Involvement of the C-terminal Domain of an ATP-binding Subunit in the Regulation of the ABC-type Nitrate/Nitrite Transporter of the Cyanobacterium Synechococcus sp. Strain PCC 7942*

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In Synechococcus sp. strain PCC 7942, an ATP-binding cassette transporter encoded by the genes nrtA, nrtB, nrtC, and nrtD mediates active transport of nitrate and nitrite, which is inhibited by ammonium, a preferred source of nitrogen for the cyanobacterium. One of the ATP-binding subunits of the transporter, NrtC, has a distinct C-terminal domain of 380 amino acid residues. A mutant NC2, constructed by removal of this domain using genetic engineering techniques, assimilated low concentrations of nitrate and nitrite and accumulated nitrate intracellularly, showing that the domain is not essential for the transporter activities. Assimilation of low concentrations of nitrate was only partially inhibited by ammonium in NC2 but was completely inhibited in the wild-type cells. Cells of NC2 and its derivative (nitrate reductase-less strain NC4) carrying the truncated NrtC but not the cells with the wild-type NrtC accumulated nitrate intracellularly in the presence of ammonium in medium. These findings indicated that the C-terminal domain of NrtC is involved in the ammonium-promoted inhibition of the nitrate/nitrite transporter. In the presence of ammonium, NC2 could not assimilate nitrate despite its ability to accumulate nitrate intracellularly, which suggested that reduction of intracellular nitrate by nitrate reductase is also subject to inhibition by ammonium.

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The abbreviations used are: NC, nitrate transporter; Nrt, nitrite reductase; kbp, kilobase pair(s); Chl, chlorophyll; HPLC, high pressure liquid chromatography.

Nitrate is a major source of nitrogen for cyanobacteria (1). It is transported into the cell by an active transport system and reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) prior to fixation into amide nitrogen of Gln. Expression of the nitrate assimilation activity is negatively regulated by ammonium (1, 2). In the unicellular non-nitrogen-fixing cyanobacterium Synechococcus sp. strain PCC 7942, the genes encoding the nitrate transport system (nrtA, nrtB, nrtC, and nrtD) (3–5), NR (narB) (6, 7), and NiR (nirA) (8, 9) form an nirA-nrtABC-narB operon (the nirA operon), and the transcription from the operon is inhibited by the addition of ammonium to the cyanobacterial cultures (9). Ammonium inhibits transcription through its fixation into Gln, but Gln is not the direct regulator of transcription (9, 10). We have proposed that cyanate, a metabolite of Gln via carbamoylphosphate, acts as the metabolic signal for the ammonium-promoted repression of the nirA operon (11).

Nitrate assimilation by cyanobacteria is subject also to post-translational regulation, being inhibited upon addition of ammonium to the cultures (12, 13). The efficiency:limiting step of nitrate assimilation in cyanobacteria is nitrate transport into the cell (3, 14), which has been shown to be inhibited by ammonium (14, 15). Because inhibition of glutamine synthetase by L-methionine-DL-sulfoximine abolishes the negative effect of ammonium on nitrate transport (14), fixation of ammonium to Gln is clearly required for the regulation. However, the metabolic signal leading to the inhibition of nitrate transport and the molecular mechanism of the regulation remain to be elucidated.

