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Nicotinamide Nucleoside Amidase Activity, a Novel Deamidating Reaction in NAD Metabolism, in *Aspergillus* Fungi*

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Cell-free extracts of *Aspergillus niger*, *A. awamori*, *A. usamii*, *A. oryzae* and *A. flavus* showed nicotinamide nucleoside amidase activity which hydrolyzes nicotinamide ribonucleoside to form nicotinic acid ribonucleoside. This enzyme was partially purified from the cell-free extract of *A. niger*. The enzyme was specific towards nicotinamide ribonucleoside and the K_m value for nicotinamide ribonucleoside was calculated to be 3.6 mM. The optimal pH of the deamidation of nicotinamide ribonucleoside was 5.0 to 5.5. Nicotinamide, NMN, NAD and other nicotinamide derivatives tested were inert as substrates, indicating the unsimilarity of this enzyme to nicotinamidase and NMN deamidase. Nicotinamide nucleoside amidase activity was found in several strains other than *Aspergillus* fungi.

The authors reported previously that both *niger* and *oryzae-flavus* groups of *Aspergillus* fungi accumulated nicotinic acid ribonucleoside extracellularly and intracellularly when they were grown in a radioactive nicotinic acid-supplemented medium.[1,2] One of the precursors of this nucleoside in *A. niger* was found to be NMN.[1] In this conversion, nicotinamide ribonucleoside, which is formed by the dephosphorylation of NMN, might be deamidated to give rise to nicotinic acid ribonucleoside. Studies on microbial deamidation of nicotinamide derivatives have been confined to nicotinamide[3] and NMN.[4] This paper deals with a novel deamidase reaction concerning the conversion of NAmR to NAR in *Aspergillus* and other fungal strains.

Materials and Methods

Materials NAD, NADP and NMN were the products of Sigma. Nicotinamide-*N*-oxide, 6-aminonicotinamide and *N*-methyl nicotinamide were obtained from Aldrich. Deamido-NAD, nicotinic acid mononucleotide, nicotinamide ribonucleoside and nicotinic acid ribonucleoside were prepared enzymatically as previously described.[2,5] DEAE-cellulose and Sephadex G-200 were the products of Whatman and Pharmacia, respectively. Hydroxyl apatite was obtained from Seikagaku Kogyo.

Organisms and culture Fungal strains were provided by Professor S. Toyama, the Department of Agricultural Chemistry, Ryukyu University, Professor T. Tochikura, the Department of Food Science and Technology, Kyoto University. These strains were grown in a glucose-peptone medium as reported previously[2] on a rotary shaker at 28°C.

Preparation of cell-free extract Mycelia were harvested from the culture after 36 to 48-h incubation by filtration and washed with cold water. The mycelial mat was ground in a chilled mortar with sea sand and 0.05 M potassium phosphate buffer (pH 7.2). Disrupted mycelia were removed by centrifugation at $9600 \times g$. The supernatant solution obtained was dialyzed against 0.01 M phosphate buffer (pH 7.2) for use as the enzyme preparation.

Enzyme assay The standard assay mixture for nicotinamide nucleoside amidase contained 0.8 μmol of nicotinamide ribonucleoside, 10 μmol of acetate buffer (pH 5.0) and enzyme solution in a final volume of 0.1 ml. Incubation was done at 37°C. After reaction for 1.5 to 3 h, an aliquot (0.03 ml) of the mixture was applied on a Toyo filter paper no. 53 and chromatographed using isobutyric acid: ammonium hydroxide (28%): water (66 : 1.7 : 33, v/v) (Solvent A). The portion corresponding to nicotinic acid ribonucleoside (R_f=0.50) was extracted with 5 ml of 0.01 N HCl at 37°C for 3 h and the amount was calculated based on the absorbance at 266 nm using

