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A nicotinamide mononucleotide adenylyltransferase with unique adenylyl group donor specificity from a hyperthermophilic archaeon, *Pyrococcus horikoshii* OT-3

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Abstract

A gene encoding a nicotinamide mononucleotide (NMN) adenylyltransferase (NMNAT, EC 2.7.7.1) homologue was identified via genome sequencing in the anaerobic hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3. The gene encoded a protein of 186 amino acids with a molecular weight of 21,391. The deduced amino acid sequence of the gene showed 59% identities to the NMNAT from *Methanococcus jannaschii*. The gene was overexpressed in *Escherichia coli*, and the produced enzyme was purified to homogeneity. Characterization of the enzyme revealed that it is an extremely thermostable NMNAT; the activity was not lost after incubation at 80 °C for 30 min. The native molecular mass was estimated to be 77 kDa. The K_m values for ATP and NMN were calculated to be 0.056 and 0.061 mM, respectively. The optimum temperature of the reaction was estimated to be around 90 °C. The adenylyl group donor specificity was examined by high-performance liquid chromatography (HPLC). At 70 °C, ATP was a prominent donor. However, above 80 °C, a relatively small, but significant, NMNAT activity was detected when ATP was replaced by ADP or AMP in the reaction mixture. To date, an NMNAT that utilizes ADP or AMP as an adenylyl group donor has not been found. The present study provides interesting information in which a di- or mono-phosphate nucleotide can be utilized by adenylyltransferase at high temperature.

Keywords: Nicotinamide mononucleotide adenylyltransferase; *Pyrococcus horikoshii*; NAD biosynthesis; Archaea; Hyperthermophile

1. Introduction

Nicotinamide mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1) catalyzes the synthesis of nicotinamide adenine dinucleotide (NAD) or nicotinic acid dinucleotide (NaAD) from nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN), respectively, by transferring the adenylyl part of ATP and concomitantly releasing pyrophosphate (PP_i) (Fig. 1). Because the reaction represents a step common to both the salvage and the de novo synthesis of NAD, NMNAT is an indispensable enzyme in the NAD biosynthetic pathway [1,2].

NMNAT has been identified, purified, and characterized from several organisms, including archaea, bacteria, and eukarya [1,2]. In contrast to most of the enzymes involved in the NAD biosynthetic pathway, the study on the NMNAT gene has been

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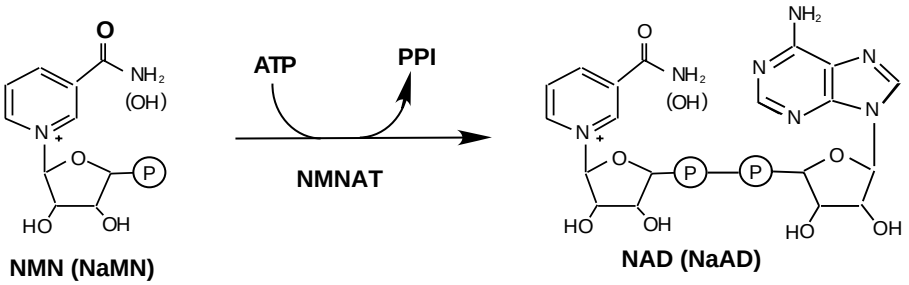

Fig. 1. NMNAT reaction scheme. NMNAT catalyzes the synthesis of nicotinamide adenine dinucleotide (NAD) from nicotinamide mononucleotide (NMN) and nicotinic acid dinucleotide (NaAD) from nicotinic acid mononucleotide (NaMN).

limited. In 1997, the gene encoding NMNAT was first identified in the hyperthermophilic archaeon *Methanococcus jannaschii* [3,4]. Genome analyses of hyperthermophilic archaeal strains, such as *Pyrococcus furiosus*, *P. abyssi*, and *Archaeoglobus fulgidus*, have revealed the presence of putative NMNAT genes on their genomes (the Kyoto Encyclopedia of Genes and Genomes; http://www.genome.ad.jp/kegg/). However, the functionality of those gene products has not been recognized.

