Posttranslational Regulation of Nitrate Assimilation in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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Assimilation of nitrate by cyanobacteria is regulated by ammonium at both transcriptional and posttranslational levels (5, 11, 30); addition of ammonium to medium promptly inhibits transcription of the relevant genes and uptake of nitrate. The inhibition of transcription of the nitrate assimilation genes is a part of global nitrogen regulation in cyanobacteria, which involves NtcA, a Crp family protein, as the transcriptional activator (40, 41). Recent in vitro experiments showed that 2-oxoglutarate (2-OG), which serves as the acceptor of the newly fixed nitrogen in the glutamine synthetase-glutamate synthase cycle, activates transcription from NtcA-dependent promoters in a concentration-dependent manner (38). Since the intracellular 2-OG concentration is low in the presence of ammonium and is increased by nitrogen deprivation (28), 2-OG is supposed to act as a coinducer of transcription of the NtcA-dependent genes, conferring ammonium sensitivity to their expression in vivo.

There are three biochemical steps essential to nitrate assimilation, i.e., transport of nitrate into the cell, reduction of nitrate to nitrite, and reduction of nitrite to ammonium (11). These are mediated by an active nitrate and nitrite transporter (NRT), ferredoxin-dependent nitrate reductase (NR), and ferredoxin-dependent nitrite reductase (NiR), respectively, in cyanobacteria (5, 30). Studies using *Synechococcus elongatus* strain PCC 7942 have shown that activities of NRT and NR are inhibited upon addition of ammonium to medium (18), whereas NiR activity is not (25). The inhibition is reversible, and assimilation of nitrate is resumed after consumption of ammonium in medium. The P_H protein and an NRT subunit (NrtC) are involved in the regulation (18, 23). NrtC is an ATP-binding subunit of the ABC-type nitrate and nitrite bispecific transporter (18, 31). It is unique among the ATP-binding subunits of bacterial ABC transporters in having a large C-terminal extension (31), which is involved in the ammonium-promoted regulation of the transport activity (18). The P_H protein (homotramer of the *glnB* gene product) has the ability to bind 2-OG, suggesting that it senses the cellular nitrogen status by binding 2-OG (6). Similar to its counterparts in proteobacteria, the cyanobacterial P_H protein is modified when nitrogen is limited, but the modification is by phosphorylation at Ser^49^ rather than by uridylylation at the Tyr^23^ residue (9); the phosphorylation state of the GlnB trimer changes from a fully dephosphorylated state in ammonium-grown cells to a highly phosphorylated state in the cells subjected to nitrogen starvation. Since P_H-deficient mutants show ammonium-insensitive nitrate assimilation (23), it is clear that P_H negatively regulates both NRT and NR in the presence of ammonium. However, it remains unclear how P_H transduces the nitrogen signal to the nitrate assimilation enzymes. In some phosphoproteins, the presence of negative electric charge at the phosphorylation site plays a role in regulating the activity of the protein (20, 27, 39, 43). Studies using site-specific mutant forms of P_H, however, revealed a complex relationship between the electric charge at the phosphorylation site and the regulation of nitrate assimilation; a *Synechococcus* mutant expressing an unphosphorylatable derivative of P_H with an S49A substitution shows negligible nitrate assimilation activity irrespective of the cellular nitrogen status, whereas a mutant expressing an S49E derivative of P_H, having a negative charge on the 49th amino acid, 

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shows ammonium-responsive regulation of nitrate assimilation like that of the wild-type strain (22). Taking into account these observations, it was hypothesized that a factor other than the electric charge at the 49th amino acid position plays a role in controlling the activity of PII(S49E), whereas PII(S49A) is fixed in a state that is inhibitory to nitrate assimilation (22).