* Metabolism of Pyridine Coenzymes in *Aspergillus* Fungi (II)

the ε value of 4.3×10^3.[1] One unit of the activity was defined as the amount of enzyme which produced 1 μmol of nicotinic acid ribonucleoside per h. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.*[6] or by A_{280} measurements on chromatographic separation of the enzyme preparation. Ammonia formed in the reaction mixture was determined by an enzymatic assay using bovine liver glutamic dehydrogenase (Sigma). Nicotinamidase activity was assayed using a reaction mixture containing 8 μmol of nicotinamide, 100 μmol of an appropriate buffer and enzyme preparation in a total volume of 1.0 ml. After reaction for 1.5 to 3 h at 37°C, an aliquot (0.05 ml) was applied on a filter paper no. 53 and chromatographed using *n*-butanol saturated with 3% ammonia (Solvent B). The portion containing nicotinic acid (R_f=0.18) was extracted from the chromatogram with 0.01 N HCl for 3 h at 37°C and absorption at 260 nm was measured. The amount of nicotinic acid was calculated from the calibration curve. Formation of nicotinic acid ribonucleoside from NMN or nicotinic acid mononucleotide was demonstrated using a reaction mixture containing 4 μmol of NMN or nicotinic acid mononucleotide, 50 μmol of acetate buffer (pH 5.0) and cell-free extract in a total volume of 0.5 ml. After reaction for 1.5 to 3 h at 37°C, an aliquot (0.05 ml) of the mixture was chromatographed using Solvent A. The formation of nicotinic acid ribonucleoside on a paper chromatogram was demonstrated by the UV absorption spectrum of the extract of the nicotinic acid ribonucleoside portion.

Results

Formation of nicotinic acid ribonucleoside from nicotinamide ribonucleoside by cell-free extracts of *Aspergillus* fungi

When nicotinamide ribonucleoside was incubated with the dialyzed cell-free extract of *A. niger* (AKU 3302), *A. awamori* (3144), *A. usamii* (3205) or *A. oryzae* (AKU 3301) at pH 5.0, the formation of a reaction product was detected by paper chromatography; the R_f value of the product was approximately 0.50 in Solvent A. This product was isolated from the reaction mixture of *A. niger* by paper chromatography and column chromatography as described previously.[1] The R_f values of the compound in paper chromatography using three different solvent systems, Solvents A and B and ammonium acetate (1 M, pH 5.0):ethanol (3 : 7, v/v) (Solvent C), and the elution properties in Dowex 1×2 (formate) column chromatography were identical with those of nicotinic acid ribonucleoside obtained from NMN. The absorption maximum of the compound in 0.01 N HCl was 266 nm and the addition of KCN gave rise to maximal absorption at 315 nm. These results indicated that the reaction product from nicotinamide ribonucleoside was identical with nicotinic acid ribonucleoside, which was produced from NMN by the cell-free extract of *A. niger* (AKU 3302).[1]

Formation of nicotinic acid ribonucleoside from nicotinamide ribonucleoside suggested the presence of a deamidase activity which is capable of acting on nicotinamide ribonucleoside in *Aspergillus* fungi. The cell-free extract of *A. niger* contained nicotinamidase, whose activity was maximal at pH 8.5, whereas the activity of formation of nicotinic acid ribonucleoside was maximal around

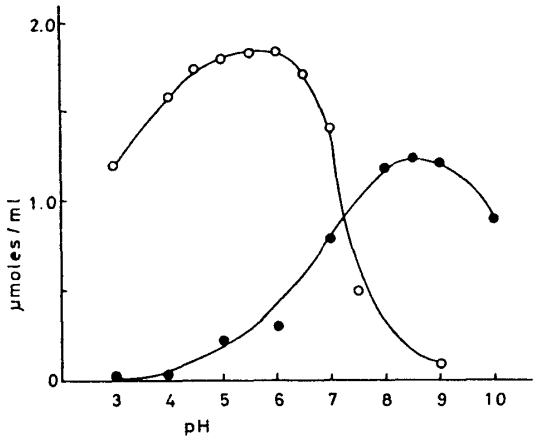

Fig. 1. Nicotinamidase and nicotinamide nucleoside amidase activities of *A. niger* (AKU 3302).
—○—, Nicotinamide nucleoside amidase activity (formation of nicotinic acid ribonucleoside from nicotinamide ribonucleoside).
—●—, Nicotinamidase activity (formation of nicotinic acid from nicotinamide).
A dialyzed solution of precipitated protein, obtained from a cell-free extract to which was added ammonium sulfate to 65 to 95% saturation, was used as an enzyme preparation. Protein was added to a reaction mixture (see *Materials and Methods*) at a concentration of 2.7 mg in a total volume of 1.0 ml. The reaction was carried out at 37°C for 1 h. The following buffers were used: glycine-HCl (pH 3.0), acetate (pH 4.0 to 5.5), potassium phosphate (pH 5.5 to 7.5), Tris-HCl (pH 7.5 to 9.0) and glycine-NaOH (pH 10.0).