Recently, we observed the presence of L-aspartate oxidase in *P. horikoshii* OT-3, an anaerobic hyperthermophilic archaeon that grows optimally at 98 °C [5]. This is the first example of the occurrence of L-aspartate oxidase either in the Archaea or in obligate anaerobic organisms. L-Aspartate oxidase is known to function as the first enzyme in the de novo NAD biosynthetic pathway in prokaryotes. By a similarity search of the database (http://www.genome.ad.jp/kegg/), the genes that encode the homologue of all other enzymes involved in the pathway were identified in the *P. horikoshii* OT-3 genome. Thus, we proposed that the de novo NAD biosynthetic pathway functions in *P. horikoshii* OT-3 under anaerobic conditions [5]. In the course of verifying this hypothesis, we investigated the function of a key enzyme, NMNAT, in the NAD biosynthetic pathway. We performed detailed characterization of the protein product of a gene encoding an NMNAT homologue from *P. horikoshii* OT-3. As a result, we found that the produced enzyme has quite a unique adenylyl group donor specificity at high temperature. We show here that the NMNAT from *P. horikoshii* OT-3 catalyzes a novel type of reaction, the transfer of the adenylyl moiety of ADP or AMP to the phosphoryl group of NMN to form NAD.

2. Materials and methods

2.1. Materials

The *E. coli* strain BL21(DE3)-CodonPlus^{TM}-RIL was obtained from Stratagene (La Jolla, CA). The plasmid DNA pET-11a was obtained from Novagen Inc. (Madison, WI). NMN, NAD, ATP, DNase I, lysozyme and BSA were purchased from Sigma–Aldrich, Japan, K.K. (Osaka, Japan). Alcohol dehydrogenase from yeast was obtained from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Assay for NMNAT activity

The routine reaction mixture, containing 50 mM glycylglycine/NaOH, pH 7.0, 25 mM $MgCl_2$, 0.5 mM ATP, 1.5 mM NMN, and the appropriate amount of the enzyme preparation in a final volume of 400 μl. NAD formed was determined either spectrophotometrically or by high-performance liquid chromatography (HPLC). After incubation for an appropriate time, the reaction was stopped with 50 μl ice-cold $HClO_4$ (30% (w/v)) and the mixture centrifuged (12,000 × *g* for 10 min). A 400 μl portion of the supernatant was neutralized by the addition of 60 μl 2 M K_2CO_3 and centrifuged (12,000 × *g* for 10 min). An aliquot of the supernatant was used for the spectrophotometric determination of NAD using ethanol and yeast alcohol dehydrogenase [3]. For the HPLC-based assay,

the supernatant was passed through a cellulose acetate filter (pore size 0.2 μm, Advantec, Tokyo, Japan), and the amount of NAD formed was determined by HPLC with a TSK gel ODS-120 Ts column (4.6 mm × 150 mm, TOSOH). Buffer A (50 mM potassium phosphate buffer, pH 6.5) and buffer B (buffer A containing 5 mM sodium 1-octanesulfonate and 25% methanol) were used as the mobile phase as follows: (i) elution with 100% buffer A for the first 8 min, (ii) linear gradient elution between buffer A and B for the next 3.5 min, and then (iii) elution with 100% buffer B for 5 min. The flow rate was 1.0 ml/min. The effluent from the column was monitored by a UV detector at a wavelength of 254 nm. One unit of activity was defined as the amount of enzyme catalyzing the synthesis of 1 μmol of NAD per minute. The spectrophotometric assay was routinely used throughout the experiments, and the reaction was carried out at 70 °C, unless otherwise noted. The protein concentration was determined by the Bradford method [6] using BSA as a standard.

