultraviolet-quenching spot was obtained for all the analogs on analysis by paper chromatography with three different solvent systems and paper electrophoresis at pH 5 (see below).

Total phosphorus was determined as described previously (4) and UV spectral data were calculated on the basis of total phosphorus. ³H-NMR spectra were obtained as described previously (3) by use of trimethylsilylpropanesulfonate (Na) as an internal marker. The assignments of the proton absorptions were made as reported (5, 6). Fab-mass spectra were obtained by using glycerol as an internal marker.

**RESULTS**

(1) General remarks on the preparation of pyridine nucleotide coenzyme analogs

As shown in the preceding paper (2), a kinetic analysis of the transglycosidation reactions catalyzed by rabbit spleen NAD glycohydrolase and guinea pig spleen NAD glycohydrolase showed that the former is more active toward NMN than the latter is, and in contrast, the latter is more active toward NAD and NADP than the former is. On the other hand, both enzymes catalyze transglycosidation reaction in the presence of a nicotinoylamine acid, but the Km value was observed to depend on the structure of the nicotinoylamine acid and the pyridine nucleotide cosubstrate. For the sake of consistency, the concentrations of nicotinoylamine acid and pyridine nucleotide cosubstrate (NMN, NAD, or NADP) in the routine reaction mixture were fixed at 0.1 m and 16 mm, respectively.

(2) Results of preparation of pyridine nucleotide analogs

When the transglycosidation reactions were carried out with the rabbit spleen NAD glycohydrolase, all NMN-analogs could be prepared in somewhat good yields, as shown in Table 1. The data are shown as the yield (%), standardized in relation to the amount of the pyridine nucleotide cosubstrate used. In contrast, limited numbers of NAD- and NADP-analogs could be prepared by using the guinea pig spleen NAD glycohydrolase, by which NAD and NADP can be transglycosylated faster than with NMN. The analogs that could be prepared in yields of greater than 10% were NAD-Gly, NAD-β-Ala, NAD-Ser, NAD-Glu; NADP-Gly, NADP-β-Ala, NADP-Ser, NADP-Asp; and NADP-GlyGly. Other dinucleotide analogs could not be practically prepared even when prolonged incubations were carried out in the presence of much more enzyme activity than that used in the routine reaction system.

(3) Chemical characterization of the pyridine nucleotide coenzymes prepared

a) Paper chromatography and paper electrophoresis

The behavior of the analogs on paper chromatography

| Nicotinamide analog | Yield (%) |
|---------------------|-----------|
| NMN | NAD | NADP |
| Nicotinoyl-Gly | 41 | 52 | 41 |
| Nicotinoyl-Leu | 46 | 0 | 0 |
| Nicotinoyl-Ser | 37 | 40 | 10 |
| Nicotinoyl-Asp | 38 | 16 | 16 |
| Nicotinoyl-Glu | 43 | 10 | 0 |
| Nicotinoyl-Asn | 38 | 0 | 0 |
| Nicotinoyl-Gln | 34 | 0 | 0 |
| Nicotinoyl-β-Ala | 50 | 38 | 20 |
| Nicotinoyl-GABA | 50 | 5 | 41 |
| Nicotinoyl-6AH | 39 | 4 | 0 |
| Nicotinoyl-GlyGly | 40 | 0 | 11 |
with the solvent system of 95% ethanol/1 M ammonium acetate, pH 7=15/6, and on paper electrophoresis at pH 5 was compared with the substrates used for the preparation, i.e., NMN, NAD, and NADP (Table 2). The data are shown as relative mobility compared with that of NMN (RNMN) and nicotinic acid mononucleotide, NaMN (MNaMN), respectively. Both on paper chromatography and electrophoresis, all the analogs gave a single UV quenching spot.