pH 5.5 (Fig. 1). This result indicated that deamidation of nicotinamide ribonucleoside was catalyzed by an enzyme which was distinct from the known nicotinamidase.[3]

Cell-free extracts of *A. awamori* (3144), *A. usamii* (3205), *A. oryzae* (AKU 3301) and *A. flavus* (1179) formed nicotinic acid ribonucleoside from NMN at pH 5.5 as in the case of *A. niger* (AKU 3302). These enzyme preparations also accumulated nicotinic acid ribonucleoside from nicotinic acid mononucleotide; nicotinic acid mononucleotide might be subjected to the degradation by acid phosphatase to form nicotinic acid ribonucleoside. The presense of nicotinic acid ribonucleoside in the mixture was demonstrated by paper chromatography; the R_f values of the products were approximately 0.05, 0.04 and 0.82 with Solvents A, B and C, respectively. The absorption maximum of the product extracted from the chromatograms was 266 nm, indicating the identity of the product with nicotinic acid ribonucleoside.

Partial purification of nicotinamide-nucleoside amidase Purification of the enzyme of *A. niger* (AKU 3302) was attempted. All steps were carried out at 0–5°C and the buffers contained 10 mM 2-mercaptoethanol.

Mycelia of a 2-day culture were ground in a chilled mortar with sea sand and 25 mM Tris-HCl buffer (pH 7.2). Cell debris was removed by centrifugation at $17,000 \times g$ and the supernatant solution was obtained [Step 1].

One and five ml of 1% protamine sulfate solution (pH 7.0) per 100 mg of protein was added to the crude extract with stirring. After 30 min, the precipitate was removed by centrifugation and the supernatant solution obtained was dialyzed against 25 mM Tris-HCl buffer (pH 7.2) [Step 2].

The enzyme solution was applied to a DEAE-cellulose column (3.0×27 cm) equilibrated with the dialysis buffer. After washing with the same buffer, the enzyme was eluted with stepwise increment of KCl concentration in the buffer. The active fraction was obtained on elution with 0.15 M KCl. These fractions were pooled and concentrated about 8-fold in an Amicon ultrafiltration cell equipped with a ultrafiltration membrane (Diaflo pM 30). This concentrate was dialyzed against 25 mM Tris-HCl buffer (pH 7.2) [Step 3].

The enzyme solution was applied to a DEAE-cellulose column (2.0×40 cm) equilibrated with the dialysis buffer. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of from 0 to 0.3 M KCl. The active fractions were pooled and concentrated about 12-fold by ultrafiltration. The concentrate was dialyzed against 1 mM potassium phosphate buffer (pH 7.2) [Step 4].

The enzyme solution was applied to a hydroxyl apatite column (1.0×13 cm) equilibrated with the same buffer. After the column was washed with the same buffer, the enzyme was eluted with 10 mM potassium phosphate buffer (pH 7.2). The active fractions were pooled and concentrated about 5-fold by ultrafiltration. The concentrate was dialyzed against 25 mM Tris-HCl buffer (pH 7.2) [Step 5].

The enzyme solution was applied to a Sephadex G-200 column (1.4×73 cm) equilibrated with the dialysis buffer. The enzyme fractions eluted with the same buffer were pooled and concentrated about 4-fold by ultrafiltration [Step 6].

Table 1 summarizes the yields and specific activities during a purification procedure. Approximately 162-fold partial purification was achieved with a yield of 24%. Further purification was unsuccessful because of the unstability of the enzyme activity. Addition of SH reagents or the substrate nicotinamide ribonucleoside and other nicotinic acid derivatives to the enzyme solution was unsuccessful for improving the stability of the enzyme activity. Disc gel electrophoresis indicated that the enzyme preparation of Step 6 contained at least 5 protein components showing different mobilities. Nicotinamidase activity was removed completely from nicotinamidenucleoside amidase activity at Step 4. Acid phosphatase activity, assayed using *p*-nitrophenyl phosphate as a substrate,[7]

Table 1. Partial purification nicotinamide nucleoside amidase from *A. niger* (AKU 3302).

