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

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Posttranslational regulation of nitrate assimilation was studied in the cyanobacterium *Synechocystis* sp. strain PCC 6803. The ABC-type nitrate and nitrite bispecific transporter encoded by the *nrtABCD* genes was completely inhibited by ammonium as in *Synechococcus elongatus* strain PCC 7942. Nitrate reductase was insensitive to ammonium, while it is inhibited in the *Synechococcus* strain. Nitrite reductase was also insensitive to ammonium. The inhibition of nitrate and nitrite transport required the P_{II} protein (*glnB* gene product) and the C-terminal domain of NrtC, one of the two ATP-binding subunits of the transporter, as in the *Synechococcus* strain. Mutants expressing the P_{II} derivatives in which Ala or Glu is substituted for the conserved Ser⁴⁹, which has been shown to be the phosphorylation site in the *Synechococcus* strain, showed ammonium-promoted inhibition of nitrate uptake like that of the wild-type strain. The S49A and S49E substitutions in GlnB did not affect the regulation of the nitrate and nitrite transporter in *Synechococcus* either. These results indicated that the presence or absence of negative electric charge at the 49th position does not affect the activity of the P_{II} protein to regulate the cyanobacterial ABC-type nitrate and nitrite transporter according to the cellular nitrogen status. This finding suggested that the permanent inhibition of nitrate assimilation by an S49A derivative of P_{II} , as was previously reported for *Synechococcus elongatus* strain PCC 7942, is likely to have resulted from inhibition of nitrate reductase rather than the nitrate and nitrite transporter.

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), where-

as NiR activity is not (25). The inhibition is reversible, and assimilation of nitrate is resumed after consumption of ammonium in medium. The P_{II} 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_{II} protein (homotrimer 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_{II} protein is modified when nitrogen is limited, but the modification is by phosphorylation at Ser⁴⁹ rather than by uridylylation at the Tyr⁵¹ 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_{II} -deficient mutants show ammonium-insensitive nitrate assimilation (23), it is clear that P_{II} negatively regulates both NRT and NR in the presence of ammonium. However, it remains unclear how P_{II} 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_{II} , 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_{II} with an S49A substitution shows negligible nitrate assimilation activity irrespective of the cellular nitrogen status, whereas a mutant expressing an S49E derivative of P_{II} , having a negative charge on the 49th amino acid,

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TABLE 1. Cyanobacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>Synechocystis</i> sp. strain PCC 6803		
GT	Glucose-tolerant derivative of strain PCC 6803, wild type	44
SNC1	GT <i>nrtC::sacB-nptI</i>	This study
SNC2	GT <i>nrtCΔC</i> , lacking the regulatory domain of NrtC	This study
SPD1	GT <i>glnB::nptI</i> Km ^r cassette	This study
SPD1S	SPD1 harboring pSGLNBS	This study
SPD1A	SPD1 harboring pSGLNBA	This study
SPD1E	SPD1 harboring pSGLNBE	This study
<i>Synechococcus elongatus</i> strain PCC 7942		
R2-SPc	Strain PCC 7942 cured of pUH24 plasmid, wild type	19
NC2	R2-SPc <i>nrtCΔC</i> , lacking the regulatory domain of NrtC	18
NC41	R2-SPc <i>nrtCΔC narB::Ω</i> Sp ^r -Sm ^r cassette	This study
NC51	NC41 harboring pSNARB	This study
NC52	NC41 harboring pNARB	This study
PD1	R2-SPc <i>glnB::Ω</i> Sp ^r -Sm ^r cassette	This study
PD1S	PD1 harboring pGLNBS	This study
PD1A	PD1 harboring pGLNBA	This study
PD1E	PD1 harboring pGLNBE	This study
Plasmids		
pSL1211	Gm ^r , <i>Synechocystis</i> shuttle vector	29
pSGLNBS	pSL1211 derivative encoding GlnB	This study
pSGLNBA	pSL1211 derivative encoding GlnB(S49A)	This study
pSGLNBE	pSL1211 derivative encoding GlnB(S49E)	This study
pSE1	Km ^r , <i>Synechococcus</i> shuttle expression vector	26
pNARB	pSE1 derivative carrying <i>Synechococcus narB</i>	This study
pSNARB	pSE1 derivative carrying <i>sll1454</i>	This study
pGLNBS	pSE1 derivative encoding GlnB(K2E)	This study
pGLNBA	pSE1 derivative encoding GlnB(K2E, S49A)	This study
pGLNBE	pSE1 derivative encoding GlnB(K2E, S49E)	This study

