A role for the signal transduction protein P_{II} in the control of nitrate/nitrite uptake in a cyanobacterium

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Abstract In the cyanobacterium *Synechococcus* sp. strain PCC 7942, ammonium exerts a rapid and reversible inhibition of the nitrate and nitrite uptake, and the P_{II} protein (GlnB) is differentially phosphorylated depending on the intracellular N/C balance. RNA/DNA hybridizations, as well as nitrate and nitrite uptake experiments, were carried out with the wild-type strain and a P_{II}-null mutant. The transcriptional control by ammonium of the expression of the *nir-nrtABCD-narB* operon remained operative in the mutant but, in contrast to the wild-type strain, the mutant took up nitrate and nitrite even in the presence of ammonium. Moreover, the wild-type phenotype was restored by insertion of a copy of the wild-type *glnB* gene in the genome of the P_{II}-null mutant. These results indicate that the unphosphorylated form of P_{II} is involved in the short-term inhibition by ammonium of the nitrate and nitrite uptake in *Synechococcus* sp. strain PCC 7942.

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Key words: Nitrogen metabolism; Ammonium inhibition; Regulation; *glnB* gene product; *Synechococcus* sp. strain PCC 7942

1. Introduction

In cyanobacteria, nitrate and nitrite are taken up by a common high affinity transport system involving the NrtABCD permease (an ABC-type transporter), and by diffusion [1–4]. Both nitrate and nitrite are reduced to ammonium in sequential reactions involving ferredoxin molecules as physiological electron donors. Nitrate is first reduced to nitrite by nitrate reductase and subsequently nitrite is reduced to ammonium by nitrite reductase. Ammonium uptake occurs both by an active transport system that depends on membrane potential and by diffusion of unprotonated molecules [1]. Finally, ammonium, irrespective of the N sources used for growth, is incorporated into glutamate mainly by the operation of the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle [1].

Ammonium, the N source preferred by cyanobacterial cells, exerts negative effects on the assimilation of alternative N sources like nitrate or nitrite, resulting in both an inhibition of their transport and a repression of the synthesis of proteins involved in their assimilation, including the products of the *nir-nrtABCD-narB* operon (nitrite reductase, nitrate/nitrite permease complex and nitrate reductase) and of the *glnA* gene (glutamine synthetase) [1]. It has recently been suggested that nitrate reductase activity could also be inhibited in vivo in response to addition of ammonium to the cells [5]. NtcA, a protein homologous to members of the CRP family of bacterial regulators, has been identified in all cyanobacteria examined [6]. This DNA binding protein is a transcriptional activator for genes subjected to nitrogen control by ammonium. In *Synechococcus* sp. strain PCC 7942, in the absence of ammonium, NtcA activates the expression of the *nir-nrtABCD-narB* operon, the *glnA* gene and its own gene [7].

In enterobacteria, the uridylyltransferase/uridylyl removing enzyme and the P_{II} protein provide a mechanism for sensing the intracellular N status. P_{II} regulates promoters dependent on the transcriptional activator NtrC, which is part of the NtrB/NtrC two-component regulatory system, and it controls glutamine synthetase activity by adenylylation/deadenylylation of the enzyme [8]. The P_{II} protein has recently been characterized in *Synechococcus* sp. PCC 7942 [9–11]. In this cyanobacterium, the active uptake of nitrate and nitrite are rapidly and effectively inhibited by ammonium. The uptake process involves transport of the substrate into the cell and subsequent intracellular reduction of nitrate or nitrite. Earlier physiological experiments (reviewed in [1]) showed that: (i) the inhibition of nitrate/nitrite uptake by ammonium requires ammonium assimilation via a functional GS/GOGAT cycle; (ii) product(s) of CO_2 fixation have a positive effect on nitrate uptake. In strain PCC 7942, the P_{II} protein is posttranslationally modified by *O*-phosphorylation at Ser^{49}, the modification state of P_{II} depending on the cellular activity of ammonium assimilation, via the GS/GOGAT cycle, relative to the rate of CO_2 fixation [10,11]. Here we demonstrate that the P_{II} protein plays a key role in the regulation of active nitrate/nitrite uptake in *Synechococcus* sp. strain PCC 7942 cells.