Studies of posttranslational regulation of nitrate assimilation by cyanobacterial cells have so far focused mainly on Synechocystis sp. strain PCC 6803 isolated by Williams (44) and commonly used for photosynthesis research (designated GT or referred to simply as the wild-type strain herein) and a derivative of Synechococcus elongatus strain PCC 7942 which is cured of the resident small plasmid pUH24 (R2-SPc; hereafter designated strain PCC 7942) were the parental strains of all of the cyanobacterial strains used in this study (Table 1). The cyanobacterial cells were grown phototrophically at 30°C under continuous illumination provided by fluorescent lamps (70 micro einsteins m\(^{-2}\) s\(^{-1}\)). The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (36) as described previously (37). Ammonium- and nitrate-containing media were prepared by addition of 3.75 mM (NH\(_4\))\(_2\)SO\(_4\) and 15 mM KNO\(_3\), respectively. (37). Ammonium- and nitrate-containing media were prepared by addition of 3.75 mM (NH\(_4\))\(_2\)SO\(_4\) and 15 mM KNO\(_3\), respectively. (37). Ammonium- and nitrate-containing media were prepared by addition of 3.75 mM (NH\(_4\))\(_2\)SO\(_4\) and 15 mM KNO\(_3\), respectively. (37). Ammonium- and nitrate-containing media were prepared by addition of 3.75 mM (NH\(_4\))\(_2\)SO\(_4\) and 15 mM KNO\(_3\), respectively. (37). Ammonium- and nitrate-containing media were prepared by addition of 3.75 mM (NH\(_4\))\(_2\)SO\(_4\) and 15 mM KNO\(_3\), respectively.

**Isolation and analysis of DNA.** Chromosomal DNA was extracted and purified from the cyanobacterial cells as described by Williams (44). Manipulations and analyses of DNA were performed according to standard protocols (35). All the cloned DNA fragments that had been generated by PCR amplification were sequenced to verify the nucleotide sequence. For Southern hybridization analysis of the genomic DNA digests of Synechocystis sp. strain PCC 6803, the following DNA fragments were amplified from the cyanobacterial DNA samples by PCR, labeled with \(^{32}\)P, and used as probes: a 443-bp Synechocystis DNA fragment carrying the glnB coding region and 107 bases of its 3' flanking sequence (Fig. 1), a 487-bp Synechocystis DNA fragment carrying the nrtC-nrtD intergenic region and 430 bases of the nrtD coding region (Fig. 1), and a 343-bp Synechococcus DNA fragment carrying the glnB coding region (see Fig. 6).

**TABLE 1. Cyanobacterial strains and plasmids used**

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<td>pSE1 derivative encoding GlnB(K2E, S49E)</td>
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**MATERIALS AND METHODS**

**Strains and growth conditions.** The glucose-tolerant derivative of *Synechococcus* sp. strain PCC 6803 isolated by Williams (44) and commonly used for photosynthesis research (designated GT or referred to simply as the wild-type strain herein) and a derivative of *Synechococcus elongatus* strain PCC 7942 which is cured of the resident small plasmid pUH24 (R2-SPc; hereafter designated strain PCC 7942) were the parental strains of all of the cyanobacterial strains used in this study (Table 1). The cyanobacterial cells were grown phototrophically at 30°C under continuous illumination provided by fluorescent lamps (70 micro einsteins m\(^{-2}\) s\(^{-1}\)). The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (36) as described previously (37). Ammonium- and nitrate-containing media were prepared by addition of 3.75 mM (NH\(_4\))\(_2\)SO\(_4\) and 15 mM KNO\(_3\), respectively, to the basal medium. Both media were buffered with 20 mM HEPES-KOH (pH 8.2). The cultures were aerated with 2% (vol/vol) CO\(_{2}\) in air. When appropriate, kanamycin, spectinomycin, and gentamicin were added to the media at 15, 15, and 5 µg/ml, respectively. E. coli strains NM522, HB101, and SL906 were grown on Luria-Bertani medium supplemented with ampicillin (50 µg/ml), spectinomycin (25 µg/ ml), streptomycin (20 µg/ml), gentamicin (30 µg/ml), or kanamycin (15 µg/ml) when appropriate.

