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Formation of pyridine nucleotides under symbiotic and non-symbiotic conditions between soybean nodules and free-living rhizobia

Takafumi Tezuka[a,*], Yuko Murayama[b]

[a]*Graduate School of Human Informatics, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan*
[b]*Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan*

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Abstract

Enzymatic regulation of pyricline nucleotide formation, under symbiotic and non-symbiotic conditions, was analyzed using soybeans (*Glycine max* L. cv. 'Akisengoku') and rhizobia (*Bradyrhizobia japonicum* strain A1017), respectively. It was found that levels of pyridine nucleotides in bacteroids in root nodules were different from those in free-living cells of rhizobia. This difference was associated with differences in activities of enzymes involved in the pathway from L-tryptophan to NAD and NADP. That is, these activities were lower in bacteroids than in free-living bacteria and lower in the nodule cytosol than in root extracts. The optimum pH for NAD synthetase in bacteroids, was 9.0. Additionally, the optimum pH for ATP-nicotinamide mononucleotide (NMN) adenyltransferase, final step enzyme in NAD formation, was estimated to be 7.6. In the bacteroid fraction, the K_m of NAD synthetase (22 μM) was ~1/22 of that of ATP-NMN adenyltransferase (482 μM). Vmax values were estimated to be almost in the same order for both NAD synthetase and ATP-NMN adenyltransferase. This is the first report on the formation of pyridine nucleotides originating from L-tryptophan in bacteroids in soybean nodules and free-living bacteria.

Keywords: *Bradyrhizobium japonicum*; *Glycine max*; Leguminosae; ATP-nicotinamide mononucleotide (NMN) adenyltransferase; Bacteroids; Free-living rhizobia; NAD synthetase; Nodules; Pyridine nucleotides [NAD)(H)/NADP(H)]

1. Introduction

Leguminous plants have nodules, a symbiotic system between rhizobia and root tissues. The nodules are composed of bacteroid zones and cortex. The bacteroid zone is mostly occupied by transforming bacteria derived from rhizobia (Ching and Hedtke, l977; Vasse et al., 1990) and bacteroids, with nitrogen being fixed by the latter (Peterson and LaRue, 1981). In the nitrogen fixation system, both NAD and NADP are essential receptors of reducing power arising from substrates such as sugars, organic acids, aldehydes and alcohols (Peterson and LaRue, 1982; LaRue et al., 1984). In this study, the formation of NAD and NADP was investigated to attempt to understand the involvement of bacteroids in nodules in the symbiotic system between soybean roots and rhizobia.

According to Nishizuka and Hayaishi (1971), tryptophan serves as a precursor of NAD in animals and in certain microorganisms, such as *Neurospora crassa* and *Xanthomonas pruni*. Magni et al. (1999) described the involvement of quinolinate phosphoribosyltransferase and ATP-NMN adenyltransferase in pyridine nudeotide metabolism, beginning with a detailed consideration of the anaerobic and aerobic pathways leading to quinolinate, a key precursor of NAD in animals. NAD synthetase, as ubiquitous enzyme catalyzing the last step in the biosynthesis of NAD from deamide-NAD, has been characterized using *Bacillus subtilis* (Rizzi et al., 1996). To our knowledge, the pathways for NAD formation between nodule bacteroids and free-living rhizobia have not been compared. In the present work, a comparison of the NAD synthetic system in free-living cells of Bradyrhizobia, bacteroids and cytosol from soybean

* Corresponding author. Tel.: +81-52-789-5022; fax: +81-52-789-4230.
E-mail address: tezuka@info.human.nagoya-u.ac.jp (T. Tezuka).

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nodules, and root extracts of soybeans was undertaken. The results were discussed with reference to the biochemical difference between symbiotic and non-symbiotic conditions.

2. Results

2.1. Levels of pyridine nucleotides

Bacteroids isolated from soybean nodules contained a considerable quantity of the pyridine nucleotides, NAD, NADH, NADP and NADPH (Fig. 1a), with the levels of the oxidized forms (NAD and NADP) being higher than those of the reduced forms (NADH and NADPH). By contrast, in the free-living cells of *B. japonicum* strain A1017 used in this study, the NADP and NADPH levels were about 10 times higher than NAD and NADH (Fig. 1b).