b) Spectrophotometrical properties of the analogs. The spectrophotometrical properties (ε) at pH 7.5 and those of the cyanide addition complex formed in 1 M KCN are listed in Table 3. The molar absorption coefficients were obtained based on the organic phosphorus analysis. The absorption maximum for NMN analogs is at 266 nm, with a molecular extinction coefficient of (5.21±0.15)×10³, i.e., slightly greater than that of NMN. The absorption maximum for AMP is at 259 nm, with a molecular extinction coefficient of 15,400. The extinction for the NAD- and NADP-analogs prepared is not simply the sum of the two single absorbances, but slightly less. This decrease is caused by an intramolecular interaction due to an oscillator system that results from a folding of the coenzyme analog molecules. Cyanide addition is a typical reaction for an intact nicotinamide-ribose linkage. However, the complexes of the analogs prepared, except for NMN-Glu, -Asp, -Gln, and -Asn, were unstable, and the spectra changed immediately after the complexes were formed (Fig. 1). Thus the molar absorption coefficients were calculated from the maximal absorbance determined with a recorder.

c) 1H-NMR spectra of the analogs. Proton NMR spectra of NMN-GABA (A), NAD-β-Ala (B), and NADP-Ala (C) are shown in Fig. 2. α-, β-, and γ-methylene protons of NMN-GABA besides NMN, α- and β-methylene protons of NADP-Ala besides NADP, and methyl protons of NADP-Ala besides NADP, respectively, were observed. The chemical shifts of the anomeric protons of pyridine nucleoside moieties were observed at 6.22 ppm, indicating that the configurations were retained in all the transglycosidation reactions. Chemical shifts suggesting chemical structures of the other prepared analogs are listed in Tables 4 and 5.

d) Fab-mass spectra. For the molecular cation analysis of the pyridine nucleotide analogs synthesized, an aqueous solution containing 0.2 μmol/25 μL was applied to the instrument. However, a 0.1 M HCl solution was used for the NADP analogs. The positive ion fast-atom bombardment mass spectra of all the analogs prepared gave a single peak at m/z that was calculated on the basis of the molecular structures expected as a monocation (data not shown).

e) Phosphodiesterase digestion of NAD and NADP
Fig. 1. Spectral change of the CN-adduct of NMN-Gly. 0.2 µmol of NMN-Gly was mixed with 2 mL of a 1 M KCN solution. The numbers in the figure indicate the reaction time (min) at room temperature after the addition of KCN: 1, 1; 2, 4; 3, 7; 4, 11; 5, 15; 6, 20; 7, 30; and 8, 60. The absorption spectra were recorded from 350 to 250 nm. The isosbestic point was observed at 307 nm.

**WAVELENGTH (nm)**

![Graph showing spectral change](image)

The substrate activity of the prepared analogs in regard to the transglycosidation reaction catalyzed by mammalian NAD glycohydrolase

The substrate activity of the pyridine nucleotide co-factor analogs in regard to the transglycosidation reactions was monitored by converting the analogs to NMN, NAD, and NADP, respectively. As shown in Table 7, NMN-Gly, NAD-Gly, NADP-Gly, NAD-β-Ala, NAD-Ala, and NAD-Ser were each observed to function as a substrate for the transglycosidation reactions catalyzed by rabbit spleen NAD glycohydrolase and guinea pig spleen NAD glycohydrolase, whereas NAD-Asp and NAD-Glu were less effective. Furthermore, a comparison of the transglycosidation velocities indicated that guinea pig spleen NAD glycohydrolase is more active than rabbit spleen NAD glycohydrolase when an NAD or NADP analog is used as the cosubstrate, which is consistent with guinea pig spleen NAD glycohydrolase being more active in the transglycosidation reactions than rabbit spleen NAD glycohydrolase is when NAD or NADP is used as the cosubstrate (2).

We detected the effect of the substituent structure on the transglycosidation velocity by using a series of NMN analogs as substrates for the transglycosidation reaction catalyzed by rabbit spleen NAD glycohydrolase. Under the same reaction conditions as those in Table 7, the transglycosidation velocity (nmol/min/unit) with NMN-Gly, NAD-β-Ala, NAD-GABA, NAD-Glu, NAD-Asp, NAD-6-AH, NAD-Asn, NAD-Leu, NAD-Ser, NAD-Asn, NAD-Gln, and NAD-Gly-Gly-Gly was 52, 69, 84, 87, 29, 42, 80, 52, 42, 57, and 61, respectively. The velocity of NaMN formation from NMN and nicotinic acid and of IsoNaMN formation from NMN and isonicotinic acid was 68 and 107, respectively.

