Nodule Protein Synthesis and Nitrogenase Activity of Soybeans Exposed to Fixed Nitrogen

K. DALE NOEL, MITCHELL CARNEOL, AND WINSTON J. BRILL
Department of Bacteriology and Center for Studies of Nitrogen Fixation, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Nitrate or ammonium was added to soybean (Glycine max L. Merrill cv Corsoy) plants grown in plastic pouches 10 days after nodules first appeared. By the third day of treatment with 10 millimolar nitrate, nitrogenase specific activity (per unit nodule weight) had decreased to 15% to 25% of that of untreated plants. Longer incubations and higher concentrations of nitrate had no greater effect. In addition, exogenous nitrate or ammonium resulted in slower nodule growth and decreased total protein synthesis in both the bacterial and the plant portion of the nodule (as measured by incorporation of 35S). Two-dimensional gel electrophoresis revealed that the nitrogenase components were not repressed or degraded relative to other bacteroid proteins. In the presence of an optimal carbon source, the nitrogenase specific activity of nodules detached from nitrate-treated plants was equivalent to that of nodules from untreated plants. These results are consistent with models that propose decreased availability or utilization of photosynthetic in root nodules when legumes are exposed to fixed nitrogen.

When fixed nitrogen is added to nodulated legume plants, Rhizobium nitrogenase activity decreases. In nonsymbiotic bacteria, the response to fixed nitrogen is mediated by repression of nitrogenase synthesis (15). However, in the Rhizobium-legume symbiosis, the bacterium is captive to the biochemical environment imposed by the surrounding plant tissue. In light of this, most theories for explaining the response to fixed nitrogen dismiss any bacterial role and focus upon factors that might change in the plant when exogenous fixed nitrogen becomes available (1, 9, 10, 13, 17, 21).

However, a possibility that has not been investigated until recently (1, 7) is that the Rhizobium might repress the synthesis of nitrogenase and other nitrogen-fixation-specific proteins in response to plant metabolites that change when fixed nitrogen is present exogenously. Using the resolution provided by O'Farrell two-dimensional gel electrophoresis (12), we have reinvestigated this response, particularly with respect to the bacterial proteins synthesized in the nodule. The results of these experiments provide further evidence that the bacterial role is passive.

MATERIALS AND METHODS

Bacterial Strains and Plant Variety. The Rhizobium japonicum strain used to inoculate the plants was strain 311b110, colony-type I-110, originally obtained from G. H. Elkan. The cultivar of soybean (Glycine max L. Merrill) was Corsoy, obtained from the Agronomy Department, University of Wisconsin.

Media. AMA is a yeast-extract mannitol broth medium (19). The plant nutrient solution was nitrogen-free RBN (19) modified to lower the sulfate content to allow for radioactive labeling of plant proteins with [35S]sulfate. Per liter of water the RBN' contained 15 mg NaFeEDTA, 23 mg K$_2$SO$_4$, 0.79 g KCl, 0.11 g NaH$_2$PO$_4$, H$_2$O, 0.17 g Na$_2$HPO$_4$, 1 ml 1 mM CoCl$_2$, and 1 ml of a micronutrient solution which contained per liter of water 3.73 g KCl, 1.55 g H$_2$BO$_3$, 0.85 g MnSO$_4$, H$_2$O, 0.13 g CuSO$_4$, 5H$_2$O, 0.18 g (NH$_4$)$_2$Mo$_7$O$_24$, 4H$_2$O, and 0.58 g ZnSO$_4$, 7H$_2$O. The plants were not noticeably affected by the lowered sulfur content.

Growth of the Plants. Seeds were surface-sterilized with commercial bleach followed by diluted HCl, as previously described (19), and germinated in the dark on sterile filter paper moistened with water. After 2 d, the seedlings were planted in growth pouches (Scientific Products) (20) containing 25 ml sterile RBN and 25 ml sterile water; two seedlings were planted per pouch. Each plant was inoculated at this time by entraining 0.5 ml of a fully-grown AMA culture of R. japonicum down the root. Sterile H$_2$O was added as needed to maintain the water level between 20 ml and 50 ml. Nodules began to appear after 10 to 11 d. At 14 d 25 ml RBN' was added.

