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Enzymes of Amide and Ureide Biogenesis in Developing Soybean Nodules[1]

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ABSTRACT

Amide and ureide biogenic enzymes were measured in the plant fraction of soybean (*Glycine max*) nodules during the period 11 to 23 days after inoculation with *Rhizobium japonicum* (USDA 3I1b142). Enzymes involved in the initial assimilation of ammonia, *i.e.* glutamine synthetase, glutamate synthase, and aspartate aminotransferase, showed substantial increases in their specific activities over the time course. These increases paralleled the induction of nitrogenase activity in the bacteroid and leghemoglobin synthesis in the plant fraction. The specific activity of asparagine synthetase, however, showed a rapid decline after an initial increase in specific activity. Following the initial increases in the ammonia assimilatory enzymes, there was an increase in the activity of 5-phosphoribosylpyrophosphate amidotransferase, the enzyme which catalyzes the first committed step of *de novo* purine biosynthesis. This was followed by a dramatic increase in the purine oxidative enzymes, xanthine dehydrogenase and uricase. Smaller increases were observed in the activities of enzymes associated with the supply of metabolites to the purine biosynthetic pathway: phosphoglycerate dehydrogenase, serine hydroxymethylase, and methylene tetrahydrofolate dehydrogenase.

The concentration of asparagine in the plant fraction decreased at the same time as the observed decrease in asparagine synthetase activity. This was followed by a recovery in plant fraction levels of asparagine in the presence of a continuing fall in the glutamine concentration and continued low asparagine synthetase activity.

The data presented are consistent with initial assimilation of ammonia into glutamine and aspartate, which are metabolized by an elevation of endogenous purine biosynthetic enzymes, and then, by the induction of a specific group of purine oxidative enzymes, directed to allantoic acid production.

The mechanism of the assimilation of fixed N into the ureides, allantoin and allantoic acid, still remains to be clearly elucidated in those leguminous plants which use these compounds for the transport of fixed N from the nodule to aerial parts of the plant. There is considerable evidence for a pathway of synthesis of allantoic acid from purines in the plant fraction of soybean and cowpea nodules (8, 18, 20, 21). However, only preliminary evidence exists which suggests that the purines used in allantoic acid biosynthesis in the nodule are, in fact, synthesized *de novo* (2, 5, 14).

In contrast, the route of assimilation of fixed N into asparagine in amide-transporting legumes is largely understood (15). A powerful tool in the elucidation of this pathway was the utilization of time-course studies during nodule development to indicate enzymes present whose activities were linked to the phenomenon of nitrogen fixation (11).

In this work, enzymes of ammonia assimilation, purine oxidation, and *de novo* purine biosynthesis were measured in the plant fraction during soybean nodule development. The levels of key amino acids present in the plant fraction were also measured and related to the various enzyme activities observed over the time course.

MATERIALS AND METHODS

Materials. [U-^{14}C]Glutamine was obtained from New England Nuclear, and [3-^{14}C]serine from Amersham. The 3a20 counting cocktail was purchased from Research Products International, Prospect, IL. All other chemicals were obtained from Sigma.

Methods. Soybean (*Glycine max* [L.] Merr. cv. Williams) seeds were germinated for 48 h on wet paper towels. The seeds were then planted, radicle down, and inoculated with *Rhizobium japonicum* (3I1b142). The plants were maintained in a Kysor Sherer growth cabinet using a Leonard jar system (19) with a nitrogen-free nutrient solution (18) on a regime of 16 h/28°C day and 8 h/24°C night. Nodules were harvested during the time period 11 to 23 d after inoculation, and the nodule plant fraction prepared (12) using an extraction buffer which consisted of 50 mM Tricine-KOH, pH 8.0; 0.4 M sucrose; 1 mM $MgCl_2$; 5 mM glutathione; 20 mM dithioerythritol. The plant fraction was centrifuged through Sephadex G-25 (10), and aliquots were used for the various enzyme assays.

Nitrogenase (EC 1.18.2.1) activity of nodules was measured by acetylene reduction (18). Leghemoglobin concentrations were determined by spectral difference (1).

