Functional Analysis of the Phosphoprotein PII (glnB Gene Product) in the Cyanobacterium Synechococcus sp. Strain PCC 7942

KARL FORCHHAMMER1,2† AND NICOLE TANDEAU DE MARSAC2*

Unité de Physiologie Microbienne, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France, and Lehrstuhl für Mikrobiologie, Institut für Genetik und Mikrobiologie der Universität München, 80638 München, Germany

Received 7 July 1994/Accepted 10 February 1995

The PII protein (glnB gene product) in the cyanobacterium Synechococcus sp. strain PCC 7942 signals the cellular N status by being phosphorylated or dephosphorylated at a seryl residue. Here we show that the PII-modifying system responds to the activity of ammonium assimilation via the glutamine synthetase-glutamate synthase pathway and to the state of CO2 fixation. To identify possible functions of PII in this microorganism, a PII-deficient mutant was created and its general phenotype was characterized. The analysis shows that the PII protein interferes with the regulation of enzymes required for nitrogen assimilation, although ammonium repression is still detectable in the PII-deficient mutant. We suggest that the phosphorylation and dephosphorylation of PII are part of a complex signal transduction network involved in global nitrogen control in cyanobacteria. In this regulatory process, PII might be involved in mediating the tight coordination between carbon and nitrogen assimilation.

As in all cyanobacteria, the utilization of sources of combined nitrogen is tightly regulated in the unicellular obligate photoautotroph Synechococcus sp. strain PCC 7942. This regulation is known as global nitrogen control (reviewed in reference 14). When cells are grown in the presence of ammonium, synthesis of enzymes required for the assimilation of other nitrogen sources is repressed, the high-affinity transport of methylammonium is inhibited, and the amount of glutamine synthetase is reduced. When ammonium is removed from the medium and dissolved inorganic carbon is available for assimilation, synthesis of these enzymes is derepressed, allowing the utilization of other N sources such as nitrate and nitrite (4, 15, 16). If no nitrogen compound is available, cyanobacteria like Synechococcus sp. strain PCC 7942 which cannot fix molecular nitrogen degrade their phycobiliproteins, a process termed chlorosis (7). Recently, a transcription factor, named NtcA, which is required for the induced expression of the genes that are subject to ammonium repression has been identified (44). The protein belongs to the family of cyclic AMP receptor protein-like gene activators, but the mechanism by which NtcA is regulated is not known to date (22).

Another putative regulatory protein involved in nitrogen control has been found in Synechococcus sp. strains PCC 7942 and PCC 6301 by in vivo 32P-labelling experiments. A strongly labelled 13-kDa polypeptide was shown to be homologous to the glnB gene product (PII protein) from different proteobacteria (42). PII functions as a central signal transmitter in nitrogen assimilation and carbon and nitrogen assimilation. In its unmodified form, PII signals nitrogen sufficiency (1). Surprisingly, we found that the cyanobacterial PII protein is modified by phosphorylation at a seryl residue (12) rather than by uridylylation at the conserved tyrosyl residue. The phosphorylated protein can be resolved by electrophoresis into any of three isomeric forms, possibly corresponding to whether the protein carries one, two, or three phosphorylated seryl residues, depending on the nitrogen status of the cell (12).

The perception of the PII-mediated signal differs in diverse organisms. In Escherichia coli, the modification state of PII is sensed by the histidine kinase NtrB, which transmits the signal to NtrC, a transcriptional activator of nitrogen-regulated genes (29). Furthermore, the modification state of PII directs the activity of the bifunctional enzyme adenylyltransferase (GlnE), which regulates the activity of glutamine synthetase by covalent modification (23). An additional role for PII-UMP as a positive effector of the expression of nitrate utilization genes (nas genes) in Rhizobium leguminosarum has been suggested recently (2). In contrast, PII in the diazotrophic bacterium Azospirillum brasilense interferes neither with its Ntr system nor with nitrate utilization. However, a null mutation of PII results in the loss of nitrogen fixation capacity (10). In cyanobacteria, glutamine synthetase is not regulated by adenylylation and no proteins homologous to NtrB or NtrC have been identified so far (41). Considering the biological diversity of PII and the fact that its modification type is not conserved between cyanobacteria and proteobacteria, it became important to identify the functions of this protein in the cyanobacterium Synechococcus sp. strain PCC 7942. Towards this goal, we characterized the signal to which the PII Phosphorylation responds in vivo. Moreover, we created a PII-deficient mutant and analyzed its general phenotype.