**DISCUSSION**

The preparation of analogs of pyridine nucleotide coenzymes has been reviewed, and 181 analogs have been listed (1). However, among them are none that have an additional residue on the nitrogen of the carboxamide group of the nicotinamide moiety. In the previous study (2), one author showed that NAD glycohydrolases is distributed ubiquitously in mammalian tissues, but that the substrate specificity of the enzymes and the $v_f/v_M$ ratio in the transglycosidation reaction vary depending on the tissue from which the enzyme is extracted. On the basis of the data obtained, we showed that several kinds of novel pyridine nucleotide analogs can be prepared by means of base replacement reactions catalyzed by the rabbit spleen and guinea pig spleen NAD glycohydrolases (Table 1). The successful preparation of the pyridine nucleotide coenzyme analogs depended on the selective utilization of the analogs. Digestion of the NAD- and NADP-analogs with venom phosphodiesterase gave the corresponding NMN-analog and 5'-AMP and 2',5'-ADP, respectively, in stoichiometric amounts.

(4) Coenzyme activity of the prepared analogs for dehydrogenases

For most of these derivatives, the inability to function as a coenzyme was confirmed experimentally, but no activity was observed for yeast alcohol dehydrogenase oxidation or Thermoaerobium brockii alcohol dehydrogenase (NADP-dependent) oxidation.

(5) Substrate activity of the prepared analogs for mammalian NAD glycohydrolase

As shown in Table 6, NMN-, NAD-, and NADP-Gly were observed to be active in regard to the hydrolysis catalyzed by both rabbit spleen NAD glycohydrolase and guinea pig spleen NAD glycohydrolase. Other NMN, NAD, and NADP analogs prepared were also active in regard to these mammalian NAD glycohydrolases, but the hydrolytic activity was less than 10% of that with NAD.

(6) Substrate activity of the prepared analogs in regard to the transglycosidation reaction catalyzed by mammalian NAD glycohydrolase

The substrate activity of the pyridine nucleotide co-factor analogs in regard to the transglycosidation reactions was monitored by converting the analogs to NMN, NAD, and NADP, respectively. As shown in Table 7, NMN-Gly, NAD-Gly, NADP-Gly, NAD-β-Ala, NAD-Ala, and NAD-Ser were each observed to function as a substrate for the transglycosidation reactions catalyzed by rabbit spleen NAD glycohydrolase and guinea pig spleen NAD glycohydrolase, whereas NAD-Asp and NAD-Glu were less effective. Furthermore, a comparison of the transglycosidation velocities indicated that guinea pig spleen NAD glycohydrolase is more active than rabbit spleen NAD glycohydrolase when an NAD or NADP analog is used as the cosubstrate, which is consistent with guinea pig spleen NAD glycohydrolase being more active in the transglycosidation reactions than rabbit spleen NAD glycohydrolase is when NAD or NADP is used as the cosubstrate (2).

We detected the effect of the substituent structure on the transglycosidation velocity by using a series of NMN analogs as substrates for the transglycosidation reaction catalyzed by rabbit spleen NAD glycohydrolase. Under the same reaction conditions as those in Table 7, the transglycosidation velocity (nmol/min/unit) with NMN-Gly, NAD-β-Ala, NAD-GABA, NAD-Glu, NAD-Asp, NAD-6-AH, NAD-Asn, NAD-Leu, NAD-Ser, NAD-Asn, NAD-Gln, and NAD-Gly-Gly-Gly was 52, 69, 84, 87, 29, 42, 80, 52, 42, 57, and 61, respectively. The velocity of NaMN formation from NMN and nicotinic acid and of IsoNaMN formation from NMN and isonicotinic acid was 68 and 107, respectively.
Pyridine Nucleotide Analogs

Fig. 2. Proton magnetic resonance spectra of the pyridine nucleotide cofactor analogs. Of the nucleotides prepared, the spectra of NMN-GABA (A), NAD-Ala (B), and NADP-β-Ala (C) are shown. Peaks: 1, pyridinéH(2); 2, pyridinéH(6); 3, pyridinéH(4); 4, pyridinéH(5); 5, H'(1); 6, adeninéH(8); 7, adeninéH(2); 8, H'(1) of adenosine; 9, γ-CH₂; 10, α-CH₂; 11, β-CH₂; 12, -CH₃; 13, β-CH₂; 14, α-CH₂; and 15, trimethylsilanepropanesulphonate (Na), as a marker.
Table 4. Chemical shifts of the pyridine mononucleotide analogs synthesized.

