Independence of carbon and nitrogen control in the posttranslational regulation of nitrate transport in the cyanobacterium *Synechococcus* sp. strain PCC 7942

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Abstract Nitrate transport by *Synechococcus* sp. strain PCC 7942 cells was inhibited by ammonium and by inhibitors of CO_2 fixation. Ammonium assimilation inhibitors, such as L-methionine D,L-sulfoximine, were known to prevent the negative effects of ammonium and of inhibitors of CO_2 fixation on nitrate uptake, leading to propose that CO_2 fixation was required to counteract the feed-back inhibition of nitrate assimilation. In NR-less mutants, L-methionine D,L-sulfoximine prevented the negative effects of ammonium on nitrate transport, but not always prevented those of inhibiting CO_2 fixation. The carboxy-terminal domain of the NrtC subunit of the nitrate transporter has recently been identified as a regulatory domain involved in N-control. The mutant strain NC2, constructed by deleting the 3′ portion of *nrtC*, showed high nitrate transport activity insensitive to ammonium but sensitive to inhibitors of CO_2 fixation. These findings indicate that the C-control and the N-control of nitrate transport are independent at both the physiological and the molecular level.

Key words: Nitrate transport; Posttranslational control; Cyanobacterium

1. Introduction

Nitrate is the major source of nitrogen for cyanobacteria. The rate-limiting step of nitrate assimilation is nitrate transport (NT) into the cell, an active process subjected to metabolic control [1–3]. Intracellular nitrate is reduced by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR), and the resulting ammonium is incorporated to carbon skeletons by the glutamine synthetase-glutamate synthase cycle, initiating amino acid biosynthesis. In the unicellular non-N_2-fixing cyanobacterium *Synechococcus* sp. strain PCC 7942, the genes encoding the NT system (*nrtA*, *nrtB*, *nrtC* and *nrtD*; [4]) as well as the enzymes NR (*narB*) and NiR (*nirA*) [5–7] are clustered together in the sequence *nirA-nrtABCD-narB* forming the so-called *nirA* operon, whose transcription is subjected to N-control, being inhibited by products of ammonium assimilation and activated by nitrate or nitrite [6–8].

Nitrate assimilation in *Synechococcus* is also subjected to posttranslational regulation by products of N and C assimilation [9]. Thus, nitrate uptake is completely and reversibly inhibited after addition of ammonium to the medium [10,11] and its rate is strictly coupled to that of CO_2 fixation [12,13]. The fact that ammonium assimilation inhibitors, such as L-methionine D,L-sulfoximine (MSX), relieved nitrate uptake from both the inhibition by ammonium and the requirement for CO_2 fixation had indicated a common link between the regulatory effects of ammonium and CO_2 assimilation on nitrate uptake. It had been proposed that CO_2 fixation products would be required to remove negative effectors derived from nitrate assimilation via ammonium [11–15]. The posttranslational control of nitrate assimilation in *Synechococcus* is exerted primarily on NT, which is inhibited within seconds after ammonium exposure, or by inhibiting CO_2 fixation at the level of the regeneration phase of the Calvin cycle with D,L-glyceraldehyde (DLG) [1,2].

The *nrtABCD* genes encoding the NT system of *Synechococcus* have been sequenced [4,16] and some of their products have been identified. NrtA is a major plasma membrane lipoprotein with an apparent molecular mass of 45–47 kDa, which binds nitrate or nitrite [17–19]. The deduced sequence of NrtB is similar to that of integral membrane components of ABC transporters, while the sequences of NrtC and NrtD are similar to those of the ATP-binding components of ABC transporters [4]. NrtC is a plasma membrane protein of 67 kDa essential for NT [20], and appears unique among the reported ATP-binding subunits of ABC transporters in that it consists of two distinct domains: the amino-terminal domain (amino acids 1–254), containing the ATP-binding motive, is strongly similar to NrtD, while the carboxy-terminal domain (amino acids 279–659) is 30% identical to NrtA [4]. Analysis of NT activity in mutants of *Synechococcus* sp. strain PCC 7942 expressing a truncated NrtC lacking its carboxy-terminal domain (NC2 and NC4 strains) has shown that this domain is involved in the ammonium-promoted inhibition of NT [20].