The nitrate transport system of Synechococcus sp. strain PCC 7942 transports nitrate as well as nitrite (16, 17) and hence is a nitrate/nitrite transporter. The product of the nrtA gene is a 45-kDa cytoplasmic membrane protein (3), which has been shown to be a nitrate/nitrite-binding lipoprotein (18). The deduced NrtB protein has structural similarities to the integral membrane components of the ABC (ATP-binding cassette) transporters, and the deduced NrtC and NrtD proteins have the sequences typical of the ATP-binding components of the ABC transporters (19), showing that the cyanobacterial nitrate/nitrite transporter belongs to the superfamily of ABC transporters (19) or traffic ATPases (20). NrtC is unique among the ATP-binding subunits of the ABC transporters in that it consists of two distinct domains, one of which (amino acids 1–254) is strongly similar to NrtD and the ATP-binding subunits of other ABC transporters, whereas the other (amino acids 279–659) is 30% identical in amino acid sequence to NrtA (5). In this work, we constructed and characterized deletion mutants of Synechococcus sp. strain PCC 7942 lacking NrtC or with a truncated NrtC lacking the C-terminal domain. Measurements of nitrate and nitrite uptake from medium and of intracellular nitrate accumulation and examination of the effects of ammonium thereon showed that NrtC is an essential component of the nitrate transporter and that the C-terminal domain of NrtC is involved in the ammonium-promoted inhibition of the transporter.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—A derivative of Synechococcus sp. strain PCC 7942 that is cured of the resident small plasmid pUH24 (R2-SPc, Ref. 6; hereafter designated simply as strain PCC 7942) and the mutant strains derived therefrom were grown photoautotrophically under CO2-sufficient conditions as described previously (11).
growth temperature was 30 °C unless otherwise stated. The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (21) as described previously (11). Ammonium-, nitrite-, and nitrate-containing media were prepared by addition of 3.75 mM (NH4)2SO4, 5 mM NaNO2, and 15 mM KNO3, respectively, to the basal medium used. All media were buffered with 0.1 M HEPES-KOH (pH 8.2). When appropriate, kanamycin was added to the media at 25 μg/ml. Expression of the nitrate assimilation genes was induced by transfer of ammonium-grown cells to the nitrate-or nitrite-containing medium as described previously (9).

Deletional Mutagenesis—Two defined mutants of Synechococcus, NC2 and NC3, were constructed by excising the 3’-flanking region of the cognate gene and expressing the EcorI recognition sequence that had been created by polymerase chain reaction amplification of the nrtB and nrtD fragments during the construction of the mutant. A NR-less derivative of NC2 (designated NC4) was constructed by inactivating nrtB according to a previously described procedure for construction of the targeted NR mutant of Synechococcus sp. strain PCC 7942 (Jarné-Bikan, Ref. 24; here designated NR1).

Isolation and Analysis of DNA and RNA—Chromosomal DNAs were extracted and purified from the Synechococcus cells as described by Williams (25). Manipulations and analyses of DNA were performed according to standard protocols (26). For Southern hybridization analysis of the genomic DNA digests, the following gene-specific probes were used (Fig. 1A): a 0.4-kbp BamHI-XhoI fragment of nrtB (probe B), a 0.6-kbp HincII fragment of the N-terminal domain of nrtC (probe C1), and a 0.8-kbp Psfl-XhoI fragment of the C-terminal domain of nrtC (probe C2). Total RNA was extracted and purified from Synechococcus cells by the method of Alba et al. (27). For Northern hybridization analysis of the 410-bp pair polymerase chain reaction-amplified nrtD fragment was used as a probe (probe D; Fig. 1A).

Expression of Plasmid-encoded NrtC in Synechococcus—A fragment of nrtC, extending from nucleotides 1 to 529 with respect to the translation start site, was amplified by polymerase chain reaction and cloned into pT7Blue T-vector (Novagen). The 14th base of the sense primer used, corresponding to the 4th base of the coding region, had been changed from Ser to Ala. pNRTC1 was transcribed into an N-terminal amino acid segment carrying six consecutive His residues. The plasmid was transformed into Escherichia coli M15 [pREP4] (Qiagen), and expression of the chimeric gene was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside. The histidine-tagged protein was used for raising antibodies in mice. Immunoblotting analysis of the cytoplasmic membrane samples prepared from Synechococcus cells was performed as described previously (18), using the antiserum against the NrtC polypeptide.

Measurements of Nitrate and Nitrite Uptake—Uptake of nitrate and nitrite by (20 mM)-grown Synechococcus cells was measured at 30 °C in the light by following the changes in concentrations of nitrate and nitrite, respectively, in the medium as described previously (18), except that the pH of the assay medium was 8.2 for the measurement of nitrate uptake; nitrite uptake was measured at 9.6 as in the previous study (18) so that passive diffusion of nitric acid (HNO2) into the cells is negligible [29].