| Nucleotide | Pyridine (ppm) | Ribose (ppm) | Amino acid residue |
|------------|----------------|--------------|--------------------|
|            | C2  | C6  | C4  | C3  | C1  | C2  | C4  | C5  | C5  |
| NMN-Gly    | 9.45 | 9.33 | 8.98 | 8.31 | 6.22 | 4.59 | 4.63 | 4.45 | 4.30/4.13 | CH$_2$; 4.01 s |
| NMN-β-Ala  | 9.38 | 9.30 | 8.91 | 8.28 | 6.21 | 4.57 | 4.63 | 4.45 | 4.30/4.13 | α-CH$_2$; 2.57, β-CH$_2$; 3.66 |
| NMN-GABA   | 9.40 | 9.30 | 8.93 | 8.30 | 6.22 | 4.57 | 4.64 | 4.45 | 4.30/4.13 | α-CH$_2$; 2.34 t, β-CH$_2$; 1.92 tt, γ-CH$_2$; 3.48 t |
| NMN-6AH    | 9.38 | 9.30 | 8.91 | 8.29 | 6.21 | 4.57 | 4.63 | 4.45 | 4.30/4.13 | α-CH$_2$; 2.25, β-CH$_2$; 1.59, γ-CH$_2$; 1.37, δ-CH$_2$; 1.65, ε-CH$_2$; 3.45 |
| NMN-Asp    | 9.41 | 9.33 | 8.96 | 8.30 | 6.22 | 4.57 | 4.62 | 4.44 | 4.29/4.15 | α-CH (hidden by 4.73 ppm peak), β-CH$_2$; 2.91/2.76 |
| NMN-Glu    | 9.41 | 9.35 | 8.97 | 8.31 | 6.23 | 4.57 | 4.62 | 4.45 | 4.30/4.15 | α-CH; 4.39, β-CH$_2$; 2.22/2.09, γ-CH$_2$; 2.39 |
| NMN-Leu    | 9.41 | 9.35 | 8.96 | 8.30 | 6.22 | 4.57 | 4.62 | 4.45 | 4.29/4.15 | α-CH; 4.45, β-CH$_2$; 1.76, γ-CH$_2$; 1.76, δ-CH$_2$; 0.94 |
| NMN-Ser    | 9.49 | 9.33 | 9.01 | 8.31 | 6.22 | 4.59 | 4.63 | 4.45 | 4.29/4.13 | α-CH; 4.54, β-CH$_2$; 3.99 |
| NMN-Asc    | 9.43 | 9.34 | 8.98 | 8.31 | 6.23 | 4.57 | 4.62 | 4.45 | 4.29/4.15 | α-CH; 4.66, β-CH$_2$; 2.95/2.82 |
| NMN-Gln    | 9.42 | 9.33 | 8.98 | 8.31 | 6.22 | 4.57 | 4.63 | 4.44 | 4.29/4.15 | α-CH; 4.66, β-CH$_2$; 2.25/2.12, γ-CH$_2$; 2.41 |
| NMN-GlyGly | 9.48 | 9.30 | 8.99 | 8.30 | 6.22 | 4.57 | 4.64 | 4.45 | 4.31/4.15 | CH$_2$; 3.56 s, 4.23 s |
| isoNaMN    | 9.23 | 9.23 | —    | 8.36 | 6.13 | 4.56 | 4.60 | 4.47 | 4.16/4.04 | — |
| β-NMN      | 9.49 | 9.30 | 8.99 | 8.31 | 6.22 | 4.58 | 4.63 | 4.45 | 4.28/4.12 | — |

Table 5. Chemical shifts of the NAD and NADP analogs synthesized.