Step	Total protein (mg)	Total units	Specific activity (units/mg)	Yield %
1. Crude extract	1672	234	0.14	100
2. Protamine treatment	634	237	0.37	101
3. First DEAE-cellulose chromatography	139	185	1.33	79
4. Second DEAE-cellulose chromatography	39	137	3.82	59
5. Hydroxyl apatite chromatography	16	108	6.93	46
6. Sephadex G-200 chromatography	2.5	56	22.70	24

of the enzyme of Step 6 was found to be less than 0.1% of that in the original cell-free extract.

Properties of nicotinamide nucleoside amidase The deamidation of nicotinamide ribonucleoside proceeded stoichiometrically with the formation of nicotinic acid ribonucleoside and ammonia. The enzyme showed maximum reactivity in the pH range of 5.0 to 5.5. The activity was stable from pH 5.0 to 6.0 on 30 min incubation at 37°C. The maximal reaction rate was obtained at 45°C, approximately 1.6 times higher than that at 37°C. The activity was specific toward nicotinamide ribonucleoside. Deamidation products of NMN, NAD, NADP, nicotinamide-*N*-oxide, 6-aminonicotinamide and *N*-methyl nicotinamide were not detected by paper chromatography. The K_m value for nicotinamide ribonucleoside was calculated to be 3.6 mM from a Lineweaver-Burk plot of the reaction. $HgCl_2$ at a concentration of 1 mM caused 52% inhibition and other divalent ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+}) did not affect the enzyme activity. *p*-Hydroxymercurobenzoate caused 26% inhibition at a concentration of 1 mM. Sodium ethylenediaminetetraacetate, sodium fluoride, sodium arsenate, sodium pyrophosphate, iodoacetic acid and urea, each at 1 mM, did not inhibit the activity.

Table 2. Distribution of nicotinamide nucleoside amidase activity in *Aspergillus* and other fungi.

Strain	Specific activity (units/mg)
Aspergillus niger (AKU 3302)	0.31
Aspergillus niger (3127)	0.32
Aspergillus niger (3123)	0.16
Aspergillus awamori (3144)	0.54
Aspergillus awamori (3146)	trace
Aspergillus awamori (3149)	0.07
Aspergillus usamii (3200)	trace
Aspergillus usamii (3203)	0.16
Aspergillus usamii (3204)	0.45
Aspergillus usamii (3205)	0.34
Aspergillus oryzae (AKU 3301)	0.25
Aspergillus oryzae (1104)	0.08
Aspergillus oryzae (1116)	0.15
Aspergillus oryzae (1151)	0.29
Aspergillus oryzae (1311)	0.09
Aspergillus flavus (0433)	0.10
Aspergillus flavus (1179)	0.33
Aspergillus flavus (1406)	trace
Aspergillus flavus (1424)	0.10
Penicillium chrysogenum (AKU 3407)	trace
Corticium rolfsii	0.40
Absidia orchidis (AKU 3153)	0
Neurospora tetrasperma (AKU 3559)	0
Gliocladium deliquescens (AKU 3835)	0.64
Fusarium oxysporum (AKU 3702)	0.11
Rhizopus oryzae (2231)	0.16
Rhizopus chinensis (AKU 3102)	0
Rhizopus japonicus (AKU 3107)	0
Mucor javanicus (AKU 3009)	0
Mucor racemosus (AKU 3002)	0

Distribution of nicotinamidenucleoside amidase activity in fungi Fungal strains were tested for the activity of nicotinamide nucleoside amidase. As shown in Table 2, many strains of *Aspergillus* fungi showed the enzyme activity. The activity was also found in several fungi other than *Aspergillus*.

