Signal Transduction Protein $P_{II}$ Phosphatase PphA Is Required for Light-Dependent Control of Nitrate Utilization in Synechocystis sp. Strain PCC 6803

Nicole Kloft and Karl Forchhammer*

Institut für Mikrobiologie und Molekularbiologie, Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany

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Cyanobacteria are oxyphototrophic bacteria capable of growing solely by using inorganic nutrients in light and using water as an abundant source of reductant for assimilatory processes. For nondiazotrophic freshwater cyanobacteria, such as Synechocystis sp. strain PCC 6803, nitrate is probably the most abundant source of combined nitrogen (14). Nitrate utilization requires uptake through an ATP-binding cassette-type transporter (NRT), which is a nitrate-nitrite bispecific transporter encoded by the nrtABCD genes (32, 33, 39, 40). Intracellular nitrate is reduced to nitrite by nitrate reductase (NR) and subsequently to ammonium by nitrite reductase (NiR). Ammonium is then assimilated into organic material via the glutamine synthetase-glutamate synthase cycle (4). Both NR and NiR use photosystem I (PSI)-reduced ferredoxin, excess reduction of nitrate along with impaired reduction of nitrite occurred in $P_{II}$ signaling mutants, resulting in excretion of nitrite to the medium. This effect could be reversed by increasing the level of PSI-reduced ferredoxin. We present evidence that nonphosphorylated $P_{II}$ controls the utilization of nitrate in response to low light intensity by tuning down nitrate uptake to meet the actual reduction capacity. This control mechanism can be bypassed by exposing cells to excess levels of nitrate. Uncontrolled nitrate uptake leads to light-dependent nitrite excretion even in wild-type cells, confirming that nitrate uptake controls nitrate utilization in response to limiting photon flux densities.

Utilization of nitrate in cyanobacteria is subject to global nitrogen control (4, 16), a process in which ammonium, through assimilation by the glutamine synthetase-glutamate synthase pathway, depresses the utilization of alternative nitrogen sources. Global nitrogen control operates both at the level of enzyme activity and at the level of gene expression, mediated by the transcriptional regulator NtcA. Addition of ammonium to cells growing in the presence of nitrate results in an immediate inhibition of NRT activity and depression of NtcA-activated gene expression (5). A central molecule for perception and signaling of the cellular nitrogen status in bacteria is the $P_{II}$ signal transduction protein (2, 7, 37). The $P_{II}$ protein family is one of the most widely distributed families of signal transduction proteins, whose members are present in all domains of life (2). $P_{II}$ proteins play ubiquitous roles in various aspects of nitrogen regulation, and they display a remarkable functional diversity with respect to the targets of regulation (2, 7, 37). The three-dimensional structure of various $P_{II}$ proteins is highly conserved, and in all cases analyzed so far, the $P_{II}$ proteins recognize adenylate nucleotides and 2-oxoglutarate proteins recognize adenylate nucleotides and 2-oxoglutarate.

In Synechocystis sp. strain PCC 6803, nitrate is probably the most abundant source of combined nitrogen (14). Nitrate utilization requires uptake through an ATP-binding cassette-type transporter (NRT), which is a nitrate-nitrite bispecific transporter encoded by the nrtABCD genes (32, 33, 39, 40). Intracellular nitrate is reduced to nitrite by nitrate reductase (NR) and subsequently to ammonium by nitrite reductase (NiR). Ammonium is then assimilated into organic material via the glutamine synthetase-glutamate synthase cycle (4). Both NR and NiR use photosystem I (PSI)-reduced ferredoxin, excess reduction of nitrate along with impaired reduction of nitrite occurred in $P_{II}$ signaling mutants, resulting in excretion of nitrite to the medium. This effect could be reversed by increasing the level of PSI-reduced ferredoxin. We present evidence that nonphosphorylated $P_{II}$ controls the utilization of nitrate in response to low light intensity by tuning down nitrate uptake to meet the actual reduction capacity. This control mechanism can be bypassed by exposing cells to excess levels of nitrate. Uncontrolled nitrate uptake leads to light-dependent nitrite excretion even in wild-type cells, confirming that nitrate uptake controls nitrate utilization in response to limiting photon flux densities.