2.3. Cloning and expression of gene

The complete sequence of the *P. horikoshii* OT-3 genome has been reported by Kawarabayasi et al. [7]. The gene, which is homologous to that of the *M. jannaschii* NMNAT, was found by a BLAST search [8]. The plasmid DNA (p2219: position 423386–425422 on the entire genome of *P. horikoshii* OT-3 has been inserted into the *Hinc*II site of pUC118) containing an open reading frame of the gene (ORF ID: PH0464, position 424245–424805 on the entire genome) which was prepared from the fosmid clone as previously described [7]. For construction of the expression plasmid, a 560-bp gene fragment, which consisted of the structural gene of the *P. horikoshii* OT-3 NMNAT homologue, and *Nde*I and *Bam*HI linkers were amplified by PCR with the following two primers. The first was designed to contain the N-terminal region of the NMNAT gene and the *Nde*I digestion sequence (5′-TCTCCATATGATAAGGGACTCTTCGT-3′), and the second was to contain the C-terminal region and the *Bam*HI digestion sequence (5′-GAGAGGATCCCTAATACTCGGGAACC-3′). The p2219 was used as the template. The amplified 560-bp fragment was digested with *Nde*I and *Bam*HI and ligated with the expression vector pET-11a linearized with *Nde*I and *Bam*HI to generate pNMNAT. *E. coli* strain BL21(DE3)-CodonPlus^{TM}-RIL cells were transformed by the recombinant plasmids and plated on Luria–Bertani medium with ampicillin (50 μg/ml).

2.4. Overexpression and purification of recombinant protein

The transformant *E. coli* was cultivated at 37 °C in 7 l of medium containing bacto tryptone (12 g/l), yeast extract (24 g/l), glycerol (5 ml/l), K_2HPO_4 (12.5 g/l), KH_2PO_4 (3.8 g/l), and ampicillin (50 μg/ml) until the optical density at 600 nm reached 0.6. The induction was carried out by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the medium and the cultivation was continued for 4 h. The cells were harvested and washed twice with 0.85% NaCl solution. The washed cells were suspended in 50 mM glycylglycine/NaOH buffer (pH 7.0) containing 0.5 M NaCl. After incubation at 37 °C for 30 min with stirring in the presence of lysozyme (1 mg/ml) and DNase (0.1 mg/ml), the cells were disrupted by ultrasonication. The cell debris was removed by centrifugation (15,000 × *g* for 10 min), and the supernatant solution was used as the crude extract. The enzyme solution was heated at 80 °C for 20 min. Denatured proteins were separated by centrifugation (15,000 × *g* for 10 min). The resulting supernatant was dialyzed against 50 mM potassium phosphate buffer (pH 6.5). The enzyme solution was placed on a Red-Sepharose CL-4B column (2.6 cm × 10 cm; Pharmacia) equilibrated with 50 mM potassium phosphate buffer (pH 6.5). After washing with 200 ml of the same buffer, protein was eluted with a 460 ml linear gradient of 0–2 M NaCl in the same buffer. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 10 mM potassium phosphate buffer (pH 6.5). The enzyme solution was placed on a hydroxyapatite column (2 cm × 10 cm) (GIGAPITE K-100, Seikagaku Kogyo, Japan) equilibrated with the 10 mM potassium phosphate buffer (pH 6.5). After washing with 300 ml of the same buffer, the enzyme was eluted with a 600 ml linear gradient of 10–600 mM potassium phosphate buffer (pH 6.5). The active fractions were pooled, dialyzed against 50 mM glycylglycine/NaOH buffer (pH 7.0), and used as the purified enzyme preparation.

2.5. Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE, 12% acrylamide slab gel, 1 mm thick) was performed by the procedure of Laemmli [9]. The protein band was stained with Coomassie brilliant blue R-250.

2.6. N-terminal amino acid sequencing

Approximately 2.5 μg of the protein was subjected to SDS-PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane. The membrane was then stained with Ponceau S and destained. A protein band was excised and subjected to automated Edman degradation using the Shimadzu Model PPSQ-10 protein sequencer.

2.7. Molecular mass determinations

The molecular mass of the native enzyme was determined by gel filtration on a Sephadex G-200 column (2.6 cm × 60 cm; Pharmacia) equilibrated with 50 mM potassium phosphate buffer (pH 6.5) containing 0.5 M NaCl. Gel filtration markers (Bio-Rad) were used as the molecular mass standards. The subunit molecular mass of the purified enzyme was determined by SDS-PAGE using eight marker proteins (New England BioLabs).