In this work, we analyzed the carbon control of NT in NR-deficient mutants of *Synechococcus* sp. strain PCC 7942 and in the NC2 strain and its NR-less derivative, NC4. The results showed that NT activity is subjected to C-control and N-control, but the regulatory mechanisms are distinct. Inhibition of ammonium assimilation by MSX prevented the ammonium inhibition but did not release NT from its strict carbon dependence. Moreover, the regulatory carboxy-terminal domain of NrtC, involved in the N-control of NT, is not involved in the C-control, which must be sensed by a different regulatory domain of the transporter.

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Abbreviations: DLG, D,L-glyceraldehyde; GA, glycolaldehyde; MSX, L-methionine D,L-sulfoximine; NiR, nitrite reductase; NR, nitrate reductase; NT, nitrate transport

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2. Materials and methods

2.1. Strains and growth conditions

Cells of *Synechococcus* sp. strain PCC 7942 and its mutant strain NC2, constructed by deletion of the 3′ portion of *nrtC* corresponding to the carboxy-terminal domain of NrtC [20], were grown photoautotrophically at 40°C under CO_2 sufficient conditions as described previously [20] with 20 mM $NaNO_3$ as nitrogen source. The mutant strains FM6 and NR1, which lack NR activity [20,21], and the NR-less derivative of NC2, strain NC4 [20], were grown as before except that 15 mM NH_4Cl was used as the nitrogen source [20]. Expression of the *nirA* operon in ammonium-grown cells (FM6, NR1 or NC4 strains) was induced by transferring the cells to media containing 4 mM $NaNO_2$. After 18 h under growing conditions, the cells were harvested by centrifugation, washed and resuspended in the buffer used for the assays. Chlorophyll *a* was determined after methanol extraction [22].

2.2. Determination of nitrate transport activity

NT activity was determined by measuring intracellular nitrate accumulation in acid lysates of cells subject to silicone-oil centrifugation [23], with the modifications described in [3]. Assays were carried out at 40°C under illumination in 25 mM Tricine-NaOH/KOH buffer, pH 8.3, containing 10 mM $NaHCO_3$, 20 μM $NaNO_3$ and an amount of cells equivalent to 33 μg of chlorophyll *a*/ml. Preincubations with MSX (1 mM), DLG (30 mM), or glycolaldehyde (GA, 30 mM) were performed as stated below. The assay was started by simultaneous addition of nitrate and illumination. Aliquots (0.3 ml) were withdrawn and rapidly transferred to 0.4 ml polyethylene microcentrifuge tubes over a silicone-oil layer (0.08 ml of a 2:1 (v/v) mixture of Versilube F50 (Serva) and silicone 14615-3 (Aldrich)), which was underlaid by 0.02 ml of 20 mM amidosulphuric acid in 2 M H_3PO_4. After rapid centrifugation (10 000 × *g*, 1 min) in a Beckman Microfuge 11, nitrate was analyzed in aliquots of the acid cell lysate by ion-exchange high pressure liquid chromatography followed by UV detection of nitrate at 210 nm [23].

2.3. Determination of nitrate uptake

Assays were performed at 40°C under illumination in 25 mM Tricine-NaOH/KOH buffer, pH 8.3, containing 10 mM $NaHCO_3$, 0.25 mM $NaNO_3$ and an amount of cells equivalent to 10 μg of chlorophyll *a*/ml. Preincubations with MSX (1 mM), DLG (30 mM), or GA (30 mM) were performed as stated below. The assay was started by simultaneous addition of nitrate, bicarbonate and illumination. Aliquots were withdrawn and, after rapid removal of cells by centrifugation, nitrate in the medium was determined as in [24].