* Corresponding author. Mailing address: Institut für Mikrobiologie und Molekularbiologie, Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany. Phone: 49 641-9935545. Fax: 49 641-9935549. E-mail: Karl.Forchhammer@ mikro.bio.uni-giessen.de.
component of NRT was regulated by PII. Studies with S49 mutants of PII, which potentially mimick the phosphorylated protein, showed that NRT was regulated by PII even without changing the PII modification status (28). A recent study confirmed that the phosphorylation status of PII does not affect the regulation of nitrate uptake in response to ammonium in Synchocystis sp. strain PCC 6803 (27). Studies by Hisbergues et al. (19) suggested that in Synechocystis sp. strain PCC 6803, high-affinity bicarbonate uptake is also regulated by PII without requiring PII modification. Moreover, NtcA-activated gene expression under conditions of nitrogen starvation was shown to depend on PII signaling (1, 41). In these cases, the direct targets of interaction with PII are not yet known at the molecular level. Recently, N-acetyl-l-glutamate kinase (NAGK) was identified as the first molecular target of PII signaling in a cyanobacterium (3, 15). NAGK catalyzes the first committed step in arginine biosynthesis and forms a tight complex with nonphosphorylated PII. Binding of PII strongly enhances the catalytic activity of this enzyme, whereas no NAGK activation or complex formation occurs with S49-modified PII (15, 34).

The cellular signal for PII phosphorylation is an elevated level of 2-oxoglutarate (11, 21), which serves as a signaling molecule of the cellular carbon/nitrogen balance (8, 36). Phosphorylated PII protein (PII-P) is dephosphorylated by a type 2C protein phosphatase, termed PphA, which was discovered in Synechocystis sp. strain PCC 6803 (22). In vitro analysis with purified components revealed that in the presence of ATP, dephosphorylation of PII-P by PphA responded in a highly sensitive manner to subtle changes of 2-oxoglutarate in the submillimolar concentration range and to a lesser extent also to oxaloacetate (7, 44). Elevated levels of these effector molecules lead to inhibition of PII-P-mediated PII-P dephosphorylation. A PphA-deficient mutant was unable to rapidly dephosphorylate PII-P in response to various signals (7, 22), in agreement with the finding that PII-P appears to be a very poor substrate for other cellular phosphatases (26). The abundance of PphA was shown to increase in response to elevated nitrate/nitrite levels, suggesting that this enzyme has an important function under these conditions (26). Detailed analysis of the phenotype of a PphA-deficient mutant should provide insights into the function(s) of the PII phosphorylation/dephosphorylation cycle in this cyanobacterium. Here, we describe a new regulatory mechanism in which PII-P dephosphorylation by PphA is required to fine-tune nitrate uptake under conditions of limiting PSI-reduced ferredoxin to prevent the formation of excess nitrite.

MATERIALS AND METHODS

Strains and growth conditions. Synechocystis sp. strain 6803T (13), the derived PphA-deficient mutant MPphA (pphA::kan) (22), and the PII null mutant ΔPII (pII::spec) (19) were grown in liquid BG11 medium (42) supplemented with 5 mM NaHCO3 and 17.6 mM NaNO3 (BG11N) as the nitrogen source. The cultures were incubated in baffled Erlenmeyer flasks capped with silicone sponge closures (Belco Glass, Vineland, NJ) and rotated with 150 rpm for efficient gas transfer. Cells were grown under photautotrophic growth conditions at 25°C at a photosynthetic photon flux density (PPFD) of 40 μmol photons s−1 m−2 from white fluorescent tubes (LUMILUX de Luxe Daylight, Osram). The mutant MPphA was maintained with kanamycin (30 μg ml−1) and the ΔPII mutant with spectinomycin (35 μg ml−1). Growth of the cultures was monitored by determination of the optical density at 750 nm (OD750).