**Isolation and analysis of DNA.** Chromosomal DNA was extracted and purified from the cyanobacterial cells as described by Williams (44). Manipulations and analyses of DNA were performed according to standard protocols (35). All the cloned DNA fragments that had been generated by PCR amplification were sequenced to verify the nucleotide sequence. For Southern hybridization analysis of the genomic DNA digests of *Synechocystis* sp. strain PCC 6803, the following DNA fragments were amplified from the cyanobacterial DNA samples by PCR, labeled with \(^{32}\)P, and used as probes: a 443-bp *Synechocystis* DNA fragment carrying the glnB coding region and 107 bases of its 3' flanking sequence (Fig. 1), a 487-bp *Synechocystis* DNA fragment carrying the nrtC-nrtD intergenic region and 430 bases of the nrtD coding region (Fig. 1), and a 343-bp *Synechococcus* DNA fragment carrying the glnB coding region (see Fig. 6).
Insertional and deleitional mutagenesis. For targeted mutagenesis of Synechocystis sp. strain PCC 6803 and Synechococcus elongatus strain PCC 7942, the genes with insertions and/or deletions were constructed with vectors that do not replicate in the cyanobacteria. The resulting plasmids were used to transform the cyanobacterial cells by replacing the wild-type gene with the mutated gene through homologous recombination. Transformation of the cyanobacteria and isolation of homozygous mutants were performed as described by Williams (44).

SNC1, an nrtC insertional mutant derivative of Synechocystis sp. strain PCC 6803, was constructed by inserting a 3.8-kbp nptI-sacB cartridge from pRL250 (3) into the NheI site located in the 3'/H11032 portion of the coding region. A mutant lacking the C-terminal domain of NrtC (SNC2) was obtained from SNC1 by deleting the 3'/H11032 portion of nrtC corresponding to the C-terminal domain by the eviction mutagenesis method using sacB as a negative selection marker (33). In SNC2, a 1,188-bp internal segment of nrtC, corresponding to nucleotides 823 to 2010 of the 2,010-nucleotide coding region (Fig. 1A), had been deleted from the genome and, as a consequence of the in-frame deletion of nucleotides, the modified nrtC encoded a protein of 274 amino acid residues, consisting of the N-terminal ATP-binding domain (amino acids 1 to 254) and a part of the linker sequence connecting the N-terminal and C-terminal domains (amino acids 255 to 274) of NrtC.

The plasmid used for inactivation of glnB in Synechocystis sp. strain PCC 6803, which was provided by T. Ogawa (Nagoya University), carried a disrupted glnB and 437 and 304 bp of its 5' and 3' flanking sequences, respectively. In this construct, the 208-bp internal fragment of glnB, extending from nucleotide +109 to +316 with respect to the translation start site, had been replaced by an nptI-kanamycin resistance gene cartridge originating from pUC4K (42).

For construction of a glnB-deficient Synechococcus elongatus strain PCC 7942 mutant, a 1.3-kbp XbaI-PstI fragment of Synechococcus DNA carrying glnB was cloned as follows. A HindIII digest of chromosomal DNA was fractionated on an agarose gel. DNA fragments of 4 to 5 kbp were recovered from the gel and ligated with a 167-bp HindIII-PvuII fragment of pT7Blue (Novagen), carrying the multiple cloning region of the plasmid. With primers specific to internal sequences of glnB and the fragment of pT7Blue, the 5'/H11032 and 3'/H11032 halves of glnB were amplified by PCR with 1.1 and 2.9 kb of the respective flanking sequences and separately cloned into pT7Blue T-Vector (Novagen). The DNA fragments carrying the 5' and 3' halves of glnB were excised from the resulting plasmids and joined on the pUC19 vector. From this plasmid, a 1.3-kbp XbaI-PstI fragment, carrying the glnB coding region and 330 and 640 bases of its 5' and 3' flanking sequences, respectively, was excised and cloned between the XbaI and PstI sites of pT7Blue. In the glnB sequence thus cloned, a 5-base sequence 5'/H11032-TAATT-3'/H11032, which was derived from the primer sequences used for PCR, replaced the A at position 152 of the coding region, resulting in a frameshift mutation. A spectinomycin and streptomycin resistance gene cassette (Ω cassette) excised from...
plasmid pRL463 (4) was subsequently inserted into the XhoI site located at nucleotide 182 of the glnB coding region. The resulting plasmid was used to inactivate glnB in wild-type Synechococcus elongatus strain PCC 7942 to yield the PD1 mutant.