3. Results

3.1. Carbon dependence of nitrate uptake in Synechococcus sp. strain PCC 7942

As previously reported in a closely related species of *Synechococcus* (strain PCC 6301, formerly *Anacystis nidulans*) [12], nitrate uptake was severely inhibited by preincubation of *Synechococcus* sp. strain PCC 7942 cells with DLG, a selective inhibitor of CO_2 fixation, for 15 min in the dark prior to the assay (Fig. 1). This illustrates the dependence of nitrate uptake upon active carbon assimilation. Also, as previously reported for *Synechococcus* sp. strain PCC 6301 [10,15], when the cells were preincubated 15 min with MSX, an inhibitor of ammonium assimilation, nitrate uptake was protected against the inhibition by ammonium (data not shown). However, when the cells were pretreated with both DLG and MSX, nitrate uptake was different depending on the preincubation conditions (Fig. 1). Thus, if the cells were preincubated with MSX for 15 min in the light prior to the preincubation with DLG for 15 min in the dark, nitrate uptake appeared practically insensitive to the negative effect of DLG and proceeded initially at rates similar to those of control untreated cells or of MSX-treated cells (Fig. 1, [12]), although as time proceeded

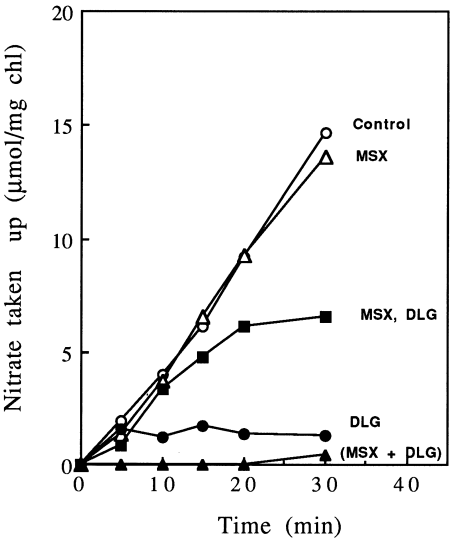

Fig. 1. Effects of DLG and MSX on nitrate uptake in *Synechococcus* sp. strain PCC 7942. All cells were preincubated in the assay medium for a period of 15 min in the light followed by another 15 min period in the dark, prior to starting the assay by nitrate and bicarbonate addition and illumination. No inhibitors were added to control cells (○). Treatment with DLG was performed by adding the inhibitor at the begining of the dark preincubation period (■, ●, ▲). Treatment with MSX was performed by adding the inhibitor either at the beginning of the light period (△, ■) or together with DLG at the begining of the dark period (▲).

the rate declined progressively and uptake stopped after 20 min of assay. This behavior had been interpreted as evidence of a common link between the N-control and the C-control of nitrate assimilation, since inhibition of ammonium assimilation seemed to release nitrate uptake from both the negative effects of ammonium and the requirement upon active CO_2 fixation [11–15]. In contrast, if the cells were preincubated during 15 min prior to the assay simultaneously with MSX and DLG, nitrate uptake was completely inhibited and no protection by MSX against DLG did appear (Fig. 1), speaking against the above interpretation.

GA is a potent inhibitor of CO_2 fixation in cyanobacteria acting at the level of transketolases [25]. As shown in Fig. 2, nitrate uptake by *Synechococcus* sp. strain PCC 7942 cells was also severely inhibited after 15 min preincubation with GA. When the cells were preincubated simultaneously with both MSX and GA, nitrate uptake was similarly inhibited (Fig. 2).

These results suggested that the carbon requirement of nitrate uptake may be maintained under conditions in which ammonium assimilation is inhibited, indicating that the C- and the N-control of nitrate assimilation may not be necessarily linked.