Treatment of the Plants with Nitrate or Ammonia. Twenty-one d after being planted, the medium in the pouches was replaced with 25 ml RBN', 25 ml water, and KNO$_3$ or NH$_4$Cl at the specified concentration. Every other day thereafter, the nitrate or ammonium salts were replenished by emptying the medium from the pouches, flushing the plants with water three times to remove loosely bound salts and replacing the RBN', water, and nitrogen salt. Control plants were treated identically except that KCl (or water) was used in place of a nitrogen salt. In all the experiments reported here 50 mg CaCO$_3$ was added to the pouch to buffer the pH between 6.5 and 7.0.

Nitrogenase Activity (Acetylene Reduction [5]) of Intact Roots. The drained roots of each pouch were incubated with 5% (v/v) acetylene in air for 1 h at room temperature. After analysis for ethylene content by GC, nodules were stripped from the roots and weighed and counted. Specific activity was calculated as the nmol acetylene reduced per hr per mg nodule fresh weight. At least six plants (three pouches) per treatment were used for each determination.

Acetylene Reduction by Detached Nodules. Nodules were picked from the root such that each set to be compared had nodules of approximately equal size. Approximately 50 mg freshly-picked nodules (10-15 in number) were weighed, were sliced once with a clean razor blade, and were placed in a 16-mm, 24-ml glass tube. They were covered with 0.10 ml of a buffer at pH 7.5 containing 50 mM Tris (hydroxymethyl) aminomethane-hydrochloride, 2.5 mM MgCl$_2$, and a carbon source (if added) at
was washed and incubated in 0.1 M NaCl/L immediately following nitrogen salts, acetylene was provided, and the tubes were shaken at 250 rpm at 30°C. At specified times, 0.3 ml of the gas phase was withdrawn for analysis on the gas chromatograph.

Table I. Nodule Parameters after 6 Days of Exposure of Plants to KNO₃

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodules/Plant</th>
<th>Acetylene Reduction</th>
<th>³⁵S Precipitable from Nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>14 ± 4</td>
<td>98 ± 6</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>5 mM KNO₃</td>
<td>16 ± 3</td>
<td>80 ± 4</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>10 mM KNO₃</td>
<td>14 ± 3</td>
<td>74 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>20 mM KNO₃</td>
<td>14 ± 2</td>
<td>67 ± 5</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>10 mM KCl</td>
<td>16 ± 4</td>
<td>92 ± 10</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>20 mM KCl</td>
<td>14 ± 3</td>
<td>78 ± 7</td>
<td>26 ± 7</td>
</tr>
</tbody>
</table>

* Value at 21 d (when salts first added) was 58 ± 8 mg.

0.1 mM (8). An atmosphere of 90% (v/v) O₂ (8) and 10% (v/v) acetylene was provided, and the tubes were shaken at 250 rpm at 30°C. At specified times, 0.3 ml of the gas phase was withdrawn for analysis on the gas chromatograph.

Analysis of Protein Synthesis. After the 4th d of the treatment with nitrogen salts, ³⁵S sulfate (0.1 mCi/plant) was added immediately following the second replacement of the root-media. Nodules were picked after 2 d of further incubation. To have a reasonably homogeneous population, the nodules were picked only from the taproots and the upper lateral roots where they first appeared. From this time onward, the nodules or their contents were maintained at 0°C to 4°C, unless otherwise specified. To 300 mg fresh weight of nodules was added 2.0 ml of PBS buffer (6.8 g KH₂PO₄, 8.7 g K₂HPO₄, and 8.7 g NaCl/L of water [pH 6.8]), and they were crushed with a glass rod. After centrifugation for 2 min at 100g, the sedimented nodule debris was crushed and
Table II. Nodule Parameters after 6 Days of Exposure of Plants to NH₄Cl