MATERIALS AND METHODS

Materials. Chemicals of analytical grade were obtained from Sigma or Merck. Restriction enzymes were purchased from New England Biolabs. The antibodies raised against glutamine synthetase from Nostoc sp. strain UCD 7801 were generously provided by J. C. Meeks (University of California, Davis), and the cloned glnA gene from Synechococcus sp. strain PCC 7942 was provided by S. J. Robinson (University of Massachusetts, Amherst).

Culture conditions. Synechococcus sp. strain PCC 7942 spc (small-plasmid cured) (18), hereafter designated Synechococcus sp. strain PCC 7942, and the
derived P$_I$-deficient mutant MP2 (ghbB::kan) were grown in light BG11 medium (30) as described previously (12). Cultures were incubated at 25°C, constantly agitated on a rotary shaker at 100 rpm, and illuminated with cool white fluorescent light under a photosynthetic photon flux density (PPFD) of 15 $\mu$mol m$^{-2}$ s$^{-1}$ measured with an LI-COR LI-185B quantum-radiator-photon sensor equipped with an LI-190SB spherical quantum sensor. Stock cultures of the mutant were maintained in the presence of kanamycin sulfate (30 $\mu$g ml$^{-1}$). Cultures used for quantitative analysis were stirred and bubbled with air containing 1% (vol/vol) CO$_2$ and continuously illuminated under a PPFD of 55 $\mu$mol m$^{-2}$ s$^{-1}$ as otherwise stated. Growth of the cultures was monitored by determination of the optical density at 750 nm (OD$_{750}$). All measurements were carried out with cells from mid-exponential-phase cultures, i.e., those collected at an OD$_{750}$ of 0.4, which corresponds to $8 \times 10^7$ cells per ml (21). For growth on plates, the medium was solidified by addition of 1% (wt/vol) agarose (Litex).

**Determination of the modification state of P$_I$.** The phosphorylation state of P$_I$ in vivo was analyzed as described previously (12). Briefly, cells were broken and the crude extract was separated by nondenaturing polyacrylamide gel electrophoresis (PAGE); the differential migration of the unphosphorylated and phosphorylated isoforms of P$_I$ was revealed by immunoblotting with a P$_I$-specific antiserum. Cross-reactive antibodies were visualized by using an enhanced chemiluminescent detection system (ECL kit; Amersham). For the generation of the P$_I$-deficient mutant, cells of Synechococcus sp. strain PCC 7942 were transformed with plasmid pPMM128 (43), which was derived from plasmid pPM119 (42) by interrupting the gdh gene at the XhoI site. Methylammonium resistance cartridge from plasmid pUC4K (Pharmacia). Transformation of the cyanobacteria and isolation of total DNA were carried out according to the method of Golden et al. (13). To distinguish between gene replacement and plasmid integration, kanamycin-resistant transformants were analyzed for the presence of vector DNA by DNA hybridization.

**RNA isolation and Northern (RNA) blot extraction.** Total RNA was isolated from mid-exponential-phase cultures was performed as described by Mazel et al. (24). A 6-μg sample of total RNA was applied per lane of a 1.2% (wt/vol) agarose–15% (vol/vol) formaldehyde gel. That the lanes contained equal loads of total RNA was verified by the intensity of the ethidium bromide fluorescence from the RNA bands under UV light. Electrophoretic conditions, transfer of RNA to nylon membranes (Hybond N; Amersham), and hybridization conditions were as described previously (6).

**Pigment, protein, and glycine determination.** Chlorophyll $a$ was spectrophotometrically determined from methanol extracts (40). Phycocyanin content was calculated from the difference spectrum of the respective culture before and after heating at 75°C for 8 min as described elsewhere (7). Protein in crude cell extracts was determined according to the dye-binding assay of Bradford (5) by using Bio-Rad protein assay solution (Bio-Rad Laboratories) and bovine serum albumin as the standard. For the determination of glycogen, about $8 \times 10^8$ cells were suspended in 200 μl of a 2.5% (vol/vol) sulfuric acid solution and heated for 40 min in a boiling-water bath. Glucose, which was produced from glycogen by acid hydrolysis, in the hydrolysate was then quantified by a colorimetric assay using the phenol–sulfuric reagent (procedure no. 635; Sigma).