GS[3] (EC 6.3.1.2) activity was assayed using the biosynthetic

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[3] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate aminotransferase; AS, asparagine synthetase; PRAT, 5-phosphoribosylpyrophosphate amidotransferase; SHM, serine hydroxymethylase; Me FH_4 DH, methylene tetrahydrofolate dehydrogenase; PGA DH, phosphoglycerate dehydrogenase; XDH, xanthine dehydrogenase.

hydroxamate method (7). GOGAT (EC 1.4.1.14), AS (EC 6.3.5.4), and AAT (EC 2.6.1.1) were assayed as described in Reference 7.

PRAT (EC 2.4.2.14) was assayed radiochemically at 25°C according to a procedure based on that of Holmes *et al.* (9). The assay system contained: 30 mM Tricine-KOH, pH 8.0; 20 mM dithioerythritol; 5 mM glutathione; 9 mM $MgCl_2$; 3 mM 5-phosphoribosylpyrophosphate; 12 mM glutamine (0.4 μCi [U-^{14}C]glutamine assay^{-1}); H_2O and enzyme solution to a final volume of 200 μl. The reaction mixture was preincubated in the absence of glutamine for 15 min and the reaction was initiated with glutamine. Aliquots (10 μl) were removed at various times and immediately dried onto Whatmann 3MM paper to stop the reaction. A glutamate marker (10 μl of a 1.5 mg ml^{-1} solution) was applied to the paper prior to the sample. Glutamate and glutamine were then separated by high voltage electrophoresis and the radioactivity in each amino acid was determined.

SHM (EC 2.1.2.1) was assayed using a modification of the method of Taylor and Weissbach (17). A mixture containing 0.25 mM pyridoxal phosphate, 2 mM tetrahydrofolate, and 25 μl nodule extract (not G-25 treated) in 50 mM potassium buffer, pH 7.5 (total volume, 375 μl) was preincubated for 10 min at 25°C, following which time 25 μl of [3-^{14}C]serine plus carrier serine was added to give a final concentration of 1 mM and 2.5 μCi ml^{-1}. After 0, 10, or 20 min, the reaction was stopped and the degree of reaction determined according to Taylor and Weissbach (17).

PGA DH (EC 1.1.1.95) activity was measured by the phosphohydroxypyruvate-dependent (0.8 mM) oxidation of 60 μM NADH in 50 mM K-phosphate, pH 7.5.

N^5,N^{10}-Me FH_4 DH (EC 1.5.1.15) activity was determined from the rate of reduction of $NADP^+$ (120 μM) when added to a mixture containing 25 μl extract, 0.5 mM formaldehyde, and 0.2 mM 2-mercaptoethanol, which had been preincubated until the *A* at 340 nm was constant. Under these conditions, methenyl tetrahydrofolate, the reaction product, hydrolyzes spontaneously to N^{10}-formyl tetrahydrofolate which does not contribute significantly to the *A* at 340 nm (M. J. Boland, unpublished results).

N^5,N^{10}-Methenyl tetrahydrofolate cyclohydrolase (EC 3.5.4.9) was measured by the rate of disappearance of absorbance due to the substrate (50 μM, E_{350} = 26,500 [13]) in 50 mM Tris-ascorbate buffer, pH 7.5, before and after the addition of nodule extract. The substrate was synthesized according to the method of Rowe (13).

XDH (EC 1.2.1.37) was measured by the rate of reduction of NAD^+ (0.3 mM) in the presence of 0.1 mM xanthine in 50 mM K-phosphate buffer, pH 7.5 (4). Uricase activity was determined from the rate of disappearance of *A* at 292 nm due to uric acid (0.1 mM) in well-aerated 50 mM Tricine-KOH buffer, pH 9.0 (16). Enzyme assays were performed in duplicate (for radiochemical assays) or triplicate (for spectrophotometric assays). The variations in measurements of activity were <2.5%, and so average values were plotted. The statistical variations between plants were minimized by pooling nodules from at least 10 plants for extraction and subsequent assay.

The amino acid analyses were performed by automated cation exchange chromatography (3). Protein was removed from samples prior to amino acid analysis with sulfosalicyclic acid. Protein in the plant fractions was determined according to the method of Bradford (6).