The procedure was identical to that of Table I. Values are ± SD, except for ³⁵S incorporation, which was determined on nodules pooled from each set.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodules/Plant</th>
<th>Acetylene Reduction</th>
<th>³⁵S Precipitable from Nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Fresh wt (mg)</td>
<td>nmol h⁻¹ fresh nodule</td>
</tr>
<tr>
<td>No addition</td>
<td>14 ± 2</td>
<td>93 ± 10</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>1 mM NH₄Cl</td>
<td>14 ± 2</td>
<td>81 ± 4</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>3 mM NH₄Cl</td>
<td>13 ± 2</td>
<td>69 ± 8</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>10 mM NH₄Cl</td>
<td>11 ± 3</td>
<td>53 ± 6</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>30 mM NH₄Cl*</td>
<td>11 ± 2</td>
<td>56 ± 7</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>10 mM KCl</td>
<td>18 ± 4</td>
<td>84 ± 8</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>30 mM KClb</td>
<td>14 ± 2</td>
<td>65 ± 8</td>
<td>25 ± 5</td>
</tr>
</tbody>
</table>

* The plant shoots were somewhat stunted and the leaf edges necrotic.

b The first trifoliate leaves were yellowed.

Fig. 2. Autoradiographs of two-dimensional gel electrophoresis of bacteroid proteins labeled with ³⁵S during growth of the plant in the presence of KNO₃, KCl or in RBN² nutrient medium alone (control). Nitrogenase component II is circled. The small arrow points to a nitrogenase component I protein. The large arrow points to bacteroid protein 3 (Table III), a major bacteroid-specific protein of unknown function. These last two proteins are just visible on the autoradiograph of bacteroid proteins from plants treated with 10 mM KNO₃. Each gel contained contents from equivalent weights of nodules, approximately 100 μg protein (Fig. 3), and each film was exposed for 7 d.

Downloaded on May 30, 2021. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.
NODULES OF SOYBEANS EXPOSED TO NITRATE

suspended once again in 1.5 ml PBS buffer. The second extract, after being separated from the debris at 100g, was combined with the turbid supernatant mixture of the first separation and centrifuged at 12,000g for 15 min. The resulting supernatant fluid, deemed the soluble plant portion, was stored at –20°C. The pellet was recentrifuged at 3,000g for 10 min. The resulting pellet, deemed the bacteroid fraction, was resuspended in 1 ml PBS buffer. A 50μl portion was saved for radioactivity measurements; the remainder was resuspended at 3,000g for 10 min and stored at –20°C.

The amount of radioactivity incorporated into acid-precipitable material was determined by adding 1.0 ml of cold 5% (w/v) TCA to 0.10 ml of the soluble plant portion with 0.10 mg BSA carrier, or to 0.050 ml of the bacteroid suspension. After 20 min on ice, each sample was filtered on GELMAN GA-6 (0.45) filters. Following two rinses of 1.0 ml cold 5% TCA and two 1.0 ml rinses with cold 70% (w/v) ethanol, the filters were dried at 60°C to 70°C in scintillation vials. Once cool, they were covered with 10 ml of scintillant cocktail, containing 15.2 g PPO, 0.19 g POPOP, and 3.8 L toluene, and counted on a Packard TriCarb 3255 liquid scintillation counter, using the 14C settings adjusted for quenching.

Bacterial pellets from approximately 300 mg fresh weight of nodules were processed for analysis by two-dimensional polyacrylamide gel electrophoresis using the method of O’Farrell (12). To the frozen pellet in a 3 ml plastic tube was added 0.15 ml of cold sonication buffer containing 0.5 mg/ml deoxyribonuclease I (Sigma), 0.1 mg/ml ribonuclease A (Sigma), and 5 mM MgCl2 in 10 mM Tris HCL (pH 7.4). The suspension was treated for 30 s with the microtip probe of a Branson Sonifier W350 at 40 W output. After 5 min further incubation on ice, the extract was re frozen on dry ice. Urea (100 mg) and 0.10 ml lysis buffer (12) was added. Following two more cycles of freezing and thawing, 0.10 ml of the sample was applied to an isoelectric focusing tube gel (12) or stored at –20°C. The acrylamide concentration in the second (SDS) dimension was 12%, with 0.32% bis-acrylamide. The second-dimension gels were stained with Coomassie Blue (12) and dried. The dried gels were placed against XR-1 film at –80°C. The radioactivity of proteins of interest was measured by the method of Helleiner and Wunner (6) in 0.7 cm × 0.5 cm squares cut from the gels.