Measurements of Intracellular Nitrate Concentration—Synechococcus cells were grown at 40 °C with ammonium as the nitrogen source. Nitrate transport activity was induced by transfer of the ammonium-grown cells to nitrate-containing medium followed by incubation under the growth conditions for 15 h. Accumulation of intracellular nitrate was lighted at 40 °C in the presence of 20 μM KNO3 and 10 mM NaHCO3, using the silicone oil centrifuging centrifugation technique and HPLC determination of nitrate as described previously (16).

Other Methods—NR and NiR activities were determined at 30 °C, using tolune-permeabilized cells with dithionite-reduced methylinologen as the electron donor (30, 31). Nitrate and nitrite were determined with a flow injection analyzer (NOX-1000W, Tokyo Chemical Industry Co., Ltd.). Ammonium was determined as described by Anderson and Little (32). Chlorophyll and protein were determined according to Mackinney (33) and Lowry et al. (34), respectively.

RESULTS

Construction of nrtC Deletion Mutants—In Southern hybridization analysis of the BamHI-XhoI digest of genomic DNA from the wild-type strain, the nrtB-specific probe hybridized with a 3.0-kbp DNA fragment (Fig. 1B, lane 1), which was recognized also by the probes specific to the 5′ portion (Fig. 1B, lane 4) and the 3′ portion (Fig. 1B, lane 7) of nrtC, as expected from the restriction map of the niaA operon (Fig. 1A). With the nrtB-specific probe, the hybridizing band in the digest of NC2 DNA was located at 1.6 kbp (Fig. 1B, lane 2), which was recognized by the probe specific to the 5′ portion of nrtC (Fig. 1B, lane 5) but not by the probe specific to the 3′ portion of nrtC (Fig. 1B, lane 8), indicating that the 3′ portion of nrtC had been deleted from the genome of NC2. In the digest of NC3 DNA, the band hybridizing to the nrtB-specific probe was located at 0.7 kbp (Fig. 1B, lane 3). The 0.7-kbp band hybridized with neither of the nrtC probes (Fig. 1B, lanes 6 and 9), indicating that the entire nrtC gene had been deleted from the genome of NC3.

Expression of the Other Nitrate Assimilation Genes in NC2 and NC3—As described previously (9), transfer of ammonium-grown wild-type cells to nitrate-containing medium induced accumulation of the niaA operon transcript, which hybridized with a probe specific to nrtD, the gene located downstream of nrtC (Fig. 2, lanes 1 and 2). The size of the hybridization signal ranged from 0.25 to >7 kilobases representing rapid degradation of the niaA operon transcript (9). The NC2 and NC3 mutants also accumulated the transcripts hybridizing with the nrtD-specific probe when transferred to nitrate-containing medium (Fig. 2, lanes 3–6), showing that nrtD is transcribed in the mutants under the regulation of the niaA operon promoter as in the wild-type strain.

In the mutants as well as in the wild-type strain, the NR and NiR activities were induced by transfer of the cells from ammonium-containing medium to nitrate-containing medium (Table I). Because NR and NiR are encoded by the last (nrbB) and the first (niaA) genes of the niaA operon, respectively, the truncation of nrtC or its deletion from the niaA operon did not essentially affect the expression of the other genes in the operon. However, the NR activity in NC2 was about 50% of the wild-type level (Table I) for an unknown reason. Because the growth rate of NC2 in nitrate-containing medium was similar to that of the wild-type cells and the rate of nitrate utilization by NC2 cells was comparable with that by the wild-type cells (see below), it was unlikely that the low NR activity was limiting nitrate assimilation in NC2.

Expression of Truncated NrtC in NC2—In immunoblotting analysis, the antibody against the NrtC polypeptide reacted with three polypeptides having apparent molecular masses of
polypeptide as the truncated NrtC lacking the C-terminal domain. These findings confirmed the expression of the N-terminal portion of NrtC and its incorporation into the cytoplasmic membrane in NC2. In accordance with its genome structure, NC3 showed neither the 67- nor the 34-kDa immunoreactive polypeptide (not shown).