For inactivation of narB in Synechococcus elongatus strain PCC 7942, a 1.7-kbp fragment of Synechococcus DNA, carrying the 3′ half of the gene, was cloned into pUC18 and the 605-bp internal fragment of narB, extending from nucleotide +1071 to +1674 with respect to the translation start site, was removed by digestion with MscI and replaced with the ϕc4164 cassette excised from pRL463 (4). The plasmid carrying disrupted narB was used to introduce narB in the Synechococcus elongatus strain PCC 7942 NC2 mutant to yield the NC41 mutant.

Expression of plasmid-borne glnB in Synechocystis. A 853-bp fragment of Synechocystis DNA, carrying the glnB coding region and 410 and 107 bp of its 5′ and 3′ flanking sequences, respectively, was amplified by PCR and cloned into pT7Blue T-Vector. The cloned fragment contained the entire promoter region of glnB (10). Two derivatives of this plasmid, encoding the S49A and S49E derivatives of GlnB, were obtained by changing the 49th codon of glnB from TCT to AGC and GAA, respectively, to create an NcoI recognition site at the sixth bases of the sense primer used, corresponding to the first and fourth bases with respect to the translation start site. The protein encoded by the amplified sequence hence had the amino acid substitutions: K2E in PD1S, K2E and S49A in PD1A, and K2E and S49E in PD1E.

For expression of the narB gene of Synechococcus elongatus strain PCC 7942, a 2,147-bp fragment of Synechocystis DNA carrying the entire sll1454 open reading frame was amplified by PCR. The second and the sixth bases of the sense primer used, corresponding to bases −1 and +4 with respect to the translation start site, had been changed from A and G in the original sequence to C and A, respectively, to create a BspHI recognition site at the translation start site. The PCR-amplified sll1454 gene was cloned into pT7Blue T-Vector and, after verification of the nucleotide sequence, was inserted into the BspHI and XbaI sites of the shuttle vector pSlL121 (29) to construct pSGSLNB (encoding the wild-type GlnB) and pSGSLNBE (encoding the S49A and S49E derivatives, respectively). The plasmids were transferred from E. coli HB101 carrying the helper plasmid to the Synechocystis sp. strain PCC 6803 glnB deletion mutant (SPD1) by conjugation to yield the SPD1A, SPD1D, and SPD1E strains, respectively.

Expression of plasmid-borne narB in Synechococcus. For heterologous expression of NR of Synechocystis sp. strain PCC 6803 in cells of Synechococcus elongatus strain PCC 7942, a 2,147-bp fragment of Synechocystis DNA carrying the entire sll1454 open reading frame was amplified by PCR. The second and the sixth bases of the sense primer used, corresponding to bases −1 and +4 with respect to the translation start site, had been changed from A and G in the original sequence to C and A, respectively, to create a BspHI recognition site at the translation start site. The PCR-amplified sll1454 gene was cloned into pT7Blue T-Vector and, after verification of the nucleotide sequence, was inserted into the BspHI and XbaI sites of the shuttle vector pSlL121 (29) to construct pSGSLNB (encoding the wild-type GlnB) and pSGSLNBE (encoding the S49A and S49E derivatives, respectively). The plasmids were transferred from E. coli HB101 carrying the helper plasmid to the Synechocystis sp. strain PCC 6803 glnB deletion mutant (SPD1) by conjugation to yield the SPD1A, SPD1D, and SPD1E strains, respectively.

For expression of the narB gene of Synechococcus elongatus strain PCC 7942, a 2,147-bp fragment of Synechocystis DNA carrying the entire sll1454 open reading frame was amplified by PCR. The second and the sixth bases of the sense primer used, corresponding to the ATG initiation codon, had been changed to GAA to create an EcoRI recognition site. The antisense primer used had an XbaI recognition sequence downstream of the stop codon. The PCR-amplified Synechocystis narB gene was digested with EcoRI and XbaI and cloned between the EcoRI and XbaI sites of pSlE1. The pSlE1 derived plasmid was electrotransferred into a strain containing a deletion in the regulatory domain of NRT, and the wild-type glnB gene was confirmed by Southern hybridization analysis of the genomic DNA samples from SNC2 and SPD1, respectively (Fig. 1). Both mutants grew normally on nitrate as well as on ammonium (data not shown), indicating that Pn2 and the C-terminal domain of NrtC are not essential for nitrate assimilation.