3.2. Carbon dependence of NT activity in a NR-deficient mutant of Synechococcus sp. strain PCC 7942

The rate-limiting step in nitrate assimilation is NT into the cell. By measuring nitrate uptake, both nitrate transport and reduction are jointly determined. NT activity can be specifically determined by measuring the intracellular nitrate accumulation under conditions in which nitrate reduction is not operative [1–3]. Fig. 3 shows the intracellular nitrate accumulation in a NR-deficient mutant of *Synechococcus* sp. strain PCC 7942, strain FM6. For an external nitrate concentration of 20 μM, FM6 cells accumulated up to 1 mM nitrate under

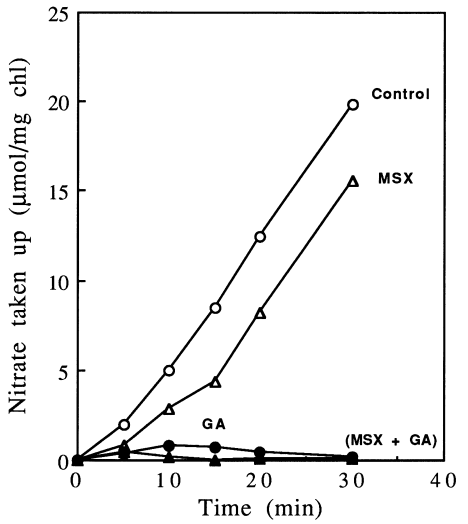

Fig. 2. Effects of GA and MSX on nitrate uptake in *Synechococcus* sp. strain PCC 7942. All cells were preincubated in the assay medium for 15 min in the dark prior to starting the assay by nitrate and bicarbonate addition and illumination. No inhibitors were added to control cells (○). Treatment with inhibitors was performed by adding either MSX (△), GA (●) or both (▲) to the cell suspension at the begining of the preincubation period.

illumination. Preincubation of the cells with DLG or GA for 15 min in the dark prior to the assay severely inhibited NT activity (Fig. 3A). When the cells were preincubated with MSX for 15 min in the light prior to the treatment with the corresponding inhibitor of CO_2 fixation, inhibition of NT by DLG was partially prevented or retarded (i.e. the initial rate of transport was similar to that of control cells [1], although the final intracellular nitrate levels were half of those of control cells), but inhibition by GA was not at all prevented (Fig. 3A). Furthermore, as observed for nitrate uptake (Figs. 1 and 2), when the cells were preincubated simultaneously with either MSX and DLG or MSX and GA, for 15 min in the dark prior to the assay, the intracellular nitrate accumulation observed in each case was extremely low and similar to that of DLG-treated or GA-treated cells, respectively (Fig. 3B), indicating that MSX is not always able to protect NT activity against the negative effects of suppressing CO_2 fixation. It should be noted that the inhibition of ammonium assimilation produced by MSX is equally effective in either preincubation conditions, as indicated by the fact that in either case MSX-treated cells were able to accumulate intracellular nitrate concentrations higher than 1 mM in the presence of ammonium (Fig. 3). It can be questioned why under certain conditions MSX may partially protect NT activity against the negative effects of DLG. Since carbon and nitrate assimilation pathways are connected at the level of ammonium assimilation, disturbance of the latter by MSX would inevitably affect the carbon status of the cell. Under the conditions in which MSX partially protected or retarded the DLG effect on NT activity (Fig. 3A, closed squares), the cells were preincubated for 15 min with MSX in the light (so they could fix CO_2 but could not reassimilate ammonium produced endogenously from protein turnover and photorespiration), and then for 15 min with DLG in the dark (during which, mobilization of glycogen reserves could take place). At the onset of NT, carbon compounds could be abundant and the effects of inhibiting CO_2 fixation with DLG might not be so rapidly or so intensely sensed by the nitrate transporter. When the cells were preincubated with both MSX and DLG for 15 min in the dark, they did not have the previous period of photosynthesis and the inhibition of CO_2 fixation might be more powerfully sensed. In MSX- and GA-treated cells, under either preincubation conditions, mobilization of glycogen during the 15 min dark period might also be hampered as a consequence of inhibition of transketolases of the oxidative pentose phosphate pathway, resulting in a more severe carbon deprivation than that of DLG treatment.