For competition experiments, cultures of Synechocystis sp. strain PCC 6803 and the mutant MPphA, both with identical ODs (0.2), were harvested by centrifugation and washed with the appropriate medium (see below) to remove the antibiotic from the MPphA culture. The strains were mixed and grown in nitrate-, ammonium-, or urea-limited medium (final concentration, 0.5 mM) at an illumination of 40 μmol photons s−1 m−2. When the nitrogen source was exhausted (as deduced from growth arrest and onset of chlorosis), an aliquot of the culture was diluted (1:5) into fresh medium containing limiting amounts of the nitrogen source. This procedure was repeated three times. Appropriate dilutions of liquid cultures were plated on BG11N plates with and without kanamycin (solidified by the addition of 0.9% [wt/vol] of Gel-Rite [Roth]) to determine the CFU of MPphA (revealed by CFU on kanamycin plates) and wild-type cells (difference of CFU between nonselective and kanamycin plates). The plates were incubated at 30°C with a PPFD of 30 μmol photons s−1 m−2.

Control experiments revealed that during the time course of the experiment, omission of the selective antibiotic did not lead to a loss of the mutation.

DNA isolation and Southern blot analysis. Extraction of chromosomal DNA from cultures of the competition experiment was performed by using the QIA-GEN DNeasy tissue kit. A 1.5-μg sample of Smal-restricted DNA was applied to each lane of a 1% (wt/vol) agarose gel. Electrophoretic conditions, transfer of DNA to a nylon membrane (Ro-Ti-Nylon plus, Roth), and hybridization conditions were according to standard protocols (45).

The pphA gene probe used in DNA-DNA hybridization, a 0.46-kb DNA fragment corresponding to nucleotides 317 to 765 of the pphA (sll1771) coding region (25), was generated by restriction of the expression plasmid pT7-7pphA (20, 22) with KpnI and Smal. This DNA probe was labeled with [32P]dCTP by using the Megaprime DNA labeling kit (Amersham Pharmacia). The hybridization signals were visualized by exposing the membrane to a phosphorimager screen (Molecular Imaging, Inc., Hercules, CA), which was recorded in a phosphorimager (Molecular Imager FX, Bio-Rad). Quantification was performed using the Bio-Rad Quantity One software.

Determination of nitrate uptake and nitrite excretion. To determine nitrate uptake and nitrite excretion, cells grown in BG11N medium were harvested by centrifugation, washed with BG11 medium without any nitrogen source, and resuspended in the same medium to an OD750 of 1. The assays were started by the addition of 200 μM NaN3O to the cell suspension. To examine the effect of ammonium, NH4Cl (2 mM final concentration) was added. The culture was incubated under light and with shaking. Nitrate uptake was determined by estimating the concentration of nitrate in 1-ml aliquots of the medium. Therefore, the cells were removed from the medium by centrifugation, and the absorbance of nitrate was measured at 210 nm. Since the absorbance at 210 nm detects both nitrate and nitrite, the apparent nitrate values were corrected for the presence of nitrite. To determine the nitrite excretion, the nitrite concentration in aliquots of the medium was quantified by colorimetric assay (47).

To analyze nitrite utilization in more detail, precultures of the wild type, MPphA, and ΔPII were grown in modified BG11 medium containing nitrite (5 mM final concentration), in which molybdate was replaced by tungstate (4.8 μM). At the mid-exponential phase of growth, the cells were harvested by centrifugation and washed in combined nitrogen-free medium (BG11N). The cells were resuspended in modified BG11N medium (containing tautoglane) to an OD750 of approximately 1, and NaN3O and/or NaNO3 (each 100 μM) was added.

Determination of the modification state of PII. The phosphorylation state of PII in vivo was analyzed by nondenaturing polyacrylamide gel electrophoresis followed by immunoblot analysis of PII as described previously (10), PII2, PII1, PII2, and PII1 represent isoforms of the trimeric PII carrying no, one, two, and three phosphate groups, respectively.

Enzyme assays. Determination of nitrate reductase (17) and nitrite reductase (18) was performed in mixed alkyltrimethylammonium bromide (MTAB)-permeabilized cells with dithionite-reduced methyl viologen as the reductant.