2.8. Stability, pH optima and kinetic parameters

To determine thermostability, the enzyme (0.07 mg/ml) in 50 mM glycylglycine/NaOH buffer (pH 7.0) was incubated at different temperatures, and the residual activity was determined by the standard assay method. The optimal pH of the enzyme was determined by running the standard assay at 70 °C using sodium acetate buffer (50 mM), potassium phosphate buffer (50 mM), glycylglycine/NaOH buffer (50 mM), and glycine/NaOH buffer (50 mM) for pH ranges of 4–5.5, 6–8, 7–9 and 8.5–10, respectively. The Michaelis constants were determined from Lineweaver–Burk plots [10] of data obtained from the initial rate of NAD production at 70 °C.

2.9. Metal ion requirement and substrate specificity

The metal ion requirement was tested by the addition of 10 mM $MgCl_2$, $CoCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, or $CaCl_2$ to the standard assay mixture for the spectrophotometric assay. Adenylyl group donor specificity was examined by HPLC. ADP, GDP, CDP, UDP, IDP, GTP, CTP, ITP, AMP, IMP, CMP, TTP, and UTP (each 0.5 mM) were used instead of ATP in the standard assay mixture for HPLC.

3. Results and discussion

3.1. Expression of the gene and purification of the recombinant enzyme

In the *P. horikoshii* entire genome sequence, we found a gene, PH0464 (560 bp), whose predicted amino acid sequence showed a 59% identity with the *M. jannaschii* NMNAT [3] (Fig. 2). We performed cloning and expression of the PH0464 gene in *E. coli* cells. The transformant *E. coli* produced some extent of the inclusion body of the enzyme. Nevertheless, a high level of NMNAT activity still remained in the supernatant solution after removing the cell debris by centrifugation. The enzyme was purified from the supernatant by three steps involving heat treatment and two chromatographies. About 3.3 mg of the purified enzyme was obtained from the cells of the seven L-culture medium (40 g wet weight). The purified enzyme showed a single protein band on SDS-PAGE (data not shown). The specific activity of the purified enzyme was 14.9 units/mg. The N-terminal sequence of the purified enzyme was determined to be M-I-R-G-L-F-V-G-R-F-Q-P-V-H-K-G-H-I-K-A. The N-terminal amino acid sequence corresponded to that predicted from the PH0464 gene sequence. This shows that the PH0464 gene codes the *P. horikoshii* NMNAT.

3.2. Molecular and catalytic properties

The subunit molecular mass was determined to be about 21 kDa and was consistent with the molecular weight (21,392) calculated from the amino acid sequence. The native molecular mass of the enzyme determined by gel filtration was about 77 kDa; this indicates that the enzyme is trimeric or tetrameric. Multiple oligomeric structures such as trimeric, tetrameric, and hexameric forms have been observed for NMNATs from bacteria and eukarya [1]. The

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P. horikoshii     1 MIRGLF-VGRFQPVHKGHIKALEF--VFSQ-VDEVIIGIGSAQASHTLKNPFTTGERMEM    56
M. jannaschii     1 M-RG-FIIGRFQPFHKGH---LEVIKKIAEEVDEIIIGIGSAQKSHTLENPFTAGER-IL    54
                    * ** *  ***** ****   **        *** ******** **** **** ***

P. horikoshii    57 LIR-ALEEAGFDKR-Y--YLIPLPDINFNAIWVPYVESMVPRFHVVFTGNSLVAQ-LFKE   111
M. jannaschii    55 MITQSLK--DY--DLTYYP-IPIKDIEFNSIWVSYVESLTPPFDIVYSGNPLV-RVLFEE   108
                     *   *             **  ** ** *** **** * *  *  ** **   ** *

P. horikoshii   112 RGYKVVVQ-P-MFK-KDILSATEIRRRMIAGEPWEDLVPKSVVEYIKEIKGVERLRNLAT   168
M. jannaschii   109 RGYE--VKRPEMFNRKE-YSGTEIRRRMLNGEKWEHLVPKAVVDVIKEIKGVERLRKL--   163
                    ***   *  * **  *   * *******  ** ** **** **  *********** *

P. horikoshii   169 NLESSEKELQAPIRVPEY                                           186
M. jannaschii   164 -AQT-DK-----------                                           168
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Fig. 2. Alignment of the amino acid sequence of NMNAT from *P. horikoshii* and *M. jannaschii*. Asterisks indicate conserved residues between the two enzymes. The sequence determined by N-terminal sequence analysis is underlined.

enzyme from thermophilic archaeon *Sulfolobus solfataricus* has been reported to form a trimeric or tetrameric structure [3]. The NMNAT from the hyperthermophilic archaeon *Methanococcus jannaschii* has a hexameric structure [11]. Thus, NMNATs from archaea may possess multiple oligomeric structures as do the enzymes from bacteria and eukarya.