These results, therefore, indicate that inhibition of ammonium assimilation by MSX released NT activity from the negative effects of ammonium, but did not release NT from its carbon requirement.

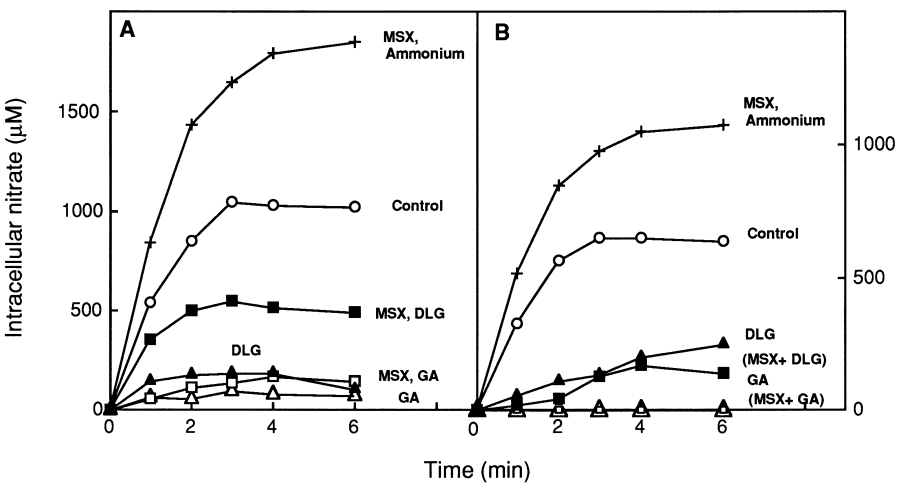

Fig. 3. Effects of DLG, GA and MSX on NT activity in the NR-less mutant strain FM6 of *Synechococcus* sp. strain PCC 7942. Cells in A were preincubated in the assay medium for a period of 15 min in the light, with or without MSX, followed by another 15 min period in the dark, with or without DLG or GA, prior to starting the assay by nitrate (20 μM) addition and illumination. Cells in B were preincubated in the assay medium during 15 min in the dark in the presence or absence of MSX, or DLG, or GA, or simultaneously with MSX and DLG or GA, prior to starting the assay by nitrate (20 μM) addition and illumination. When indicated, ammonium (0.25 mM) was added at zero time.

3.3. Carbon dependence of NT activity in nitrogen-deregulated mutants of Synechococcus sp. strain PCC 7942

We have recently reported the characterization of a mutant of *Synechococcus* sp. strain PCC 7942, strain NC2, constructed by removal of the 3′ portion of *nrtC* [20]. Cells of NC2, which express a truncated NrtC with an apparent molecular mass of 34 kDa, lacking the distinct carboxy-terminal domain, exhibit a very high NT activity insensitive to ammonium, showing that the carboxy-terminal domain of NrtC is a regulatory domain involved in the N-control of NT [20]. As Fig. 4 shows, nitrate-grown NC2 cells were able to accumulate transiently very high intracellular nitrate concentrations in the absence of ammonium, although the intracellular nitrate was subsequently depleted presumably due to reduction by NR. In the presence of ammonium, NC2 cells accumulated and maintained even higher intracellular nitrate levels, illustrating that the removal of the carboxy-terminal regulatory domain of NrtC resulted in an ammonium-insensitive NT activity. The maintenance of the intracellular nitrate levels in this situation also suggests that ammonium inhibits NR activity [20]. Interestingly, when NC2 cells were pretreated with GA and assayed either in the absence or in the presence of ammonium, NT activity was completely inhibited, indicating that the C-control is operative in this N-deregulated mutant (Fig. 4). Furthermore, a NR-less derivative of NC2 (strain NC4), constructed by inactivating *narB*, also accumulated intracellular nitrate in the presence of ammonium, although at lower rates than in its absence, indicating ammonium-insensitive NT activity [20]. Fig. 5 shows that the targeted NR-less mutant with truncated *nrtC*, NC4, accumulated and maintained intracellular nitrate concentrations similarly to the NR-less targeted mutant NR1 having wild-type *nrtC*. As it was the case for FM6 and NC2 cells, preincubation of NR1 and NC4 cells with GA resulted in complete inhibition of NT activity (Fig. 5).

