Enzymes of Ammonia Assimilation and Ureide Biosynthesis in Soybean Nodules: Effect of Nitrate

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ABSTRACT

The effect of nitrate on N2 fixation and the assimilation of fixed N2 in legume nodules was investigated by supplying nitrate to well established soybean (Glycine max L. Merr. cv Bragg)-Rhizobium japonicum (strain 311b110) symbioses. Three different techniques, acetylene reduction, 15N2 fixation and relative abundance of ureides ([ureides/(ureides + nitrate + α-amino nitrogen)] × 100) in xylem exudate, gave similar results for the effect of nitrate on N2 fixation by nodulated roots. After 2 days of treatment with 10 millimolar nitrate, acetylene reduction by nodulated roots was inhibited by 48% but there was no effect on either acetylene reduction by isolated bacteroids or in vitro activity of nodule cytoplasmic glutamine synthetase, glutamine oxoglutarate aminotransferase, xanthine dehydrogenase, uricase, or allantoinase. After 7 days, acetylene reduction by isolated bacteroids was almost completely inhibited but, except for glutamine oxoglutarate aminotransferase, there was still no effect on the nodule cytoplasmic enzymes. It was concluded that, when nitrate is supplied to an established symbiosis, inhibition of nodulated root N2 fixation proceeds the loss of the potential of bacteroids to fix N2. This in turn precedes the loss of the potential of nodules to assimilate fixed N2.

In the cytoplasm of legume nodules, ammonia produced by the N2 fixing bacteroids is incorporated into glutamine and glutamate via the GS, GOGAT pathway (19). Glutamate subsequently undergoes transamination reactions to yield aspartate and other amino acids. In tropical legumes, such as soybean, glutamine, aspartate, and glycine, are incorporated into purines which are subsequently oxidatively degraded to form the ureides, allantoin and allantoic acid. Purine oxidation proceeds via xanthine dehydrogenase and uricase to produce allantoin and finally allantoinase catalyzes the conversion of allantoin to allantoic acid (19). The ureides are the principal form in which fixed nitrogen is transported from soybean nodules (19). Furthermore, since ureide biosynthesis in soybean is virtually unique to the nodule, levels of these compounds in the xylem sap and shoot axes have been used to estimate nitrogen fixation (13, 16).

When nitrate is supplied to a legume inoculated with Rhizobium bacteria, root infection, nodule development, N2 fixation and synthesis of bacteroid proteins (including nitrogenase) are inhibited (7, 18, 19). In soybean, ureides as a proportion of total N2 in the xylem sap and shoot axes also decline (13, 16). The mechanisms by which nitrate affects these parameters are not well understood. The purpose of this study was to investigate the relationship between N2 fixation and the bacteroid and nodule cytoplasmic enzymes involved in N2 fixation and the assimilation of fixed N2, as they are affected by nitrate. The experimental approach involved supplying nitrate to well established symbioses rather than from the time of planting. This simplified the system under investigation since it excluded the inhibitory effects of nitrate on root infection and differentiation of the root tissue to form nodules.

MATERIALS AND METHODS

Plant Materials and Chemicals. Soybean plants (Glycine max L. Merr. cv Bragg) inoculated with Rhizobium japonicum strain 311b110 (supplied by J. C. Streeter Department Agronomy, Ohio State University, Wooster, OH 44691) were grown in 81 pots of either vermiculite or sand (see legends to figures and tables) in a naturally lit glasshouse. Plants were watered daily with tap water and twice weekly with a N-free nutrient solution (12). When the symbioses was well developed, the nutrient solution was supplemented with either KCl (control plants) or KNO3 (treated plants). Daily watering with the supplemented nutrient solution was continued for up to 14 d. Plants were in vegetative growth phase for the duration of each experiment. Chemicals used in nodule extraction buffers and enzyme assays were obtained from Sigma Chemical Co. All other chemicals were analytical reagent grade and were obtained from various sources.