2.2. Activities of enzymes associated with the formation of pyridine nucleotides

The activities of some enzymes involved in the formation of NAD from L-tryptophan, and NAD kinase which converts NAD into NADP were examined. Specific activities of NADPH- and NADH-kynurenine hydroxylases, 3-hydroxyanthranilic acid oxygenase, quinolinate phosphoribosyltransferase, NAD synthetase, ATP-NMN adenyltransferase and NAD kinase in fractions prepared from the bacteroids and free-living bacteria are shown in Table 1. The activities of NADPH- and NADH-kynurenine hydroxylases in bacteroids were

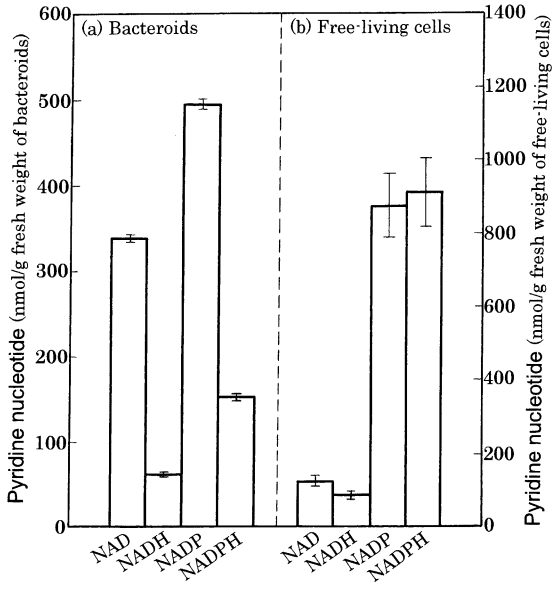

Fig. 1. The levels of pyridine nucleotides in bacteroids of *Glycine max* root nodules and in free-living cells of *Bradyrhizobium japonicum*. Vertical bars represent the means±S.E. ($n=3$).

Table 1
Specific activities of various enzymes involved in the pathway for the formation of pyridine nucleotides in bacteroids from nodules of *Glycine max* and free-living cells of *Bradyrhizobium japonicum*

	Bacteroids	Free-living bacteria
[nkat (mg protein)$^{-1}$]		
NADPH-kynurenine hydroxylase	60.0±1.7	1200.0±10
NADH-kynurenine hydroxylase	41.7±3.3	2301.7±285.0
3-Hydroxyanthranilic acid oxygenase	13.3±0.0	2900.0±8.3
Quinolinate phosphoribosyltransferase	5.0±0.0	380.0±23.3
[pkat (mg protein)$^{-1}$]		
NAD synthetase	0.8±0.0	12.1±0.4
ATP-NMN* adenyltransferase	1.9±0.1	6.2±0.1
NAD kinase	35.9±1.7	82.6±1.1

The activities of several key enzymes in the pathway for the formation of NAD and NADP from L-tryptophan were measured. Bacteroids and free-living bacteria were prepared as described in the text. Values are represented as the means±S.E. ($n=3$). *NMN: nicotinamide mononucleotide.

lower than those in free-living bacteria. Furthermore, in bacteroids, the activity of NADPH-kynurenine hydroxylases was higher than that of NADH-kynurenine hydroxylases and in the free-living bacteria, the activity of both enzymes was just the opposite.

The activity of 3-hydroxyanthranilic acid oxygenase was relatively lower in the bacteroids than in the free-living bacteria, like the case of quinolinate phosphoribosyltransferase. NAD synthetase and ATP-NMN adenyltransferase in bacteroids showed lower activities than those in the free-living bacteria. Furthermore, in the bacteroids, ATP-NMN adenyltransferase showed higher activities than NAD synthetase, whereas in the free-living bacteria NAD synthetase was about 2 times more active than ATP-NMN adenyltransferase. The NAD kinase activity in bacteroids was lower than that in free-living bacteria.

In roots, the NADH-kynurenine hydroxylase showed higher activity (about 14 times) than NADPH-kynurenine hydroxylase (Table 3), as in free-living bacteria (Table 1). The other enzymes such as 3-hydroxyanthranilic acid oxygenase, quinolinate phosphoribosyltransferase, NAD synthetase, ATP-NMN adenyltransferase and NAD kinase in the pathway of NAD and NADP formation showed higher activities in root extract than in nodule cytosol (Table 3).