2. Materials and methods

2.1. Strains and culture conditions

Synechococcus sp. strain PCC 7942 spc (small-plasmid cured strain) [12], hereafter designated *Synechococcus* sp. PCC 7942 throughout this report, and the derived P_{II}-null mutant MP2 and the MP2-S strains were grown in liquid $BG11_0$ medium [13] containing 0.4 mM Na_2CO_3 and supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 8.0. Either $NaNO_3$ (17.6 mM) or NH_4Cl (5 mM) was used as the N source. The P_{II}-null mutant MP2 was grown with kanamycin (30 μg/ml) and MP2-S with both kanamycin and streptomycin (10 μg/ml). Precultures were incubated for 3–4 days (OD_{750} of approximately 0.6) at 30°C in air and illuminated with fluorescent lamps (Osram L18W/25 universal white) providing a photosynthetic photon flux density (PPFD) of 50 μmol/m^2/s measured with a LI-COR LI-185B quantum/radiometer/photometer equipped with a LI-190SB quantum sensor. Experimental cultures were incubated at 35°C under the same PPFD, in a

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culture medium supplemented with $NaHCO_3$ (10 mM) and with a constant bubbling with air-3% (v/v) CO_2. All the measurements were carried out with cells from mid-exponential-phase cultures (OD_{750} of 0.4).

Plasmids were maintained in the *Escherichia coli* strains DH5α or DH5α Mcr$^-$. Strains were grown at 37°C in Luria-Bertani medium supplemented with appropriate antibiotics and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when required.

2.2. Nucleic acid methods

Standard methods were used for *E. coli* plasmid DNA isolation and cell transformation. Restriction endonucleases (New England Biolabs or Pharmacia) and other DNA-modifying enzymes (New England Biolabs or Amersham) were used according to the manufacturers' recommendations. Extraction of total RNA from experimental cultures grown with nitrate or ammonium was performed as described by Liotenberg et al. [14] with the following modifications: cells (~250 μg of chlorophyll *a*) were harvested by centrifugation at 25°C and 5000×*g* for 15 min, resuspended in 3 ml of cold buffer (10 mM sodium acetate pH 4.5, 200 mM sucrose, 55 mM Na_2EDTA), frozen in liquid nitrogen and kept at −20°C; cell aliquots were thawed on ice in the presence of 0.4% (w/v) Bentone MA (Rheox Company, NL Industries, USA) in 0.1 M Tris-HCl pH 8.0, 0.5% (w/v) sodium dodecyl sulfate, 3.5 ml saturated phenol, 3.5 g of sterile glass beads and disrupted in a Mickle disintegrator five times for 1 min at 4°C. DNA and RNA gel electrophoresis, blotting and hybridizations were carried out on Hybond-N membrane (Amersham) as described by Damerval et al. [15] and Liotenberg et al. [14], respectively. Prehybridization (4 h) and hybridization (16 h) experiments were performed at 65°C (high stringency conditions) for DNA/DNA analyses and at 42°C in the presence of 50% (v/v) formamide for RNA/DNA analyses. The probes internal to *nrtA* (763 bp) and *nrtD* (737 bp) were obtained by PCR amplification of the corresponding fragments from the plasmid pCS3 carrying the *nir-nrtABCD-narB* operon [16] and labelled with [α-^{32}P]dATP (110 TBq/mmol) by using a Megaprime random labelling kit (Amersham).