These data clearly indicate that the regulatory carboxy-terminal domain of NrtC involved in the N-control of NT activity is not involved in the C-control, and therefore the two mechanisms of negative and positive control act at different sites on the nitrate transporter.

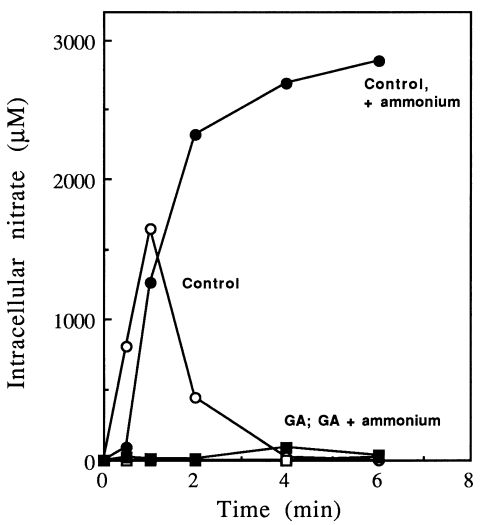

Fig. 4. Insensitivity to ammonium and sensitivity to GA of NT activity in the mutant strain NC2 of *Synechococcus* sp. strain PCC 7942, expressing truncated NrtC. Cells were preincubated in the assay medium for 15 min in the dark with (squares) or without GA (circles), prior to starting the assay by nitrate (20 μM) addition and illumination in the absence (open symbols) or presence (closed symbols) of ammonium (0.25 mM), added at zero time.

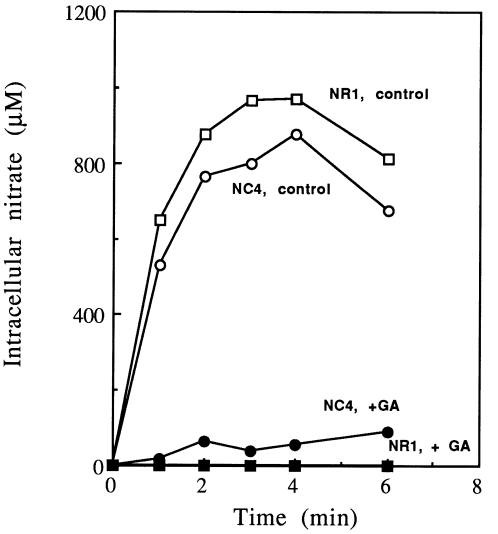

Fig. 5. Sensitivity of NT activity to GA in the mutant strains NC4 (NR-less derivative of NC2, with inactivated *narB*), and NR1 (with inactivated *narB*), of *Synechococcus* sp. PCC 7942. Cells of NC4 (circles) or of NR1 (squares) were preincubated in the assay medium for 15 min in the dark with (closed symbols) or without (open symbols) GA, prior to starting the assay by nitrate (20 μM) addition and illumination.

4. Discussion

To date, research on the control of nitrate assimilation in cyanobacteria has paid much more attention to the study and implications of the ammonium-promoted inhibition of NT, than to its carbon dependence. Negative N-control was thought to involve the primary regulatory logics of nitrate assimilation, i.e. when a reduced form of inorganic nitrogen is available in the medium, or organic N compounds accumulated into the cell, the uptake of oxidized forms such as nitrate or nitrite are inhibited in the short-term at the level of transport activity and in the long-term at the level of gene expression [15]. Within this framework, the carbon dependence of NT activity appeared as a manifestation of a secondary mechanism for counteracting the negative N-control: a flow of carbon compounds would prevent the accumulation of nitrate (ammonium) assimilation products acting as feed-back inhibitors of nitrate transport [1,10–15].