2.3. pH dependence of NADPH-kynurenine hydroxylase, NAD synthetase and ATP-NMN adenyltransferase from bacteroids

The pH dependence of NADPH-kynurenine hydroxylase, NAD synthetase and ATP-NMN adenyltransferase in the bacteroids is shown in Fig. 2. The pH optima for NADPH-kynurenine hydroxylase, NAD

Table 2
Kinetic parameters of some enzymes in bacteroids from nodules of *Glycine max*

	Bacteroids
NADPH-kynurenine hydroxylase	
K_m NADPH (μM)	11.0±0.4
V_{max} (μmol NADP formed mg protein^{-1} min^{-1})	3.6±0.3
NAD synthetase	
K_m Deamide NAD) (μM)	22.0±0.7
V_{max} (nmol NAD formed mg protein^{-1} h^{-1})	3.1±0.2
ATP-NMN adenyltransferase*	
K_m Nicotinamide mononucleotide (μM)	482.0±37.3
V_{max} (nmol NAD formed mg protein^{-1} h^{-1})	7.4±0.4

The activities of some key enzymes in the pathway for the NAD from L-tryptophan were measured. Bacteroid fraction was prepared as described in the text. Values are represented as the means±S.E. ($n=3$). *NMN: nicotinamide mononucleotide.

Table 3
Specific activities of various enzymes in the pathway for the formation of NAD in nodule cytosol and root extract of *Glycine max* plants

	Nodule cytosol	Root extract
[nkat (mg protein)$^{-1}$]		
NADPH-kynurenine hydroxylase	56.7±1.7	91.7±3.3
NADH-kynurenine hydroxylase	100.0±3.3	1266.7±31.7
3-Hydroxyanthranilic acid oxygenase	10.0±0.0	26.7±1.7
Qunolinate phosphoribosyltransferase	21.7±0.0	138.3±1.7
[pkat (mg protein)$^{-1}$]		
NAD synthetase	0.3±0.0	6.5±0.1
ATP-NMN* adenyltransferase	1.2±0.0	5.6±0.1

The activities of several key enzymes in the pathway for the formation of NAD from L-tryptophan were measured. Nodule cytosol and root extract were prepared as described in the text. Values are represented as the means±S.E. ($n=3$). *NMN: nicotinamide mononucleotide.

synthetase, and ATP-NMN adenyltransferase were estimated to be 8.0, 9.0 and 7.6, respectively.

2.4. Kinetic analysis

Lineweaver–Burk plots were made from the isotherms of NADPH-kynurethne hydroxylase, NAD synthetase and ATP-NMN adenyltransferase (Table 2). The K_m and V_{max} values of NAD synthetase for NAD formation were smaller than those of ATP-NMN adenyltransferase for NAD formation.

3. Discussion

The soybeans (cv. 'Akisengoku') used in this experiment were late cultivars. Although soybean cultivars other than late cultivars are generally used for biochemical analysis at 4–5 weeks (at anthesis) after sowing, we used cv. Akisengoku at 7 weeks when at the flowering stage.