2.3. Construction of the MP2-S strain

The MP2-S strain was derived from the P_{II}-null mutant MP2 (*glnB::kan*) [10] in which the wild-type *glnB* gene was reintroduced into the chromosome. Plasmid pPM119 containing the *glnB* gene from *Synechococcus* sp. PCC 7942 [9] was digested with *Ppu*10I, blunt-ended with mung bean nuclease, digested with *Nhe*I and the resulting 768 bp fragment subcloned at the *Sma*I-*Xba*I sites of pTZ18R to generate pPM241. Plasmid pPM241 was then digested with *Hin*dIII, blunt-ended with mung bean nuclease, digested with *Sac*I and the resulting 802 bp fragment cloned into the *Sma*I-*Sac*I sites of plasmid pAM1303 (gift from S.S. Golden, USA) to generate pPM243. The non-replicative plasmid pAM1303 contains a Sp^R/Sm^R omega cassette inserted into an approximately 2 kb long sequence of *Synechococcus* sp. PCC 7942 genomic DNA that permits the introduction of any cloned DNA fragment by recombination in the corresponding region of the genome [17]. Plasmid pPM243 was used to transform P_{II}-null mutant cells [11]. Transformation was carried out as described [18], cells being plated on solid $BG11_0$ medium supplemented with 17.6 mM $NaNO_3$ and kanamycin (30 μg/ml). After incubation for 24 h at 25°C under a PPFD of 15 μmol/m^2/s, streptomycin (10 μg/ml) was added under the agar to select for transformants. Total genomic DNAs from positive transformants were isolated as described [19] from 35 ml liquid cultures incubated for 1–2 weeks at 25°C under a PPFD of 15 μmol/m^2/s. To check for appropriate gene replacement, DNA/DNA hybridizations were performed by using three probes, a 284 bp *Afl*II-*Bgl*II fragment containing most of the *glnB* gene, a 400 bp *Sac*I-*Pst*I fragment from pAM1303 corresponding to a portion of the region where insertion took place in the *Synechococcus* sp. PCC 7942 genome and a 1.4 kb *Acc*I-*Pst*I fragment from pBR322 corresponding to a part of pAM1303 which is lost after recombination. The first probe revealed a 4.5 kb *Hin*dIII fragment in the wild-type and two DNA fragments of 3.8 kb and 2.0 kb in the P_{II}-null mutant MP2. An additional 7.5 kb *Hin*dIII fragment was present in the MP2-S strain that corresponds to the integration into the MP2 genome of the wild-type *glnB* gene. The second probe revealed a 7.5 kb *Hin*dIII fragment in MP2-S and a 6.6 kb *Hin*dIII fragment in the wild-type and the MP2 genomes. As expected, the third probe did not hybridize with the MP-S genome.

2.4. Determination of nitrate and nitrite uptake activities

For nitrate uptake assays, cells from experimental cultures grown with either nitrate or ammonium were harvested by centrifugation, washed twice with 5 mM Tricine-NaOH pH 8.3/10 mM $NaHCO_3$ buffer and resuspended in the same buffer to a concentration corresponding to 10 μg of chlorophyll *a* per ml (spectroscopically determined from methanolic extracts as described in [20]). Uptake assays were started by addition of $NaNO_3$ (100 μM final concentration) to the cell suspensions preincubated with or without NH_4Cl (500 μM final concentration) for 5 min at 35°C under a PPFD of 200 μmol/m^2/s and were carried out under the same incubation conditions. Nitrate uptake activity was determined by estimating the concentration of nitrate in aliquots of the incubation medium after rapid removal of the cells by filtration (Millipore HA 0.45 μm filter). Nitrate was determined by absorbance at 210 nm in acid solution according to Cawse [21]. In some experiments, nitrate was separated from other substances that might be present in the sample by HPLC on an analytical column (Whatman Partisil-10 Sax) according to Romero et al. [22].