**Growth and Nitrate Uptake Capability of the Mutants**—In a medium containing 2 mM nitrate as the nitrogen source, NC2 grew as rapidly as the wild-type strain, whereas NC3 grew only poorly (Fig. 4A). The cell suspensions of the wild-type strain and NC2 (5 μg Chl/ml) used 120 μM of nitrate until its exhaustion in 30 min, whereas the NC3 cells could not utilize the low concentration of nitrate (Fig. 4B). These findings indicated that NC2 is capable of active transport of nitrate but NC3 is not. A derivative of NC3 carrying plasmid-borne nrtC (designated NC31) utilized nitrate until its exhaustion (Fig. 4B), indicating that NrtC itself is essential for nitrate transport. The ability of NC2, having the truncated NrtC, to transport nitrate therefore indicated that the N-terminal ATP-binding domain of NrtC but not the C-terminal domain is essential for the activity of the transporter.

**Regulation of Nitrite and Nitrate Uptake in NC2**—NC2 assimilated low concentrations of nitrite as effectively as the wild-type strain (Fig. 5, A and B). As previously reported in a closely related species of Synechococcus (strain PCC 6301, for-

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**TABLE I**

| Strain | NR activity | NiR activity |
|--------|-------------|-------------|
|        | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ |
| WT     | 17 (15–20) | 259 (250–268) | 10 (9–11) | 47 (41–58) |
| NC2    | 19 (17–23) | 129 (126–131) | 11 (9–14) | 82 (83–87) |
| NC3    | 11 (9–13) | 258 (254–263) | 9 (6–11) | 98 (97–99) |

**FIG. 2.** Northern blot analysis of total RNA from the wild-type strain and the nrtC mutants using an nrtD-specific probe. Ammonium-grown cells were transferred to nitrate-containing medium, and RNA samples were extracted from the cells before and 30 min after the transfer. The RNA samples (10 μg/lane) from wild type (WT, lanes 1 and 2), NC2 (lanes 5 and 6), and NC3 (lanes 3 and 4) extracted before (lanes 1, 3, and 5) and after (lanes 2, 4, and 6) the transfer were denatured by treatment with formamide, fractionated on a 1.2% agarose gel that contained formaldehyde, transferred to positively charged nylon membrane (Hybond N⁺; Amersham Corp.), and hybridized with the 32P-labeled nrtD-specific probe.
merely *Anacystis nidulans* (29), nitrite uptake by the wild-type PCC 7942 cells was inhibited by the addition of ammonium to the medium (Fig. 5A) and resumed after consumption of the ammonium in the medium (data not shown). By contrast, nitrite utilization by NC2 was only partially inhibited by ammonium (Fig. 6B). Although the nitrite uptake rate was reduced by 60% by ammonium, the cells utilized nitrite and ammonium simultaneously and eventually used up nitrite in the presence of ammonium (Fig. 5B). A nitrite-specific transporter was reconstituted from the wild-type strain and NC2, respectively. As previously reported in other strains of cyanobacteria (12, 13), nitrate utilization by NC2 was completely and reversibly inhibited by ammonium (Fig. 5D) as in the wild-type strain.

**Accumulation of Intracellular Nitrate and Its Regulation**—Uptake of nitrate, as measured by following the nitrate concentration in medium, includes jointly transport and reduction. To determine specifically nitrate transport activity, intracellular nitrate accumulation in the light was measured in the NR-deficient mutants NR1 (24) and NC4, which were constructed from the wild-type strain and NC2, respectively. As observed previously in a mutant of strain PCC 7942 with no appreciable NR activity (15), NR1 accumulated about 1.5 mM of nitrate intracellularly at 20 μM external nitrate concentration in the absence of ammonium but did not accumulate nitrate in the presence of ammonium (Fig. 6A). The targeted NR mutant with truncated *nrtC*, NC4, accumulated nitrate to a concentration similar to that observed in NR1 in the absence of ammonium and, unlike the NR1 mutant having wild-type *nrtC*, accumulated nitrate in the presence of ammonium as well, although the initial rate of nitrate accumulation and the final intracellular nitrate concentration were reduced by 60% by ammonium (Fig. 6B). These findings showed that nitrate is actively transported into NC4 cells in the presence of ammonium.