The positions of components I and II of nitrogenease on the two-dimensional gels were determined by using nitrogenease components purified from nodules containing R. japonicum strain 110. These samples of pure component I and component II were kindly supplied by Daniel Arp, Biochemistry Department, University of Wisconsin. The pure components and bacteroid samples were analyzed separately, and they were analyzed together after being combined such that the concentration of added pure component I or component II was at least 3 times that contained in the bacteroid sample itself (KD Noel, G Stacey, SR Tandon, LE Silver, WJ Brill, unpublished).

RESULTS

Kinetics of Nitrogenase Suppression. Nitrogenase activity declined during the first day of nitrate treatment but full inhibition did not occur until the 2nd or 3rd d (Fig. 1). This inhibition was readily reversed by flushing out the nitrate; almost complete recovery was achieved in 3 d (Fig. 1). Somewhat different kinetics resulted from treatment with 5 mM NH4Cl. Full inhibition was reached in general 1 d later than with nitrate and recovery after removing ammonium was slower (data not shown).

Other Nitrogenease Parameters Affected. As indicated by nodule fresh weight, nodule growth rate decreased as a function of increasing nitrate (Table I), as seen previously in pea (9), Siratro, and clover (4). Nonnitrogen salt (KCl) also affected growth but higher concentrations were necessary to exert the same effect. Total protein synthesis, as measured by the incorporation of ³⁵S sulfate into TCA-precipitable material, declined even more abruptly than nitrogenase activity as nitrate concentration increased (Table I). A concentration of 10 mM was consistently equivalent to 20 mM nitrate in its effect on protein synthesis and nitrogenase activity. Comparable results were observed after applying NH4Cl (Table II).

Relative Synthesis of Nitrogenase. The plants were exposed to [³⁵S]sulfate during the last 2 d of 6-d treatments with nitrate. Proteins synthesized in the nodule bacteria during this period were analyzed by two-dimensional polyacrylamide gel electrophoresis. The position of nitrogenease component II (cll) of R. japonicum strain 110 had been identified by the migration of the purified protein ("Materials and Methods"). It was a prominent protein on these two-dimensional gels (Fig. 2). As mentioned previously, the total bacteroid protein synthesis appeared to decrease quite dramatically as the nitrate concentration was increased (Fig. 2, Table I), but the amount of nitrogenease cll relative to the other bacterial proteins did not decrease (Fig. 2, Table III). In other words, it was not totally repressed. Nitrogenase cll was not so readily identified. It appeared to migrate in a crowded region of these gels, and it very likely had several iso-species. However, no protein in this area was obviously repressed. Gels stained for total protein content (Fig. 3) confirmed that the effect of nitrate was to decrease further synthesis of protein (or at least incorporation of ³⁵S) rather than to decrease the preexisting protein content. Again the effect of NH4Cl was similar in all respects.

Nodules Detached from the Root. The nitrogenase activity measurements thus far presented were obtained from intact roots. Measurements of activity also were made after nodules had been detached from roots (8) (Fig. 4). The addition of succinate or sucrose stimulated the initial rate of ethylene reduction between 2- and 6-fold; the stimulation of total ethylene production was usually greater (2- to 10-fold).

As a test that this ethylene production was due to Rhizobium and to nitrogease, several control assays were performed with nodules of strain SM5, a R. japonicum mutant with a defect in component II of nitrogenease (11). In each case, these nodules failed to produce any ethylene (less than 0.5 nmol/mg nodule fresh weight) regardless of the carbon source.

Nodules from plants grown in nonnitrogen nutrient responded more strongly to succinate; whereas nodules from plants grown in the presence of 10 mM nitrate responded more strongly to sucrose.

