Energy State and Dinitrogen Fixation in Soybean Nodules of Dark-grown Plants

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ABSTRACT

Dark treatment of 25-day-old greenhouse-grown plants of inoculated soybean (Glycine max var. Chippewa) for 1 day reduced ATP by 70%, sucrose by 60%, total adenosine phosphates by 60%, ATP/ADP ratio by 55%, nitrogenase activity by 50%, and energy charge by 15% in nodules. The close correlation between nitrogenase activity and these energy parameters indicates that they may play a major role in regulating dinitrogen fixation in the symbiotic system.

The fixation of atmospheric dinitrogen by nitrogenase in nodules requires an energy supply (ATP) and reductant (NADPH) (6, 7). Based on enzymatic and physiochemical analyses, about four ATPs are needed for one pair of electrons transferred by nitrogenases isolated from various sources (3, 17). For 1 mole of nitrogen fixed, therefore, at least 12 moles of ATP would have to be provided. Even though it is not a tremendous energy drain reaction in biological processes, the possible controlling role of ATP on biological nitrogen fixation is evident and known in bacterial cells (5, 9, 12, 15). Other potential regulators of nitrogenase activity in the symbiotic system could be the ATP/ADP ratio and energy charge because a positive correlation of the ratio and the activity has already been demonstrated by preparations of nitrogenase in vitro (1, 5, 6, 9, 12, 15). The ATP content, the ATP/ADP ratio, and energy charge, however, have never been investigated in soybean nodules. Also, the possible regulatory role of these parameters in N fixation in vivo (6) is unknown. This paper provides experimental data to fill this void.

MATERIALS AND METHODS

Soybean (Glycine max var. Chippewa) seeds were inoculated with Rhizobium japonicum, planted in pots containing perlite, watered with nutrient solution, and grown for 25 days under greenhouse conditions with a 16-hr light (10-30 klx) period at 26°C, and an 8-hr dark period at 21°C. The plants had four to six trifoliate leaves, and the root nodules varied in diameter from 2 to 4 mm. The detailed cultural method already has been published (8). In March and April of 1974, groups of pots for the dark treatment were covered with four layers of black cloth or with a black cardboard box in the greenhouse. The experimental period was 5 days. Decapitated roots from three replicate cultures of control and dark-treated plants were collected at 9:00 AM and assayed for nitrogenase activity utilizing the acetylene reduction procedure (11).

For the analysis of adenosine phosphates, four replications of 2-g samples of nodules were quickly collected in liquid nitrogen around 8:00 AM and extracted by hand grinding each sample three times with a total of 20 ml of 0.25 N perchloric acid. The extract was neutralized and diluted 100-fold with 0.25 M each of HEPES and MgSO4 (pH 7.5). The total amount of nucleotides extracted after this dilution procedure was greater than that recovered after interfering polyphenols were removed by PVP (7). ATP was determined in the diluted extract by the luciferin-luciferase system with an AminoChem-glowluminometer. ADP was converted to ATP by P-enolpyruvate and pyruvate kinase (EC 2.7.1.40) and then assayed by luciferase. AMP was converted to ADP with endogenous ATP by the use of adenylyl kinase (EC 2.7.4.3) and the resulting ADP was converted to ATP and assayed (4). EC was calculated as: Energy charge = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]). Extraction was conducted at 0 to 3 C, and extracts were kept in an ice bath to prevent deterioration.

For estimation of transported photosynthate in nodules, glucose plus glucose-6-P and sucrose were determined in the original extracts. Glucose was assayed by a modification of the "glucose stat pack" procedure of Calbiochem. In this procedure, 50 ml of the original extract were added to 2 ml of reaction mixture containing buffer, hexokinase (EC 2.7.1.1), glucose-6-P dehydrogenase (EC 1.1.1.49) and NAD. The zero time absorbance at 340 nm was read immediately to correct for absorbance of the extract. The absorbance was measured again after 15 min of incubation at 30 C, and the increment from 0 time was calculated as glucose plus glucose-6-P. Sucrose in each extract was hydrolyzed by invertase (EC 3.2.1.26) in 0.1 M acetate buffer (pH 5) at 30 C for 15 min then assayed as glucose. The difference in the glucose content of the original extract and invertase-hydrolyzed extract was multiplied by a factor of 1.9 and considered as sucrose.

The water content of the nodules was determined by the difference of fresh and dry weight (24 hr at 100°C).