Nitrite uptake was determined in 25 mM MOPS-glycine buffer at two different pH conditions (pH 7.2 and pH 9.6). Cells from experimental cultures grown with nitrate were washed twice with 1 mM Tricine-NaOH pH 8.3 and resuspended in 1 ml of the same buffer. This concentrated cell suspension was diluted in the reaction buffer to a final concentration corresponding to 10 μg of chlorophyll *a* per ml. The procedure for this assay was the same as for determining nitrate uptake, except that the reaction was started by addition of $NaNO_2$ (100 μM final concentration). Nitrite disappearance in aliquots of the cell suspension clarified by rapid filtration as above was measured according to Snell and Snell [23].

3. Results

Previous analysis of the phenotypic properties of the P_{II}-null mutant MP2 led to the conclusion that P_{II} might be a key element in the coordination of N and C assimilation in *Synechococcus* sp. PCC 7942 [11]. To get deeper insights into the role of the P_{II} protein, further comparative analyses were performed with the wild-type, the P_{II}-null mutant MP2 and the MP2-S strains. The latter is the P_{II}-null mutant MP2 in which a copy of the wild-type *glnB* gene has been inserted at another location in the genome (see Section 2 for details). In the P_{II}-null mutant MP2, no protein was detected with the specific antibodies raised against P_{II} (Fig. 1). In contrast, in the MP2-S strain, the P_{II} protein was present and responded to the nitrogen regime as in the wild-type, the four isoforms of P_{II} carrying zero (P_{II}^0), one (P_{II}^1), two (P_{II}^2) or three phosphate groups (P_{II}^3) being obtained in nitrate-grown cells and

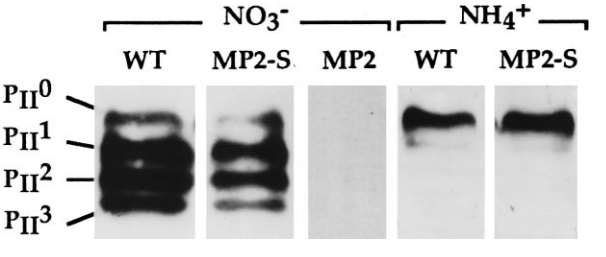

Fig. 1. In vivo phosphorylation of P_{II} in response to nitrogen assimilation in the wild-type *Synechococcus* sp. PCC 7942 (WT) and MP2 cells containing a copy of the wild-type *glnB* gene (MP2-S) inserted in the genome. A lane corresponding to the P_{II}-null mutant (MP2) has been included. Cells were grown in the presence of nitrate (NO_3^-) or ammonium (NH_4^+). The isoforms were separated on non-denaturing PAGE and revealed by immunoblotting with a P_{II}-specific antiserum. P_{II}^0 indicates unphosphorylated protein and P_{II}^1, P_{II}^2 and P_{II}^3 represent the isoforms of P_{II} carrying one, two and three phosphate groups, respectively [10].

