Nitrogen Starvation and the Regulation of Glutamine Synthetase in *Agmenellum quadruplicatum*¹

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ABSTRACT

The level of glutamine synthetase activity in *Agmenellum quadruplicatum* strain PR-6 was dependent on the nitrogen source used for growth and on the nutritional status of the cells. During exponential growth, glutamine synthetase activity was low in cells grown on ammonia, urea, or nitrate. During the transition from nitrogen replete to nitrogen starved growth, glutamine synthetase activity began to rise. With ammonia as a nitrogen source, glutamine synthetase activity as determined in whole cells increased from 1 nanomole per minute per milliliter during exponential growth to 22 nanomoles per minute per milliliter during severe nitrogen starvation. In cells grown on nitrate the increase was from 5 to 39 nanomoles per minute per milliliter, and in cells grown on urea the increase was from 4 to 31 nanomoles per minute per milliliter.

The rise in glutamine synthetase activity corresponded with the rapid decline in the nitrogen and c-phycocyanin content of the cells. Prior to nitrogen starvation, the nitrogen content of the cells was 140, 90, and 83 micrograms nitrogen per milligram dry weight for ammonia, urea, and nitrate grown cells, respectively. During nitrogen starvation where glutamine synthetase activity was highest, the nitrogen content of cells had declined to 35 to 40 micrograms nitrogen per milligram dry weight of cells. At the same time, the c-phycocyanin content of cells dropped by 95%.

Unlike many cyanobacteria such as *Anabaena* species strain CA (17) and *Anabaena cylindrica* (19), *Agmenellum quadruplicatum* strain PR-6 is incapable of nitrogen fixation (11). However, Kapp et al. (6) demonstrated that a wide variety of nitrogen compounds, both organic and inorganic, could be used by PR-6 as a source of nitrogen for growth with NO₃⁻, NO₂⁻, and NH₃ supporting the fastest growth rates.

Most of the work on the nitrogen metabolism of PR-6 has centered on studying mutants with lesions in the reduction of NO₃⁻ or NO₂⁻ (20, 22–24). These studies led to the conclusion that the reduction of NO₃⁻ to NO₂⁻ was tightly regulated by NH₃ (22, 23). A logical extension of these studies was to examine the incorporation of NH₃ into glutamate. Wolk et al. (29) have shown that glutamine was the first organic product to become labeled with ¹⁵N after fixation of [¹⁵N]N₂ by *A. cylindrica*. Further experiments by Thomas et al. (26) using preparations of isolated heterocysts from *A. cylindrica* demonstrated that glutamine was the first organic product labeled with ¹⁵N when the heterocysts were supplied with [¹⁵N]NH₃. These experiments indicated that NH₃ entered amino acid metabolism via a reaction catalyzed by GS². Subsequently, GS was isolated from *Anabaena* CA (18) and *A. cylindrica* (13–15) and characterized. Herein, we report the outcome of an investigation to determine the result of nitrogen starvation with respect to GS levels, elemental nitrogen content and GS activity of PR-6 when grown on three different nitrogen sources.

MATERIALS AND METHODS

Organism and Growth Conditions. *A. quadruplicatum* strain PR-6 is a marine cyanobacterium isolated by Van Baalen (28). PR-6 was grown in medium A of Provasoli et al. (9) as modified by Van Baalen (28) and Stevens et al. (21) with the appropriate nitrogen source. Nitrogen sources were filter-sterilized using a type GSWP, 0.22 µm filter (Millipore Corporation) and were added to the medium yielding a final concentration of 39 µg N/ml. Cultures were bubbled with 0.6% CO₂ in air (ν/ν) and the temperature was maintained at 39°C. Illumination consisted of 4 F7T12 CW fluorescent lamps (two on each side of the water bath) providing an intensity of 3.8 × 10⁻³ µE cm⁻² s⁻¹ incident on each growth tube.

Measurement of Physiological Parameters. Growth was routinely measured photometrically with a Bausch and Lomb Spectronic 20 at 550 nm. Values of the specific growth rate constant, k, are reported in terms of log₁₀ U/day. When k = 0.301, the generation time is 24 h. A cell suspension with an OD = 1.0 contains 6.9 × 10⁴, 6.5 × 10⁴, or 4.5 × 10⁴ cells/ml when grown on NH₃, NO₃⁻, or urea, respectively, as determined by viable cell counts. The standard estimate of error was 2%. The above viable cell counts although different were each equal to a dry weight of cells of 0.15 mg/ml.

