Regulation of Glutamine Synthetase Activity in the Unicellular Cyanobacterium *Synechocystis* sp. Strain PCC 6803 by the Nitrogen Source: Effect of Ammonium

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Glutamine synthetase activity from *Synechocystis* sp. strain PCC 6803 is regulated as a function of the nitrogen source available in the medium. Addition of 0.25 mM NH₄Cl to nitrate-grown cells promotes a clear short-term inactivation of glutamine synthetase, whose enzyme activity decreases to 5 to 10% of the initial value in 25 min. The intracellular levels of glutamine, determined under various conditions, taken together with the results obtained with azaserine (an inhibitor of transamidases), rule out the possibility that glutamine per se is responsible for glutamine synthetase inactivation. Nitrogen starvation attenuates the ammonium-mediated glutamine synthetase inactivation, indicating that glutamine synthetase regulation is modulated through the internal balance between carbon-nitrogen compounds and carbon compounds. The parallelism observed between the glutamine synthetase activity and the internal concentration of α-ketoglutarate suggests that this metabolite could play a role as a positive effector of glutamine synthetase activity in *Synechocystis* sp. Despite the similarities of this physiological system to that described for enterobacteria, the lack of in vivo ³²P labeling of glutamine synthetase during the inactivation process excludes the existence of an adenylylation-deadenylylation system in this cyanobacterium.

Glutamine synthetase (GS; EC 6.3.1.2) plays a central role in nitrogen metabolism by prokaryotes. This central role requires that both the synthesis and activity of this enzyme be strictly regulated in response to the available nitrogen source. In enterobacteria this regulation takes place at two different levels, first by modulating enzyme activity by an adenylylation-deadenylylation system (35) and second by regulating GS synthesis through transcription from different promoters (18). The adenylylation of GS makes it more susceptible to a cumulative feedback inhibition by metabolites derived from ammonium assimilation, such as amino acids or nucleotides (35). In contrast, most of the gram-positive bacteria lack this system, and the regulation of the enzyme appears to involve a single feedback mechanism, in which GS is strongly inhibited by glutamine (5, 14).

In cyanobacteria, ammonium assimilation takes place mainly by the sequential action of GS and glutamate synthase (GOGAT) (GS-GOGAT pathway) (21). The regulation of GS synthesis in *Anabaena* sp. strain PCC 7120 has been well characterized; several promoters have been found for the structural GS gene, *glnA* (37). Selective transcription from these promoters determines that in ammonium-grown cells the level of GS protein is about 50% lower than that observed under dinitrogen-fixing conditions (37). Recently, we have reported a repression of GS synthesis by ammonium in the cyanobacteria *Synechocystis* sp. and *Calothrix* sp. (22).

The existence of an adenylylation-deadenylylation system, as observed in the majority of gram-negative bacteria, has been previously discounted for the genus *Anabaena* (8), and it has been proposed that regulation of GS activity in these cyanobacteria could be similar to that shown in gram-positive bacteria (6, 29, 34). At present, the regulation of GS activity in cyanobacteria, especially in the unicellular species, is still not well characterized.

In this work we report for the first time a clear short-term regulation of GS activity by the nitrogen source in a *Synechocystis* sp. We also show data supporting an important role of the intracellular C-N balance in the modulation of GS activity in this cyanobacterium.

MATERIALS AND METHODS

Organisms and growth conditions. *Synechocystis* sp. strain PCC 6803 was grown photoautotrophically at 35°C on the medium previously described (13), containing 20 mM NaNO₃ as the nitrogen source with continuous fluorescent illumination (25 W · m⁻², white light). The cultures were bubbled with 1.5% (vol/vol) CO₂ in air.

Cell extracts. Cells were harvested by centrifugation at 3,000 × g for 10 min, washed in nitrogen-free medium, and resuspended at a concentration of 1 g of cells per 3 ml of 50 mM N-2-hydroxyethylpiperezine-N'2-2ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.0). Cells were disrupted by sonication (20 KHz; 75 W) for 4 min (in 30-s bursts) with a Branson sonifier model B 12. The homogenate was centrifuged at 41,000 × g for 20 min, and the resulting supernatant constituted the cell extract.

