Plant Physiol. (1980) 66, 139-141
0032-0889/80/66/0139/03/$00.50/0

# Asparagine Formation in Soybean Nodules

Received for publication December 3, 1979 and in revised form March 20, 1980

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## ABSTRACT

**$^{15}NH_4^+$ and [$^{15}$N](amide)-glutamine externally supplied to detached nodules from soybean plants (cv. Tamanishiki) were incorporated within nodule tissues by vacuum infiltration and metabolized to various nitrogen compounds during 60 minutes of incubation time. In the case of $^{15}NH_4^+$-feeding, the $^{15}$N abundance ratio was highest in the amide nitrogen of glutamine, followed by glutamate and the amide nitrogen of asparagine. In $^{15}$N content (micrograms excess $^{15}$N), the amide nitrogen of asparagine was most highly enriched after 60 minutes. $^{15}NH_4^+$ was also appreciably assimilated into alanine.**

**When [$^{15}$N](amide)-glutamine was fed to detached nodules, $^{15}$N was almost entirely in the amide nitrogen of asparagine. The result indicates the presence of active glutamine-linked asparagine synthesizing systems in soybean root nodules.**

The results from numerous *in vivo* studies with metabolic inhibitors (14) and from *in vitro* enzymic analyses (9-11, 14) have established glutamine-linked asparagine synthesis in higher plants, although the pathway providing the carbon skeleton to asparagine remains open (11). In most legumes so far considered, asparagine is the principal assimilation product of symbiotic nitrogen fixation (7, 8, 10, 16). Scott *et al.* (10) found glutamine-linked asparagine synthetase in the cytosol fraction of lupin nodules and proposed a sequence for asparagine formation from atmospheric nitrogen fixed by nodule bacteroids. Bleeding sap of soybean nodules also contains high concentrations of asparagine (16), indicating the presence of active asparagine synthesizing and transporting systems in soybean nodules. Extensive attempts by Streeter (12) to demonstrate asparagine biosynthesis from various radioactive precursors have, however, failed, possibly because of the presence of a very active asparaginase in soybean nodules (12). We observed that soybean nodule bacteroids had a high ability to transform asparagine to aspartate (unpublished data). The existence of such an active asparagine-decomposing system in nodules makes *in vitro* enzymic or tracer studies on asparagine synthesis difficult. In the present *in vivo* tracer study, asparagine formation in soybean nodules was examined especially in regard to the source of the amide nitrogen of asparagine.

The data from isotopic analysis in this communication clearly indicate that the amide nitrogen of glutamine is efficiently utilized as nitrogen donor of amide nitrogen of asparagine in soybean nodules.

## MATERIALS AND METHODS

**Nodules.** Soybean plants (*Glycine max* cv. Tamanishiki) were grown in Vermiculite applied with a mineral nutrient solution lacking nitrogen (2) in a greenhouse. Plants were nodulated with strain J-5033 of *Rhizobium japonicum*. After 7 weeks, fresh nodules were detached from the roots and immediately employed for tracer study.

**Vacuum Infiltration and Extraction.** Detached nodules (2g) were transferred into 20-ml vials fitted with a rubber cap. After addition of 2 ml Krebs-Ringer bicarbonate buffer (pH 7) containing 5 mM DTT (Sigma), and 10 μmol ($^{15}NH_4)_2SO_4$ (70 atom % $^{15}$N, Hikari Kogyo Co.) or 20 μmol [$^{15}$N](amide)-glutamine (95 atom % amide $^{15}$N, Hikari Kogyo Co.), vials were evacuated with a rotary pump for 2 min and Ar gas was introduced immediately. After reevacuation for 2 min, the atmosphere of the vials was quickly replaced with mixed gas consisting of 20% $O_2$ and 80% Ar, and incubated at 30 C for 1 h. Reaction was terminated by the addition of 2 ml cold 0.4 N $HClO_4$ to each vial. The contents of the vials were immediately transferred into a cold mortar, ground with a pestle, washed into a centrifuge tube, and centrifuged at 15,000*g* for 30 min at 0 C. The acid precipitate was washed in cold 0.2 N $HClO_4$, centrifuged again, and the combined supernatant fluid was neutralized to pH 6.5. The cleared solution was used for the fractionation of each nitrogen constituent.