**Light-dependent oxygen evolution.** Rates of light-induced oxygen production were measured by using a Clark-type oxygen electrode (model 5300; Yellow Springs Instruments). To obtain maximal rates of photosynthetic O$_2$ evolution, the cells were illuminated with a PPFD of 300 $\mu$mol m$^{-2}$ s$^{-1}$ provided by a slide projector lamp.

**Enzyme assays.** Determination of nitrate reductase (15) and nitrite reductase (16) in permeabilized cells was performed with dithionite-reduced methyl viologen as the reductant. Glutamine synthetase activity (transferase assay) in permeabilized cells was measured as described elsewhere (3). Methylammonium transport was assayed by measuring the uptake of [3$^2$P]dCTP by using the Megaprimerandom priming kit (Amersham). For the generation of the P$_I$-deficient mutant, cells of Synechococcus sp. strain PCC 7942 were transformed with plasmid pPMM128 (43), which was derived from plasmid pPM119 (42) by interrupting the gdh gene at the XhoI site. Methylammonium resistance cartridge from plasmid pUC4K (Pharmacia). Transformation of the cyanobacteria and isolation of total DNA were carried out according to the method of Golden et al. (13). To distinguish between gene replacement and plasmid integration, kanamycin-resistant transformants were analyzed for the presence of vector DNA by DNA hybridization.

**results**

Response of the phosphorylation state of P$_I$ to ammonium assimilation and CO$_2$ fixation. The phosphorylation state of P$_I$ can be analyzed by electrophoretic separation of the different isoforms generated by phosphorylation followed by immunoblot detection with P$_I$-specific antibodies (12). As shown in Fig. 1A, the most slowly migrating form of P$_I$ (P$_I^\text{III}$), which corresponds to unmodified P$_I$ protein, was present in ammonium-starved cells. Phosphorylation of P$_I$ in cells grown with nitrate as an N source or in cells which are starved for nitrogen can be monitored by the resolution of the fast-migrating forms P$_I^\text{I}$, P$_I^\text{II}$, and P$_I^\text{III}$. The addition of only 0.5 mM ammonium to nitrogen-starved cells was sufficient to cause complete dephosphorylation of the P$_I$ protein (data not shown). In another set of experiments (Fig. 1B), cells grown in the presence of ammonium were transferred either to a medium lacking combined nitrogen or to a medium containing the inhibitors of glutamine synthetase and glutamate synthase, MSX (33, 38) and 6-diazo-5-oxo-L-norleucine (DON) (26, 28, 39), respectively. After 30 min and 1, 2, and 4 h, samples were removed and the phosphorylation state of P$_I$ was analyzed as mentioned above. As shown in Fig. 1B, removal of ammonium and inhibition of glutamine synthetase activity with MSX led to an increase in phosphorylated forms of P$_I$. P$_I$ phosphorylation was almost complete after 1 h. In contrast, inhibition of glutamate synthase caused a more rapid phosphorylation of P$_I$, as it could be detected after 30 min. These experiments demonstrate that the modification state of P$_I$ correlates with
the activity of ammonium assimilation through the glutamine synthetase–ferredoxin-dependent glutamate synthase pathway.

In nitrate-assimilating cells, the degree of P II phosphorylation also depends on the fixation of CO₂ (Fig. 2). When a cyanobacterial culture is not stirred and aerated, the concentration of dissolved inorganic carbon (CO₂, HCO₃⁻) decreases substantially (25) and becomes limiting for growth. Under such conditions, the degree of P II phosphorylation was low but increased as soon as the cells were provided with 1% (vol/vol) CO₂ in air (Fig. 2A). The dependence of P II phosphorylation on CO₂ fixation was further demonstrated by inhibiting CO₂ fixation with 20 mM DL-glyceraldehyde. At this concentration of inhibitor, photosynthetic oxygen evolution was reduced by 80% (data not shown). In the experiment shown in Fig. 2B, DL-glyceraldehyde was added to nitrate-grown cells provided with 1% (vol/vol) CO₂ in air. The phosphorylation state of P II was analyzed at various times following this addition. After 5 min, a substantial part of phosphorylated P II was already converted to the unmodified form; 30 min after the addition of DL-glyceraldehyde, P II was almost completely dephosphorylated.