Unlike the SNC2 mutant, the nrtC insertional mutant SNC1 (the parental strain of SNC2) showed negligible NR activity (data not shown) and failed to utilize nitrate as the nitrogen source even when the nitrate concentration in the medium was as high as 60 mM (Fig. 2A), suggesting that insertion of the nrtC gene had caused polar inhibition of expression of the putative NR structural gene sll1454. SNC1 grew normally in a medium containing 5 mM nitrate or 7.5 mM ammonium (Fig. 2A) but failed to take up <100 μM nitrite in a medium at pH 9.6 (Fig. 2B). Under the conditions of low nitrite concentration and high pH, passive entry of nitrous acid (HNO₂, the protonated form of nitrite) into the cell is negligible and up-
the operation of an active transport system (25). The results therefore indicated that SNC1 is defective in active transport of nitrite. Because eviction of the 3′ region of nrtC and the nptI-sacB gene cartridge from the genome of SNC1 to construct SNC2 restored the ability to grow on nitrate (see above) and to take up low concentrations of nitrate and nitrite (see below), we concluded that the truncation of nrtC had no inhibitory effect on expression of the genes located downstream.

**Ammonium-insensitive NRT activity in the Synechocystis mutant having truncated nrtC.** The cell suspensions of the wild-type Synechocystis strain efficiently used 100 μM nitrate and nitrite until its exhaustion in the medium at pH 9.6 (Fig. 3A), indicating operation of a high-affinity active transport system for nitrite as well as nitrate. During nitrate assimilation, there was no accumulation of nitrite (Fig. 3A, a) or ammonium (not shown) in medium, indicating that the rate of net nitrate uptake was equal to the rate of nitrate assimilation. As in Synechococcus elongatus strain PCC 7942 (18), nitrate and nitrite uptake by the wild-type cells was completely inhibited by addition of ammonium to the medium (Fig. 3A). The inhibition was reversible, and nitrate and nitrite uptake was resumed after consumption of the ammonium in the medium (data not shown). The SNC2 mutant, having a truncated nrtC, also exhibited high-affinity nitrate and nitrite uptake activity (Fig. 3B), but the uptake was not inhibited by ammonium (Fig. 3B). This indicated that, as in Synechococcus elongatus strain PCC 7942, NrtC is involved in the transport of both nitrate and nitrite, with its C-terminal domain being required for regulation of the transport activity (18). During the nitrate uptake measurements, transient accumulation of low concentrations of nitrite was observed in the cultures of SNC2 (Fig. 3B, a), indicating that there was an imbalance between the rate of nitrite production by NR and that of nitrite consumption by NiR, with the former being faster than the latter. Since no nitrite was detected in the cultures of the wild-type cells assimilating nitrate (Fig. 3A, a), it was deduced that the C-terminal domain of NrtC modulates NRT activity in the absence of exogenously added ammonium as well, presumably responding to the ammonium generated intracellularly by nitrate reduction.

**Insensitivity of Synechocystis NR to ammonium.** Because modification of an NRT subunit abolished the inhibition of nitrate and nitrite assimilation by ammonium (Fig. 3B), it was deduced that NR and NiR are not inhibited by ammonium in...
Nitrate assimilation pathway in the monium (Fig. 3C, a). Since NRT is the regulatory step of the constructed in the present study (SPD1) was not affected by am monium in P II-deficient Nitrate assimilation has been shown to be unaf fected by ammonium in the presence of ammonium, the NC41 derivative expressing Synechocystis narB of Synechococcus elongatus strain PCC 6803. This was in contrast with the results obtained previously with Synechococcus elongatus strain PCC 7942; the Syneechococcus NC2 mutant, which lacks the C-terminal domain of NrtC and which has ammonium-resis tant NRT activity, could not assimilate nitrate in the presence of ammonium, because its NR was inhibited by ammonium. To gain insight into the mechanism of inhibition of NR by ammonium, the Synechocystis NR enzyme was expressed in Synechococcus elongatus strain PCC 6803 and the effect of ammonium was examined (Fig. 4). Expression of the sll1454 gene of Synechocystis (Fig. 1A) in an NR-null derivative of the Synechococcus NC2 mutant (NC41 nrtCA narB::Ω) restored the ability of the cells to assimilate nitrate (Fig. 4a) as the Synechococcus narB gene did (Fig. 4b), verifying that sll1454 is the NR gene (narB) of Synechocystis sp. strain PCC 6803. In the presence of ammonium, the NC41 derivative expressing Synechococcus narB (NC51) continued nitrate assimilation (Fig. 4a), whereas nitrate assimilation by the NC41 derivative expressing Synechococcus narB (NC52) was inhibited (Fig. 4b). These results showed that NR of Synechocystis sp. strain PCC 6803 has ammonium-resistant activity not only in Synechocystis cells but also in Synechococcus cells.