Purification and assays of GS. GS was purified by DEAE-cellulose chromatography and subsequent affinity chromatography on 2',5'-ADP-Sepharose by using the procedure previously described (22). GS activity was determined, either in vitro or in situ, by the formation of γ-glutamylhydroxamate (transferase assay) or glutamine (biosynthetic assay). The Mn²⁺-dependent in vitro γ-glutamyltransferase activity was determined as described previously (32), using a modified reaction mixture which contained, in a final volume of 1 ml, 60 μmol of HEPES-NaOH buffer (pH 7.0), 40 μmol of L-glutamine, 4 μmol of MnCl₂, 60
μmol of hydroxylamine, 1 μmol of ADP, 20 μmol of sodium arsenate, and 0.05 ml of cell extract (containing ca. 2 mg of protein). The reaction was started by adding the sodium arsenate, and the amount of γ-glutamylhydroxamate formed after 10 min of incubation at 30°C was determined by its A_{400} value. For the in situ assay, a sample of whole cells containing about 0.4 mg of protein was added to the reaction mixture supplemented with 20 μl of 1.25% (wt/vol) mixed allyltrimethylammonium bromide.

GS biosynthetic activity in situ was determined from the rate of glutamine formation in a reaction mixture containing, in a final volume of 1 ml, 60 μmol of HEPE-NaOH buffer (pH 7.0), 25 μmol of MgCl₂, 25 μmol of glutamate, 10 μmol of NH₄Cl, and 6 μmol of ATP, in addition to a sample of whole cells permeabilized as above. The reaction was started by the addition of ATP and was continued at 30°C for 20 min. Glutamine was determined by the fluorescence of its α-phthalaldehyde derivative by reverse-phase high-performance liquid chromatography (HPLC) on a μBondapack C₁₈ column as described previously (20).

Electroimmunoassay. Immunoelectrophoresis was performed by the method of Laurell and McKay (17), except that the buffer consisted of 100 mM glycine, 37.5 mM Tris, and 1% (vol/vol) Triton X-100 (pH 8.6). Monospecific antibodies (90 μl) raised against pure Synechocystis GS were mixed with 25 ml of buffered 1% (wt/vol) agarose gel and poured to a 1.5-mm thickness on a polyester film (11.5 by 12.5 cm). Sample wells (diameter, 4 mm) were cut 20 mm from one side of the gel. Samples of 10 μl were applied to the wells and electrophoresed at 5 V/cm in a Pharmacia flat bed chamber for 16 h at 10°C. The gel was finally washed with 0.15 M NaCl to remove unprecipitated proteins, dried with filter paper, and stained as described previously (17).

In vivo ³²P-labeling experiments. An 80-ml culture was grown to mid-logarithmic phase in medium with nitrate as the nitrogen source, and cells were harvested by centrifugation, washed twice with culture medium lacking phosphate, and resuspended in culture medium containing 20 μM sodium phosphate and buffered with 6 mM HEPE-NaOH (pH 7.5) (the phosphate concentration in the standard culture medium was 12 mM). Cells were incubated under growth conditions for 12 h and then supplemented with 0.4 μCi of ³²P (orthophosphoric acid in water, 8.8 mCi/mmol). After further incubation for 16 h, 8 mM NH₄Cl was added, and the culture was harvested 2 h later. The cells were resuspended in 1 ml of 50 mM HEPE-NaOH buffer (pH 7.0) and disrupted by freezing in liquid air on a precooled mortar and pestle.

A 0.3-ml aliquot of cell extract was incubated at 4°C with rabbit preimmune serum for 12 h and then centrifuged for 10 min at 12,000 × g. The supernatant was incubated at 4°C with rabbit monospecific antibodies raised against pure Synechocystis GS for 12 h and centrifuged at 12,000 × g for 10 min. The supernatant was dialyzed against 0.1 M phosphate buffer (pH 7.0) and then electrophoresed in a 12% (wt/vol) polyacrylamide gel containing 1% (wt/vol) sodium dodecyl sulfate (SDS) as described previously (16). The gel was stained with 0.1% Coomassie brilliant blue R-250 in 10% acetic acid containing 40% methanol and finally autoradiographed for 48 h.

Analytical methods. For the determination of intracellular amino acids, cell lysates were obtained by adding aliquots of cell suspensions equivalent to 0.4 mg of protein into ice-cold 0.2 N HCl followed by centrifugation at 12,000 × g for 20 min at 4°C. The amino acid concentration in the samples was determined by HPLC as described previously (20).