RESULTS AND DISCUSSION

Detection of Enzymes of Ammonia Assimilation, *de Novo* Purine Biosynthesis, and Purine Oxidation in the Plant Fraction of Soybean Nodules. The various enzymes that were assayed and their specific activities in 21-d-old plants are shown in Table I. The enzymes involved in the initial assimilation of ammonia into amino acids (Table I, nos. 2–4) had specific activities comparable to those reported previously (11) and were present at levels consistent with the observed rate of nitrogen fixation. AS (Table I, no. 5) showed an extremely low value in these 21-d-old plants.

Table I. *Enzyme Activities in the Plant Cytosol Fraction of 21-Day-Old Soybean Nodules*

Enzyme	Activity
	nmol min^{-1} mg^{-1} protein
1. Nitrogenase[a]	8.4
2. Glutamine synthetase	232
3. Glutamate synthase	11.5
4. Aspartate aminotransferase	644
5. Asparagine synthetase	0.5
6. 5-Phosphoribosylpyrophosphate amidotransferase	13.0
7. Phosphoglyceric acid dehydrogenase	104
8. N^5,N^{10}-Methylene tetrahydrofolate dehydrogenase	11.5
9. Serine hydroxymethylase	2.4
10. N^5,N^{10}-Methenyl tetrahydrofolate cyclohydrolase	21.5
11. Xanthine dehydrogenase	11.5
12. Uricase	74.0

[a] This value for nitrogenase specific activity was obtained by applying a conversion factor of $4C_2H_2$ reduced per mol of N_2 fixed to the data in Figure 1.

This paper reports the detection of enzymes involved in *de novo* purine biosynthesis and glycine biosynthesis in soybean nodules (Table I, nos. 6–10). The first enzyme of the *de novo* purine biosynthetic pathway, PRAT (Table I, no. 6), showed a specific activity comparable to that of GOGAT. Enzymes involved in the supply of glycine and one-carbon units for purine biosynthesis (Table I, nos. 7–10) were also present.

Enzymes of purine oxidation (Table I, nos. 11 and 12) have already been reported in soybean nodules (14, 18) and were present at comparable levels in these plants (Table I).

Activities of Various Soybean Nodule Enzymes during Nodule Development. During the period 11 to 23 d after inoculation, the specific activities of acetylene reduction and GS increased 4- and 9-fold, respectively (Fig. 1A). During the same period, leghemoglobin showed a 26-fold increase in the plant fraction. Leghemoglobin concentration and GS specific activity increased in parallel with acetylene reduction. Nodule weight and protein concentration in the plant fraction are shown in Figure 1B. These data are used to calculate specific activity values for acetylene reduction and leghemoglobin concentration.

The other enzymes involved in the initial incorporation of NH_3 into amino acids, GOGAT and AAT, showed a 6- and 2.5-fold increase in specific activity, respectively, and paralleled the increase in GS specific activity (Fig. 2A). AS also showed an increase in specific activity, although this increase lagged behind that of the other ammonia assimilatory enzymes. Interestingly, the activity of AS showed a dramatic decrease after day 17 (Fig. 2A), falling to values as low as those at the beginning of the time period assayed. This fall in activity occurred at the same time that the purine biosynthetic enzyme, PRAT, and the purine oxidative enzymes, XDH and uricase, were approaching their maximum values (Fig. 2B). These changes in enzyme activity may reflect a 'switch-over' in the metabolism of the soybean nodule from the production of asparagine to the production of the ureides, allantoin and allantoic acid.

PRAT showed a 6-fold increase in specific activity by day 21 (Fig. 2B). This preceded the strong induction of the purine oxidative enzymes, XDH specific activity increasing 12-fold and uricase activity 25-fold (Fig. 2B).