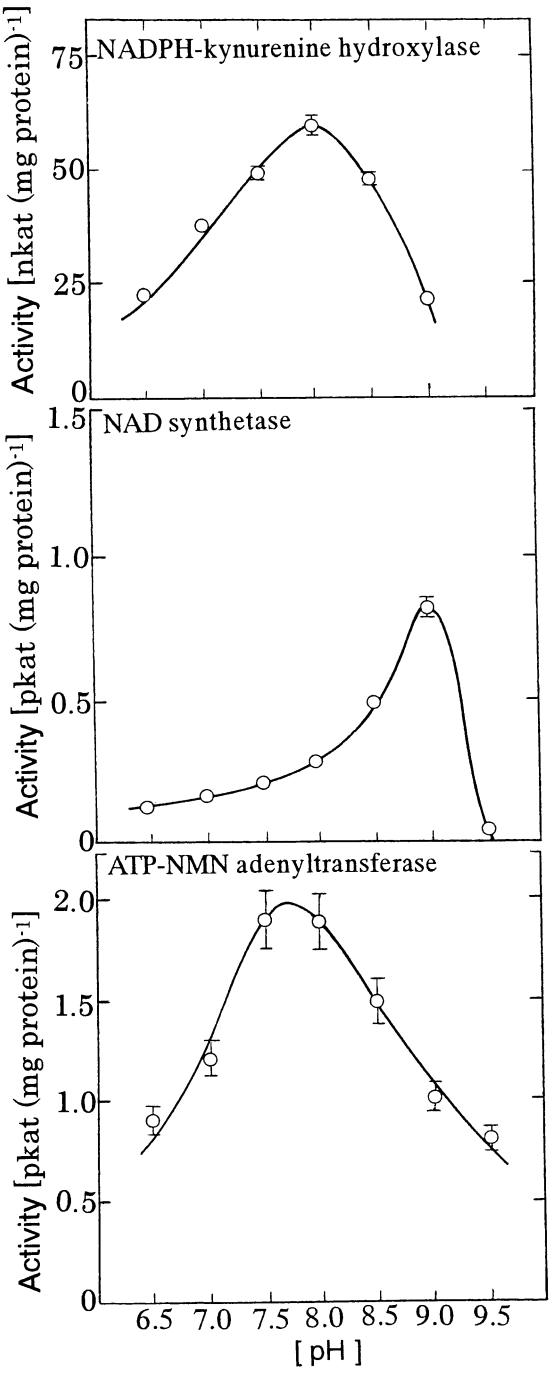

Fig. 2. The pH dependence of NADPH-kynurenine hydroxylase, NAD synthetase and ATP-NMN adenyltransferase in the enzyme fraction of bacteroids from root nodules of *Glycine max*. The enzyme fraction was prepared as described in the text. Vertical bars represent the means±S.E. ($n=3$). NMN: nicotunamide mononucleotide.

Bacteroids contained NAD and NADP at high levels, whereas in free-living cells (bacteria) NADP and NADPH were more predominant (Fig. 1). The levels of pyridine nucleotides in nodule cytosol were similar to those in free-living bacteria (data not shown). These

phenomena suggest that the pyndine nucleotide level is regulated differently in free-living bacteria and bacteroids. Indeed, the activities of the enzymes in the pathway from L-tryptophan to NM) and NADP in bacteroids were different from those in free-living bacteria (Table 1).

Given that bacteroids are derived from free-living bacteria (Ching and Hedtke, 1977) and associated with nodule cells (of plant origin), pyridine nucleotides in bacteroids may be involved in symbiotic work between nodule cells and bacteroids, such as nitrogen fixation. The levels of NADH and NADPH in bacteroids were lower than those of NAD and NADP, probably because mtrogenase converts N_2 into NH_3. The ~2-fold lower levels of pyridine nucleotides [NAD(P) + NAD(P)H] per g fr. wt of bacteroids than that of free-living cells may also be due to their consumption for nitrogen fixation.

The ratios of NAD/NADH and NADP/NADPH in the bacteroids were 5.56 and 3.26, which are higher than the values reported by Tajima and Kouzai (1989), i.e. 2.17 and 0.37, respectively. The difference between their NAD(P)/NAD(P)H ratios and ours may be due to differences in the procedures used for the extraction and assay of pyndine nucleotides in bacteroids. In the earlier work, pyridine nucleotides in bacteroids were extracted with 40 mM NaOH and the oxidized form [NAD(P)] was destroyed by heating for 10 min at 60 °C to estimate the amount of only the reduced form [NAD(P)H]. To estimate the amount of only NAD(P), pyridine nucleotides were extracted with the acidic solution of 20 mM H_2SO_4/0.1 M Na_2SO_4 and heated for 30 min at 60 °C. The amount of pyridine nudeoticles was estimated by the enzymatic cycling assay (Passonneau and Lowry, 1974) after incubation for 60 min. In contrast, our procedure (see Experimental) was based on a method described elsewhere (Nisselbaum and Green, 1969; Tezuka et al., 1994; Shiozaki, 2000).