The effect of pH on activity was examined, and the highest activity was observed around pH 6.5. We examined the enzyme activity at temperatures from 37 to 100 °C, and the optimum temperature was about 90 °C. The enzyme was highly thermostable: upon heating at 80 °C for 30 min, the enzyme retained its full activity. The enzyme lost 20 and 40% of its activity after 30 and 60 min incubation at 90 °C, respectively. Complete inactivation was observed upon incubation at 100 °C for 100 min. The enzyme was stable over a wide range of pH; on heating at 70 °C for 30 min, the enzyme did not lose activity at pH 5.5–8.5. The enzyme required divalent cations for the activity. $MgCl_2$ was the most effective additive, and it could be replaced by $CoCl_2$, $ZnCl_2$, $CuCl_2$, and $MnCl_2$ to some extent (Table 1). The ability of the *P. horikoshii* NMNAT for the utilization of various tri-, di-, or mono-phosphate nucleotides was examined using the standard assay by HPLC. Under the standard assay conditions (70 °C for 10 min), the enzyme acted exclusively on ATP. The following substrates were inert: ADP, GDP, CDP, UDP, IDP, GTP, CTP, ITP, AMP, IMP, CMP, TTP, and UTP. In contrast to the NMNATs from *M. jannaschii* and *S. solfataricus* [3], the *P. horikoshii* enzyme exhibited a linear kinetic behavior. *Km* values for ATP and NMN were determined to be 0.056 and 0.061 mM, respectively.

3.3. Adenylyl donor specificity

The ability of the *P. horikoshii* NMNAT for the transfer of the adenylyl moiety of ADP or AMP to NMN was examined by HPLC. Fig. 3 shows the elution patterns of the substrates and the reaction product. NMN, ATP, ADP, AMP, and NAD were separated on the column at retention times of about 2.4, 3.9, 4.5, 6.4, and 11.7 min, respectively (Fig. 3A). Fig. 3B clearly indicates the formation of NAD and the decrease in the amount of ATP and NMN after incubation of the standard assay mixture for 10 min at 70 °C. To our surprise, the formation of NAD was also observed when ATP was replaced by ADP or AMP. Fig. 3C and D,

Table 1
Divalent cation requirement

Divalent cation (10 mM)	Relative activity (%)
Mg^{2+}	100
Co^{2+}	51
Mn^{2+}	8
Cu^{2+}	28
Zn^{2}	30
Ca^{2+}	0
None	0