Regulation of NRT by nonmodifiable derivatives of PII in Synechocystis. Nitrate assimilation has been shown to be unaffected by ammonium in PII-deficient Synechococcus elongatus strain PCC 7942 (23) and Synechocystis sp. strain PCC 6803 (14) mutants. In accordance with the previous observations, nitrate assimilation by the Synechocystis glnB mutant constructed in the present study (SPD1) was not affected by ammonium (Fig. 3C, a). Since NRT is the regulatory step of the nitrate assimilation pathway in the Synechocystis strain (see above), the results indicated that the PII protein is required for regulation of NRT. Similar to the SNC2 mutant having truncated NrtC, SPD1 excreted nitrite into medium during nitrate assimilation (Fig. 3C, a), indicating the imbalance between the rates of nitrate reduction and nitrite reduction. In accordance with the involvement of NRT in transport of nitrite as well as nitrate (see above), nitrite assimilation by the mutant, measured under high-pH and low-nitrite conditions, was also insensitive to ammonium (Fig. 3C, b). An SPD1 derivative (SPD1S) carrying a plasmid-borne wild-type glnB gene showed ammonium-promoted inhibition of nitrate uptake like that of the wild-type strain (Fig. 5a). The SPD1A and SPD1E strains, expressing the S49A and S49E derivatives of PII, respectively, also showed ammonium-responsive inhibition of nitrate uptake (Fig. 5b and c). These findings demonstrated that the presence or absence of a negative charge at amino acid position 49 does not affect the ability of PII to regulate the NRT activity in an ammonium-responsive manner.

Regulation of NRT by nonmodifiable derivatives of PII in Synechococcus. To see whether or not the responsiveness of NRT to ammonium is independent of the presence or absence of a negative charge at the 49th amino acid position in Synechococcus elongatus strain PCC 7942 as well, we examined nitrite uptake activity and its regulation in the Synechococcus mutants expressing modified glnB. A glnB-deficient Synechococcus mutant (PD1) was constructed by insertion of a spectinomycin and streptomycin resistance gene cassette (Fig. 6A), and segregation of the homozygous mutant was confirmed by Southern hybridization analysis (Fig. 6B). Overexpression of PII, the glnB derivatives were cloned in the shuttle expression vector pSE1 to make a transcriptional fusion with the Ptrc promoter, and the resulting plasmids were introduced into the PD1 mutant to yield the PD1S, PD1A, and PD1E strains (Table 1). Unlike the plasmid-encoded GlnB expressed in Synechocystis (see above), the plasmid-encoded Synechococcus GlnB carried a K2E mutation introduced for the purpose of cloning into pSE1. Figure 6C compares the polypeptide compositions of the soluble proteins extracted from the wild-type and the mutant strains. The three PD1 derivatives carrying plasmid-borne glnB accumulated large amounts of a polypeptide with apparent molecular mass of 13 kDa, which was close to the calculated molecular mass of GlnB (12.4 kDa) (Fig. 6C, a, SPD1S, b, SPD1A, c, SPD1E). Nitrate was added at time zero to the cell suspensions containing 5 μg of Chl per ml, and ammonium (500 μM) was added immediately after the addition of nitrate. Changes in the nitrate and nitrite concentrations in the medium are shown. FIG. 4. Ammonium-resistant activity of Synechocystis NR in the cells of Synechococcus elongatus strain PCC 7942. The NR structural genes of Synechocystis sp. strain PCC 6803 (sll1454) and Synechococcus elongatus strain PCC 7942 (narB) were introduced into the Synechococcus elongatus mutant that lacks NR and has ammonium-resistant NRT (NC41) to construct NC51 (a) and NC52 (b), respectively. Nitrate was added at time zero to the cell suspensions containing 5 μg of Chl per ml, and ammonium (500 μM) was added immediately after the addition of nitrate. Changes in the nitrate concentration in the medium are shown. Circles, control; triangles, suspensions with added ammonium.
Dots, positions of the weakly labeled bands.