**Generation of a P II-deficient mutant.** As a first step to elucidate targets of the P II-mediated signal, we created a P II-deficient mutant of *Synechococcus* sp. strain PCC 7942 by inactivating the *glnB* gene (encoding the P II protein) on the chromosome. Plasmid pPM128, in which the *glnB* gene had been interrupted by insertion of a kanamycin resistance cartridge, was used to transform cells of *Synechococcus* sp. strain PCC 7942. As the plasmid does not replicate in the recipient cells, kanamycin resistance can be acquired only by gene replacement or plasmid integration into the chromosome. To distinguish between these two possibilities, kanamycin-resistant transformants were screened by DNA-DNA hybridization for the absence of vector DNA. Following segregation, three identical clones were obtained in which all chromosomal *glnB* alleles were replaced by the disrupted gene. Fig. 3 shows the restriction map of the *glnB* region and a DNA-DNA hybridization analysis of total DNA from the mutants and the parental strain. The clone in lanes 2 of Fig. 3B was taken as representative and was named MP2. RNA-DNA hybridization and immunoblot analysis confirmed that no *glnB* transcript and no *glnB* gene product could be detected in MP2 cells (data not shown).

**Phenotype of the P II-deficient MP2 cells.** The P II-deficient MP2 cells can utilize nitrate, glutamine, and ammonium as nitrogen sources. With nitrate and glutamine, growth rates were only slightly decreased (by about 10%) in comparison with that of the parental strain. However, in the presence of...
ammonium, the mutant could only grow in a medium buffered to pH less than 8; at more alkaline pH, cells ceased to grow and degraded chlorophyll \(a\) (data not shown). To further characterize the general phenotype of the \(P_{II}\)-deficient mutant, the quantities of glycogen and protein, which are the major products of carbon and nitrogen assimilation, were compared with those produced by the wild type. Moreover, the light-harvesting antennae, phycocyanin and chlorophyll \(a\), were quantified since their synthesis is tightly regulated in response to the environment (41). The analysis was performed with nitrate- and ammonium-supplemented medium. At time zero (indicated by the arrow), cells in the exponential phase of growth in a nitrate-containing medium were harvested, washed, and resuspended in a medium supplemented with 5 mM ammonium chloride. OD\(620\) and maximal rate of light-dependent \(O_2\) evolution were determined. Rates of \(O_2\) evolution are given as percentages of the maximal rate (taken as 100%) determined immediately before the shift experiment.

![Graph showing the response of the \(P_{II}\)-deficient mutant MP2 to transfer from nitrate- to ammonium-supplemented medium. At time zero (indicated by the arrow), cells in the exponential phase of growth in a nitrate-containing medium were harvested, washed, and resuspended in a medium supplemented with 5 mM ammonium chloride. OD620 and maximal rate of light-dependent O2 evolution were determined. Rates of O2 evolution are given as percentages of the maximal rate (taken as 100%) determined immediately before the shift experiment.](http://jb.asm.org/)

### TABLE 1. Determination of chlorophyll \(a\), phycocyanin, total protein, and glycogen in cells of Synechococcus sp. strain PCC 7942 and of the \(P_{II}\)-deficient mutant

<table>
<thead>
<tr>
<th>Cell type and growth conditions (a)</th>
<th>Amt of:</th>
<th>Chlorophyll (a)</th>
<th>Phycocyanin (b)</th>
<th>Protein (b)</th>
<th>Glycogen (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, NO(_3) (b)</td>
<td>6.7</td>
<td>513</td>
<td>158</td>
<td>31.1</td>
<td></td>
</tr>
<tr>
<td>MP2, NO(_3) (b)</td>
<td>6.3</td>
<td>363</td>
<td>137</td>
<td>28.7</td>
<td></td>
</tr>
<tr>
<td>WT, NH(_4) (b)</td>
<td>7.2</td>
<td>450</td>
<td>154</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>MP2, NH(_4) (b)</td>
<td>5.0</td>
<td>273</td>
<td>108</td>
<td>36.8</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Cells were grown with nitrate (NO\(_3\)) or ammonium (NH\(_4\)) as N source, under a PPFD of 70 \(\mu\)mol \(\cdot\) \(m^{-2}\) \(\cdot\) \(s^{-1}\). WT, Synechococcus sp. strain PCC 7942; MP2, \(P_{II}\)-deficient mutant.