RESULTS

Growth phenotype of a PphA-deficient mutant. Previous investigations showed that PphA is required for the rapid dephosphorylation of PII-P by various treatments, such as addition of ammonium, depletion of inorganic carbon, or inhibition of photosynthetic electron flow (7, 22). Despite impaired dephosphorylation of PII, PphA-deficient mutants retained their capacity to acclimate rapidly to the addition of ammonium, as revealed by glutamine synthetase assays (26), and their growth, as deduced from growth rates, was not obviously impaired with ammonium as the nitrogen source (22). This imposed the ques-
tion of the physiological significance of PphA-mediated dephosphorylation of P$_H$-P. To answer this, we examined the growth phenotype of PphA-deficient mutants (MPphA) in more detail by performing growth competition experiments in mixed cultures (see Materials and Methods). When the cells were competing for limiting amounts of nitrate, wild-type cells outgrew PphA-deficient mutants. After three repetitions of dilution and growth, only approximately 10% of the CFU were derived from the MPphA cells (Fig. 1A). By contrast, when the experiment was performed with ammonium- or urea-limited medium the proportion of wild-type and mutant cells did not change. In accord with plating analysis, Southern blot analysis of isolated chromosomal DNA from samples of the nitrate-limited mixed culture revealed the gradual disappearance of the PphA mutant while the relative proportion of wild-type cells increased (Fig. 1B and C).

Nitrates utilization and nitrite excretion in mutants of the PII signaling system. The competitive disadvantage of MPphA cells relative to wild-type cells in nitrate-supplemented medium suggested a subtle impairment in nitrate utilization. Previous analysis of P$_H$-deficient mutants of *Synechococcus* strain PCC 7942 revealed the accumulation of nitrite during nitrate-supplemented growth (12). Determination of nitrite in the culture medium of MPphA cells indeed showed increased formation of nitrite compared to wild-type cells. In standard BG11 medium (with a final concentration of 17.6 mM nitrate), six times more nitrite was detected in MPphA cultures in the exponential phase of growth than in wild-type cultures (Fig. 2). To analyze the rate of nitrate consumption and concomitant formation of nitrite in vivo, cells were incubated in medium containing 200 μM nitrate, and at different times, nitrate depletion and nitrite accumulation were analyzed. As shown in Fig. 3A, MPphA cells took up nitrate even more rapidly than the wild type but excreted the excess of reduced nitrate back to the medium in the form of nitrite. Addition of ammonium resulted in a stop of nitrate uptake in both wild-type and MPphA cells (Fig. 3B), and as a consequence, MPphA cells produced almost no nitrite. Further experiments demonstrated that the responses of NRT activity toward various ammonium concentrations were identical in wild-type and MPphA cells (data not shown). In the P$_H$-deficient strain (ΔP$_H$), however, nitrate consumption was not affected by the presence of ammonium (Fig. 3B, triangles). Part of the consumed nitrate was reexcreted to the medium as nitrite, confirming previous reports that the P$_H$ protein is required for the control of nitrate utilization in response to a short-term exposure to ammonium.
(19, 27, 29), albeit by a mechanism that does not require P II dephosphorylation.

Excretion of nitrite by MPphA cells indicates an imbalance of nitrate reduction compared to nitrite reduction. Therefore, the activities of nitrate and nitrite reductases (NR and NiR, respectively) were measured in MTAB-permeabilized cells, using methyl viologen as the electron donor. The activities of NR and NiR were not significantly different in nitrate-grown wild-type and MPphA cells. Wild-type cells exhibited activities for NR and NiR of 291 ± 24 and 101 ± 6 (activities are given in nmol substrate minute⁻¹ mg chlorophyll a⁻¹), respectively, compared to 284 ± 24 and 101 ± 6 in MPphA cells (the chlorophyll a contents of 1-ml cell suspensions at an OD₇₅₀ of 1 corresponded to 5.5 μg for the wild type and 5.0 μg for MPphA cells). This argued against the possibility that the PphA mutation affects the levels of NR and NiR and thereby causes the excretion of nitrite. Furthermore, the intracellular concentrations of nitrite, determined in nitrate-grown cells, were almost identical in wild-type and PphA-deficient cells, indicating that differential accumulation of nitrite cannot account for the observed phenotype (data not shown).