In the pathway for formation of NAD from L-tryptophan, in bacteroids the activity of NADPH-kynurenine hydroxylase was higher than that of NADH-kynurenine hydroxylase, while that in free-living bacteria was quite the reverse (Table 1). This phenomenon suggests that the activities of NADPH- and NADH-kynurenine hydroxylase under symbiotic conditions are regulated differently from those under non-symbiotic conditions. According to Saito et al. (1957), kynurenine hydroxylase in animals is NADPH-dependent. In bacteroids and free-living bacteria, kynurenine hydroxylase was not only NADPH-dependent but also NADH-dependent (Table 1). Furthermore, kynurenine hydroxylase in the nodule cytosol and root extract showed activities of both NADPH- and NADH-enzymes (Table 3). Thus, organisms other than animals and certain microorganisms seem to have both NADPH- and NADH-kynurenine hydroxylases. This suggests that bacteroids, free-living rhizobia and soybean roots form NAD from L-tryptophan, as well as animals and some microorganisms such as *N. crassa* and *X. pruni* (Nishizaki and Hayaishi, 1971).

The activities of NAD synthetase and ATP-NMN adenyltransferase in the nodule cytosol were similar to those in bacteroids, whereas those in roots were higher than those in bacteroids (Tables 1 and 3). Therefore, it appears that these enzymes have lower activities in symbiotic than non-symbiotic conditions. Similarly, the activity of NAD kinase which forms NADP from NAD in bacteroids and free-living bacteria was also lower in bacteroids than in free-living bacteria (Table 1). Hence, these phenomena may be common to enzymes in the pathway for the formation of pyridine nucleotides.

The activities of other enzymes, which are not concerned in the formation of pyridine nucleoticles, also differ between symbiotic and non-symbiotic conditions. For example, lipoxygenase and hydroperoxide lyase from soybeans (cv. 'Wilkin'), were higher in the root extract than in the nodule cytosol (data not shown), similar to what was observed with the activities of 6-phosphogluconate dehydrogenase in soybean (Copeland et al., 1989) and aldolase and malate dehydrogenase in pea (Smith, 1985). Furthermore, the activities of isocitrate dehydrogenase in soybean (Suganuma and Yamamoto, 1987) and alfalfa plants (Irigoyen et al., 1990), as well as glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, aldolase and pyruvate kinase in the latter (Irigoyen et al., 1990) were also higher in free-living bacteria than in bacteroids. By contrast, according to Copeland et al. (1989), the activities of sucrose synthetase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase pyruvate kinase, phosphoenolpyruvate carboxylase and α,β-amylase were lower in the root extract than in the nodule cytosol. Moreover, malate dehydrogenase in soybean (Suganuma and Yamamoto, 1987) and alfalfa plants (Irigoyen et al., 1990) were also lower in free-living bacteria than in bacteroids. All of these phenomena may be caused by differences in symbiotic and non-symbiotic metabolism in leguminous plants.

As shown in Fig. 2, the optimum pH for NADPH-kynurenine hydroxylase and ATP-NMN adenyltransferase was in the region of relatively weak alkali (pH 8.0 and ~7.6, respectively). However, the optimum pH of NAD synthetase was different from that of the above enzymes. That is, the optimum pH was in the region of relatively strong alkali (pH 9.0). Kinetic analysis suggests that the formation of NAD in bacteroids may be dominated by NAD synthetase, as compared with ATP-NMN adenyltransferase, because the K_m of NAD synthetase, (22 μM) was ~1/22 of that of ATP-NMN adenyltransferase (482 μM), as shown in Table 2.

In conclusion, the levels of pyridine nucleotides in bacteroids and free-living cells of rhizobia depended on the activities of enzymes in the pathway from L-tryptophan

to NAD and NADP. A possible pathway for the formation of NM) and NADP in bacteroids and free-living bacteria, i.e., in symbiotic and non-symbiotic systems, is shown in Fig. 3. Since the principal function of bacteroids in soybean nodules is nitrogen fixation which needs NADH and NADPH as reducing power, the formation of NAD and NADP under symbiotic conditions (bacteroids) is different from that under non-symbiotic conditions (free-living bacteria). Accordingly, the activities of enzymes involved in the formation of pyridine nucleotides differ between symbiotic and non-symbiotic systems. This is the first report on the formation of