Cyanobacterial cells with functional NR reduce intracellular nitrate in the light and usually accumulate only low concentration of nitrate (20–30 μM) (14). NC2 cells, however, accumulated nitrate under illumination to a concentration as high as 1 mM in the absence of ammonium (Fig. 6C), although the intracellular nitrate was subsequently depleted presumably due to reduction by NR (Fig. 6C). Similar to NC4, NC2 accumulated nitrate in the light and was not capable of utilizing nitrate exogenously (Fig. 6C). Unlike nitrite uptake, nitrate uptake by NC2 was completely and reversibly inhibited by ammonium (Fig. 5D) as in the wild-type strain.

**Fig. 3.** Immunoblotting analysis of the products of the wild-type and the mutant *nrtC* genes. Ammonium-grown cells were transferred to nitrate-containing medium, and cytoplasmic membrane samples were extracted and purified from the cells before and 16 h after the transfer. The membrane samples (10 μg of protein/lane) from wild type (WT, lanes 1 and 2) and NC2 (lanes 3 and 4) extracted before (lanes 1 and 3) and 16 h after (lanes 2 and 4) the transfer were electrophoresed in a 10% SDS-polyacrylamide gel. After the electrophoresis, polypeptides in the gel were electrotransferred to polyvinylidene difluoride membrane for immunostaining using the antiserum against the NrtC polypeptide.

**Fig. 4.** Growth and nitrate uptake capability of the wild-type strain and the *nrtC* mutants. A, growth curves in a medium containing 2 mM KNO₃. B, changes in nitrate concentration in the medium after addition of nitrate to the cell suspensions containing 5 μg of Chl/ml. ○, wild type (WT); △, NC2; □, NC3; ◊, NC31.

**Fig. 5.** Effects of ammonium on the uptake of nitrite and nitrate by the cells of the wild-type strain and the NC2 mutant. A, nitrate uptake by wild type (WT). B, nitrate uptake by NC2. C, nitrate uptake by wild type. D, nitrate uptake by NC2. Nitrite or nitrate (200 μM) was added at time 0 to the cell suspensions containing 5 μg of Chl/ml and ammonium (500 μM in A and B; 150 μM in C and D) was added at the time indicated by the arrows. Changes in the nitrate/nitrite concentration (circles) and the ammonium concentration (triangles) in the medium are shown. Nitrite uptake was measured at pH 9.6, whereas nitrate uptake was measured at pH 8.2 (see “Experimental Procedures”). Open circles, control; closed circles, plus ammonium.
nitrates in the presence of ammonium, showing no depletion of the intracellular nitrate (Fig. 6C). The maintenance of intracellular nitrate accumulation in NC2 cells in the presence of ammonium but not in its absence suggested inhibition by ammonium of NR activity, in accordance with the complete inhibition by ammonium of nitrate uptake in NC2 (Fig. 5D).

**DISCUSSION**

In NC2, the uptake of nitrite from the medium proceeded in the presence of ammonium (Fig. 5B), suggesting that the nitrate/nitrite transporter of NC2 is much less susceptible to ammonium than the wild-type transporter. This, however, apparently contradicted with the complete inhibition by ammonium of nitrate uptake by the mutant (Fig. 5D). Direct measurements of intracellular nitrate concentration, performed by HPLC determination of nitrate in acid lysates of the cell (16), showed that NC2 and its NR-less derivative (NC4) accumulate high concentrations of nitrate intracellularly (Fig. 6C), despite its inhibition by ammonium (Fig. 6A). The maintenance of intracellular nitrate (Fig. 6C) is probably contradicted with the complete inhibition by ammonium of nitrate uptake by the mutant (Fig. 5D).

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**FIG. 6. Effects of ammonium on intracellular accumulation of nitrate by Synechococcus cells.** A. NR1. B. NC4. C. NC2. Nitrate (20 μM) was added at time 0 to the cell suspensions containing 33.3 μg of Chl/ml with or without 250 μM ammonium. Changes in the intracellular nitrate concentration are shown. Open circles, control; closed circles, plus ammonium.