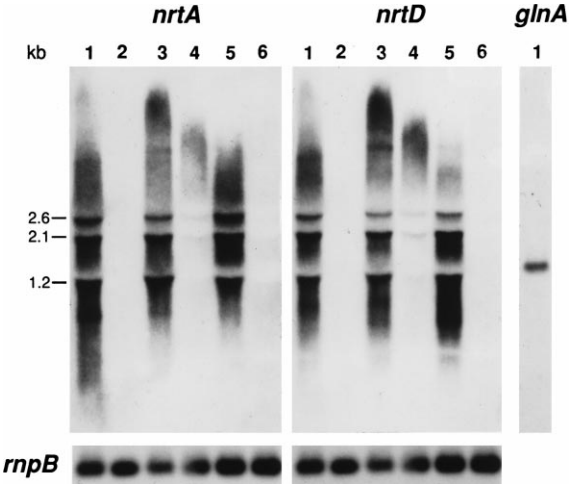

Fig. 2. RNA blot analysis of the *nrtA* and *nrtD* transcripts from *Synechococcus* sp. PCC 7942 cells grown with nitrate (lanes 1, 3 and 5) or ammonium (lanes 2, 4 and 6): wild-type (lanes 1 and 2), P_{II}-null mutant MP2 (lanes 3 and 4) and complemented strain MP2-S (lanes 5 and 6). The same blot (50 μg of total RNA per lane) was successively hybridized with different probes: fragments internal to the *nrtA* and *nrtD* genes; a 0.6 kb *Kpn*I-*Xho*I fragment containing the *rnpB* gene [34] as a control for RNA loading and transfer to the membrane; and a 0.9 kb *Eco*RI fragment internal to the *glnA* gene [11] as a control for the quality of the RNA preparations. A single and sharp hybridizing band was obtained with the *glnA* probe whatever the strain and culture conditions used (only one lane is presented as an example).

mostly the unphosphorylated P_{II}^0 form in ammonium-grown ones (Fig. 1).

3.1. Expression of the nir-nrtABCD-narB operon

In the three strains, nitrate reductase and nitrite reductase-specific activities were found to be high in nitrate-grown cells and no activity was detectable in ammonium-grown cells ([11] and data not shown). Therefore, the expression of the *nir-nrtABCD-narB* operon (which includes genes for both the reductases and the permease complex) might take place in nitrate-grown cells of the P_{II}-null mutant MP2 and of the MP2-S strain, as in the wild-type. This was confirmed by RNA/DNA hybridization experiments. Fig. 2 shows hybridizations performed with two probes, *nrtA* and *nrtD*, corresponding to two components of the nitrate/nitrite permease complex [2]. As previously observed [5,7], the transcripts corresponding to the *nir-nrtABCD-narB* operon were rapidly degraded generating hybridization signals ranging in size from 0.25 to >7 kb with molecules accumulating in the regions that correspond to ribosomal RNAs. In the P_{II}-null mutant MP2 and the MP2-S cells adapted to nitrate, transcripts accumulated as in the wild-type. No transcripts, or only very low levels, were observed in ammonium cultures of the three strains. Repression by ammonium of the expression of the genes encoding the nitrate/nitrite permease complex was therefore operative in the P_{II}-null mutant MP2.

3.2. Comparison of the nitrate and nitrite uptake activities

The nitrate and nitrite uptake activities were determined in cells from the wild-type, the P_{II}-null mutant MP2 and the MP2-S strains adapted to nitrate. Wild-type cells could efficiently take up nitrate, but addition of ammonium to such cells drastically reduced the uptake within less than 10 min

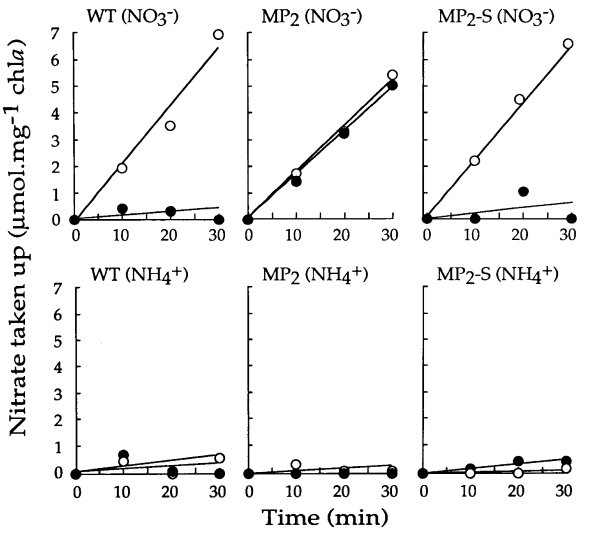

Fig. 3. Nitrate uptake by *Synechococcus* sp. PCC 7942 cells: wild-type (WT), P_{II}-null mutant (MP2) and complemented strain (MP2-S). Uptake assays were performed on nitrate- or ammonium-adapted cells incubated for 0–30 min in the presence of nitrate, with (closed symbols) or without (open symbols) addition of ammonium. Data are representative of five independent experiments.