The A at 625 nm due to C-PC was determined from whole cell spectra by use of the equation of Sigalat and de Kouchkovsky (16). The concentration of C-PC was then calculated by use of the extinction coefficient of Svedberg and Katsurui (25).

Total cellular nitrogen was determined on a 150-µl sample of cells that had been dried to constant weight at 80°C for 48 h. The analysis was done by the Micro-Dumas method using a Coleman model 29 nitrogen analyzer. Analysis of standards indicated a variation of ±0.1% in our nitrogen estimates.

Glutamine synthetase was assayed using a whole cell biosynthetic assay and was done within 24 h of sampling. Samples were stored at 4°C until assayed. No loss in activity was observed during storage. The reaction mix contained: 10 mM ATP, 20 mM MgCl₂, 20 mM NH₄Cl, 100 mM Hepes buffer (pH 7.6), 0.5% Nonidet P-40, 50 mM L-glutamate containing 0.18 µCi of 1-[¹³C]glutamate (292 mCi/mmol). The reaction was initiated by the addition of

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³ Abbreviations: GS, glutamine synthetase; C-PC, c-phycocyanin.
whole cells to give a final assay volume of 0.1 ml. Temperature during the assay was maintained at 39 C. The assay was terminated by the addition of 2.0 ml of ice-cold double-distilled H2O. Glutamine was separated from glutamate by a modification of the procedure of Prusiner and Milner (10) using Dowex 1 X 8 (chloride), 400 mesh ion-exchange resin. After separation, 10 ml of Tritosol (3) scintillation cocktail was added and samples were counted on a Packard Tri-Carb Liquid Scintillation Counter. One unit of enzyme activity is defined as the amount of enzyme needed to catalyze the formation of 1 nmol glutamine/min.

Ammonia was measured using the phenol-hypochlorite method (12) as modified by Greenbaum et al. (5). Fifty nmol of NH3 gave an A of 0.1.

Urea was measured by the NH3 assay procedure as above after treatment with 1 U/ml of jack bean urease at 28 C in Tris buffer (pH 9.2), containing 2 mm EDTA for 45 min.

Nitrate was measured as NO3− (8) after reduction using the cadmium-copper method of Wood et al. (30). A 10-ml sample containing between 10 to 50 nmol NO3− was added to the cadmium-copper column and washed through with 0.12 M NH4Cl. Fifty nmol NO3− after reduction to NO3− resulted in an A of 0.99.

Resagents. ATP, glutamate, Hepes, Tris, Dowex 1 X 8, and jack bean urease were obtained from Sigma. l-[U-14C]glutamate was obtained from New England Nuclear Corp. Nonidet P-40 was obtained from Bethesda Research Laboratories. All other biochemicals and chemicals used were reagent grade.

RESULTS

When PR-6 was grown on a limiting concentration of NO3− (2.8 μmol/ml) the cells grew exponentially (Fig. 1a) from 0 to 16.5 h. During this period the specific growth rate, k (7) was 2.06 day−1 representing a doubling time of 3.5 h. After 16 h, PR-6 entered a period during which the growth rate progressively decreased.

To determine at which point in growth NO3− became limiting, the time course of NO3− disappearance was determined (Fig. 1a). The rate of NO3− disappearance was constant at 0.29 μmol/ml-h and was linear with time from 7.5 h to depletion at 16.5 h. The time of NO3− depletion corresponded with the end of the exponential growth phase of PR-6.

The effect of NO3− limitation (starvation) on the accessory pigment C-PC over the time course of the experiment is shown in Figure 1b. The amount of C-PC was at its maximum level very early during growth. There was a decline in the C-PC content from 81.6 to 64 μg/mg dry weight during the first 14 h. Between 14 and 22.5 h there was a large decrease in the C-PC content dropping from 64 to 7.5 μg/mg dry weight.

Inasmuch as NO3− was depleted from the medium by 16.5 h and the culture continued to grow for an additional 8.5 h, the question was raised as to where the cell was finding nitrogen for growth. Consequently, the nitrogen content of the cells was measured during growth. Between 0 and 17.5 h the nitrogen content declined slowly to 73.3 μg N/mg dry weight. Over the next several hours the nitrogen content dropped rapidly and reached a low level of 28 μg N/mg dry weight at 25 h.