Light and reductant dependence of nitrite excretion. In the search for factors that were involved in nitrite excretion by MPphA cells, we observed that the extent of nitrite production strongly depended on the illumination conditions. To investigate the relation between PPFD and nitrite formation systemically, cultures of the wild type and the mutants MPphA and ΔP₉ were incubated in medium containing 200 μM NaNO₃ at PPFDs of 10, 40, and 120 μmol photons s⁻¹ m⁻². From the slopes of nitrite formation or nitrate removal (compare Fig. 3), the values shown in Table 1 were derived. Under low-light conditions, both mutants utilized more nitrate than the wild type and produced the largest amounts of nitrite. Thirty-three and 43% of the consumed nitrate was converted to nitrite and was excreted to the medium by MPphA and ΔP₉ cells, respectively. As expected from the fact that nitrate utilization consumes PSI-reduced ferredoxin in cyanobacteria, elevated illumination increased nitrate consumption in all strains. Concomitantly with the increased PPFD, nitrite formation declined in both mutants. At an illumination of 40 μmol photons s⁻¹ m⁻², MPphA converted only 13% and ΔP₉ 24% of the consumed nitrate to nitrite that was excreted to the medium. Under high-light conditions, nitrite production in all strains was barely detectable. To analyze the phosphorylation status of P₉ under the different light conditions, samples of wild-type and MPphA cultures were removed after 30 min of incubation (Fig. 4). Under low-light conditions (10 μE), an intermediate level of P₉ phosphorylation was observed, with appreciable levels of the nonphosphorylated form (P₀) and predominantly the forms with one and two phosphorylated subunits (P₁a and P₁b). At higher light intensity, the phosphorylation status was.

**FIG. 2. Nitrite formation by PphA-deficient cells in BG11N medium.** Wild-type Synechocystis sp. strain PCC 6803 (squares) and MPphA (diamonds) cells were grown in BG11N medium to the mid-exponential phase of growth, harvested by centrifugation, washed twice in BG11N, and resuspended in BG11N to an OD_750 of 1. Cells were incubated under standard growth conditions, and after different times, aliquots were removed and nitrite in the supernatant was determined.

**FIG. 3.** (A) Consumption of 200 μM nitrate (filled symbols) and formation of nitrite (open symbols) by wild-type *Synechocystis* sp. strain PCC 6803 (squares) and PphA-deficient mutant MPphA (diamonds) cells. (B) Consumption of 200 μM nitrate (filled symbols) in the presence of 2 mM NH₄Cl and formation of nitrite (open symbols) by cells of wild-type *Synechocystis* sp. strain PCC 6803 (squares), the PphA-deficient mutant MPphA (diamonds), and the P II-deficient mutant ΔP₉ (triangles). The experiments were performed at a PPFD of approximately 35 μmol photons s⁻¹ m⁻². A representative of three independent experiments is shown.

**TABLE 1.** Light-dependent nitrate consumption and nitrite formation (nmol min⁻¹ mg chlorophyll a⁻¹) by cells of wild-type *Synechocystis* sp. strain PCC 6803, the PphA-deficient mutant MPphA, and the P II-deficient mutant ΔP₉.

<table>
<thead>
<tr>
<th>PPFD (a)</th>
<th>WT</th>
<th>MPphA</th>
<th>ΔP₉</th>
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<tbody>
<tr>
<td>10</td>
<td>118 ± 2.5</td>
<td>25 ± 1.2</td>
<td>137 ± 12.5</td>
</tr>
<tr>
<td>40</td>
<td>203 ± 10</td>
<td>7.5 ± 3.7</td>
<td>250 ± 5.0</td>
</tr>
<tr>
<td>120</td>
<td>296 ± 1.2</td>
<td>2.5 ± 3.7</td>
<td>340 ± 1.2</td>
</tr>
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(a) In μmol photons s⁻¹ m⁻².
clearly increased, with only traces of unmodified \( P_{II} \) and high levels of fully phosphorylated \( P_{II} \) (\( P_{II}^{3} \)). By contrast, in MPpH\( A \) cells, \( P_{II} \) protein was present in its highly phosphorylated forms (\( P_{II}^{2} \) and \( P_{II}^{3} \)) with no unmodified isoform detectable under any light conditions.