(Fig. 3, top). In contrast, in the P_{II}-null mutant cells, nitrate uptake activities remained high both without and upon addition of ammonium. The MP2-S strain behaved like the wild-type *Synechococcus* sp. PCC 7942 strain. The wild-type *glnB* gene inserted in the genome of the P_{II}-null mutant could therefore functionally complement the mutation of strain MP2. These results clearly indicated that P_{II} controls the nitrate uptake activity in response to a short-term exposure to ammonium. As expected from the repression by ammonium of the *nir-nrtABCD-narB* operon, none of the strains adapted

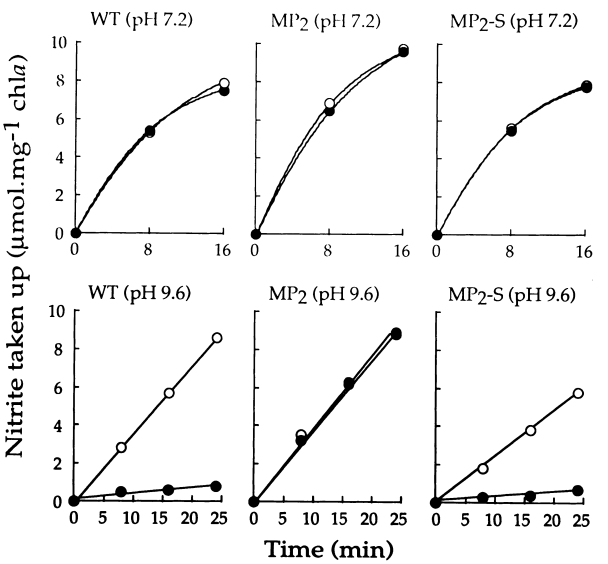

Fig. 4. Nitrite uptake by *Synechococcus* sp. PCC 7942 cells: wild-type (WT), P_{II}-null mutant (MP2) and complemented strain (MP2-S). Uptake assays were performed on nitrate-adapted cells incubated for 0–30 min in the presence of nitrite with (closed symbols) or without (open symbols) addition of ammonium. Data are representative of three independent experiments.

to growth in the presence of ammonium took up nitrate (Fig. 3, bottom).

The three strains adapted to nitrate showed similar nitrite uptake activities with and without ammonium when determined at pH 7.2, at which nitrite enters the cells by diffusion as nitrous acid (Fig. 4, top). Diffusion of nitrous acid decreases, and active transport of nitrite ions increases, as the pH of the medium is raised [24]. At pH 9.6, nitrite ions can be actively transported into the cells through the same permease operating active transport of nitrate [3,4]. Similar to what occurred for nitrate uptake, a strong short-term inhibition by ammonium of nitrite uptake was observed in both the wild-type and the complemented MP2-S cells but not in the P_{II}-null mutant MP2 which displayed a high nitrite uptake activity both in the presence and in the absence of ammonium (Fig. 4, bottom).

4. Discussion

The results presented in this report demonstrate that, in *Synechococcus* sp. PCC 7942, the P_{II} protein is required for the short-term inhibition by ammonium of nitrate/nitrite uptake known to occur in cyanobacteria [1]. Indeed, nitrate-grown cells of the P_{II}-null mutant MP2 do take up nitrate and nitrite even in the presence of ammonium, and the short-term inhibition by ammonium can be restored by complementation with the wild-type *glnB* gene. The P_{II} protein can be differentially phosphorylated in response to N availability in the wild-type cells. P_{II} being predominantly unphosphorylated under the physiological conditions that determine the inhibition of nitrate/nitrite uptake, this form of the protein is most likely the one involved in this process. It is known that the inhibition by ammonium of nitrate/nitrite uptake takes place via a posttranscriptional control of the activity of the nitrate/nitrite permease [24,25], but an additional effect on nitrate reductase can be considered [5]. The fact that nitrate/nitrite uptake proceeds freely in the P_{II}-null mutant MP2 in the presence of ammonium indicates that P_{II} would be required for the regulation of both the permease and the nitrate reductase. Whether P_{II} directly binds to one of the components of the NrtABCD transport system and to nitrate reductase or acts indirectly, e.g. by catalyzing the dephosphorylation of another partner in the control system in a way similar to the dephosphorylation of NtrC by NtrB in enterobacteria [8], is currently unknown. The C-terminal domain of NrtC has recently been shown to be involved in the inhibition by ammonium of the nitrate/nitrite transporter [5]; the possibility that this domain represents a target site for P_{II} is worth being examined.