When NH3 was used as a nitrogen source for the growth of PR-6, a pattern of growth similar to that on NO3− was observed (Fig. 2a). Under this condition, growth of PR-6 was exponential from 0 to 13 h with a specific growth rate of 2.44 days−1 and a doubling time of 3.0 h. After 13 h the growth rate progressively decreased.

For growth to occur, NH3 must be taken up from the medium and assimilated into cellular material. The time course of NH3 removal from the medium is shown in Figure 2a. During the first 5 h of growth the disappearance of NH3 proceeded slowly. After 5 h the rate of disappearance increased 16-fold and was maintained until depletion occurred at 12.5 h.

As was observed in the NO3−-grown culture of PR-6, NH3 limitation caused a drastic effect on the C-PC level (Fig. 2b). During the first 6 h of the experiment there was a 30% increase in the C-PC content of the cells. Between 6 and 14 h there was a moderate decrease in the C-PC content. After 14 h there was a large decline in the C-PC content of the cells reaching a low value of 6 μg/mg dry weight.

It was expected that as the NH3 became depleted from the medium, a decline in the nitrogen content of the cell would occur. The data presented in Figure 2b support this notion. During the initial 12.5 h of the experiment, the nitrogen content of the cells declined to 120 μg N/mg dry weight. After 12.5 h, the nitrogen content of PR-6 declined rapidly attaining a low level of 27.5 μg N/mg dry weight by 25 h.

To compare the effects of nitrogen starvation on cells grown on inorganic versus organic sources of nitrogen, urea was chosen as a third nitrogen source. Growth of PR-6 on urea was exponential from 0 to 15 h (Fig. 3a) with a specific growth rate of 1.96 days−1 (doubling time = 3.7 h). The growth rate of PR-6 began to decline progressively 8 h before depletion of urea from the medium. This was unlike the results observed for growth on NO3− or NH3 (see Figs. 1a and 2a).

The time course of urea disappearance from the medium is shown in Figure 3a. Urea disappearance from the medium was constant at 0.05 μmol/ml-h. Depletion of urea from the medium occurred at 22.5 h.

The effect of a limiting concentration of urea on C-PC concentration is shown in Figure 3b. As in the NH3-grown culture there was a period where the C-PC content of the cell was still increasing. By 5 h a maximum C-PC concentration of 63 μg/mg dry weight had been reached. After 7.5 h of growth the C-PC level
low, but increased at an exponential rate during the entire experiment. A maximum of 40 U/ml was reached by 25 h. Ammonia-grown cells exhibited low GS activity during the first 12 h of growth. Afterwards GS activity began to increase rapidly. A GS activity of 22.5 U/ml at 25 h was attained. Glutamine synthetase activity in the urea grown culture, as in the NH₃-grown culture, did not increase substantially during the first 15 h. After 15 h, a rapid rise in GS activity was observed. Activity continued to increase during the remainder of the experiment. At 25 h, 32 U/ml of GS activity was observed.

To determine the relationship between GS activity and cell mass, the log of the GS activity was plotted against the log of cell dry weight (Fig. 5). When data are plotted in this manner, a doubling of either cell mass or enzyme activity will result in an increase on either axis of 0.301 U. If the enzyme activity is doubling at the same rate as the cell mass, a slope of one is observed. If the enzyme activity is increasing faster than the cell mass, a line with a slope greater than one will be seen. A slope of less than one indicates cell mass is increasing faster than enzyme activity.

In the NO₃⁻-grown culture the change in GS activity was initially faster, becoming equal to, then once again faster than the increase in cell dry weight. In the NH₃- or urea-grown cultures the change in GS activity was initially slower, than faster, and finally equal to the increase in cell dry weight.

**DISCUSSION**

In heterotrophic bacteria NH₃ is considered a good nitrogen source and its use is reflected by low levels of GS activity (27). Nitrate is considered a poor nitrogen source for bacteria and its use is reflected by high levels of GS activity (1). A nitrogen source such as urea which must undergo catabolism to NH₃ prior to assimilation into glutamine would be expected to be a poorer

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**Fig. 3.** Growth, urea disappearance, nitrogen content, and C-PC concentration in PR-6. a, Growth (*) and urea disappearance (■). b, Nitrogen content (□) and C-PC concentration (○).