Comparative Acetylene Reduction and 15N2 Fixation Assays. Plants were grown in pots of vermiculite (10 plants/pot) for 38 d prior to the commencement of treatment with 5 mm nitrate. Three plants from each of two pots per treatment were harvested for either acetylene reduction or 15N2 fixation assays. The remaining plants were retained in their pots for xylem sap collection (see later). Single root systems trimmed to the well nodulated crown region were placed in 37 ml tubes which were then capped with Suba-seals. Six tubes per treatment were injected with 3.0 ml N2 (98% 15N2) and 0.8 ml O2 and another six with 4.0 ml acetylene and 0.8 ml O2. After 30 min, samples were removed from the acetylene tubes for ethylene determination by FID-GC and the 15N2 tubes were opened and vented. Nodule and root fresh weights were determined. The material exposed to 15N2 was digested and analyzed for 15N (6) using a Micromass 903 (V. G. Micromass, Winsford, Cheshire, U.K.) mass spectrometer.

Standard Acetylene Reduction Assay. Plants were grown in pots of vermiculite (10 plants/pot) for 41 d prior to the commencement of treatment with 10 mm nitrate. Five plants from each of two pots per treatment were harvested to be assayed for

1 Supported by an Agrigenetics Corporation sponsored research program and an Australian Government Postgraduate Research Award to K. A. S.
2 Abbreviations: GS, glutamine synthetase; GOGAT, glutamine oxoglutarate aminotransferase; XDH, xanthine dehydrogenase; β-HBDH; β-hydroxybutyrate dehydrogenase; FID, flame ionization detector.

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acetylene reduction activity. Xylem sap was collected from the remaining plants (see later). Pairs of intact root systems were placed in 1 L gas tight jars fitted with a rubber stopper. Acetylene (50 ml) was added to give a concentration in air of 5% (v/v). After 20 min, gas samples were removed and analyzed for ethylene by FID-GC. Nodule fresh weights were determined and nodules stored at 70°C for later analysis for enzyme activities.

**“Root Bleeding” Xylem Sap Collection.** Xylem sap was collected from the tops of decapitated root systems over a period of 20 min beginning at approximately 10:00 h each sampling day. Sap samples were stored frozen and later analyzed for ureides, α-amino-N and nitrate. Nodules harvested from these plants were used for nodule nitrogen analyses.

**Nodule Extracts for N Analyses.** Nodules (0.3 g) were homogenized with a mortar and pestle in 3 ml of 0.25 N HClO4 (1). The homogenate was centrifuged at 10,000g for 15 min and the supernatant neutralized with the required volume of 2 N KOH. The resultant KClO4 precipitate was removed by centrifugation and the supernatant retained for analysis.

**Nitrification, α-Amino Nitrogen and Ureide Analyses.** Nitrification was determined by the salicylic acid method (8) α-amino-N by a modification (14) of the ninhydrin method (28) and ureides as the phenylhydrazone of glyoxylate (26).

**Enzyme Assays.** Nodules (0.3 g) to be assayed for GS and GOGAT activity were homogenized with a mortar and pestle in 3 ml of 100 mM Mes buffer (pH 6.8), 100 mM succrose, 2% (v/v) 2-mercaptoethanol, and 15% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000g for 30 min and the supernatant was retained for enzyme assays. Transferase activity of GS was determined using the γ-glutamyl transferase assay as described by Cullimore and Sims (10) with the exception that Mes buffer replaced Tris/acetate buffer. GOGAT activity was assayed as described by Groth and Vance (11). Nodules (0.3 g) to be assayed for XDH and uricase activity were homogenized in 3 ml 25 mM Tes buffer (pH 7.5), 0.3 M sorbitol, 1 mM DTE, and insoluble PVP (0.1 g per g nodule fresh weight) and the homogenate centrifuged at 75,000g. The supernatant was retained for enzyme assays. DTE was excluded from the extraction buffer for allantoinase assays because it inhibits allantoinase activity. All three enzymes were assayed as described by Schubert (21).