For the determination of the intracellular α-ketoglutarate concentration, cells from 130-ml cultures were harvested by centrifugation at 20,000 × g for 10 min at 4°C. The pellet was resuspended in 2 ml (final volume) of cold 0.3 M HClO₄ and incubated for 15 min at 4°C. The cell lysates thus obtained were centrifuged at 12,000 × g for 20 min at 4°C, and α-ketoglutarate in the supernatant was analyzed by using glutamate dehydrogenase as described previously (31).

The amount of protein in cell extracts was estimated by the method of Bradford (2), and the amount in whole cells was estimated by the method of Lowry et al. as modified by Bailey (1). Ammonium was determined enzymatically with commercial glutamate dehydrogenase.

Chemicals. L-Amino acids used as standards and azaserine were from Sigma Chemical Co., St. Louis, Mo.; Coomassie brilliant blue R-250 was from Bio-Rad, Richmond, Calif.; DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, United Kingdom; and 2',5'-ADP—Sepharose was from Pharmacia Biotechnology, Uppsala, Sweden. Inorganic salts and other biochemicals were obtained from E. Merck AG, Darmstadt, Germany. Radioactive (³²P-labeled) orthophosphoric acid was purchased from NEN, Du Pont de Nemours, Dreieich, Germany.

RESULTS

Inactivation of GS on addition of ammonium to nitrate-grown cells. Addition of 0.25 mM NH₄Cl to Synechocystis cells growing in nitrate as the nitrogen source caused a rapid decrease of GS activity, which fell to 5 to 10% of the initial level in 25 min (Fig. 1). The initial activity was completely recovered if the cells were washed and transferred to an ammonium-free medium, or after ammonium was consumed from the culture medium. It is worth noting that the decay of GS activity can be detected not only by using the transferase but also by using the biosynthetic assay, confirming the physiological significance of this inactivation in Synechocystis sp. Using rocket immunoelectrophoresis with polyclonal...
monospecific antibodies raised against pure Synechocystis GS, we determined that the amount of GS protein in nitrate-grown cells was the same before and 1 h after ammonium addition (3.9 and 3.7 μg of GS per mg of protein, respectively). However, the GS activity in ammonium-treated cells represented only 9% of that in untreated cells (74 and 813 mU/mg of protein, respectively). We conclude that ammonium assimilation exerts a reversible, short-term inactivation of the enzyme.

Alteration of amino acid pools after ammonium addition. The changes in the intracellular concentrations of amino acids were examined after addition of ammonium to Synechocystis cells growing with nitrate as the nitrogen source. Figure 2 shows that 30 s after ammonium addition, the pool of glutamate (the most abundant amino acid in Synechocystis sp.) had drastically decreased, while the glutamine level had increased reciprocally. This alteration was transitory, since 1 h later the levels of these amino acids were reestablished at values close to the initial ones. Aspartate showed a slight and slower increase, probably as a result of transamination from glutamate (see below). Other amino acids such as serine, glycine, and alanine showed negligible alterations (data not shown).

The rapid change and restoration of the intracellular levels of glutamate and glutamine could be directly attributed to GS activity, since addition of ammonium to nitrate-grown cells with fully active GS would lead to a rapid conversion of glutamate to glutamine. Subsequently the gradual inactivation of the enzyme should allow the cells to restore the amino acid pools to their initial levels (Fig. 2).

Intracellular concentration of amino acids in nitrate- and ammonium-grown cells. We have determined the intracellular amino acid concentrations in Synechocystis cells growing on either nitrate or ammonium to assess their differences in relation to the nitrogen source. As shown in Table 1, glutamine levels were nearly the same in the presence of either nitrogen source. However, glutamate and aspartate levels in ammonium-grown cells were 40% lower than those observed in nitrate-grown cells. Other amino acids, such as serine, remained unchanged.

Role of glutamine in GS inactivation: effect of azaserine. The intracellular glutamine level has previously been suggested to be a metabolic signal that could regulate the nitrogen metabolism in several organisms (7, 11, 23). In Synechocystis sp., the intracellular concentration of glutamine is only slightly affected by the available nitrogen source (Table 1); therefore, it is not likely to play such a regulatory role. In addition, we have obtained evidence against a regulatory role for glutamine by using azaserine, an inhibitor of GOGAT and other transaminases (28). The addition of azaserine (final concentration, 0.1 mM) to ammonium-treated Synechocystis cells (containing inactivated GS) resulted in a rapid GS reactivation, reaching levels close to those present before ammonium treatment (Fig. 3) despite the great increase observed in the intracellular glutamine pool (Table 1). A parallel decrease in the glutamate pool was noted since glutamate could not be replenished because of the absence of GOGAT activity.