Under low-light conditions, PSI-reduced ferredoxin, the electron donor for NR and NiR, is the limiting factor for the activities of these enzymes. Therefore, deficiency of electrons may cause the observed deregulation of nitrate/nitrite reduction, in spite of the identical activity levels of these enzymes as measured with artificial electron donors (see above). If this was the case, donation of electrons under light-limiting conditions should decrease nitrite formation in MPpH\( A \) and \( \Delta P_{II} \). This possibility was investigated by addition of glucose, which can be utilized as a source of reductant in this facultatively photoheterotrophic organism. One half of each culture was incubated in medium containing 200 \( \mu \)M NaNO\( _{3} \), and the other half was incubated in medium containing 200 \( \mu \)M NaNO\( _{3} \) together with 0.1% glucose at an illumination of 10 \( \mu \)mol photons s\( ^{-1} \) m\( ^{-2} \) (Table 2). In the presence of glucose, both mutants took up nitrate more rapidly and excreted less nitrite, such that the ratio of nitrite production per nitrate consumption decreased by a factor of two. The phosphorylation state of \( P_{II} \) under those conditions is shown in Fig. 5. In the absence of glucose, three phosphorylated \( P_{II} \) isoforms were observed in wild-type cells, the unmodified form (\( P_{II}^{0} \)) and the phosphorylated forms of \( P_{II} \) with one and two phosphorylated subunits (\( P_{II}^{1} \) and \( P_{II}^{2} \)), whereas only highly phosphorylated \( P_{II} \) forms (\( P_{II}^{2} \) and \( P_{II}^{3} \)) were observed in MPpH\( A \). The addition of glucose resulted in an increased phosphorylation of \( P_{II} \), with \( P_{II}^{0} \) disappearing in cells of the wild type.

**In vivo competition of nitrate and nitrite reductase.** To elucidate whether the excretion of nitrite at 10 \( \mu \)mol photons s\( ^{-1} \) m\( ^{-2} \) in \( P_{II} \) signaling mutants was caused by an impaired in vivo nitrite reductase activity, the utilization of nitrite in the absence or presence of nitrate was analyzed in a time course experiment. Both strains, the wild type and MPpH\( A \), exhibited almost identical consumptions of nitrite (Fig. 6A), revealing that in vivo nitrite reductase activity was not impaired in MPpH\( A \) as long as nitrate was absent. In the presence of nitrate and nitrite at 100 \( \mu \)M (Fig. 6B), wild-type cells were able to utilize nitrite concomitantly with the utilization of nitrate. By contrast, MPpH\( A \) cells utilized only nitrate, whereas the utilization of nitrate was impaired. The same result was obtained for the \( \Delta P_{II} \) strain (data not shown). To clarify whether the activity of NR affects nitrite utilization in the mutants or whether nitrate had other effects, NR was poisoned by growing the cells in a medium in which molybdate was replaced by tungstate. Under these conditions, the cells expressed a non-active nitrite reductase (18). Using such NR-poisoned cells, no difference in nitrite reduction could be detected between the wild type and the \( P_{II} \) signaling mutant in the presence of nitrate (Fig. 6C). This confirmed that the impaired in vivo nitrite reduction in the mutants was indeed due to the competing activity of NR under conditions of limited PSI-reduced ferredoxin. Furthermore, these results implied that wild-type cells are able to balance the reduction of nitrate and nitrite by controlling the amount of nitrite reduction in response to the availability of reductant, a process that is defective in the \( P_{II} \) mutants. To distinguish whether this process operates at the level of NR activity or whether the amount of nitrate reduction is controlled by nitrate uptake, the dependence of nitrate utilization on active transport was bypassed by increasing the nitrate concentration in the medium to 60 mM, a concentration at which nitrate enters the cells by diffusion (38). Under these conditions, nitrite formation was tested again at 10, 40, and 120 \( \mu \)mol photons s\( ^{-1} \) m\( ^{-2} \). If nitrate reduction is controlled by regulation of NR activity, no nitrite accumulation should occur. When, however, nitrate transport controls nitrate reduction, the wild-type cells should lose control. As shown in Fig. 7, wild-type cells were indeed no longer able to prevent nitrite formation with decreasing PPFD, implying that light control of nitrate utilization operates at the level of nitrate uptake.