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 P_{II}(S49E), whereas P_{II}(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 *Synechococcus elongatus* strain PCC 7942. *Synechocystis* sp. strain PCC 6803 is potentially useful for molecular genetic analysis of the regulatory mechanisms, because its genome is the best characterized among the cyanobacterial genomes (Cyanobase, <http://www.kazusa.or.jp/cyano/cyano.html>; CYORF, <http://cyano.genome.jp/>), but information about the regulation of nitrate and nitrite uptake at the posttranslational level is limited (14, 16). To expand our knowledge on the regulation of nitrate assimilation in this cyanobacterium, we characterized the mutants expressing modified NRT and P_{II} in the present study. The C-terminal portion of NrtC is shown to act as the regulatory domain of NRT, as in *Synechococcus elongatus* strain PCC 7942. It is shown that NR is not regulated in *Synechocystis* sp. strain PCC 6803. Characterization of the *Synechocystis* mutants having amino acid substitutions at the 49th amino acid position of GlnB suggested that regulation of NRT is independent of the phosphorylation of P_{II}. This finding led us to investigate the specific effects of GlnB modification on the regulation of NRT in *Synechococcus elongatus* strain PCC 7942.

MATERIALS AND METHODS

Strains and growth conditions. The glucose-tolerant derivative of *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 [19]; 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 photoautotrophically at 30°C under continuous illumination provided by fluorescent lamps (70 microeinsteins 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 (NH₄)₂SO₄ and 15 mM KNO₃, 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) CO₂ in air. When appropriate, kanamycin, spectinomycin, and gentamicin were added to the media at 15, 15, and 5 μg/ml, respectively. *Escherichia coli* strains NM522, HB101, and SL906 were grown on Luria-Bertani medium supplemented with ampicillin (50 μg/ml), spectinomycin (25 μg/ml), streptomycin (30 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* and *Synechococcus*, the following DNA fragments were amplified from the cyanobacterial DNA samples by PCR, labeled with ³²P, 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).

FIG. 1. Construction of the *Synechocystis* sp. strain PCC 6803 *nrtC* and *glnB* mutants. (A and B) Physical maps of the *nrtA*-to-*narB* region and the *glnB* region, respectively, of the genomes of the wild-type strain (GT) and the mutants. Bars above the maps in panels A and B, probes used for Southern hybridization analysis in panels C and D, respectively; inverted triangle (A), site of insertion of the *nptI-sacB* gene cassette in the *nrtC* insertional mutant SNC1. Restriction endonuclease sites are abbreviated as follows: Sp, SpeI; Nh, NheI; Af, AflII. (C and D) Southern hybridization analysis of genomic DNA from wild-type and mutant strains, with *nrtD* and *glnB*, respectively, as probes. DNA samples (2 μ g/lane) were digested with SpeI (C) or AflII (D), fractionated on a 1% agarose gel, transferred to a positively charged nylon membrane (Hybond N+; Amersham), and hybridized with the 32 P-labeled probes.

Insertional and deletional 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' portion of the coding region. A mutant lacking the C-terminal domain of NrtC (SNC2) was obtained from SNC1 by deleting the 3' 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' and 3' 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'-TAATT-3', 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 Ω cassette excised from pRL463 (4). The plasmid carrying disrupted *narB* was used to inactivate *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 GCT and GAA, respectively, with the QuikChange site-directed mutagenesis kit (Stratagene). After verification of the nucleotide sequence, the 853-bp inserts carrying the wild-type and the modified *glnB* genes were excised from the plasmids with BamHI and XbaI and cloned between the BamHI and XbaI sites of the shuttle vector pSL1211 (29) to construct pSGLNBS (encoding wild-type GlnB) and pSGLNBA and pSGLNBE (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 SPD1S, SPD1A, 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, excised from the plasmid with BspHI and XbaI and cloned between the NcoI and XbaI sites of the *Synechococcus* shuttle expression vector pSE1 (26). Due to the G4A replacement in the nucleotide sequence, the protein encoded by *sll1454* in the resulting plasmid (pSNARB) carried a D2N mutation.

For expression of the *narB* gene of *Synechococcus elongatus* strain PCC 7942 from the shuttle vector pSE1, a 2,210-bp *Synechococcus* DNA fragment carrying the entire *narB* open reading frame was amplified by PCR. The sixth to eighth 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 *Synechococcus narB* gene was digested with EcoRI and XbaI and cloned between the EcoRI and XbaI sites of pSE1. The protein encoded by the resulting plasmid (pNARB) had an additional glutamic acid inserted next to the initiator methionine residue.