Enzymes involved in the supply of glycine and 1-carbon units

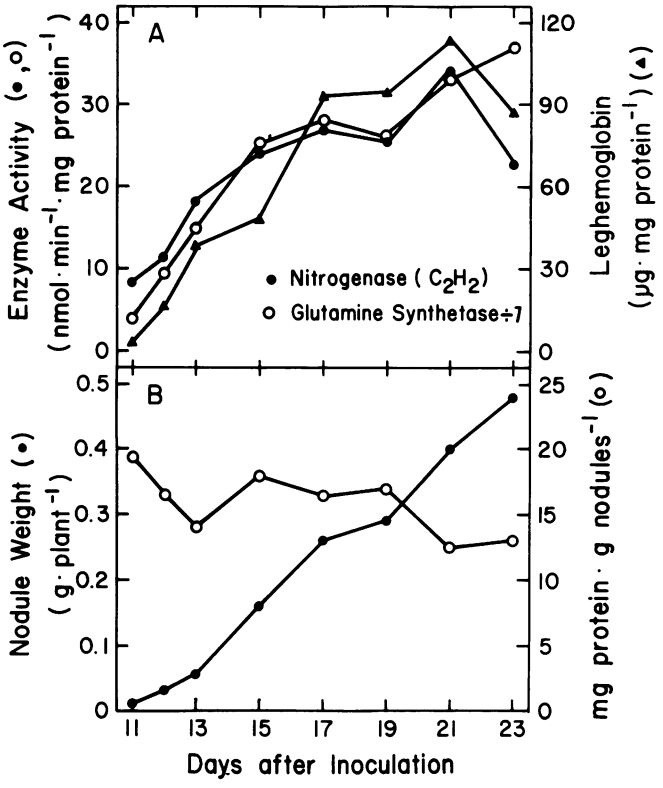

FIG. 1. A, Acetylene reduction rate (●), leghemoglobin concentration (▲), and GS activity (○) during soybean nodule development. B, Nodule weight (●) and protein concentration (○) during soybean nodule development.

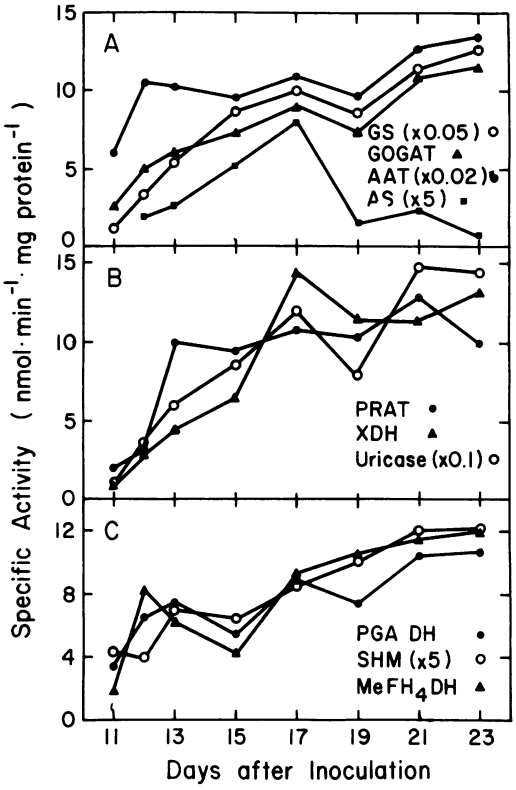

FIG. 2. Specific activities of ureide biogenic enzymes in the plant cytosol fraction during soybean nodule development.

to the purine biosynthetic pathway (Fig. 2C) also demonstrated increases in their specific activities over the time course. Generally, however, the increases shown by these enzymes were not as dramatic. PGA DH, the first enzyme of the serine biosynthetic pathway, and SHM, which catalyzes the synthesis of glycine from serine and produces N^5,N^{10}-Me FH_4, increased 3-fold over the time period. That the specific activities of these enzymes increased is significant as they were already present in the nodule (Fig. 2C) at comparatively high levels early in the time period. Synthesis of glycine and serine is important as a part of the increased protein biosynthesis occurring during nodule growth. The later increases in the activities of these enzymes could be the result of higher demands for serine, glycine, and Me FH_4 in increased *de novo* purine biosynthesis for ureide production.