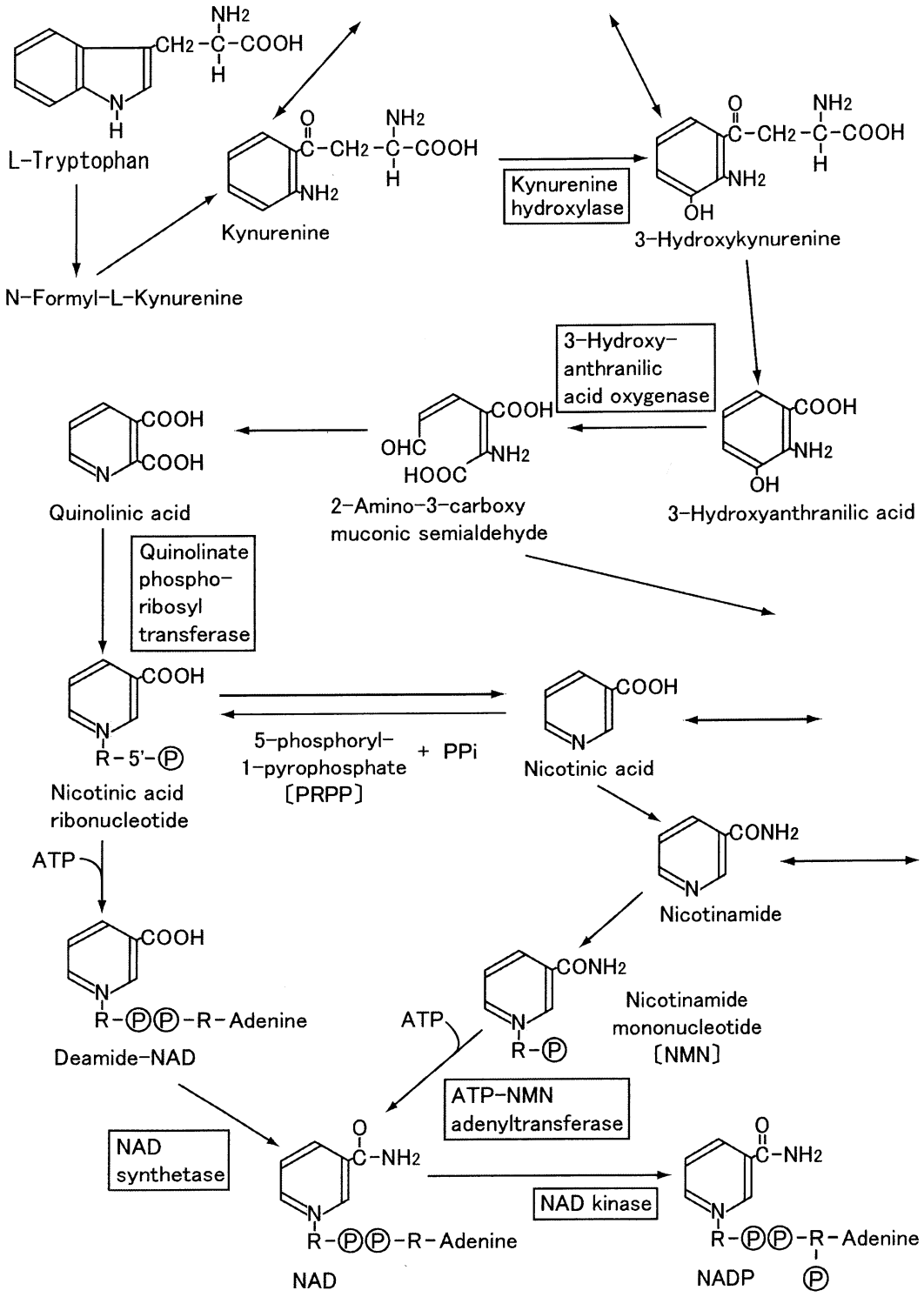

Fig. 3. A possible pathway for the formation of NAD and NADP from L-tryptophan in bacteroids from root nodules of *Glycine max* and free-living cells of *Bradyrhizobium japonicum*. Enzymes in six rectangles were assayed in the present study.

NAD and NADP originating from L-tryptophan in symbiotic bacteroids in soybean and non-symbiotic free-living bacteria.