**TABLE 2.** Nitrate consumption and nitrite formation (nmol min\(^{-1}\) mg chl\(^{-1}\)) by cells of wild-type Synechocystis sp. strain PCC 6803, the Pph\( A \)-deficient mutant MPpH\( A \), and the \( P_{II} \)-deficient mutant \( \Delta P_{II} \) in the presence or absence of 0.1% glucose

<table>
<thead>
<tr>
<th>Cell type</th>
<th>( NO_{3}^{-} ) consumption</th>
<th>( NO_{3}^{-} ) formation</th>
<th>( NO_{3}^{-} : NO_{2}^{-} ) ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+ Glucose</td>
<td>- Glucose</td>
<td>220 ± 29</td>
</tr>
<tr>
<td>MPh( A )</td>
<td>+ Glucose</td>
<td>- Glucose</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>( \Delta P_{II} )</td>
<td>+ Glucose</td>
<td>- Glucose</td>
<td>41.1</td>
</tr>
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</table>
DISCUSSION

The present study reveals a novel regulatory link between photosynthetic electron transport and nitrate utilization in the cyanobacterium *Synechocystis* sp. strain PCC 6803. During photoautotrophic growth, assimilation of nitrate is a genuine photosynthetic process through the dependence of nitrate and nitrite reductases on PSI-reduced ferredoxin (4). The majority of photosynthetically generated reductant is required for CO₂ fixation. In nitrate-grown cells, approximately 20% of reductant is utilized for conversion of nitrate to ammonium. At low PPFD, the availability of reductant limits assimilatory reactions, and nitrate and nitrite reductases compete for limiting reductant. To avoid the accumulation of the intermediary product nitrite, nitrite reduction has to keep pace with nitrate reduction. In *Synechocystis* sp. strain PCC 6803 wild-type cells, no nitrite excretion occurs when the ambient nitrate concentration is so low that nitrate uptake depends on active transport. However, when the environmental concentration of nitrate rises to high levels, nitrite excretion occurs preferentially at lower light intensities. Under these conditions, nitrite enters the cells in a nitrate transport-independent manner (38). This implies that under low-light conditions, the in vivo activity of nitrate reductase exceeds nitrite reductase activity, resulting in excess formation of nitrite, which is then excreted to the medium. In contrast, no nitrite formation occurs in the presence of submillimolar concentrations of nitrate. From this it follows that *Synechocystis* wild-type cells regulate the reduction of nitrate in response to low light at the level of nitrate uptake. This is the same regulatory principle the cells use to control nitrate utilization in response to ammonium (27). Nitrate transport is rapidly inhibited upon ammonium treatment, a process that depends on the PII signal transduction protein, whereas nitrate reductase activity is not affected (27). However, the dependence of nitrate transport on the PII protein differs with respect to mode of signal transduction. Whereas the present study revealed that nonphosphorylated PII is necessary for the low-light control of nitrate uptake (see below), regulation of nitrate uptake in response to ammonium does not depend on the phosphorylation state of PII. This was previously shown in mutants of *Synechococcus elongatus* PCC 7942 in which the site of phosphorylation, Ser49, was mutated to aspartate or glutamate (28) and was recently corroborated in *Synechocystis* sp. strain PCC 6803 (27). In accord with these data, this study shows that the PphA-deficient mutant, which is impaired in rapid PII-P dephosphorylation, is not affected in ammonium-responsive control of nitrate uptake, whereas the PII-deficient mutant has lost this control. Most likely, binding or dissociation of effector molecules to PII is sufficient to mediate this response.