The pSE1 derivatives pSNARB and pNARB were transformed into the *Synechococcus elongatus* strain PCC 7942 NC41 mutant (*nrtCΔCΔnarB::Ω*) to yield the spectinomycin-resistant transformants NC51 and NC52, respectively. Expression of plasmid-borne *narB* was induced by treatment of the cells of the transformants with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 16 h in nitrate-containing medium.

Expression of plasmid-borne *glnB* in *Synechococcus*. A fragment of *Synechococcus* DNA carrying the entire *glnB* coding region (from nucleotide -2 to +341 with respect to the translation start site) was amplified by PCR. The third and sixth bases of the sense primer used, corresponding to the first and fourth bases of the coding region, had been changed from T and A in the original *glnB* sequence to A and G, respectively, to create an NcoI recognition site at the translation start site. The protein encoded by the amplified sequence hence carried a K2E mutation. Two other derivatives of *glnB*, carrying additional base replacements to create S49A and S49E mutations, were generated by overlap extension PCR (15) with oligonucleotide primers carrying mismatches with the wild-type sequence. The PCR-amplified DNA fragments were cloned into pT7Blue T-Vector, and, after verification of the nucleotide sequences, the modified *glnB* genes were excised from the plasmids with NcoI and XbaI and cloned between the NcoI and XbaI sites of pSE1 (26) to place the *glnB* coding sequence under the control of the P_{trc} promoter. The resulting plasmids were transformed into the *glnB*-deficient mutant PD1 to yield the strains PD1S, PD1A, and PD1E, and expression of the modified *glnB* genes was induced by 16-h treatment with

1 mM IPTG. The GlnB derivatives thus expressed had the following amino acid substitutions: K2E in PD1S, K2E and S49A in PD1A, and K2E and S49E in PD1E.

In vivo labeling of P_{II} in *Synechococcus*. Nitrate-grown *Synechococcus* cells were collected by centrifugation at $5,000 \times g$ for 5 min at 25°C, washed by resuspension and recentrifugation in phosphate- and nitrogen-free medium prepared by omitting K₂HPO₄ from the basal medium, and resuspended in the medium supplemented with 2 mM KNO₃ at a chlorophyll (Chl) concentration of 5 µg per ml. A 2-ml aliquot of the cell suspension, to which carrier-free H₃³²PO₄ (Amersham) at 5.5×10^7 dpm was added, was placed under illumination at 30°C. After incubation for 1.5 h, a 0.5-ml aliquot of the cell suspension was removed from the test tube, mixed with 0.5 ml of 10% trichloroacetic acid in a 1.5-ml centrifuge tube, and placed on ice for 30 min. To the remainder of the cell suspension, (NH₄)₂SO₄ was added to a final concentration of 0.25 mM and the cells were incubated for additional 1 h under illumination, after which another aliquot of 0.5 ml was removed from the cell suspension and processed as described above. From the cell samples treated with trichloroacetic acid, total protein was pelleted by centrifugation at $14,000 \times g$ for 5 min. The pelleted protein was washed twice with acetone, dried, suspended in 40 µl of the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21), and lysed by heat treatment at 100°C for 5 min. Twenty microliters of the protein solution, containing approximately 70 µg of protein, was electrophoresed in an SDS-10 to 20% polyacrylamide gradient gel in the buffer system of Laemmli (21). The fractionated polypeptides were electrotransferred to a Hybond C Extra membrane (Amersham), and the ³²P-labeled polypeptides were detected by autoradiography on X-ray film. Soluble fractions from unlabeled *Synechococcus* cells were prepared as described previously (32), and their polypeptide compositions were analyzed by SDS-PAGE as described above.

Measurements of nitrate and nitrite uptake. Uptake of nitrate and nitrite by the cyanobacterial cells was measured at pH 9.6 by monitoring the decrease in the extracellular concentration of nitrate and nitrite, respectively, in cell suspensions containing 5 µg of Chl per ml, as previously described (18). When the effects of ammonium on the uptake of nitrate and nitrite were examined, 250 µM (NH₄)₂SO₄ was added to the cell suspensions immediately after the addition of nitrate or nitrite. Nitrate and nitrite were determined with a flow injection analyzer (NOX-1000W; Tokyo Chemical Industry Co., Ltd.). Chl was determined as described by Mackinney (24).