**Separation of Nitrogen Compounds and Conversion to Ammonia.** The supernatant solution was shaken vigorously with 10% (w/v) of Permutit for several min and centrifuged at 10,000*g* for 10 min. The ammonia free supernatant fluid was run onto a Dowex 1-X8 column. Aspartate and glutamate adsorbed on this column were eluted with 2 N formic acid, and after evaporating the formic acid this fraction was used for further separation of aspartate and glutamate. An aliquot of the effluent passed through the above column was used for the separation of allantoin, and another aliquot was used for stepwise enzymic hydrolysis of glutamine and asparagine. The sample solution was subjected to alkaline hydrolysis to decompose allantoin to allantoic acid as described by Young and Conway (17). After hydrolysis, the solution was neutralized with HCl and the resultant allantoic acid was adsorbed on a Dowex 1-X8 column. After evaporating formic acid, the residue was dissolved in 0.1 M phosphate buffer (pH 7), and urea derived from allantoic acid was converted to ammonia by urease (from jack bean, Sigma). Recovery of the ammonia in acid solution was performed by the usual Conway microdiffusion method.

Amide nitrogen of glutamine and asparagine were released by glutaminase (from *Escherichia coli*, Sigma) and asparaginase (from *E. coli*, gift of K. Miura), respectively, and recovered separately in acid solution as follows. Sample solution was placed in a Conway microdiffusion vessel, and incubated at 37 C for 5 h with 100 mM acetate buffer (pH 4.9) and glutaminase solution.

Ammonia released from glutamine was volatilized by the addition of 2 M $K_2CO_3$ and collected in $H_2SO_4$ in an inner vessel. After removal of the amide nitrogen of glutamine, the solution was neutralized with HCl, incubated at 37 C for 2 h with 100 mM borate buffer (pH 7.6) and asparaginase solution, and ammonia released from asparagine was collected in the same manner as described above. The solution remaining in the Conway vessel

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was acidified by HCl, placed on a Dowex 50W-X8 column and amino acids held on this column were eluted with 6 N HCl. Separation of amino acids was carried out in an automatic amino acid analyzer. The eluate from the analyzer column was pooled after the reaction with ninhydrin, concentrated $H_2SO_4$ was added for destruction of the ninhydrin-ammonia complex, and the resulting free ammonia was steam-distilled.

**$^{15}N$ Analysis.** For isotopic analysis, ammonia recovered in $H_2SO_4$ from each nitrogen compound was further converted to $N_2$ gas with sodium hypobromite and the abundance of $^{15}N$ in $N_2$ gas was determined with Hitachi RMU-6 mass spectrometer. Estimation of ammonia in sample solution was made by Nessler's reagent.

To compare the result from $^{15}NH_4^+$-feeding experiment with that from [$^{15}$N](amide)-glutamine-feeding experiment, the data in Table I were expressed in terms of 100 atom % $^{15}N$ of $^{15}NH_4^+$ and [$^{15}$N](amide)-glutamine, *i.e.* atom % $^{15}N$ values of nitrogen compounds measured were corrected by multiplying by 100/70 for $^{15}NH_4^+$-feeding and by 100/95 for [$^{15}$N](amide)-glutamine feeding, respectively.

## RESULTS AND DISCUSSION

According to Streeter (12), soybean nodule asparaginase is very stable and active even in 75% ethanol solution at room temperature. In the present experiment, extraction of nitrogen compounds from soybean nodules was performed by cold $HClO_4$. From a preliminary examination it was shown that release of amide nitrogen of asparagine and glutamine during acid extraction could be avoided by using ice-cold 0.4 N $HClO_4$ followed by immediate neutralization with KOH after removal of protein and other cell debris by centrifugation.

$^{15}NH_4^+$ and [$^{15}$N](amide)-glutamine externally supplied to detached nodules were incorporated within nodule tissues by vacuum infiltration and metabolized to various nitrogen compounds during 60 min of incubation time. Table I presents the data of $^{15}N$ incorporation into early products of ammonia assimilation and ureide allantoin which is the major nitrogen constituent of soybean nodules. In the case of $^{15}NH_4^+$-feeding, the highest atom % excess $^{15}N$ value was obtained in amide-N of glutamine, followed by glutamate and amide-N of asparagine. Rapid incorporation of $^{15}NH_4^+$ into amide-N of glutamine is explained by the presence of glutamine synthetase having high affinity for ammonia usually found in large amounts in leguminous root nodules (7). As for $^{15}N$ content ($\mu$g excess $^{15}N$), amide-N of asparagine was most highly enriched after 60 min. High $^{15}N$ enrichment in asparagine apparently indicates the presence of active asparagine-synthesizing systems in soybean nodules, and labeling sequence in this experiment implies that $^{15}NH_4^+$ was incorporated into amide-N of asparagine via glutamine amide-N. $^{15}NH_4^+$ was also incorporated into alanine in appreciable amounts, the $^{15}N$ abundance is comparable to the level of glutamate. On the other hand, aspartate, which is one of the early products of ammonia assimilation and a precursor of asparagine biosynthesis, contained little $^{15}N$.

