Enzymes of Amide and Ureide Biogenesis in Developing Soybean Nodules

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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 311b142). 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 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-14C]Glutamine was obtained from New England Nuclear, and [3-14C]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 (311b142). The plants were maintained in a Kysor Sherr 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 mM sucrose; 1 mM MgCl2; 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* (EC 6.3.1.2) activity was assayed using the biosynthetic

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1 Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate aminotransferase; AS, asparagine synthetase; PRAT, 5-phosphoribosylpyrophosphate amidotransferase; SHM, serine hydroxymethylase; Me FH, 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 in accordance
with 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 MgCl₂; 3 mM 5-phospho-
ribosylpyrophosphate; 12 mM glutamine (0.4 µCi [U-¹⁴C]glutamine
assay⁻¹); H₂O₂ and enzyme solution to a final volume of 200 µl.
The reaction mixture was preincubated in the absence of gluta-
mine 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 gluta-
mate 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 [3-¹⁴C]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 phos-
phohydroxypyruvate-dependent (0.8 mM) oxidation of 60 µM
NADH in 50 mM K-phosphate, pH 7.5.
N⁵,N¹⁰-Me FH₄ DH (EC 1.5.1.15) activity was determined from
the rate of reduction of NADPH (120 µM) when added to a mixture
containing 25 µl extract, 0.5 mM formaldehyde, and 0.2 mM 2-
mercaptoethanol, which had been preincubuated until the A₄₃⁴
nm was constant. Under these conditions, methenyl tetrahydro-
folate, the reaction product, hydrolyzes spontaneously to N⁵-
formyl tetrahydrofolate which does not contribute significantly
to the A₄₃⁴ nm (M. J. Boland, unpublished results).
N⁵,N¹⁰-Methyl tetrahydrofolate cyclohydrolase (EC 3.5.4.9)
was measured by the rate of disappearance of absorbance due to
the substrate (50 µM, E₃₅₀ = 26,500 [13]) in 50 mM Tris-acetate
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). Uracil activity was determined from
the rate of disappearance of A₂₉₂ 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 mini-
mized 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 sulfosalicylic 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.
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 biosyn-
thetic 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, leghemo-
globin showed a 26-fold increase in the plant fraction. Leghemo-
globin 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 NH₃
into amino acids, GOGAT and AAT, showed a 6- and 2.5-fold
increase in specific activity, respectively, and paralleled the in-
crease 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),
failing 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, allani-
toin 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 oxidi-
native 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

<p>| Table 1. Enzyme Activities in the Plant Cytosol Fraction of 21-Day-Old Soybean Nodules |
|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nitrogenase*</td>
<td>8.4</td>
</tr>
<tr>
<td>2. Glutamine synthetase</td>
<td>232</td>
</tr>
<tr>
<td>3. Glutamate synthase</td>
<td>11.5</td>
</tr>
<tr>
<td>4. Aspartate aminotransferase</td>
<td>644</td>
</tr>
<tr>
<td>5. Asparagine synthetase</td>
<td>0.5</td>
</tr>
<tr>
<td>6. 5-Phosphoribosylpyrophosphate amidotransferase</td>
<td>13.0</td>
</tr>
<tr>
<td>7. Phosphoglyceric acid dehydrogenase</td>
<td>104</td>
</tr>
<tr>
<td>8. N⁵,N¹⁰-Methylene tetrahydrofolate dehydrogenase</td>
<td>11.5</td>
</tr>
<tr>
<td>9. Serine hydroxymethylase</td>
<td>2.4</td>
</tr>
<tr>
<td>10. N⁵,N¹⁰-Methyl tetrahydrofolate cyclohydrolase</td>
<td>21.5</td>
</tr>
<tr>
<td>11. Xanthine dehydrogenase</td>
<td>11.5</td>
</tr>
<tr>
<td>12. Uricase</td>
<td>74.0</td>
</tr>
</tbody>
</table>

* This value for nitrogenase specific activity was obtained by applying a conversion factor of 4C/H₂ reduced per mol of N₂ fixed to the data in Figure 1.
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 \( N^\alpha,N^\alpha\text{-Me FH}_4 \), 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 \textit{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 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 allantioic 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 synthase 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 allantioic and allantioic 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, 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 19d 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, 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 allantioic 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 allantioic 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.

**Acknowledgments**—We wish to thank Larry L. Wall and Joe Absheer for their amino acid analyses.

**LITERATURE CITED**


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13. ROWE PB 1968 A simple method for the synthesis of N\(^{\gamma}, N\^{\gamma}\)-methenyltetrahydrofolic acid. Anal Biochem 22: 166-168
20. WOO KC, CA ATKINS, JS PATE 1980 Biosynthesis of ureides from purines in a cell-free system from nodule extracts of cowpea. Plant Physiol 66: 735-739