|                      | Pyridine (ppm) | Pyridine nucleoside (ppm) | Adenosine (ppm) | Side chain |
|----------------------|----------------|--------------------------|-----------------|------------|
|                      | C2  | C6  | C4  | C3  | C1  | C2  | C4  | C5  | C5  | C1  | C2  | C3  | C4  | C5  | C6  | C7  | C8  | C9  | C10 | C11 |
| NAD-Gly              | 9.18 | 9.03 | 8.67 | 8.05 | 5.95 | 5.90 | 8.06 | 8.27 | α-CH$_2$; 4.01 |
| NAD-β-Ala            | 9.28 | 9.19 | 8.78 | 8.18 | 6.11 | 6.06 | 8.21 | 8.45 | α-CH$_2$; 2.61, β-CH$_2$; 3.67 |
| NAD-Ala              | 9.32 | 9.18 | 8.82 | 8.17 | 6.10 | 6.05 | 8.20 | 8.42 | α-CH$_2$; 3.17, β-CH$_2$; 2.93 |
| NAD-Asp              | 9.33 | 9.20 | 8.84 | 8.19 | 6.11 | 6.07 | 8.21 | 8.51 | α-CH$_2$; 3.17, β-CH$_2$; 2.93 |
| NAD-Glu              | 9.31 | 9.19 | 8.83 | 8.19 | 6.11 | 6.06 | 8.18 | 8.43 | α-CH$_2$; 3.17, β-CH$_2$; 2.93 |
| NAD-Ser              | 9.22 | 9.04 | 8.73 | 8.03 | 5.95 | 5.91 | 8.07 | 8.30 | α-CH$_2$; 3.17, β-CH$_2$; 2.93 |
| isoNaAD              | 8.95 | 8.95 | —    | 8.21 | 6.04 | 6.00 | 8.13 | 8.43 | — |
| NAD-Gly              | 9.46 | 9.16 | 8.82 | 8.21 | 6.18 | 6.07 | 8.19 | 8.41 | α-CH$_2$; 4.01 |
| NAD-β-Ala            | 9.25 | 9.14 | 8.74 | 8.15 | 6.18 | 6.06 | 8.18 | 8.49 | α-CH$_2$; 2.58, β-CH$_2$; 3.67 |
| NAD-GABA             | 9.39 | 9.27 | 8.92 | 8.26 | 6.31 | 6.21 | 8.29 | 8.45 | α-CH$_2$; 2.49, β-CH$_2$; 1.96, γ-CH$_2$; 3.49 |
| NAD-Asp              | 9.31 | 9.15 | 8.83 | 8.17 | 6.20 | 6.08 | 8.29 | 8.47 | β-CH$_2$; 2.85 |
| NAD-Ser              | 9.36 | 9.16 | 8.97 | 8.19 | 6.20 | 6.08 | 8.20 | 8.45 | β-CH$_2$; 2.40 |
| isoNaADP             | 8.91 | 8.91 | —    | 8.22 | 6.12 | 5.96 | 8.11 | 8.43 | — |

mammalian NAD glycohydrolases: the rabbit spleen enzyme for preparing NMN analogs and the guinea pig spleen enzyme for preparing NAD and NADP analogs. Although the yields of the NMN-analogs synthesized were greater than 30% in all instances, limited numbers of NAD- and NADP-analogs could be prepared, and no transglycosidation product was obtained when nicotinoyl-6-aminohexanoic acid, nicotinoyl-Leu, nicotinoyl-Asn, or nicotinoyl-Gln were used as the cosubstrate, suggesting that the ADP-ribosyl or the ATP-ribosyl moiety of the intermediary AD(T)P-ribosyl-enzyme complex might inhibit the access of the pyridine bases to the active center.

Changes in the structures of the various components of pyridine nucleotide molecules can have pronounced effects on the reactions catalyzed, and the modification of these components to produce structural analogs constitutes an effective experimental approach for the study of the mode of action of these molecules. One characteristic property of the prepared pyridine nucleotide coenzymes is that they retain the original structures along with the configuration of the pyridinium C-N glycoside linkage, except for the carboxamide group, and they have a carboxylic acid group at the end of the alkyl chain. Therefore these analogs can be easily coupled to commercial resins, which have introduced free primary amino groups, and used as ligand for affinity chromatography of NAD(P)-requiring enzymes, whether the biological function involves oxidation-reduction or ADP-ribosylation.
Table 6. Substrate activity of NMN-Gly, NAD-Gly, and NADP-Gly in regard to the hydrolysis catalyzed by rabbit spleen NAD glycohydrolase and guinea pig spleen NAD glycohydrolase. The hydrolytic velocity was monitored at the substrate concentration of 4 mM as described under Materials and Methods. The data are shown as the relative activity standardized to the activity with NAD.