In the case of [$^{15}$N](amide)-glutamine-feeding, amide-N of glutamine was almost entirely incorporated into amide-N of asparagine. Apparent $K_m$ value of asparagine synthetase for glutamine is reported to be much lower than that for ammonia in several plant species (9–11). Although in soybean nodules the existence of asparagine synthetase has not yet been ascertained, the result presented herein indicates the presence of glutamine-linked asparagine synthetase in soybean nodules. NAD or NADP-dependent glutamate synthase, which produces glutamate by the reaction transferring amide-N of glutamine to $\alpha$-ketoglutarate, is commonly detected in various nodules (7). However, in the present *in vivo* study, amide-N of glutamine was not significantly incorporated into amino-N of glutamate, whereas relatively high incorporation of $^{15}N$ into glutamate was observed in $^{15}NH_4^+$-feeding experiment.

Soybean plants contain large quantities of ureide allantoin and allantoic acid (2, 5, 13), and it has been confirmed that these compounds are mainly produced within nodule tissues via xanthine-uric acid (3, 15). From the evidence that atmospheric nitrogen was significantly incorporated into these ureides (4, 6), it has been suggested that ureides are the assimilation products of fixed $N_2$ in actively $N_2$-fixing soybean nodules. Additionally, it was indicated that glutamine was a precursor for the ureide biosynthesis, since glutamine antagonist azaserine inhibited ureide formation from ammonia in soybean nodules (unpublished data). Tracer studies showed that the incorporation of glutamine amide-N into allantoin was very low compared to that into asparagine, although glutamine was found to be more efficient than ammonia as nitrogen donor in allantoin formation (Table I). These facts are suggestive of the biosynthetic sites involved in the formation of asparagine and allantoin. It appears that asparagine and ureide allantoin respectively are formed at different sites within the soybean nodule. There are two (or more) pools of ammonia within root nodules (1). One is a very small pool which directly connects with $N_2$ fixation and is rapidly saturated with newly fixed nitrogen, and the other is a relatively large pool whose origin and location within the nodule is ambiguous. It is of interest to speculate that in actively $N_2$-fixing soybean nodules, the production of allantoin might associate with the small ammonia pool related to $N_2$ fixation, while the production of asparagine might associate with the large ammonia pool. Relatively high activity of asparaginase and glutaminase detected in soybean nodule bacteroids (12) will be disadvantageous for glutamine-linked asparagine formation within bacteroids. Further studies on the location of the enzymes related to asparagine biosynthesis within soybean nodules are necessary.

Table I. *Incorporation of $^{15}N$ into Main Nitrogen Compounds by Soybean Nodules*

Detached nodules (2 g fresh weight) from 7-week-old soybean plants were immersed in 2 ml Krebs-Ringer bicarbonate buffer (pH 7) containing 5 mM DTT and 10 $\mu$mol $(^{15}NH_4)_2SO_4$, or 20 $\mu$mol [$^{15}$N](amide)-glutamine. Each tracer was vacuum-infiltrated into nodule tissues. Reaction vials were replaced with mixed gas (20% $O_2$, 80% Ar) and incubated at 30 C for 1 h. The details of the procedures of vacuum infiltration, extraction with cold $HClO_4$ and separation of each compound are described under Materials and Methods. Amide nitrogen of glutamine and asparagine were released separately and recovered by glutaminase (from *E. coli*) and asparaginase (from *E. coli*), respectively. The average and variation of duplicate experiments are given.

| Nitrogen Compound | Source of $^{15}N$ Supplied to Nodules | | | |
|---|---|---|---|---|
| | $^{15}NH_4^+$ | | [$^{15}$N](amide)-glutamine | |
| | *atom % excess* | *$\mu$g excess*[a] | *atom % excess* | *$\mu$g excess*[a] |
| Glutamine amide-N | 6.30 ± 0.48 | 2.50 ± 0.34 | | |
| Asparagine amide-N | 2.47 ± 0.40 | 3.30 ± 0.27 | 6.42 ± 0.43 | 7.40 ± 1.05 |
| Glutamate | 3.86 ± 0.29 | 1.29 ± 0.28 | 0.52 ± 0.16 | 0.13 ± 0.05 |
| Aspartate | 0.50 ± 0.05 | 0.02 ± 0.01 | 0.09 ± 0.02 | 0.01 ± 0.01 |
| Glycine | 0.54 ± 0.07 | 0.39 ± 0.06 | 0.07 ± 0.02 | 0.05 ± 0.01 |
| Alanine | 2.86 ± 0.27 | 2.31 ± 0.20 | 0.93 ± 0.11 | 0.81 ± 0.08 |
| Allantoin | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.25 ± 0.08 | 0.20 ± 0.03 |

[a] Excess ($\mu$g) $^{15}N$ reaction vial which contains 2-g nodules.

*Acknowledgment*—The authors are grateful to K. Miura of the Department of Agricultural Chemistry, University of Osaka Prefecture, for supplying crystalline asparaginase.

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