\(b\) Expressed as micrograms per OD\(620\) unit of cell suspension. Values are means of two independent determinations.

### TABLE 2. Light-dependent decrease of phycocyanin and chlorophyll \(a\) contents in cells of Synechococcus sp. strain PCC 7942 and of the \(P_{II}\)-deficient mutant

<table>
<thead>
<tr>
<th>Cell type and growth conditions (a)</th>
<th>Content (%) at indicated PPFD ((\mu)mol (\cdot) (m^{-2}) (\cdot) (s^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phycocyanin (b)</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>WT, NO(_3) (b)</td>
<td>100</td>
</tr>
<tr>
<td>MP2, NO(_3) (b)</td>
<td>76</td>
</tr>
<tr>
<td>WT, NH(_4) (b)</td>
<td>100</td>
</tr>
<tr>
<td>MP2, NH(_4) (b)</td>
<td>74</td>
</tr>
</tbody>
</table>

\(a\) Cells were grown in the presence of either nitrate (NO\(_3\)) or ammonium (NH\(_4\)). WT, Synechococcus sp. strain PCC 7942; MP2, \(P_{II}\)-deficient mutant.

\(b\) The percentages of phycocyanin and chlorophyll \(a\) were calculated relative to their amounts in the wild-type cells. Values are means of two independent determinations.

### Regulation of enzymes involved in nitrogen assimilation

The synthesis of glutamine synthetase, nitrate reductase, and nitrite reductase is generally prevented or decreased by the presence of ammonium in cyanobacteria (14). To determine whether this regulation requires the function of \(P_{II}\), we compared the activities of these enzymes in permeabilized cells from the wild type and the mutant grown in the presence of nitrate or ammonium (Table 3). For nitrate-grown cells, the activities of the three enzymes were higher in the mutant than in the wild type. In contrast, for ammonium-grown cells the activities of nitrate and nitrite reductase decreased in both the mutant and the wild type but glutamine synthetase activity was increased twofold in the mutant. As an approach to understanding the molecular basis of the increased activities of glutamine synthetase in the mutant cells, we examined the steady-state level of \(glnA\) mRNA by RNA-DNA hybridization experiments and the quantity of glutamine synthetase protein...
by immunoblotting. As shown in Fig. 5A, the amount of immunodetectable glutamine synthetase protein parallels the levels of enzyme activity presented in Table 3, indicating that, both in the mutant and the wild-type cells, the increased levels of glutamine synthetase activity are, at least in part, due to an overproduction of the protein. Preliminary results showed that the increased activity of glutamine synthetase correlated with a twofold increase in the intracellular pool size of glutamine and a parallel decrease in glutamate in the mutant cells whatever the nitrogen source provided for growth (data not shown). Finally, the steady-state level of glnA mRNA was higher in the mutant under both culture conditions, suggesting that the regulation occurs at a transcriptional or posttranscriptional level (Fig. 5B). Note that with the mutant cells, growth in the presence of ammonium, rather than nitrate, still resulted in a significant reduction of the glnA transcript abundance.