In contrast to ammonium response, the PII-deficient and PphA-deficient mutants exhibit the same phenotype with respect to nitrate utilization under low-light conditions. In these mutants, conditions of limiting PPFD lead to the excretion of

FIG. 6. (A) Consumption of 100 μM nitrite by cells of wild-type *Synechocystis* sp. strain PCC 6803 (squares) and the PphA-deficient mutant MPphA (diamonds). (B) Consumption of a mixture of 100 μM nitrate (filled symbols) and 100 μM nitrite (open symbols) by wild-type *Synechocystis* sp. strain PCC 6803 cells (squares) and the PphA-deficient mutant MPphA (diamonds). (C) Consumption of a mixture of 100 μM nitrate (filled symbols) and 100 μM nitrite (open symbols) by NR-poisoned cells of wild-type *Synechocystis* sp. strain PCC 6803 (squares) and of the PphA-deficient mutant MPphA (diamonds). The assays were performed at an illumination of 10 μmol photons s⁻¹ m⁻². The data are from a representative of three independent experiments yielding nearly identical results.

FIG. 7. Nitrite formation by wild-type cells of *Synechocystis* sp. strain PCC 6803 incubated at an illumination of 10 (circles), 40 (squares), or 120 (triangles) μmol photons s⁻¹ m⁻² in BG11 medium containing 60 mM nitrate.
nitrite, even at low nitrate concentrations. Nitrite formation is the typical phenotype of an impaired nitrite reductase activity (48). Indeed, it could be shown that in spite of almost identical activity levels of NR and NiR, in vivo nitrite utilization is impaired under low-light conditions when nitrate is present. This impairment results from the competing activity of nitrate reductase under conditions of limiting reductant: when nitrate reductase was poisoned with tungstate, in vivo nitrite reduction in the presence of nitrate was not affected, and nitrite excretion could also be lowered by increasing the availability of reductant. In the PphA-deficient mutant, the P$_{II}^*$ signaling protein is present at wild-type levels. Moreover, P$_{II}^*$ signaling processes, which do not depend on the phosphorylation state of P$_{II}^*$, are not affected in this mutant (26). For this reason, the mutant allows the identification of cellular processes in which the phosphorylation state of P$_{II}^*$ plays a role. The fact that nitrite excretion at low PPFD occurs in MPphA as well as in P$_{II}^*$null mutants implies that nonphosphorylated P$_{II}^*$ is required for this regulatory process. Indeed, the nonphosphorylated form of P$_{II}^*$ could be detected in wild-type cells under those conditions, where the mutants excreted nitrite. The inability to control nitrate utilization effectively explains the observed competitive disadvantage to wild-type cells. By excreting nitrite, the mutant wastes reductant, which can be used by wild-type cells.

In mechanistic terms, the different regulation of nitrate transport by P$_{II}^*$, which is independent of its phosphorylation state in response to ammonium but is dependent on nonphosphorylated P$_{II}^*$ in response to low light, remains elusive. However, it reminds one of the different regulation of NRT activities toward ammonium or CO$_2$ fixation. In a C-terminal truncated NrtC mutant of *Synechococcus* sp. strain PCC 7942 (43), the response of NRT activity toward ammonium was lost, whereas regulation in response to CO$_2$ fixation was still functional, indicating the existence of at least two independent mechanisms in the regulation of nitrate transport. Biochemical analyses with purified components are necessary to resolve this issue and to reveal a potential link to P$_{II}^*$ signaling. As shown in this study, low PPFD results in a preferential dephosphorylation of P$_{II}^*$ by PphA. Decreasing 2-oxoglutarate or ATP levels are likely to be the primary signals to which PphA-mediated P$_{II}^*$ dephosphorylation responds (6, 44). Although subtle changes in the effector molecule levels may not be sufficient to allow a direct regulation of P$_{II}^*$ targets independent of the P$_{II}^*$ phosphorylation status, they may be sufficient to modulate the reactivity of P$_{II}^*$ toward PphA. In this context, it is interesting that the amount of PphA increases in the presence of nitrite (26), which might contribute to efficient dephosphorylation of P$_{II}^*$ under conditions of nitrite accumulation. Nonphosphorylated P$_{II}^*$, in turn, controls nitrate transport to prevent further production of nitrite. Therefore, one function of PphA would be to amplify subtle changes in effector molecule concentrations by adjusting the phosphorylation status of P$_{II}^*$, leading to a fine-tuned regulation of nitrate utilization.

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