The results presented here confirm that NT activity in *Synechococcus* is strictly dependent on active CO_2 fixation, and show that this positive C-control of the rate-limiting step of nitrate assimilation proceeds independently of the negative feed-back regulation promoted by ammonium assimilation. First, the carbon dependence of nitrate uptake and NT activity is evident in cells unable to assimilate ammonium after MSX treatment, indicating that products of CO_2 fixation are required for active NT even in the absence of feed-back N-regulation (Figs. 1–3). Second, the regulatory carboxy-terminal domain of NrtC involved in the N-control of NT [20], is not involved in its C-control. Cells of NC2 and its NR-less

derivative NC4, which expressed truncated NrtC lacking this regulatory domain, have NT activity insensitive to ammonium [20] but highly sensitive to CO_2 fixation inhibitors (Figs. 4 and 5). The exact role played by the carboxy-terminal domain of NrtC on the ammonium-promoted inhibition of NT remains to be elucidated. In *Synechococcus* sp. strain PCC 6301, a plasma membrane protein kinase activity has been shown to be rapidly inactivated, whereas a soluble phosphatase activity is activated, after exposure of the cells to ammonium [26]. Because the ammonium-sensitive protein kinase activity phosphorylates in vitro several plasma membrane proteins including those comigrating with NrtA and NrtD, the regulation of NT activity may involve phosphorylation/dephosphorylation of the transporter [26]. The carboxy-terminal domain of NrtC, by being either a sensor of the dephosphorylation cascade triggered by ammonium or an effector-binding domain, plays an essential role in the response of NT to ammonium, but is not involved in the C-control, which must be sensed or effected by other regulatory domain(s). Another regulatory protein involved in N-control, the *glnB* gene product (P_{II} protein), has been identified in *Synechococcus* sp. strains PCC 7942 and 6301 [27–29]. P_{II} is phosphorylated at a seryl residue in nitrate-grown cells with full capacity for nitrate assimilation, and is rapidly dephosphorylated in vivo after exposure of the cells to ammonium or by inhibiting CO_2 fixation with DLG [27,28]. Although the targets of P_{II} signalling have not yet been identified, the phosphorylation state of the P_{II} protein seems to respond in the same way to N-excess or to C-deficiency. In vitro phosphorylation of the P_{II} protein is stimulated by 2-oxoglutarate [29]. Actually, it has been shown that P_{II} binds 2-oxoglutarate and ATP in a mutually dependent manner, and it has been proposed that only the liganded P_{II} could be phosphorylated, acting as a sensor for 2-oxoglutarate, the carbon skeleton primarily required for N assimilation [30]. The exact mechanism of the interplay between the positive C-control and the negative N-control of nitrate assimilation is complex and may involve different signal-transduction pathways and sensors. Much research is still needed to unravel their common features and the specific independent paths of C and N modulation of the nitrate transporter.

The independence of the C-control of NT (and hence of nitrate assimilation) with respect to the N-control in cyanobacteria, may have important implications in the understanding of the evolution of the posttranslational control of nitrate assimilation in photosynthetic organisms. In plants, the high capacity to store nitrate in vacuoles has shifted the main posttranslational control of nitrate assimilation from NT to NR, which is widely recognized as the rate-limiting step of the pathway. In plant leaves, NR activity is not sensitive to ammonium and responds to light-dark modulation, being active in the light and partially inactive in the dark [31]. Evidence has accumulated indicating that the light dependence of NR activity is in fact a reflection of a positive regulation by products of CO_2 fixation, i.e. hexose phosphate [32,33]. In most herbaceous species ammonium is primarily assimilated in the roots and is not transported to the leaf. Consequently, NR activity has conserved the sensitivity to C-control but not to N-control, which appears restricted to gene expression [34]. The independence of the mechanisms of C-control from those of N-control on the main rate-limiting step of nitrate assimilation in cyanobacteria may represent the origin of the molecular basis for the shift in regulatory strategies that govern the interplay between carbon and nitrogen assimilation in photosynthetic organisms.

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