Surprisingly, we found that the mutant excretes nitrite into the medium when grown in the presence of nitrate. About 0.2 mM nitrite was detected in the medium from a mid-exponen-
tial-phase culture, and up to 1.8 mM nitrite was detected in the medium from a culture in the early stationary phase. In cultures of the wild-type strain, only about 3 μM nitrite could be detected, regardless of the growth phase. The enhanced excretion of nitrite pointed to a deregulation of nitrate assimilation under these growth conditions. In cyanobacteria, nitrate reduction is directly coupled to photosynthetic electron transport (27, 31). Thus, to further examine the metabolism of nitrate in intact cells, nitrate reduction was measured by the determination of nitrate-dependent oxygen evolution. In order to specifically achieve nitrate-dependent oxygen evolution, CO2-dependent oxygen evolution has to be suppressed (e.g., by the addition of D,L-glyceraldehyde) and glutamine synthetase activity must be inhibited (e.g., by the addition of MSX). Indeed, if the activity of the latter enzyme is not inhibited, nitrate reduction is turned down in response to the inhibition of CO2 fixation (32). Wild-type cells treated with D,L-glyceraldehyde effectively showed nitrate-dependent oxygen evolution in the presence of MSX (19.7 μmol of O2·min·OD750 unit) but not in the absence of this inhibitor (0 μmol of O2·min·OD750 unit). In contrast, the mutant cells exhibited nitrate-dependent oxygen evolution to nearly the same extent as in the presence of MSX (17.5 and 22.6 μmol of O2·min·OD750 unit, respectively), suggesting that the regulatory mechanism which links nitrate reduction to CO2 fixation is impaired in the absence of PII. (Values given above are means for two independent determinations, and the experiment was carried out as described in Materials and Methods.)

Methylammonium transport is another cellular activity subjected to nitrogen control in cyanobacteria. In contrast to the situation with the other nitrogen-regulated enzymes described above, no methylammonium transport activity could be detected in the mutant cells grown in the presence of nitrate, whereas the activity was fully induced in the wild-type cells under these conditions (Table 4). Following transfer of the mutant cells grown in the presence of nitrate to a medium lacking combined nitrogen, methylammonium transport activity increased rapidly after approximately 3 h to finally reach a level nearly fivefold greater than that of the wild-type cells. In the presence of ammonium, methylammonium transport was undetectable in both the mutant and the wild-type cells.

**DISCUSSION**

In the cyanobacterium *Synechococcus* sp. strain PCC 7942, the PII protein is phosphorylated at a seryl residue in response to the state of nitrogen and carbon assimilation. In contrast, the proteobacterial PII protein is modified by uridylylation; in enterobacteria, a uridylyltransferase–uridylyl-removing enzyme

---

**TABLE 3. Activity levels of nitrate reductase, nitrite reductase, and glutamine synthetase in cells of *Synechococcus* sp. strain PCC 7942 and of the PII-deficient mutant**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Nitrate reductase</th>
<th>Nitrite reductase</th>
<th>Glutamine synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO₃⁻</td>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>WT</td>
<td>11</td>
<td>&lt;1</td>
<td>7.6</td>
</tr>
<tr>
<td>MP2</td>
<td>16</td>
<td>11</td>
<td>18.5</td>
</tr>
</tbody>
</table>

* The enzymes were assayed in cetyltrimethylammonium bromide-permeabilized cells from cultures grown in the presence of nitrate (NO₃⁻) or ammonium (NH₄⁺). Specific activities of nitrate and nitrite reductases are given in nanomoles per minute per OD750 unit of cell suspension. Glutamine synthetase (transferase) activity is given in micromoles per minute per OD750 unit. Estimates of the specific activities per microgram of chlorophyll a or per milligram of protein can be calculated from the values given in Table 1. Each assay was performed with triplicate samples taken from three independent cultures. Mean values are reported. In all cases, the standard deviations are less than 10%.

---

**TABLE 4. Methylammonium transport activity in cells of *Synechococcus* sp. strain PCC 7942 and of the PII-deficient mutant**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>NO₃⁻</th>
<th>NH₄⁺</th>
<th>NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.7</td>
<td>0.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MP2</td>
<td>&lt;0.01</td>
<td>3.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* WT, *Synechococcus* sp. strain PCC 7942; MP2, PII-deficient mutant. 

Activity is expressed as nanomoles per minute per OD750 unit of cell suspension and was determined from cells grown with nitrate (NO₃⁻) or ammonium (NH₄⁺) as N source or from nitrate-adapted cells transferred to a medium lacking combined nitrogen for 4 h (–N). Each assay was performed with triplicate samples taken from three independent cultures. Mean values are reported. In all cases, the standard deviations are less than 10%.