**Leghemoglobin and Total Soluble Protein Determinations.**

Extractions were analyzed for leghemoglobin and total soluble protein. Leghemoglobin was determined by the pyridine-hemochrom method (2) and total soluble protein by the method of Lowry et al. (15).

**Bacteroid Isolation and Assay for Acetylene Reduction.** Plants were grown in pots of sand (5 plants/pot) for 8 to 9 weeks prior to the commencement of treatment with 10 μM nitrate. Nodules were harvested from 5 to 10 plants. Bacteroids were isolated anaerobically as previously described (5, 20). Aliquots (0.5 ml) of the resultant bacteroid suspension (2 mg protein/ml) to be assayed for acetylene reduction were injected into 22 ml glass scintillation-vials sealed with a Suba-seal. The vials contained 0.5 ml of wash buffer (20) plus 0.2, 0.5, 1.0, 1.5, or 2.0% O2 (v/v) in N2 or argon. Malate (10 mm) was added to the wash buffer where indicated in Table IV. The assay was initiated by the addition of 1 ml of acetylene and the vials were sealed at 25°C. After 20 min, gas phase samples were removed and analyzed for ethylene by FID-GC. Protein content of the bacteroid isolates was determined by the method of Lowry et al. (15).

**Estimation of Bacteroid Content of Nodules.** The bacteroid content of nodules was estimated using β-HBDH as a bacteroid specific marker (27). The β-HBDH activity of bacteroid preparations and nodule homogenates was measured by following the oxidation of NADH in the presence of acetoacetate. The reaction mixture (1 ml) contained 50 mM Tes buffer (pH 7.0), 0.2 mM KCN, 20 μM NADH, and 50 μl of sonicated (2 min, 300 W) bacteroids or nodule homogenate. The reaction was initiated by adding acetoacetate (5 mM, final concentration) and the decrease in absorbance measured with a Pye-unicam P8800 spectrophotometer. The bacteroid protein content of nodules was estimated by expressing bacteroid β-HBDH activity per mg bacteroid protein and nodule β-HBDH activity per g nodule fresh weight.

**RESULTS**

**Effect of Nitrate on N2 Fixation: Comparison of Estimates.**

Table I illustrates the effect of 5 μM nitrate on acetylene reduction and 15N2 fixation by nodulated roots. Acetylene reduction, was inhibited by 44% and 60% after 2 and 6 d, respectively, of nitrate treatment. Similar levels of nitrate induced inhibition (47% after 2 d and 59% after 6 d) were observed when N2 fixation was assayed using 15N2.

The effect of 5 μM nitrate on xylem sap nitrogen content is shown in Table II. Ureide concentration in the xylem sap of treated plants had decreased by 66 and 46% after 2 and 6 d, respectively, of nitrate treatment. Thus, the magnitude of the effect of nitrate on xylem ureide concentration was greater than that for acetylene reduction and 15N2 fixation (Table I) after 2 d but lesser after 6 d. The sum of ureides, α-amino-N, and nitrate accounted for approximately 95% of the total Kjeldahl-N in the xylem sap of soybean (16). Therefore, for the purposes of the following analysis this sum will be referred to as total N. Ureides constituted 79 and 63% of total N in the xylem sap of control plants after 2 and 6 d, respectively, of nitrate treatment. The corresponding figures for the nitrate treated plants were 17 and 31%. This decline in ureides as a proportion of total N was largely attributable to the increase in total N upon the appearance of nitrate in the xylem sap. Nitrate accounted for 71 and 64% of total N in the xylem sap of nitrate treated plants after 2 and 6 d, respectively, of nitrate treatment.

Table III illustrates the effect of 5 μM nitrate on nodule N content. Only low concentrations of nitrate were detectable and α-amino-N levels were significantly lower in the nodules of nitrate treated plants relative to control plants. There was no significant effect of a 2-d nitrate treatment on nodule ureide level. A 6-d nitrate treatment, on the other hand, resulted in a significant accumulation of ureides in the nodule and this coincided with the increase in ureide content of xylem sap collected from treated plants.