The 13-kDa polypeptide that accumulated in the PD1S cells (Fig. 6D, lane 3) was also labeled with $^{32}$P in a nitrogen-responsive manner (Fig. 6D, lanes 5 and 6), verifying that the overexpressed 13-kDa protein is GlnB. The results also showed that the K2E mutation did not affect the nitrogen-responsive modification of the $P_H$ protein. Though PD1 lacked the wild-type copy of glnB, a weak $^{32}$P signal was often detected in the 13-kDa region (e.g., Fig. 6D, lane 4). Since glnB is a single-copy gene in *Synechococcus*, this signal was ascribed to a polypeptide other than GlnB. There were also several other weakly labeled polypeptides (Fig. 6D), the nature of which is currently unknown. In agreement with the previous report that Ser$^{49}$ is the only site for $P_H$ phosphorylation in *Synechococcus elongatus* strain PCC 7942 (8), no significant incorporation of $^{32}$P into the 13-kDa polypeptide that accumulated in the PD1A and PD1E strains was observed (Fig. 6C, lanes 4 and 5, and D, lanes 7 to 10).

Figure 7 compares the effects of ammonium on nitrite uptake by the wild-type and the mutant strains of *Synechococcus* at pH 9.6 and nitrite concentrations of <100 μM. Nitrite uptake by the wild-type cells was inhibited by ammonium, as previously shown (Fig. 7a) (18). Since ammonium does not affect in vivo NiR activity of *Synechococcus* (25), the results indicate inhibition of NRT activity by ammonium. In the glnB-deficient mutant PD1, ammonium reduced the rate of nitrite uptake but the cells exhibited ammonium-resistant NRT activity and continued nitrite uptake in the presence of ammonium (Fig. 7b), confirming that $P_H$ is required for the ammonium-promoted inhibition of NRT. The PD1S strain, expressing the K2E derivative of GlnB, showed ammonium-responsive inhibition of NRT like that of the wild-type strain (Fig. 7c), indicating that the substitution of the second amino acid residue did not affect the activity of $P_H$ to regulate NRT. The PD1A and PD1E strains, expressing nonmodifiable forms of $P_H$, also showed nitrite uptake activity similar to that in the wild-type strain, and the activity was inhibited by ammonium as in the wild-type strain (Fig. 7d and e). These results showed that changes in the electric charge at the 49th amino acid position do not affect the ability of $P_H$ to respond to cellular nitrogen status and to regulate the NRT activity accordingly in *Synechococcus elongatus* strain PCC 7942 as well as in *Synechocystis* sp. strain PCC 6803.

**DISCUSSION**

In *Synechocystis* sp. strain PCC 6803, the sl1450-sll1451-sll1452-sll1453 gene cluster has been believed to encode an NRT, because (i) it is strongly similar to the *Synechococcus elongatus nrtABCD* gene cluster, which codes for an ABC-type NRT; (ii) its expression is regulated by the nitrogen conditions of the cell (1); and (iii) it forms an operon with the NR gene *nrbB* (sl1454) (1). The defect of the sl1452 inserional mutant SNC1 in active uptake of nitrite (Fig. 2B) and the ammonium-insensitive uptake of nitrate and nitrite in the SNC2 mutant, which has a truncated sl1452 gene (Fig. 3B), verify the involvement of this gene cluster in transport of nitrate and nitrite, providing the first experimental evidence for the identification of sl1450, sl1451, sl1452, and sl1453 as *nrtA*, *nrtB*, *nrtC*, and *nrtD*, respectively, of *Synechocystis* sp. strain PCC 6803.