Relationship of Developmental Changes in Enzyme Specific Activities to Nitrogenase Levels. If the specific activity (activity per mg protein) of an enzyme is constant, then the ratio of enzyme activity divided by nitrogenase activity will decrease as nitrogenase activity increases. If the value of this ratio increases, then the specific activity of an enzyme is not constant but increasing.

If GS, XDH, or PRAT activities were expressed as a function of nitrogenase activity, an increase was observed in the values of the ratios between days 11 and 17 (Fig. 3). Furthermore, these increases were sequential. The GS ratio showed a large increase between days 11 and 12. This is consistent with the important role of this enzyme in ammonia assimilation and its increase during nodule development paralleling that of nitrogenase activity (Fig. 1).

The PRAT ratio showed a large increase between days 12 and 13, and the increase in the ratio for XDH followed this. A somewhat different pattern was seen for SHM. This enzyme is one of those associated with the provision of metabolites to the purine

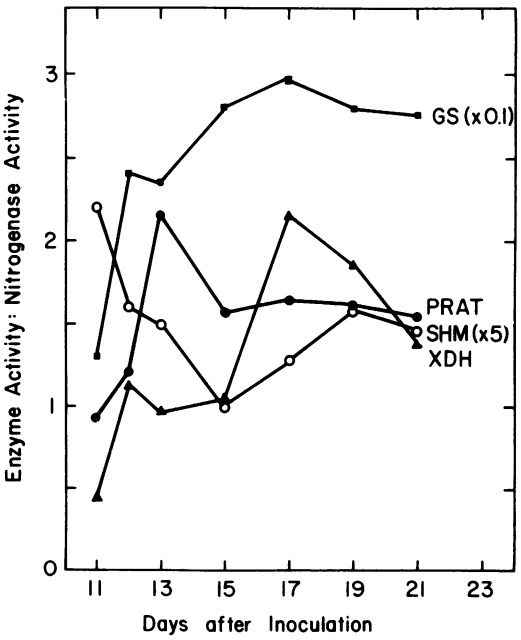

FIG. 3. Specific enzyme activity divided by specific nitrogenase activity during soybean nodule development. Nitrogenase specific activity values were obtained by applying an acetylene reduction-nitrogenase activity conversion factor of 4 to the data in Figure 1.

biosynthetic pathway and demonstrated constant specific activity in the early stages of nodule development. The increase in the already established levels of this enzyme did not occur until between 15 and 19 d after inoculation. The pattern seen for this enzyme is representative of that seen for the other enzymes involved in the supply of metabolites for purine biosynthesis

(Table I, nos. 7–10; Fig. 2C).

These data are consistent with a flow of nitrogen from ammonia to allantoic acid in the plant fraction of developing soybean nodules, and are in agreement with the hypothesis that products of purine biosynthesis *de novo* act as precursors for ureide biosynthesis. The ordered nature of the increases observed poses interesting questions as to the overall control of this pathway. It may be necessary for precursors to accumulate before the next set of enzymes appears. For example, purine accumulation may trigger the induction of xanthine dehydrogenase. On the other hand, a general depletion of existing serine may be required before the serine-glycine enzymes appear. The apparent induction of the ureide biogenic enzymes in distinct groups argues for this type of sequential control mechanism.

Amino Acid Levels in the Plant Cytosol Fraction of Developing Soybean Nodules. The plant fractions of plants aged between 15 and 23 days were subjected to amino acid analysis. The concentrations of key amino acids are given in Table II. Glutamate and aspartate levels were constant over the time period, whereas the level of glutamine showed a constant decline. The amount of asparagine showed a dramatic decrease between days 17 and 19. This was at the same time as the large decrease in the specific activity of AS in the plant fraction (Fig. 2A). However, the asparagine levels showed a recovery after this drop, rising to 80% of the level on day 15 by day 23. This could be due to retention of this amino acid in the plant fraction of the nodule, or to a non-glutamine dependent asparagine synthesis in the nodule. A similar fall and rise in asparagine concentration was observed by Schubert (14) in the xylem sap of developing soybean plants. Asparagine concentration decreased in the sap between days 14 and 20. This was followed by a recovery in xylem asparagine concentration to greater than 50% of the original levels.