**Effect of Nitrate on Enzymes of Ammonia Assimilation and Ureide Biosynthesis in the Nodule.** In these experiments nitrate was supplied at a concentration of 10 μM, rather than 5 μM, due to the more marked effect on N2 fixation. N2 fixation, as measured by acetylene reduction was inhibited by 48, 94, and 92 after 2, 7, and 14 d, respectively, of nitrate treatment (Fig. 1A). Similar, though less pronounced, inhibitions of N2 fixation were indicated by xylem ureide concentration. Values for treated plants were 23, 70, and 88 less than the relevant controls after 2, 7, and 14 d, respectively, of nitrate treatment (Fig. 1B). Total soluble protein content of the nodule, expressed on a nodule fresh weight basis, was not significantly affected by nitrate treatment for the 14-d duration of the treatment (Fig. 1C). Nodule leghemoglobin content, expressed on a mg nodule soluble protein basis, declined significantly during the 2nd week of nitrate treatment but was unaffected during the 1st week (Fig. 1D).
Table I. Effect of Nitrate on N₂ Fixation by Nodulated Roots

Plants grown in vermiculite during November–December 1984 were supplied daily with either 5 mM KCl (control) or 5 mM KNO₃ (treated) from 38 d after planting. Plants were harvested 2 and 6 d after commencement of the treatment and assayed for acetylene reduction using the “comparative” assay (see “Materials and Methods”). Acetylene reduction is expressed as μmol C₂H₂ produced h⁻¹ g⁻¹ nodule fresh weight and ¹⁵N₂ fixation as μmol N₂ fixed h⁻¹ g⁻¹ nodule fresh weight. Data are the mean ± sd (n = 6). Figures in parentheses are percent inhibition.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
<th>Control</th>
<th>Treated</th>
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<tr>
<td>Acetylene reduction</td>
<td>11.9 ± 3.9</td>
<td>6.6 ± 2.0</td>
<td>64 (44%)</td>
<td>13.2 ± 2.6</td>
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<td>¹⁵N₂ fixation</td>
<td>3.8 ± 0.8</td>
<td>2.1 ± 0.8</td>
<td>47 (7%)</td>
<td>5.8 ± 2.4</td>
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</tbody>
</table>

Table II. Effect of 5 mM Nitrate on N Content of Xylem Sap

Experimental details are outlined in the legend to Table I. Data are the mean ± sd and are expressed as μmol ml⁻¹ xylem sap. Figures in parentheses are the number of replicates.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 d</th>
<th>6 d</th>
<th>2 d</th>
<th>6 d</th>
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</thead>
<tbody>
<tr>
<td>Ureides</td>
<td>5.6 ± 1.0 (5)</td>
<td>1.9 ± 0.3 (5)</td>
<td>5.2 ± 1.5 (7)</td>
<td>2.8 ± 1.1 (7)</td>
</tr>
<tr>
<td>α-NH₂-N</td>
<td>1.5 ± 0.5 (5)</td>
<td>1.3 ± 0.3 (5)</td>
<td>3.0 ± 0.3 (5)</td>
<td>0.5 ± 0.7 (4)</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0 (5)</td>
<td>8.0 ± 1.5 (5)</td>
<td>0 (6)</td>
<td>5.8 ± 1.4 (7)</td>
</tr>
</tbody>
</table>

Table III. Effect of 5 mM Nitrate on N Content of the Nodule

Experimental details are outlined in the legend to Table I. Data are the mean ± sd (n = 4) and are expressed as μmol g⁻¹ nodule fresh weight.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 d</th>
<th>6 d</th>
<th>2 d</th>
<th>6 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ureides</td>
<td>7.2 ± 1.0</td>
<td>5.9 ± 0.5</td>
<td>8.5 ± 0.6</td>
<td>12.5 ± 1.2</td>
</tr>
<tr>
<td>α-NH₂-N</td>
<td>32.1 ± 2.6</td>
<td>22.9 ± 1.0</td>
<td>28.5 ± 1.5</td>
<td>18.2 ± 1.7</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0</td>
<td>1.1 ± 0.1</td>
<td>0</td>
<td>3.9 ± 1.0</td>
</tr>
</tbody>
</table>