Since the extent of $P_H$ phosphorylation reflects the nitrogen status of the cell, it has been presumed to act as the major biochemical signal for regulation of nitrate assimilation (7).
However, the ammonium-responsive regulation of NRT by the S49A and S49E derivatives of PII (Fig. 5 and 7) indicates that PII can sense the cellular nitrogen status and control the NRT activity accordingly, irrespective of the presence or absence of negative charge or of a change in the size of the side chain at the 49th amino acid position. It is therefore unlikely that changes in the phosphorylation state of PII play a role in regulation of NRT. The binding of ATP and 2-OG to phospho-PII, on the other hand, has been shown to inhibit dephosphorylation of the protein by PII phosphatase, verifying that the effectors provoke a conformational change in PII (34). Since the intracellular 2-OG concentration is high in cells grown with nitrate and is decreased by ammonium (28), we hypothesize that the conformational change of PII, provoked by the binding of 2-OG, controls the activity of the protein to inhibit NRT. It remains to be elucidated how PII and the C-terminal domain of NrtC regulate the NRT activity.

Recent studies of Synechococcus elongatus strain PCC 7942 revealed the binding of PII to N-acetyl-L-glutamate (NAG) kinase, the key enzyme of the arginine biosynthetic pathway (2, 12). Detailed in vitro and in vivo analyses have shown that PII strongly enhances NAG kinase activity by binding to the enzyme (12). This interaction requires the nonphosphorylated Ser49 residue of PII, indicating that NAG kinase activity is regulated by phosphorylation and dephosphorylation of PII (12). The independence of NRT regulation from modification of Ser49 indicates that PII has two distinct modes of action for regulation of different targets: one involving modification of the Ser49 residue and the other depending on the binding of the effector molecules.

In a previous study of Synechococcus elongatus strain PCC 7942 (22), the mutant having an S49A derivative of PII was shown to express negligible activity of nitrate assimilation irrespective of the nitrogen status of the cell, whereas the mutant having an S49E derivative of PII showed ammonium-sensitive nitrate assimilation activity like that of the wild-type strain. These observations suggest that PII(S49A) permanently inhibits either NRT or NR. The present results show that PII(S49A) of Synechocystis inhibits NRT only in the presence of ammonium (Fig. 5b). Also, PII(K2E, S49A) of Synechococcus was shown to require the presence of ammonium for inhibition of NRT even when overexpressed from the Ptc promoter (Fig. 7d). These findings suggest that it is the NR that is permanently inhibited by PII(S49A) in Synechococcus. On the other hand, the ammonium-sensitive assimilation of nitrate in the Synechococcus mutant expressing PII(S49E) (22) can be accounted for by regulation of NRT; PII(S49E) clearly does not inhibit NR in the absence of ammonium either. Thus the presence or absence of a negative charge at the 49th position of GlnB, and hence the changes in the phosphorylation state of PII, seem to have a role in the regulation of NR activity in Synechococcus.

Whereas NR of Synechococcus elongatus strain PCC 7942 is inhibited by ammonium (18), the present results show that NR of Synechocystis sp. strain PCC 6803 is resistant to ammonium in the Synechococcus cells as well as in the Synechocystis cells (Fig. 4A). Thus Synechocystis NR does not respond to the NR regulatory mechanism of the Synechococcus cell. This is presumably due to a structural difference(s) between Synechocystis NR and Synechococcus NR. Although the deduced Synechococcus NR protein (X74597 in the EMBL, GenBank, and DDBJ databases) is 61% identical in amino acid sequence to the Synechocystis enzyme (17), there are several regions of poor similarity (not shown), which might be involved in the posttranslational regulation of the enzyme activity. The Synechocystis NR can be thus regarded as a naturally occurring mutant enzyme insensitive to ammonium, which will be useful for further molecular genetic analysis of the mechanism of NR regulation in Synechococcus elongatus strain PCC 7942.

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**FIG. 7. Effects of ammonium on nitrite uptake by the cells of the wild-type Synechococcus elongatus strain PCC 7942 (a), the PII-deficient strain PD1 (b), and the PD1S (c), PD1A (d), and PD1E (e) strains expressing the plasmid-encoded GlnB derivatives.** Nitrite was added at time zero to the cell suspensions containing 5 μg of Chl per ml, and ammonium (500 μM) was added immediately after the addition of nitrite. Changes in the nitrite concentrations in the medium are shown. PD1S, PD1A, and PD1E cells were treated with 1 mM IPTG for 16 h prior to the experiment.
REFERENCES