4. Experimental

4.1. Plant culture

Soybean (*Glycine max* L. cv. 'Akisengoku') seeds were sown in pots containing a mixture of fine vermiculite and coarse sand (1:1, v/v). Each pot contained 135 mg N, as KNO_3, added at planting to promote early growth and good nodulation, and was watered with a nutrient solution, pH 5.8–6.2, lacking N containing salts (Summerfield et al., 1977). Seeds were inoculated with *Bradyrhizobium japonicum* strain A1017 that had been cultured in liquid culture medium (Rowsthorn and LaRue, 1986) with shaking for 5 days at 30 °C. Plants were grown in a glasshouse [with a fan (50 × 50 cm) operating automatically at a temperature of 35 °C or above] at 14–35 °C from April to the end of June and at 7–35 °C from October to December. After emergence, seedlings were thinned to one per pot and watered with nutrient solution via a basal saucer as required until 14 days after planting. Pots then received nutrient solution with free drainage each day. Plants were harvested at 7 weeks after planting. The yield of nodule was about 2–3 g plant^{-1}.

4.2. Isolation of bacteroids and root extracts

All procedures were carried out below 4 °C and under Ar which had been purged of O_2 (Peterson et al., 1982). Nodules (10 g) were ground in a mortar with a pestle with potassium phosphate buffer (50 mM, 30 ml, pH 7.4) containing Na-ascorbate (200 mg) and polyvinylpolypyrrolidone (2 g) (Polyclar AT) in an ice bath. The homogenate was squeezed through four layers of cheesecloth and centrifuged for 5 min at 370 *g* to remove starch and cell debris. The supernatant was again centrifuged for 10 min at 5000 *g*. The resulting supernatant was used as nodule cytosol. The pellet containing bacteroids was resuspended three times in washing buffer [50 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)/KOH, pH 8.2] and again centrifuged for 10 min at 5000 *g* to provide the washed bacteroids. Final pellet (washed bacteroids) was resuspended in TES/KOH (100 mM, 3.3 ml, pH 8.2) and stored at −80 °C until use. Root extracts were obtained from roots (10 g) by the same procedures as used for the preparation of the nodule cytosol.

After thawing, the bacteroid suspension was sonicated for (15×30 s) in an ice bath using a Branson Sonifer (Model 200, Branson Sonic Power Co., USA) with an output of 20 W and centrifuged for 20 min at 20,000 *g*. The supernatant (1.5 ml) was loaded onto a Sephadex G-25 column (1 × 14 cm) with and eluted with TES/KOH (50 mM, pH 7.5). After discarding 20 drops of the eluate, the protein-rich (enzyme) fraction (3 ml) was collected to assay enzyme activities.

4.3. Free-living bacteria

Free-living cells of *B. japonicum,* strain A10l7 were cultured in a liquid culture medium (Rowsthorn and LaRue, 1986) by shaking for 10 days at 30 °C. The culture medium with the free-living bacteria was centrifuged for 10 min at 20,000 *g* to remove polysaccharides exuded from bacteria during culture. The pellet (collected free-living bacteria) was washed with TES/KOH (50 mM, 30 ml, pH 7.5), then suspended in TES/KOH (50 mM, 20 ml, pH 7.5) and centrifuged for 10 min at 10,000 *g*. The resulting pellet (washed free-living bacteria) was used for extraction of pyridine nucleotides and preparation of enzymes. For preparation of enzymes, the above pellet (washed free-living bacteria) was suspended in TES/KOH (50 mM, 2 ml, pH 7.5), sonicated in the same way as bacteroid suspension mentioned above, and centrifuged at 20,000 *g* for 20 min. The supernatant was treated in the same way as the preparation of enzymes from bacteroids, affording the enzyme fraction of free-living bacteria. The fraction was used to assay enzyme activities.

4.4. Extraction and quantitation of pyridine nudeotides

All procedures were carried out below 4 °C and under Ar which had been purged of O_2 (Peterson et al., 1982). Nodules (6 g) were homogenized with potassium phosphate buffer (5 mM, 18 ml, pH 6.8) in a mortar with a pestle on ice, squeezed through 4 layers of cheesecloth and centrifuged at 370 *g* for 5 min. The supernatant was recentrifuged at 5000 *g* for 10 min. The resulting pellet was suspended in potassium phosphate buffer (5 mM, 18 ml, pH 6.8) and again centrifuged at 5000 *g* for 10 min. This step was repeated twice. The final pellet was resuspended in the same buffer (5 ml) as a bacteroid fraction. The bacteroid fractions were divided into two rations of equal wet weight to isolate the oxidized (NAD and NADP) and reduced (NADH and NADPH) forms of pyridine nucleotides, respectively. The washed free-living bacteria mentioned above was also weighed and divided into two rations of equal weight (free-living cell fraction), one for the extraction of NAD and NADP, and the other for the extraction of NADH and NADPH.