Effect of Nitrate on Acetylene Reduction by Isolated Bacteroids. The results of a typical experiment in which acetylene reduction by bacteroids isolated from the nodules of nitrate treated plants was compared with that of bacteroids isolated from the nodules of control plants are presented in Table IV. After 2 d of nitrate treatment, acetylene reduction activity of bacteroids isolated from both control and nitrate treated plants, was stimulated 3.0- to 3.5-fold when malate was supplied to supplement endogenous substrates. When expressed on a mg bacteroid protein basis acetylene reduction activity of control, nodulated roots was similar to the malate-supported activity of isolated bacteroids, demonstrating that little activity was lost during bacteroid isolation. There was no effect of a 2-d nitrate treatment on either endogenous or malate-supported acetylene reduction by isolated bacteroids. This was in contrast to the effect on acetylene reduction by nodulated roots, which was inhibited by 70% at this stage. After 7 d of nitrate treatment, endogenous substrate-supported acetylene reduction activity of isolated bacteroids was not detectable. This coincided with a 90% inhibition of nodulated root acetylene reduction activity. A low level of isolated bacteroid acetylene reduction was recovered when endogenous bacteroid substrates were supplemented with malate. However, this was only 21% of the activity of bacteroids isolated from control plants.

DISCUSSION

Bacteroids isolated from the nodules of nitrate treated plants retained full N₂ fixation capacity despite a strong inhibition of nodulated root N₂ fixation. These results confirm those obtained previously with soybean bacteroids (17) but are in conflict with results obtained with bacteroids isolated from French bean nodules (25). This conflict may arise because the Rhizobium species which infects soybean differs from that which infects French bean or because French bean nodule metabolism is more susceptible to nitrate treatment than it is that of soybean. Alternatively, if nitrate-induced inhibition of N₂ fixation is due to a direct effect of NO₃⁻, as has been postulated (25), then differences between soybean and French bean may be attributable to differences in nodule NO₃⁻ levels or compartmentation. Whatever the explanation, it is clear that, in soybean, nitrate-induced inhibition of nodulated root, N₂ fixation precedes loss of functional bacteroid nitrogenase protein. This is in accord with results of previous studies with soybean and pea in which nitrate treatment...
EFFECT OF NITRATE ON UREIDE BIOSYNTHESIS IN SOYBEAN NODULES

The in vitro activities of XDH, uricase, and allantoinase were not inhibited for at least 7 d after the commencement of nitrate supply to an established soybean-R. japonicum symbiosis. This observation confirms previously reported results of experiments in which nitrate was supplied from the time of planting (29).

Given that a mean half-life for nodule bacteroid proteins of 5 d and for soluble plant proteins of 15 to 20 d has been reported for lupin nodules (9) it is not surprising that loss of nitrogenase activity of isolated bacteroids precedes the loss of in vitro activity of the plant derived enzymes involved in ureide biosynthesis in soybean nodules.

Depriving cowpea plants of N2 by exposing them to an atmosphere of 80% Ar:20% O2 does not affect acetylene reduction activity but results in a decline in the activity of enzymes involved in ureide biosynthesis indicating that ammonia plays a role in the regulation of these enzymes (3). This observation is not in conflict with the results presented here since in the present study nitrate treated plants continued to fix N2 albeit at a lower level.

The accumulation of ureides in the nodules of nitrate treated plants, observed in the present study, has been reported previously (24). There are several possible explanations for this, none of which have been thoroughly investigated. First, ureides may be synthesized from nitrate in either the root or the nodule or both. Results of previous studies (22-24) indicate that the necessary enzyme activities are expressed in the nodule. Second, nitrate may have an inhibitory effect on the export of ureides from the nodule (24). A third alternative is that ureides may arise from nucleotide breakdown in response to nitrate induced nodule senescence (3, 14). Whatever the explanation for ureide accumulation in the nodules of nitrate-treated soybean plants it is delayed relative to the inhibition of N2 fixation and therefore cannot be involved in the primary mechanism of inhibition of N2 fixation.