| Substrate | Rabbit spleen | Guinea pig spleen |
|-----------|---------------|-------------------|
| NAD       | 100           | 100               |
| NAD-Gly   | 13.0          | 12.6              |
| NADP-Gly  | 11.1          | 8.0               |
| NMN-Gly   | 8.9           | 4.5               |

As shown in Tables 6 and 7, the prepared analogs, except for the isonicotinic acid analogs, can function as substrates for the hydrolysis and transglycosidation reaction catalyzed by the mammalian NAD glycohydrolases, though they were inactive in regard to the reduction by either yeast alcohol dehydrogenase or Thermoanaerobium brochii alcohol dehydrogenase. This indicates that the transglycosidation reactions between nicotinamide and the nicotinoylamino acids are reversible ones. Thus it will be possible to monitor the transglycosidation reactions with a nondissociable nicotinamide, isonicotinamide, or isonicotinic acid-hydrazide with the use of the nicotinoyl-Gly analogs of NMN, NAD, and NADP because the substrate and products could easily be separated and analyzed spectrophotometrically. Metabolic studies on isonicotinic acid-hydrazide and isonicotinic acid-hydrazidemethanesulfonate with mammalian NAD glycohydrolases are in progress in our laboratory.

Acknowledgments
The authors wish to thank Mr. Shigeyuki Kitamura for measurement of the Fab-mass spectra of the synthesized nucleotides, and also Ms. Kyoko Yamada, Ms. Minori Kato, and Mr. Hiroyuki Mori for their help in the preparation of the pyridine nucleotide analogs.

Table 7. Substrate activity of the synthesized nicotinoyl analogs in regard to the transglycosidation reaction catalyzed by mammalian NAD glycohydrolases. Reaction mixtures consisting of 4 mM of a synthesized pyridine nucleotide, 0.1 mM nicotinamide, 0.05 mM Tris-maleate (pH 6.5), and 20 mU of rabbit spleen NAD glycohydrolase or guinea pig spleen NAD glycohydrolase, respectively, in a total volume of 50 μL were incubated at 39°C for 1 h. The transglycosidation products, i.e., NMN, NAD, and NADP, were separated by Method B described in the text and assayed spectrophotometrically.

| Substrate | Rabbit spleen enzyme | Guinea pig spleen enzyme |
|-----------|----------------------|--------------------------|
| NMN-Gly   | 52                   | 51                       |
| NAD-Gly   | 23                   | 77                       |
| NADP-Gly  | 27                   | 61                       |
| NAD-β-Ala | 25                   | 63                       |
| NAD-λ-Ala | 7.3                  | 16                       |
| NAD-Ser   | 7.7                  | 22                       |
| NAD-Asp   | 2.3                  | 5.0                      |
| NAD-Glu   | 3.3                  | 5.5                      |

REFERENCES
1) Anderson BM. 1982. Analogs of pyridine nucleotide coenzymes. In: The Pyridine Nucleotide Coenzymes (Everse J. Anderson BM, You KS, eds), p 91–133. Academic Press, New York.
2) Imai T. 2002. Substrate specificity of mammalian pyridine nucleotide transglycosidases. J Nutr Sci Vitaminol 48: 171–176.
3) Imai T. 1995. Synthesis of pyridine nucleotide analogs using rabbit spleen pyridine nucleotide glycohydrolase and stereospecificity of the transglycosidation reaction. J Biochem 118: 196–203.
4) Imai T, Okuda S, Suzuki S. 1969. Natural occurrence of a new pyridine nucleotide consisting of nicotinamide adenine dinucleotide and adenosine diphosphate ribose joined through a ribosidic link. J Biol Chem 244: 4547–4554.
5) Oppenheimer NJ, Arnold IJ, Kaplan NO. 1971. A structure of pyridine nucleotides in solution. Proc Natl Acad Sci USA 68: 3200–3205.
6) Oppenheimer NJ, Kaplan NO. 1976. Proton magnetic resonance study of the intermolecular association and conformation of the α and β pyridine mononucleotides and nucleosides. Biochemistry 15: 3981–3989.