Pyridine nucleotides were extracted from bacteroid and free-living cell fractions by a modified version of the method of Tezuka et al. (1994). One part of each fraction was homogenized at 90–95 °C for 2 min with 0.1 N HC1 (10 ml) to extract oxidized coenzymes (NAD and

NADP). The other part of each fraction was similarly treated with 0.1 N NaOH to extract reduced coenzymes (NADH and NADPH). Each of the four homogenates was then rapidly chilled in an ice bath and the extracts for oxidized and reduced pyridune nudeotides were adjusted to pH 6.5 with NaOH and pH 7.5 with HC1, respectively, and then 0.2 M glycylglycine buffer (0.5 ml) at pH 6.5 and pH 7.5, respectively, were added. Total volumes were measured, and then each homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C. Supernatants were stored at −80 °C prior to the assay of pyndine nucleotides. To estimate possible losses of dinucleotides during extraction, authentic standards (NADH: 2 nmol; others: 10 nmol) were added to the alkaline and acidic extracts before homogenization. Recovery of NAD(P) and NAD(P)H was 98–103 and 75–88%, respectively. Pyridine nucleotides were quantified by a modified version (Tezuka et al., 1994) of the method of Nisselbaum and Green (1969).

4.5. Enzyme assays

Kynuremine hydroxylase was assayed at 25 °C in a reaction mixture (0.6 ml) that contained 0.1 M TES/KOH (pH 8.0), 10 mM KC1, 0.33 mM NADH or NADPH, 3.33 μM L-kynurenine and an enzyme fraction by a modified version of the method of Okamoto et al. (1967). 3-Hydroxyanthranilic acid oxygenase was assayed at 25 °C in Tris/acetate (pH 8.0) by the method of Henderson and Swan (1971). Quinolinate phosphoribosyltransferase was assayed at 25 °C in potassium phosphate (pH 7.0) by the method of Packman and Jakoby (1967). NM) synthetase was assayed at 30 °C for 30 mm in a reaction mixture (1 ml) that contained 25 mM TES/KOH (pH 9.0), 1 mM deamide NAD, 20 mM L-glutamine, 4 mM ATP, 0.164 M nicotunamide, 50 μM $MgCl_2$ and an enzyme fraction by a modified version of the method of Preiss and Handler (1958). Immediately after the assay, 1 N HC1 (0.1 ml) was added to the mixture, which was then heated at 95 °C for 2 min and cooled in an ice bath. After cooling, 1 N NaOH (0.1 ml) and TES/KOH (0.25 M, 0.1 ml, pH 7.6) were added to the mixture. ATP-NMN adenyltransferase was assayed at 30 °C for 30 min in a reaction mixture (0.33 ml) that contained 30 mM TES/KOH (pH 7.6), 15 mM $MgCl_2$, 7.6 mM ATP, 0.2 M nicotinamide, 6 mM nicotinamide mononucleotide and an enzyme fraction by a modified version of the method of Körnberg (1950). Immediately after the assay, 0.18 N HCl (0.42 ml) was added to the mixture, and then the mixture was heated at 95 °C for 2 min and cooled in an ice bath. Then 1.5 N NaOH (0.05 ml) and 0.25 M TES/KOH (0.2 ml), pH 7.6 were added to the mixture. NAD kinase was assayed at 30 °C for 30 min in a reaction mixture (1 ml) that contained 34 mM Tricine/KOH (pH 7.6), 1 mM NAD, 5 mM ATP, 6 mM nicotinamide, 7 mM $MgCl_2$ and an enzyme fraction by a modified version of the method of Tezuka and Yamamoto (1972). Immediately after the assay, the mixture was heated at 95 °C for 2 mm, cooled in an ice bath and centrifuged at 10,000 *g* for 10 min. Finally, NAD formed by NAD synthetase and ATP-NMN adenyltransferase and NADP formed by NAD kinase were measured by the method of Tezuka et al. (1994). Protein was determined according to Lowry et al. (1951) using bovine serum albumin as the standard. All experiments were repeated three times with similar results and representative results are shown.

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