CONCLUDING REMARKS

Nitratre treatment of an established soybean-R. japonicum symbiosis leads to severe inhibition of nodulated root N2 fixation within 2 d. However, isolated bacteroid N2 fixation and in vitro activity of enzymes involved in ammonia assimilation and ureide biosynthesis in the nodule is not affected until much later (7-14 d). The eventual inhibition of isolated bacteroid N2 fixation and the in vitro activity of some of the enzymes involved in the

fig. 2. Effects of nitrate treatment on nodule GOGAT (A), XDH (B), uricase (C), and allantoinase activity (D). Each point is the mean ± so of three replicate nodules extracts. Control plants are represented by (O) and treated plants by (■). Nodules were collected from the plants used to obtain the data presented in Figure 1.

failed to repress the synthesis of nitrogenase relative to other bacteroid proteins (7, 18).

Nodule GS activity was not affected by nitrate treatment for at least 14 d and the decline in GOGAT activity was delayed relative to the inhibition of N2 fixation. Similar results were obtained with alfalfa (4, 11) in that GOGAT activity was more susceptible to nitrate treatment than GS activity and inhibition of N2 fixation preceded loss of in vitro GS and GOGAT activity.

Table IV. Effect of 10 mm Nitrate on Acetylene Reduction by Nodulated Roots and Isolated Bacteroids

<table>
<thead>
<tr>
<th></th>
<th>2 d Control</th>
<th>2 d Treated</th>
<th>7 d Control</th>
<th>7 d Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nodulated Roots</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>μmol Ethylene·h⁻¹·g⁻¹ nodule fresh wt</td>
<td>11.3 (70%)</td>
<td>3.4 (70%)</td>
<td>16.2 (91%)</td>
<td>1.5 (91%)</td>
</tr>
<tr>
<td>nmol Ethylene·h⁻¹·mg⁻¹ bacteroid protein</td>
<td>336 (68%)</td>
<td>106 (68%)</td>
<td>432 (89%)</td>
<td>46 (89%)</td>
</tr>
<tr>
<td><strong>Isolated Bacteroids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmol Ethylene·h⁻¹·mg⁻¹ bacteroid protein</td>
<td>Endogenous</td>
<td>128 (%)</td>
<td>27</td>
<td>0 (100%)</td>
</tr>
<tr>
<td></td>
<td>5 mm Malate</td>
<td>435 (0%)</td>
<td>453</td>
<td>96 (79%)</td>
</tr>
</tbody>
</table>

Soybean plants, grown in sand during January–February 1985 and supplied daily with 10 mm KNO3 (treated) or 10 mm KCl (control) from 8 to 9 weeks after planting, were harvested 2 and 7 d after the commencement of nitrate treatment. Nodulated roots were assayed for acetylene reduction using the “standard” assay (see “Materials and Methods”) and activity was expressed on a mg bacteroid protein basis. The bacteroid protein content of nodules was estimated by using β-HBDH as a bacteroid specific marker (see “Materials and Methods”). This activity (80 μmol NAD·min⁻¹·mg⁻¹ protein) did not change significantly during the period of treatment, nor upon nitrate application (DA Day, unpublished data). The bacteroid content of nodules was 34.1 ± 2.3 mg bacteroid protein, g⁻¹ nodule fresh weight (± SD, n = 4) and was unaffected by nitrate treatment.

Bacteroids were isolated and assayed for acetylene reduction as described in “Materials and Methods.” The bacteroid acetylene reduction activities shown were measured in the presence of endogenous substrates or added malate (5 mm), at the optimal O2 concentration for acetylene reduction (determined separately for each preparation). Figures in parentheses are percent inhibition.
assimilation of fixed N₂ are secondary responses to nitrate treatment. The primary mechanism by which nitrate inhibits N₂ fixation remains unknown.

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