Other methods. In vitro activities of NR in toluene-permeabilized cells were determined at 30°C with dithionite-reduced methylviologen as the electron donor (13).

RESULTS

Construction of the *nrtC* and *glnB* *Synechocystis* mutants. To study the roles of the C-terminal domain of NrtC and the P_{II} protein in the regulation of NRT in *Synechocystis* sp. strain PCC 6803, a mutant having truncated *nrtC* (SNC2) and a P_{II}-deficient mutant (SPD1) were constructed (Table 1 and Fig. 1). The absence of the 3' portion of *nrtC*, encoding the putative 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 P_{II} 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 insertional interruption of *nrtC* had caused polar inhibition of expression of the putative NR structural gene *sll1454*. SNC1 grew normally in a medium containing 5 mM nitrite 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 entrance of nitrous acid (HNO₂, the protonated form of nitrite) into the cell is negligible and up-

FIG. 2. (A) Growth of the insertional *nrtC* (*sll1452*) mutant (SNC1) on 3.75 mM (NH₄)₂SO₄ (circles), 5 mM NaNO₂ (triangles), and 60 mM KNO₃ (squares). (B) Nitrite uptake capability of the wild-type stain (GT) and the SNC1 mutant. Nitrite was added at time zero to the cell suspensions containing 5 µg of Chl per ml. Changes in the concentrations of nitrite are shown. OD₇₃₀, optical density at 730 nm.

take of nitrite requires 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

FIG. 3. Effects of ammonium on the uptake of nitrate and nitrite by the wild-type strain (A) and the mutants having truncated *nrtC* (SNC2) (B) and disrupted *glnB* (SPD1) (C). Nitrate (a) or nitrite (b) 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 and nitrite. Changes in the nitrate and nitrite concentrations in the medium are shown. Circles, control; triangles, suspensions with added ammonium.

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 μ g of Chl per ml, and ammonium (500 μ M) 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.

Synechocystis sp. strain PCC 6803. This was in contrast with the results obtained previously with *Synechococcus elongatus* strain PCC 7942; the *Synechococcus* NC2 mutant, which lacks the C-terminal domain of NrtC and which has ammonium-resistant NRT activity, could not assimilate nitrate in the presence of ammonium, because its NR was inhibited by ammonium (18). To gain insight into the mechanism of inhibition of NR by ammonium, the *Synechocystis* NR enzyme was expressed in *Synechococcus elongatus* strain PCC 7942 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 *nrtCΔ 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 *Synechocystis 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 P_{II} in *Synechocystis*. Nitrate assimilation has been shown to be unaffected by ammonium in P_{II} -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 P_{II} 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 P_{II} , 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 P_{II} to regulate the NRT activity in an ammonium-responsive manner.

Regulation of NRT by nonmodifiable derivatives of P_{II} 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). For overexpression of P_{II} , the *glnB* derivatives were cloned in the shuttle expression vector pSE1 to make a transcriptional fusion with the P_{trc} 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,

FIG. 5. Effects of ammonium on nitrate uptake by the cells of the SPD1S (a), SPD1A (b), and SPD1E (c) strains, which have the wild-type P_{II} , the S49A derivative of P_{II} , and the S49E derivative of P_{II} , respectively. Nitrate was added at time zero to the cell suspensions containing 5 μ g of Chl per ml, and ammonium (500 μ M) was added immediately after the addition of nitrate. Changes in the nitrate and nitrite concentrations in the medium are shown.