Discussion

The enzyme system which is involved in the formation of nicotinic acid ribonucleoside

by *Aspergillus* was investigated in this study. As reported previously, growing mycelia of *A. niger, A. awamori, A. usamii, A. oryzae* and *A. flavus* accumulated nicotinic acid ribonucleoside as a metabolite of nicotinic acid.[1,2] NMN was shown to be one of the precursors of nicotinic acid ribonucleoside using cell-free extracts of *A. niger*[1] and other *Aspergillus* fungi. Direct formation of nicotinic acid ribonucleoside from nicotinamide ribonucleoside was also demonstrated in these fungi in this experiment. These results suggested the presence of the following metabolic sequence leading to nicotinic acid ribonucleoside: NMN→ nicotinamide ribonucleoside→nicotinic acid ribonucleoside. Besides NMN, nicotinic acid mononucleotide was also a substrate for the formation of nicotinic acid ribonucleoside; intense activity of acid phosphatase was thought to be involved in the dephosphorylation of nicotinic acid mononucleotide.

In the conversion of nicotinamide ribonucleoside to nicotinic acid ribonucleoside, a sort of deamidase (amidase) was supposed to be involved. The first candidate is nicotinamidase, which participates essentially in the first step of NAD synthesis from nicotinamide by deamidation of this precursor to nicotinic acid. Since this enzyme was first demonstrated in lactic acid bacteria,[8] the enzyme has been identified in several microorganisms.[9-11] The fungal enzyme was first found in *A. niger*.[3] However, the partially purified enzyme preparation was specific towards nicotinamide and did not deamidate NAD or other pyridine nucleotides. NMN deamidase found in *Propionibacterium shermanii*[4] is the only example of the deamidation at the nucleotide level, and specified by the activity towards NMN. Although the enzyme was inactive on free nicotinamide and NAD, it was not clarified if this enzyme deamidated nicotinamide ribonucleoside or not. Because the activity of NMN deamidase was not detected in *A. niger*, the possibility of the presence of the metabolic sequence NMN →nicotinic acid mononucleotide→nicotinic acid ribonucleoside can be excluded.

Our experiment showed the presence of

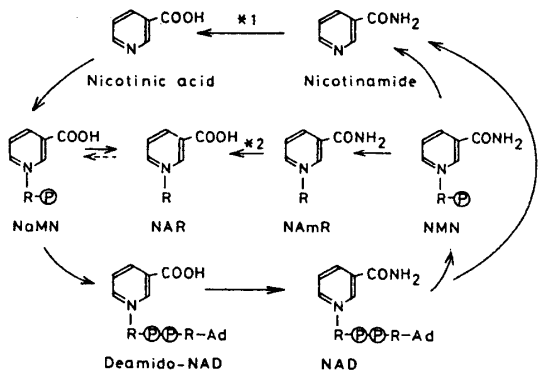

Fig. 2. Formation of nicotinic acid ribonucleoside in the nicotinic acid metabolism of *Aspergillus* fungi.
The abbreviations: NaMN, nicotinic acid mononucleotide; NAmR, nicotinamide ribonucleoside; NAR, nicotinic acid ribonucleoside; R, ribosyl moiety; Ⓟ, phosphoryl moiety; Ad, adenine.
*1 nicotinamidase
*2 nicotinamide nucleoside amidase

the third type of deamidation in the nicotinamide metabolism of microorganisms concerning the conversion of nicotinamide ribonucleoside to nicotinic acid ribonucleoside. During the purification procedure of the enzyme from mycelium of *A. niger*, nicotinamidase activity was separated completely from nicotinamide nucleoside amidase. The partially purified enzyme preparation was specific towards nicotinamide ribonucleoside, whereas nicotinamide, NAD and other pyridine nucleotides tested were inert as substrates. As shown in Table 2, this enzyme is thought to be widely distributed in fungi.

It is supposed from this experiment that a kind of pyridine nucleotide cycle, in which nicotinic acid ribonucleoside is involved, is operating in *Aspergillus* fungi as shown in Fig. 2. It is known that *Escherichia coli*[12] and *Salmonella typhimurium*[13] possess an NAD cycle in which nicotinamide formed from NAD by the action of NAD glycohydrolase is reutilized *via* a salvage pathway. *P. shermanii* was suggested to possess another type of NAD cycle in which deamidation of NMN to nicotinic acid mononucleotide was involved.[4] It is necessary to demonstrate the presence of the activity to phosphorylate nicotinic acid ribonucleoside to nicotinic acid mononucleotide for the completion of

the novel NAD cycle in *Aspergillus* fungi. However, attempts to show regeneration of nicotinic acid mononucleotide from nicotinic acid ribonucleoside have been unsuccessful so far.

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