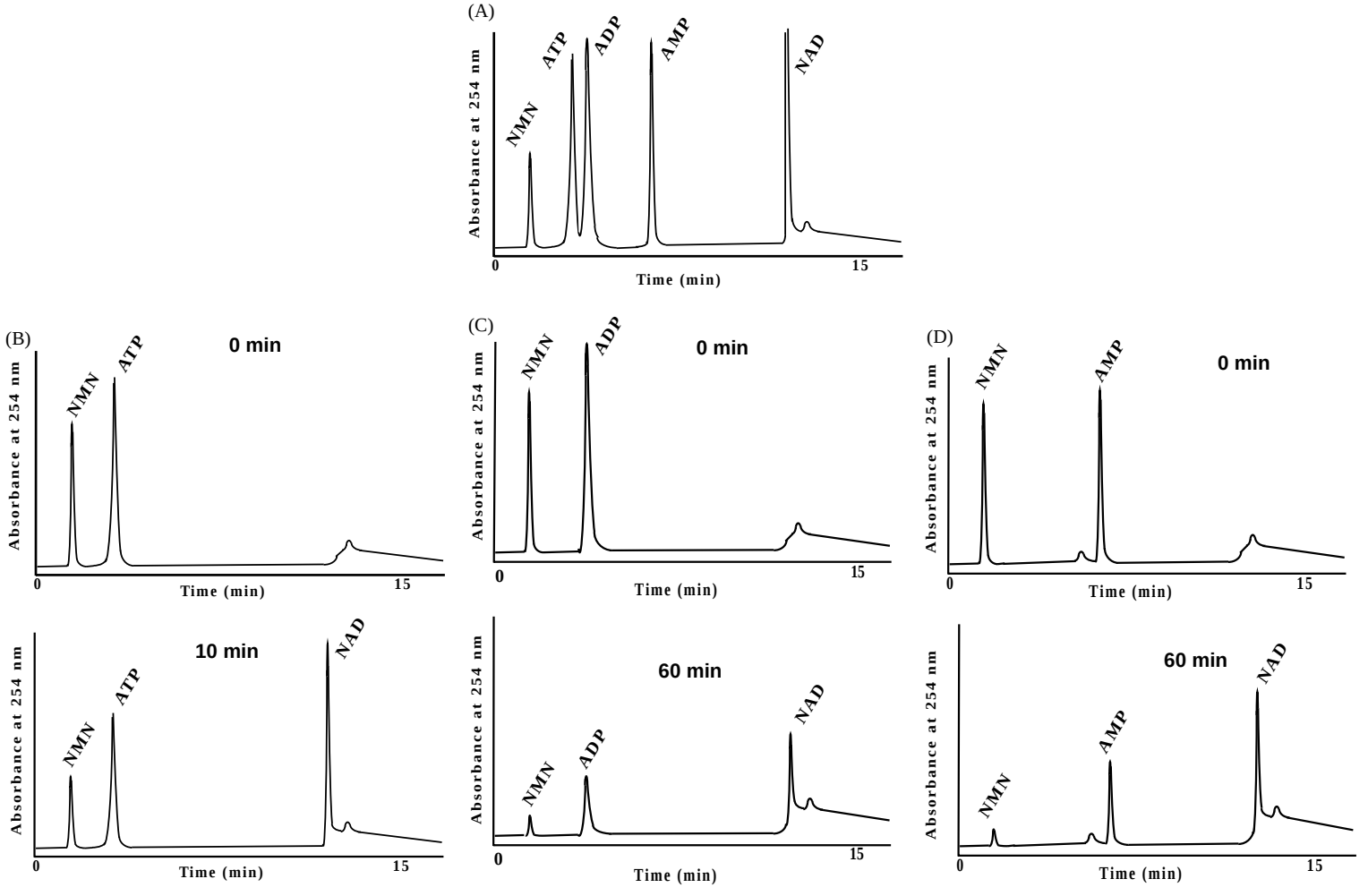

Fig. 3. HPLC analysis of the NMNAT reaction. (A) Separation of a standard mixture of NMN, ATP, ADP, AMP, and NAD (5 nmol each). (B) Elution profiles of the standard assay mixture. ATP was used as an adenylyl group donor, and the reaction was carried out at 70 °C for 10 min. (C) Elution profiles of the reaction mixture in which ADP was used as an adenylyl group donor. The reaction was carried out at 90 °C for 60 min. (D) Elution profiles of the reaction mixture in which AMP was used as an adenylyl group donor. The reaction was carried out at 90 °C for 60 min.

respectively represent elution profiles of the reaction mixture in which ADP and AMP were used as adenylyl group donors. The reaction was carried out at 90 °C for 60 min. In both cases, the retention time of the reaction product coincided with that of authentic NAD. When these samples were injected with NAD, an enhancement of the peak was observed. The relative activities for ADP and AMP were estimated to be 17.5 and 50.6%, respectively, compared with that for ATP under the same conditions. The formation of NAD from ADP or AMP was prominently observed at a reaction temperature higher than 80 °C. These results indicate that the *P. horikoshii* NMNAT catalyzes the transfer reaction of the adenylyl moiety of ADP or AMP to the phosphoryl group of NMN at a high temperature. An NMNAT that utilizes ADP or AMP as an adenylyl group donor has not been reported so far. The present study provides interesting information in which a di- or mono-phosphate nucleotide can be utilized by adenylyltransferase, and the information may be useful for the development of novel applications of the enzyme.

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