Glycine levels were high and did not change significantly over the time period. Serine levels also remained constant. This may be because when the pools of these amino acids were drawn on by ureide biogenesis, the enzymes supplying them also increased. The overall pattern suggests a change in enzyme levels to ensure a smooth changeover to a ureide-exporting system with minimum disruption of nodule amino acid levels.

A Route for Ureide Biogenesis in Soybean Nodules. The time course data presented in this paper support a pathway of nitrogen assimilation via *de novo* purine biosynthesis and subsequent purine oxidation to allantoin and allantoic acid. The enzymes involved in this process can be placed into four distinct groups: (a) the ammonia assimilatory enzymes—GS, GOGAT, AAT, and AS; (b) PRAT, the first enzyme of the *de novo* purine biosynthetic pathway; (c) enzymes involved in the supply of glycine and 1-carbon fragments to purine biosynthesis—PGA DH, SHM, and Me FH_4 DH; and (d) the purine oxidative enzymes—XDH and uricase.

The group a enzymes (except AS) are responsible for the initial assimilation of ammonia, incorporating it into glutamine, glutamate, and aspartate which are utilized by other ureide biogenic enzymes. AS activity during nodule development stands apart from all the other enzyme activities followed over the time course. The marked decrease in the specific activity of this enzyme between 17 and 19 d after inoculation is reflected in a marked drop in the concentration of asparagine in the nodule. The decrease in the enzyme activity together with the low concentrations of asparagine, clearly show that this enzyme does not provide the major route for ammonia assimilation in the nodules of these plants. The recovery of asparagine levels in the nodule plant fraction after the decline in AS activity could reflect accumulation due to less transport or an alternative method of synthesis of this amino acid, particularly as glutamine levels continue to fall. A non-glutamine-dependent asparagine synthesis together with an asparagine-utilizing PRAT activity could represent another route for the flow of fixed atmospheric N in soybean nodules.

The data suggest that the group b enzyme, PRAT, utilizes the glutamine produced from the GS/GOGAT pathway and directs the flow of N through *de novo* purine biosynthesis. The specific activity of this enzyme increased substantially (6-fold), and this increase occurred early in the time course. The large early increase in PRAT activity (well before the other enzymes of purine metabolism) could implicate it in control.

Although PGA DH, SHM, and Me FH_4 DH are also involved in purine biosynthesis, these enzymes fall into a separate group (group c) as they are not as strongly induced as PRAT. There seemed to be established levels of these enzymes, and their activities did not appear to increase until after PRAT activity, and thus the demand for glycine and 1-carbon fragments was increased.

The purine oxidative enzymes (group d), XDH and uricase, increased last and were by far the most strongly induced activities involved with purine metabolism. The strong increase in these enzymes, together with the smaller increases in purine biosynthetic enzymes, is consistent with other comparative data (P.H.S. Reynolds, unpublished results) suggesting an elevation of endogenous purine biosynthesis which is further directed to allantoic acid production by the induction of a specific group of purine oxidative enzymes which are found only in ureide-transporting legume nodules. Indeed, concentrations of allantoic acid in the nodule plant fraction and in stem sections show increases after these enzyme activities are established (data not shown). The strong induction of the purine oxidative enzymes is due to their extremely low initial levels. Unlike the other enzymes of ureide biogenesis, they are not required for the metabolism associated with nodule growth.

Table II. *Amino Acids in the Plant Cytosol Fraction during Soybean Nodule Development*

Amino Acid	Amino Acid in Nodules				
	Day 15	Day 17	Day 19	Day 21	Day 23
			μmol/g		
Glutamate	3.8	3.5	3.1	3.2	2.5
Aspartate	1.9	1.8	1.7	2.1	1.8
Glutamine	0.22	0.18	0.08	0.04	0.04
Asparagine	0.52	0.45	0.18	0.36	0.42
Glycine	7.2	7.9	9.1	9.0	7.2
Serine	1.4	1.4	1.5	1.4	1.3
Alanine	1.8	1.8	1.8	1.6	1.4
β-Alanine	0.8	0.9	1.0	1.0	0.9

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