The effect of azaserine on GS activity did not result from an inhibition of NH₄⁺ uptake. In fact, NH₄⁺ levels in the medium increased after the addition of azaserine, since this relieves the inhibition of nitrate uptake by ammonium, as previously described (9). Nitrate taken into the cell is reduced to ammonium and then released. Probably it could not be assimilated as a result of depletion of the intracellular glutamate pool.

Effect of nitrogen starvation on ammonium-mediated GS inactivation. Synechocystis cells subjected to different periods of nitrogen starvation showed a delay in the ammonium-promoted GS inactivation. The degree of inactivation was found to be inversely related to the incubation time in the absence of a nitrogen source. As shown in Fig. 4, cells starved of nitrogen for 4 h displayed only 50% GS inactivation 1 h after ammonium addition, in contrast with non-starved cells, which showed 95% GS inactivation. In all cases, the GS inactivation finally reached the level observed in the control cells (data not shown). The intracellular levels of glutamate, glutamine, and aspartate were also determined under the conditions of the experiment in Fig. 4. In general, these levels were lower than those found in nitrate- or ammonium-grown cells, although the ratio between them remained unchanged (Table 1). The addition of ammonium...
to nitrogen-starved cells promoted changes in glutamate and glutamine levels, but the recovery of the initial ratio between these two amino acids was slower than that observed in cells that were not nitrogen deficient (data not shown). Addition of nitrate instead of ammonium resulted only in a slow increase of amino acid levels to reach a normal C-N balance, but their respective ratios remained unchanged. During this adjustment, the GS activity was constant (data not shown).

**Effect of nitrogen availability on the intracellular concentration of α-ketoglutarate.** The reduced inactivation found in nitrogen-starved cells could result from the accumulation of a carbon metabolite that attenuates the GS inactivation promoted by ammonium. In cyanobacteria, the tricarboxylic acid cycle is incomplete (27, 33), lacking α-ketoglutarate dehydrogenase activity. Therefore, α-ketoglutarate is used primarily as a carbon skeleton for ammonium assimilation and thus might accumulate in nitrogen-starved cells. The intracellular levels of α-ketoglutarate determined under different metabolic conditions are shown in Table 1. In ammonium-grown cells, the α-ketoglutarate level was 40% of that in nitrate-grown cells. In nitrogen-starved cells, the α-ketoglutarate concentration was about sevenfold higher than in nitrate-grown cells. It should be noted that azaserine addition to ammonium-treated cells resulted in a recovery of GS activity and a simultaneous increase in the intracellular α-ketoglutarate concentration (about sevenfold).

**In vivo 32P labeling of GS.** As stated above, GS from most gram-negative bacteria is regulated by an adenylation-deadenylylation system. In this case the modified enzyme is radiolabeled in vivo in the presence of 32P (8). We have carried out 32P-labeling experiments with *Synechocystis* cells to study the molecular mechanism of ammonium-promoted GS inactivation. Ammonium was added to nitrate-grown cells in the presence of 32P, and after GS had been completely inactivated, extracts were prepared and precipitated with specific antibodies. SDS-polyacrylamide gel of the immunoprecipitated proteins and their respective autoradiograms are shown in Fig. 5. In nitrate-grown cells treated with ammonium for 2 h, the inactivated GS protein did not show any radioactive labeling. Although this exper-

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**FIG. 3.** Effect of azaserine on ammonium-treated *Synechocystis* cells. GS transferase activity was determined in situ in nitrate-grown cells, as described in Materials and Methods, at the indicated times after the addition of 2 mM NH₄Cl (●) or with no addition (○). The arrow indicates the addition of 0.1 mM azaserine to one of the ammonium-treated cultures (●). The level of 100% GS activity corresponds to 0.98 U/mg of protein.

**FIG. 4.** GS inactivation by ammonium in nitrogen-starved *Synechocystis* cells. Cells grown on nitrate as the nitrogen source were transferred to nitrogen-free medium, incubated for 0 min (○), 15 min (▲), 90 min (●), and finally supplemented with 2 mM NH₄Cl. The GS transferase activity was determined in situ as described in Materials and Methods. The level of 100% GS activity corresponds to 0.97, 0.99, 1.2, or 1.4 U/mg of protein from 0 to 240 min in nitrogen-free medium, respectively.