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**Fig. 4.** The time course of the appearance of GS activity in cells during growth on NO₃⁻, NH₃, and urea. Assays for GS were done as described under "Materials and Methods." (▲) and (----), GS activity levels in NO₃⁻-grown cells. (●) GS activity levels in cells grown on NH₃. (■) GS activity levels in cells grown on urea.

began to decrease. By 12 h the C-PC level dropped to 57 μg/mg dry weight and a constant rate of decline was subsequently observed.

The nitrogen content of the urea culture was initially 97 μg N/mg dry weight. Over the first 10 h there was a very slow decline in the nitrogen content of the cells; but, after 12 h of growth the rate of decline increased 7-fold. By 27.5 h a minimum level of 38.4 μg N/mg dry weight was reached.

It has been shown previously in other micro-organisms that when nitrogen was limiting or they were growing on a "poor" nitrogen source, levels of GS were elevated (27). The activity of GS in cells grown on the three nitrogen sources over the course of growth shown in Figures 1a, 2a, and 3a is shown in Figure 4. In the NO₃⁻-grown culture, the enzyme activity was initially very

![Graph](https://via.placeholder.com/150)

**Fig. 5.** The effect of growth as evidenced by an increase in cell dry weight and nitrogen starvation on the levels of GS in PR-6. The data are plotted in a log versus log manner since a doubling in cell mass or enzyme activity results in a 0.301 unit increase on the specific axis. A slope of one, as illustrated by the dotted line (---), indicates a doubling of cell mass at the same rate as a doubling of enzyme activity. A slope of less than one indicates cell mass increasing faster than GS activity and a slope greater than one indicates GS activity increasing faster than cell mass. (▲) and (----), NO₃⁻ culture. (●), NH₃ culture. (■), urea culture.
nitrogen source than NH₃ and its use would be reflected by an intermediate level of GS activity (4).

The activity of GS in PR-6 was low while cells were in exponential growth (see Fig. 4), regardless of the nitrogen source used in our study. There was little difference in the level of GS activity. These results seemed to be at odds with the pattern of GS activity in response to different nitrogen sources established for heterotrophic bacteria (see above). As the growth rate of PR-6 began to decline in response to nitrogen depletion from the medium, the activity of GS began to increase dramatically (compare Figs. 1a, 2a, and 3a with Fig. 4). The magnitude of the increase in GS activity depended on the nitrogen source used for growth and on the extent of starvation for that nitrogen source. The highest level of GS activity was observed in cells of PR-6 grown on NO₃⁻. An intermediate level of GS activity was observed in cells grown on urea. A lower level of GS activity was observed in cells grown on NH₄ (note the levels of GS at 25 h in Fig. 4). Thus, when PR-6 was in a nitrogen-depleted state, the observed pattern of GS activity corresponded to the pattern reported for exponentially growing heterotrophic bacteria (1, 4, 27).

Our results are in contrast to the results reported in Figure 1, of Emond et al. (2). They reported no change in GS activity per optical density unit during growth and nitrogen starvation of Anacystis nidulans (compare with Fig. 5 of this paper). From the results (2) we have calculated a doubling time of 66 h for Anacystis nidulans. This is 33 times greater than the doubling time for this strain of A. nidulans reported by Kratz and Myers (7) for the same growth medium. Clearly, the cells used in the experiments of Emond et al. (2) were already severely limited for either light, CO₂, or both; otherwise, their growth rates would have been more nearly comparable to the rates observed by Kratz and Myers (7). Rapid growth dramatizes physiological changes, and we rationalize our quite different results on this basis.

At approximately the same time that GS activity began to rise in nitrogen-depleted cultures of PR-6 we observed that the nitrogen and C-PC content of these cells began a steep decline. It was evident from the still increasing OD and the decline in nitrogen content based on dry weight that a fixed amount of nitrogen was being redistributed among "new" cells. A large change in GS activity with only a small change in cell mass occurred after the onset of nitrogen starvation. This was most clearly observed in cells of PR-6 grown on NH₄ (see Fig. 5).

Our results suggest that GS activity is regulated in PR-6. They do not indicate how GS activity is regulated. The degradation of C-PC is intertwined with changes in the level of GS activity and the nitrogen content of PR-6. This presents itself as an interesting interrelationship between the energy yielding process of photosynthesis and nitrogen metabolism.

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