In the P_{II}-null mutant MP2, the activities of the nitrate and nitrite reductases are high in the absence of ammonium [11]. The expression of the corresponding operon that includes not only *nir* and *narB*, but also the *nrtABCD* genes encoding the permease complex, is repressed by ammonium (see Fig. 2) and thus remains under the control of the transcriptional activator NtcA in the P_{II}-null mutant MP2. The P_{II} protein is therefore not required for the transcriptional regulation of N assimilation mediated by NtcA in *Synechococcus* sp. PCC 7942 or some other compounds can substitute for it in the control of NtcA activity. In *E. coli*, GlnK, a P_{II}-like protein whose gene expression depends on the presence of UTase, the nitrogen regulator NtrC and the absence of ammonia, has been found which may ensure at least some of the functions of the P_{II} protein [26]. No second P_{II} protein has been found, however, in either *Synechocystis* sp. PCC 6803 whose complete genome sequence is known [27] or *Synechococcus* sp. PCC 7942 (Fig. 1).

In cyanobacteria, both CO_2 assimilation [28] and nitrate utilization [1] are photosynthetic processes and it was proposed that nitrate uptake would be controlled by a system integrating both N and C metabolisms, with CO_2 fixation products counteracting the inhibitory effects of certain ammonium derivatives [29]. This hypothesis was later confirmed by Romero et al. [30] who showed that a specific inhibition of CO_2 fixation by D,L-glyceraldehyde (DLG) led to a simultaneous inhibition of nitrate uptake, this dependence being released by treatment of the cells with MSX. On the other hand, Forchhammer and Tandeau de Marsac [11] observed that DLG and MSX had counteracting effects on the phosphorylation state of P_{II}, this protein being totally unphosphorylated with the former treatment and highly phosphorylated with the latter one. Moreover, nitrate assimilation was uncoupled from its dependence upon active CO_2 fixation in the P_{II}-null mutant MP2. These results are in good agreement with a role for CO_2 fixation in removing the inhibitory form of P_{II} in the wild-type cells. The P_{II} protein would thus be able to integrate information about N and C metabolisms for the regulation of the activity of nitrate/nitrite uptake in *Synechococcus* sp. PCC 7942.

In vitro, the phosphorylation of P_{II} is strictly dependent upon the addition of α-ketoglutarate to the reaction mixture which contains cell-free extract, ATP and purified P_{II} [31]. This is fully consistent with earlier physiological observations showing that some amino acids that would readily act as amino donors in transamination reactions to α-ketoglutarate were inhibitory for nitrate uptake in *Synechococcus* sp. cells in the absence of CO_2 [32]. Therefore, α-ketoglutarate, which has been shown to bind to P_{II} in the presence of ATP [33], is a key metabolite in the regulation of the phosphorylation state of P_{II} and of the nitrate uptake in cyanobacteria. These microorganisms, lacking the α-ketoglutarate dehydrogenase, have an incomplete tricarboxylic acid cycle and the α-ketoglutarate produced can only be used for biosynthetic purposes [28]. This property constitutes a major difference with the prokaryotes in which the P_{II} protein has been studied up to now and has to be taken into account in the integration of the regulatory pathways for N and C metabolisms in cyanobacteria.

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