FIG. 6. Construction of a *Synechococcus elongatus* strain PCC 7942 *glnB* mutant and overexpression of the *glnB* derivatives. (A) Physical map of the *glnB* region of the genome of the wild-type strain and the *glnB*-deficient mutant PD1. Bar, probe used for Southern hybridization analysis in panel B. Restriction endonuclease sites are abbreviated as follows: Ps, PstI; Xh, XhoI. (B) Southern hybridization analysis of PstI-digested genomic DNA samples (2 μ g per lane) from the wild-type strain and the PD1 mutant, with *glnB* as a probe. (C) Polypeptide compositions of the soluble fraction extracted from the wild-type (lane 1), PD1 (lane 2), PD1S (lane 3), PD1A (lane 4), and PD1E (lane 5) strains. Samples (30 μ g of protein per lane) were fractionated by electrophoresis in an SDS-10 to 20% polyacrylamide gradient gel, and the polypeptides were stained with Coomassie blue. (D) Autoradiogram showing the 32 P-labeled proteins extracted from cells of the wild-type (lanes 1 and 2), PD1 (lanes 3 and 4), PD1S (lanes 5 and 6), PD1A (lanes 7 and 8), and PD1E (lanes 9 and 10) cells. Labeling with $H_3^{32}PO_4$ was performed for 1.5 h in nitrate (17.5 mM)-containing medium (lanes 1, 3, 5, 7, and 9) or for 1.5 h in the nitrate-containing medium and then for an additional 1 h after addition of ammonium (0.5 mM) to the medium (lanes 2, 4, 6, 8, and 10). Total cellular protein (70 μ g of protein per lane) was pelleted with trichloroacetic acid, solubilized with SDS, and fractionated on an SDS-10 to 20% polyacrylamide gradient gel. For panels C and D, PD1S, PD1A, and PD1E cells were treated with 1 mM IPTG for 16 h prior to the experiment. Dots, positions of the weakly labeled bands.

lanes 3 to 5). Incubation of the wild-type cells with $H_3^{32}PO_4$ in nitrate-containing medium resulted in phosphorylation of a 13-kDa polypeptide (Fig. 6D, lane 1). Addition of ammonium to the medium prevented the labeling of the 13-kDa polypeptide (Fig. 6D, lane 2), indicating that the polypeptide is GlnB. The 13-kDa polypeptide that accumulated in the PD1S cells (Fig. 6C, 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_{II} 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 Ser⁴⁹ is the only site for P_{II} 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 μ M. Nitrite uptake by the wild-type cells was inhibited by ammonium, as previously shown (Fig. 7a) (18). Since ammonium does not affect in vivo NRT 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_{II} 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_{II} to regulate NRT. The PD1A and PD1E strains, expressing nonmodifiable forms of P_{II} , 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_{II} 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 *sll1450-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 *narB* (*sll1454*) (1). The defect of the *sll1452* insertional 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 *sll1452* 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 *sll1450*, *sll1451*, *sll1452*, and *sll1453* as *nrtA*, *nrtB*, *nrtC*, and *nrtD*, respectively, of *Synechocystis* sp. strain PCC 6803.

Since the extent of P_{II} 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).

FIG. 7. Effects of ammonium on nitrite uptake by the cells of the wild-type *Synechococcus elongatus* strain PCC 7942 (a), the P_{II} -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 μ g of Chl per ml, and ammonium (500 μ M) 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.

However, the ammonium-responsive regulation of NRT by the S49A and S49E derivatives of P_{II} (Fig. 5 and 7) indicates that P_{II} 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 P_{II} play a role in regulation of NRT. The binding of ATP and 2-OG to phospho- P_{II} , on the other hand, has been shown to inhibit dephosphorylation of the protein by P_{II} phosphatase, verifying that the effectors provoke a conformational change in P_{II} (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 P_{II} , provoked by the binding of 2-OG, controls the activity of the protein to inhibit NRT. It remains to be elucidated how P_{II} and the C-terminal domain of NrtC regulate the NRT activity.

Recent studies of *Synechococcus elongatus* strain PCC 7942 revealed the binding of P_{II} 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 P_{II} strongly enhances NAG kinase activity by binding to the enzyme (12). This interaction requires the nonphosphorylated Ser⁴⁹ residue of P_{II} , indicating that NAG kinase activity is regulated by phosphorylation and dephosphorylation of P_{II} (12). The independence of NRT regulation from modification of Ser⁴⁹ indicates that P_{II} 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 P_{II} 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 P_{II} showed ammonium-sensitive nitrate assimilation activity like that of the wild-type strain. These observations suggest that P_{II} (S49A) permanently inhibits either NRT or NR. The present results show that P_{II} (S49A) of *Synechocystis* inhibits NRT only in the presence of ammo-

nium (Fig. 5b). Also, P_{II} (K2E, S49A) of *Synechococcus* was shown to require the presence of ammonium for inhibition of NRT even when overexpressed from the P_{trc} promoter (Fig. 7d). These findings suggest that it is the NR that is permanently inhibited by P_{II} (S49A) in *Synechococcus*. On the other hand, the ammonium-sensitive assimilation of nitrate in the *Synechococcus* mutant expressing P_{II} (S49E) (22) can be accounted for by regulation of NRT; P_{II} (S49E) clearly does not inhibit NR in the absence of ammonium in the medium and may not inhibit NR in the presence 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 P_{II} , 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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