---

**FIG. 5.** (A) Immunoblot analysis of glutamine synthetase protein. Cells of *Synechococcus* sp. strain PCC 7942 (WT) and of the PII-deficient mutant (MP2) were grown in the presence of either nitrate (NO₃⁻) or ammonium (NH₄⁺). The sodium dodecyl sulfate (SDS) lysates of the cells, equivalent to 1 ml of culture, were separated by SDS-PAGE. Glutamine synthetase (GS) was revealed by using antibodies raised against glutamine synthetase from *Nostoc* sp. strain UCD 7801. (B) Northern blot hybridization of total RNA from cells of *Synechococcus* sp. strain PCC 7942 (WT) and of the PII-deficient mutant (MP2) grown under the conditions described in the legend to panel A. The RNA blot was probed with a 0.9-kb *EcoRI* fragment internal to the *glnA* gene.
Global nitrogen control in cyanobacteria operates in response to the same stimuli as those shown in the present study to affect PII phosphorylation. Assimilation of ammonium and inhibition of CO₂ fixation similarly lead to the repression of several enzymes involved in nitrogen assimilation (glutamine synthetase, methylammonium transport, and nitrate and nitrite reductases) (32, 45); these same conditions result in the dephosphorylation of PII, while an increased level would correlate with its phosphorylation. PII phosphorylation is currently being analyzed in vitro to determine whether these different metabolites indeed act as signal molecules for the PII kinase-phosphatase activities.

Global nitrogen control in cyanobacteria operates in response to the same stimuli as those shown in the present study to affect PII phosphorylation. Assimilation of ammonium and inhibition of CO₂ fixation similarly lead to the repression of several enzymes involved in nitrogen assimilation (glutamine synthetase, methylammonium transport, and nitrate and nitrite reductases) (32, 45); these same conditions result in the dephosphorylation of PII. In contrast, under conditions of nitrate utilization or nitrogen starvation or in the presence of the inhibitors MSX and DON, which were previously shown by others to induce the synthesis of the enzymes mentioned above (22, 39), PII was shown to be phosphorylated. It is thus reasonable to suppose that PII might be involved in the signal transduction pathway underlying global nitrogen control in cyanobacteria.

Nitrogen control is exerted at different levels of regulation. A transcriptional activator, NtcA, has been identified as responsible for the induction of the expression of the ntrA operon and the glnA gene in the absence of ammonium (22). Furthermore, nitrate and methylammonium uptakes have been shown to be inhibited by ammonium. To identify a possible function of the PII protein in this regulatory system, a mutant of Synechococcus sp. strain PCC 7942 lacking this protein was created. The inactivation of the glnB gene leads to a pleiotropic phenotype indicative of a metabolic deficiency. In cells which were adapted to ammonium, however, the level of nitrate and nitrate reductase activities was as depressed in the mutant as in the wild type (Table 3). This suggests that the synthesis of these enzymes is subject to ammonium repression even in the absence of PII. The situation is slightly different for glutamine synthetase; both the amount of protein and the glnA mRNA steady-state level are increased in the mutant whatever the ammonium source provided for growth. Nevertheless, the same twofold decrease of glnA expression upon addition of ammonium is observed in the mutant. From these data it can be concluded either that PII does not directly regulate NtcA activity in response to the nitrogen status or that its function is redundant for this purpose. If phosphorylated PII were absolutely required for the activation of NtcA, then the phenotype of the PII-deficient mutant would be similar to that of the NtcA-deficient mutant, which constitutively expresses at a low level the genes coding for nitrate and nitrite reductases and for glutamine synthetase, irrespective of the nitrogen source (45).

Furthermore, the ability of the mutant cells to adapt to a shift from ammonium to nitrate suggests that the induced synthesis of the enzymes required for nitrate assimilation is not impaired in the absence of PII. If the unmodified PII form acted as an inhibitor of NtcA activity, causing an effect analogous to the effect of PII on the two-component regulatory system NtrB-NtrC in enterobacteria (29), then one should expect some relief from ammonium repression of nitrate and nitrite reductases in the mutant. Furthermore, if PII acted via NtcA, it should be expected that the glnB mutation similarly affects the expression of nitrate and nitrite reductases and that of glutamine synthetase. The high abundance of glnA mRNA in the mutant could indicate that PII acts on a yet- unidentified factor which is involved in glnA gene expression. The identification of such a factor is a subject for further investigations.