Table III. Relative Synthesis of Nitrogenase Component II

<table>
<thead>
<tr>
<th>Treatment of Plants</th>
<th>Total*</th>
<th>cII/Total</th>
<th>cII/BP3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm × 10⁻³</td>
<td>ratio × 100b</td>
<td>ratio⁴</td>
</tr>
<tr>
<td>No addition</td>
<td>91.8</td>
<td>63.0</td>
<td>1.8</td>
</tr>
<tr>
<td>20 mM KCl</td>
<td>69.2</td>
<td>4.5</td>
<td>3.3</td>
</tr>
<tr>
<td>5 mM KNO₃</td>
<td>33.0</td>
<td>5.8</td>
<td>2.1</td>
</tr>
<tr>
<td>10 mM KNO₃</td>
<td>6.4</td>
<td>6.8</td>
<td>2.7</td>
</tr>
<tr>
<td>20 mM KNO₃</td>
<td>7.0</td>
<td>4.2</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Radioactivity in entire gel (total of all bacteroid proteins).
⁴ Mean deviation was ± 1.6.
⁵ Mean deviation was ± 0.5.
(Fig. 4). Nodules from nitrate-treated plants had nearly the same specific activity as nodules from control plants, when each set was incubated in its respective optimal carbon source. Even in the presence of succinate, the specific activity of nodules from control plants decreased through detachment to 30% to 60% of the specific activity of nodules attached to roots. In contrast, detachment of nodules from 10 mM nitrate-treated plants and their exposure to sucrose led to an initial nitrogenase specific activity 2- to 4-fold greater than that observed when the nodules were attached to the roots. It must be noted that the conditions for measuring activity of the detached nodules were very different from those used to measure the activity of intact roots.

**DISCUSSION**

Bisseling et al. (1), studying pea and using a method with less resolution than that used in the present study, also concluded that nitrogenase synthesis is not repressed relative to other bacteroid proteins when nitrate or ammonium is added to the plant. Inasmuch as the nitrogenase proteins are not degraded (Fig. 3) and retain full, but cryptic, activity that can be detected in isolated bacteroids supplied with energy (7), the decrease in specific nitrogenase activity of the nodules must be due to some other cause.

Our observations are fully consistent with a long-standing hypothesis (9, 13, 21) that the nodule is competing for energy with the areas of the plant that are assimilating nitrate or ammonium. It would explain the general inhibition or nodule metabolism, as reflected by retarded nodule growth and decreased incorporation of $^{15}$N into both plant and bacteroid proteins, as well as the decreased nitrogenase activity. This hypothesis is attractive because of its simplicity and because it can explain the effects of both nitrate and ammonium (9). It is difficult to prove experimentally but is supported by considerable circumstantial evidence, including the observed decrease in carbon flow to the nodule after the application of fixed nitrogen (10, 17). However, there are
detectors to this view (1, 2, 14), and it has been pointed out (4) that energy flow would decrease if an inhibitory mechanism in the nodule diminished its capacity as a sink for energy. Any mechanism that blocked energy utilization within both the plant and bacterial portions of the nodule would explain our results also.

As a test of whether the nodules of nitrate-treated plants merely required sufficient carbon to perform as well as control nodules, we detached the nodules and supplied them with equal quantities of carbon. Such experiments must be interpreted with caution, for not enough is yet known of nodule metabolism in situ nor of changes caused by excision from the root and subsequent manipulation (16). Inasmuch as the nodules of our experiments were immersed in liquid, O2 was a limitation (8); therefore, we applied an atmosphere of 90% O2 (8). The nodules were sliced in half to facilitate the access of carbon sources, but presumably most of the bacteroids were still encased within peri-bacteroid membranes surrounded by plant cytoplasm (16). Others have seen stimulation of the nitrogenase activity of excised nodules by sucrose (18) or succinate (8), but the activities we observed were perhaps higher than usually reported.

One way to interpret the results with detached nodules is to say that they supported the prediction of the energy competition models. When supplied with a potential energy source, the detached nodules from nitrate-treated plants were restored to a nitrogenase-specific activity equivalent to that of nodules of non-treated plants (Fig. 4). However, in contrast to the nodules of nontreated plants, only sucrose was effective. Succinate was not. Together with previous observations, such as the decrease in leghemoglobin-heme content (1, 2) and anaplerotic CO2 assimilation (3), this result urges more attention to metabolic shifts in the nodules of plants exposed to fixed nitrogen and determination of whether the shifts are primary (1, 2, 14) or secondary (9, 13) effects.

Acknowledgments—We thank Lin E. Silver for doing most of the gel electrophoresis and Diane Northrup for typing the manuscript.

LITERATURE CITED

10. LATMORE M JR, J GIDDENS, DA ASHLEY 1977 Effect of ammonium and nitrate nitrogen upon photosynthate supply and nitrogen fixation by soybeans. Crop Sci 17: 399-404
21. WILSON PW 1940 The Biochemistry of Symbiotic Nitrogen Fixation. The University of Wisconsin Press, Madison

Downloaded on May 30, 2021. - Published by https://plantphysiol.org Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.