**FIG. 5.** In vivo 32P labeling of ammonium-inactivated GS from *Synechocystis* sp. 32P labeling of nitrate-grown cells was carried out as described in Material and Methods. The cell extracts thus obtained were incubated with rabbit preimmune serum and then with rabbit monospecific anti-GS antibodies, and the immunoprecipitated proteins were electrophoresed. Panels A and B show, respectively, the Coomassie blue-stained gel and the autoradiogram of the same gel. (A) Lanes: 1, immunoprecipitated extract of non-ammonium-treated cells (containing active GS); 2, immunoprecipitated extract of cells treated with 8 mM NH₄Cl (containing inactive GS); 3, sample of pure GS (6 μg) from *Synechocystis* sp. (B) Lanes 1 and 2 are the autoradiograms of lanes 1 and 2 of panel A, respectively. Abbreviation: IgG, immunoglobulin G.
The small difference found between glutamine pools present in nitrate- and ammonium-grown cells indicates that glutamine per se is not the (only) metabolic signal that causes GS regulation. In fact, addition of azaserine to ammonium-treated cells promoted GS reactivation despite the 10- to 15-fold increase in the glutamine pool (Table 1). Also, nitrogen starvation seems to attenuate the effect of ammonium on GS activity, so the longer the nitrogen starvation period, the slower the GS inactivation (Fig. 4). It has been shown that in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 6301, nitrate uptake is independent of ammonium inhibition in nitrogen-deficient cells (10). In this case, it has been proposed that a sensitive regulatory system integrating the metabolism of both carbon and nitrogen would modulate this step of nitrogen assimilation. As can be seen from Table 1, low GS activity levels correspond to high ratios between carbon-nitrogen metabolites (glutamate and glutamine) and carbon metabolites (α-ketoglutarate) of the GS-GOGAT pathway. In addition, metabolic conditions that diminish this ratio (growth with nitrate as the sole nitrogen source, nitrogen starvation, or azaserine treatment) correspond to high GS activity levels. The similarities between the regulation patterns of both processes, nitrate uptake and GS activity, point to the existence of a general regulatory system of nitrogen assimilation in cyanobacteria. In fact, the presence in *Synechococcus* sp. strain R2 of a gene involved in the regulation of the synthesis of nitrate assimilation elements (nitrate transport-implicated protein, nitrate reductase, and nitrite reductase) and glutamine synthetase has been shown recently (38).

The role of glutamine and α-ketoglutarate as negative and positive effectors, respectively, of GS activity in particular and of nitrogen metabolism in general in enterobacteria has been clearly demonstrated (35). These metabolites operate as allosteric modulators of a uridylyl transferase enzyme (3, 19) that sets off a regulatory cascade which determines GS inactivation by adenylylation, as well as other events such as repression of $glnA$ expression (4, 24). GS synthesis is decreased in ammonium-grown *Synechococcus* cells (22), and, as a result, the GS protein level is 50% lower than in nitrate-grown cells. This result, taken together with those reported in this study, suggest the existence in *Synechococcus* sp. of a metabolic sensor system similar to that described for enterobacteria, involving modulation of GS activity by the internal balance between nitrogen compounds and non-nitrogen compounds of the GS-GOGAT pathway.

It has been shown that in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 6301, a protein similar to the P$_{II}$ protein which regulates the GS-adenyllyltransferase activity in enterobacteria exists (12). However, the ammonium-inactivated GS from *Synechococcus* sp. does not contain any bound AMP molecule, as can be deduced from the in vivo $^{32}$P-labeling experiments reported here (Fig. 5). Furthermore, incubation with phosphodiesterase, a treatment that reactivates adenylylated GS (35), has no effect on ammonium-inactivated *Synechococcus* GS. Therefore, although the presence of a P$_{II}$-like protein and the analogy between the metabolic sensor system that regulates GS activity suggest the existence in cyanobacteria of a regulatory bicyclic cascade similar to that described for enterobacteria, our data indicate that this system, if it exists, is incomplete and the ammonium-mediated GS inactivation in *Synechococcus* sp. is not due to adenylylation of the enzyme.

The data from in vivo $^{32}$P-labeling experiments also exclude other systems of regulation of enzyme activity, such as phosphorylation or ADP-ribosylation. At present, our goal is...
to study the nature of the molecular modification that causes GS inactivation.

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