The pleiotropic phenotype of the mutant described in this work could in part be attributed to the increase in glutamine synthetase activity. The reduced intracellular pool size of glutamate and the elevated level of glutamine observed to be present in the mutant are consistent with an increased consumption of glutamate due to the high-level activity of glutamine synthetase. A reduction in the pool size of glutamate might be responsible for the reduced synthesis of protein and pigments in the mutant, as glutamate is the precursor for tetrapyrrole synthesis and is of central importance in amino acid biosynthesis. The necessity to tightly regulate the activity of glutamine synthetase and to maintain the large cellular glutamate pool had been demonstrated with a glnE mutant of Salmonella typhimurium (19). This mutant is unable to reduce the activity of glutamine synthetase upon a shift from nitrogen limitation to medium containing an excess of ammonium. As a consequence, the glutamate pool of the cells drains out, resulting in a large growth defect. A similar reaction to ammonium is shown by the PII-deficient Synechococcus cells, suggesting that the elevated levels of glutamine synthetase activity might indeed be responsible for the observed growth defect.

The ratio of glycogen to protein reflects the partitioning of newly fixed carbon either to carbon storage products or to the glycolytic pathway yielding amino acids and chromophores (8). The increase of the glycogen reserves relative to the protein and pigment content in the mutant provides an indication that this partitioning is imbalanced in the absence of PII.

The dependence of nitrate utilization upon the availability of CO₂ is well documented for cyanobacteria and green algae (9, 11, 23). Here we provide evidence that PII is involved in the coordination of these processes, since in the absence of PII, photosynthetic nitrate reduction is uncoupled from CO₂ fixation. According to Romero et al. (32), the control of nitrate utilization by CO₂ occurs at the level of nitrate uptake; PII might therefore interfere with this biological process.
methylammonium uptake. It is not obvious, however, why the lack of phosphorylated PII protein would lead to an inhibition of methylammonium transport in the presence of nitrate but to an increase of activity under conditions of nitrogen starvation. Possibly, the absence of methylammonium transport could be due to the accumulation of an inhibitory compound in the mutant cells grown in the presence of nitrate. In contrast, when these cells are starved of nitrogen, the inhibitor would be consumed, thereby relieving methylammonium transport activity.

There might be some other targets of PII-mediated regulation, as suggested by the sensitivity of the mutant in adapting to decreasing light intensity. This phenotype indicates that the mutant might be impaired in controlling its energy metabolism. In analysis of the PII-deficient mutant, the fact that unmodified and modified PII proteins could have counteracting functions must be considered. The simultaneous lack of the putative antagonists could mask clearer responses which would become apparent if PII were locked in either its unmodified or its modified state. Similar problems have been encountered with PII mutants of Klebsiella pneumoniae (17). Site-directed mutagenesis of the glnB gene would provide a means of obtaining PII variants which no longer can be modified, facilitating the further identification of PII-regulated functions in Synechococcus sp. strain PCC 7942.

Together, the results presented in this work provide evidence that PII is involved in the regulation of nitrogen assimilation and in its coordination with carbon assimilation in cyanobacteria. PII phosphorylation responds to the same stimuli that dictate global nitrogen control. However, PII is not the only signal transducer in this system. At least for the regulation of NtcA activity, PII appears to be dispensable. Thus, there might exist several branched signalling pathways originating from the primary sensor to control nitrogen assimilation at different levels of regulation in cyanobacteria.

ACKNOWLEDGMENTS

We thank J. C. Meeks for providing antibodies directed against glutamine synthetase and S. J. Robinson for the clone of the Synechococcus sp. strain PCC 7942 glnA gene. We gratefully acknowledge J. Houmard for helpful discussions and G. Sawers for critical reading of the manuscript.

K.F. was supported by a grant from the Deutsche Forschungsgemeinschaft. This work was also supported by the Institut Pasteur and by the French Centre National de la Recherche Scientifique (URA 1129).

REFERENCES

laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, N.Y.
43. Tsinoremas, N. F., and N. Tandeau de Marsac. Unpublished data.