RESULTS AND DISCUSSION

In control materials, an age-related increase of acetylene reduction (Fig. 1), ATP content (Fig. 4), and total adenosine phosphates (Fig. 5) are shown. This developmental trend had already been reported for this variety (13). Exposure of the
soybean plant to a 1-day dark treatment resulted in reductions in nodule tissue of 50% in the nitrogenase activity (acetylene reduction) (Fig. 1), 15% of the energy charge (Fig. 2), 60% of sucrose content (Fig. 3), 70% of ATP content (Fig. 4), 60% of total adenosine phosphates (APS) content (Fig. 5), and 55% of the ratio of ATP/ADP (Fig. 6). When a longer dark stress was imposed (up to 5 days), further gradual decreases were observed. A dark treatment of 8 hr reduced 20 to 25% of all the measurements in another group of plants. The reduction of N2-fixing ability and sucrose content by dark treatment and shading is well known (6, 13, 16, 18). Whereas this report is the first one known of the effect of dark treatment on changes of the energy state in the symbiotic system. It would be of interest to study the energy state in nodules of plants grown under CO2 enrichment as the treatment increased N2 fixation by 4-fold (10). The parallel decrease of acetylene-reducing activity with the energy charge certainly indicates a close relationship of the two in vivo and suggests a controlling mechanism. In enzyme extracts of Clostridium, adding ADP and AMP to a reaction mixture already containing an optimum amount of ATP, decreased acetylene reduction by 50% (15). However, energy charge was not determined in these reaction mixtures.

The ATP concentration in control soybean nodule containing 72 to 78% water was 0.5 to 0.6 mM, whereas that of dark-treated plants was 0.1 mM. The Km value of nitrogenase for ATP ranges from 0.1 to 0.3 mM (2, 9). It appears that the reduction of ATP concentration in nodules of dark-grown plants was sufficient to limit nitrogenase activity (Fig. 1). In the cultures of Clostridium pasteurianum with sucrose as a carbon source, 55% of the total ATP derived from sucrose was utilized for biosynthesis, 28% for N2 fixation, 11% for auxillary metabolism, and 6% for catabolism (5). In the symbiotic system, such as soybean nodules, the distribution must be different because the weight of bacteroids alone amounts only to 20 to 25% of the nodule weight. Also, the metabolism of plant tissue and bacteroids are different from that of free living bacteria. The only available information somewhat related to the energetics of N2 fixation by legume is the carbon budget investigation of pea plants (14). About 32% of the carbon fixed by photosynthesis in peas was found in nodules, of which 12% of the total carbon was consumed for nodule respiration which presumably produced ATP. The absolute concentration of ATP surrounding bacteroid nitrogenase, of course, is difficult to discern. It is clear, however, that the reduction of total ATP in the tissue of dark-treated plants was sufficient to inhibit the nitrogenase activity.

A positive correlation of acetylene reduction activity and the ratio of ATP/ADP in the range of 0.5 to 2.5 was reported for the bacteroid nitrogenase from legume nodule (1). In cell-free preparations of blue-green algae (9), adding sufficient nucleotides to obtain an ATP/ADP ratio of 10 or more resulted in 8% or less inhibition of the acetylene reduction of adding ATP alone. When the ATP/ADP ratio was reduced to 4, 1, or 0.4, inhibition of 23, 56, or 86%, respectively, was observed in the crude nitrogenase preparation. In soybean nodules, the over-all ATP/ADP ratio varied a great deal. In control material, the ratio generally fluctuated from 5 to 11, whereas a small range of 1.5 to 3.5 was found in nodules of dark-treated plants (Fig. 6). Based on the results of blue-green algae, the low ATP/ADP ratio found in dark materials would reduce the nitrogenase activity by 25 to 50%. The experimental finding was 50% inhibition (Fig. 1), and therefore, the ratio appears to be regulating the N2 fixation activity.

There was little difference observed in glucose and glucose-6-P content of soybean nodule from plants grown under control or dark conditions. An average of 3 to 4 μmoles of glucose and glucose-6-p was found per g fresh weight. The glucose may be

![Fig. 1. Acetylene reducing activity of soybean nodules (μmole acetylene reduced/g fresh weight) from plants grown under controlled greenhouse conditions (O) and in the dark (X).](image1)

![Fig. 2. Energy charge ratio in soybean nodules from plants grown under control conditions (O) and in the dark (X).](image2)

![Fig. 3. Sucrose content in soybean nodules (μmole/g fresh weight) from plants grown under control conditions (O) and in the dark (X).](image3)

![Fig. 4. ATP content in soybean nodules (nmole/g fresh weight) from plants grown under control conditions (O) and in the dark (X).](image4)

![Fig. 5. Total adenosine phosphates in soybean nodules (nmole/g fresh weight) from plants grown under control conditions (O) and in the dark (X).](image5)

![Fig. 6. ATP/ADP ratio of soybean nodules from plants grown under control conditions (O) and in the dark (X).](image6)

mobilized from other parts of the plant to maintain the pool for basal metabolism of nodule cells, as plants did survive after the 5-day dark treatment.

From these experimental data, we conclude that the dark treatment of soybean plants limits photosynthesis, and this, in turn, reduces the supply of sucrose in nodules. This reduction of substrate results in decreased phosphorylation of ADP and less synthesis of nucleotides, thus both pools of ATP and total adenosine phosphates were decreased in the dark. Nitrogenase activity is apparently limited as a consequence of the reduced energy supply, changed energy state, and decreased reductants under the dark condition.

**LITERATURE CITED**