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# Protein PII regulates both inorganic carbon and nitrate uptake and is modified by a redox signal in *Synechocystis* PCC 6803

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**Abstract In *Synechocystis* PCC 6803 as in other cyanobacteria, involvement of protein PII in the co-regulation of inorganic carbon and nitrogen metabolism was established based on post-translational modifications of the protein resulting from changes in the carbon/nitrogen regimes. Uptake of bicarbonate and nitrate in response to changes of the carbon and/or nitrogen regimes is altered in a PII-null mutant, indicating that both processes are under control of PII. Modulation of electron flow by addition of methyl viologen with or without duroquinol, or in a NAD(P)H dehydrogenase-deficient mutant, affects the phosphorylation level of PII. The redox state of the cells would thus act as a trigger for PII phosphorylation.**

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*Key words:* Protein PII; Cyanobacterium; Nitrate uptake; Bicarbonate uptake; Phosphorylation; Redox state

## 1. Introduction

Concerted regulation of nitrogen and inorganic carbon (Ci) metabolism in photosynthetic cells has long been studied. Dependence of nitrate uptake upon $CO_2$ fixation has been demonstrated in the cyanobacterium *Anacystis nidulans* by physiological approaches [1]. Understanding the molecular mechanism of the control for Ci and nitrogen assimilation has only recently been attempted. In the purple bacterium *Rhodospirillum rubrum*, Joshi and Tabita [2] have proposed a model involving a two-component system as the first step of a phosphorylation integrating the regulation of the three essential metabolic processes, Ci assimilation via ribulose-bisphosphate carboxylase, photosynthetic electron transfer and nitrogen fixation. A protein, PII, identified in a wide number of eubacteria in which it plays a central role in the regulation of nitrogen metabolism, was proposed to mediate the co-regulation of Ci and nitrogen metabolism in the cyanobacterium *Synechococcus* PCC 7942 [3].

The mode of action of protein PII, also known as GlnB, has been extensively studied in *Escherichia coli* ([4], for review). In this organism, the protein is covalently modified by uridylylation of a conserved tyrosyl residue (Tyr-51). Modification is via phosphorylation on the Ser-49 residue in the corresponding protein of *Synechococcus* PCC 7942 [3], although the protein also carries a conserved tyrosyl residue at position 51 [5]. In vitro experiments have shown that the PII proteins from *E. coli* and from *Synechococcus* PCC 7942, in spite of their different modification types, share the property of binding two small molecule effectors, ATP and 2-oxoglutarate, in a positive cooperative manner [6–8]. The cellular concentration of the latter metabolite has been postulated to be a crucial factor for the control of the modification level of PII in both organisms. Since cyanobacteria lack 2-oxoglutarate dehydrogenase, 2-oxoglutarate is used solely as carbon skeleton for the incorporation of nitrogen into organic molecules. It is thus critical to coordinate synthesis of 2-oxoglutarate with nitrogen availability. This interdependence raised the possibility that PII could play a role as a coordinator between 2-oxoglutarate production, thence Ci assimilation, and nitrogen metabolism. As a support for this hypothesis, it has been found that growth of *Synechococcus* PCC 7942 on ammonium as nitrogen source with a high Ci concentration (HC), or in the presence of a low Ci concentration (LC) with nitrate, led to the unphosphorylated form of PII [3,9,10].

In *Synechococcus* PCC 7942, the control of nitrogen metabolism by PII occurs at the nitrate and nitrite uptake step [9,11]. When PII is unphosphorylated, these processes are inhibited in the wild-type strain (WT) while in a PII-null mutant, uptake is active regardless of the nitrogen regime. A parallel action of PII on Ci uptake could be expected. Ci uptake in *Synechocystis* PCC 6803 is regulated according to the Ci regime of the cells [12]. At least two transport systems are involved. A low affinity system ($K_{m,app} = 300$ μM) is expressed constitutively, while an additional system with a higher affinity for Ci ($K_{m,app} = 60$ μM) is observed under LC conditions. The Ci uptake characteristics have thus been analyzed in parallel to the nitrate uptake activity in *Synechocystis* PCC 6803 in conditions corresponding to different levels of PII phosphorylation, i.e. by growing the cells under different Ci and nitrogen regimes, and in a PII-null mutant.

When PII was first identified in the cyanobacteria *Synechococcus* PCC 6301 and 7942, the authors suggested that the extent of reduction of the plastoquinone (PQ) pool could act as a trigger in the control of PII phosphorylation [13,14]. However, actual evidences were lacking in support of such a hypothesis. In the present work, this hypothesis was challenged by analyzing the PII phosphorylation level in two situations leading to modified electron flow: (1) by the establishment of an artificial electron transfer route, to methyl viologen (MV), leading to the oxidation of the interchain carriers PQ and cytochrome $b_6f$; re-reduction of the PQ pool can be achieved by subsequent addition of duroquinol;

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(2) by use of a mutant of *Synechocystis* PCC 6803 deficient in NAD(P)H dehydrogenase (NDH1) activity, M55 [15]. This enzymatic complex is the first step of the respiratory pathway and one of the main carriers for cyclic electron flow around PS1 [16], both processes proceeding via the interchain carriers. Mi et al. [17] have compared the extent of reduction of the quinone $Q_A$ mediated by the NAD(P)H generated by respiratory substrates and/or PS1 cyclic electron transfer between the mutant M55 and the WT. The mutant shows a low reduction level of $Q_A$. Adaptation of *Synechocystis* PCC 6803 to a high salinity leads to the induction of an alternative route for cyclic electron transfer [18]. This treatment partly restores the impairment of respiratory and PS1 cyclic electron transfer in the M55 mutant [19].

## 2. Materials and methods

### 2.1. Strains and growth conditions

The WT *Synechocystis* PCC 6803, the NDH1-deficient mutant M55 (kindly provided by T. Ogawa) and the PII-null (ΔPII) mutant (see below) were grown as in [12]. The nitrogen source was either $NH_4Cl$ (5 mM) or $NaNO_3$ (35 mM), as indicated. HEPES (20 mM, pH 8.5) was added when ammonium was used as nitrogen source. The Ci regimes corresponded to low (LC, $CO_2$ provided by the air) or high (HC, 12 mM $NaHCO_3$) Ci concentrations, with additional bubbling with air enriched with 5% v/v $CO_2$ when specified. Standard and high salt conditions were adjusted to 50 and 550 mM NaCl in the medium, respectively. When necessary, kanamycin or streptomycin were added at 100 and 30 μg/ml, respectively.

### 2.2. Cloning of the *glnB* gene and construction of a PII-null mutant in *Synechocystis PCC 6803*

A partial library of the *Synechocystis* PCC 6803 genome was constructed by cloning 3–4 kb DNA fragments resulting from digestion by *Hin*dIII into the pK19 vector (Boehringer). The *glnB* gene from *Synechococcus* PCC 7942 was used as a probe for hybridization with the *Synechocystis* partial library. A subclone, obtained as a *Hin*dIII-*Nco*I restriction fragment from the insert of a positive clone, was transferred into the pUCBM20 plasmid (Boehringer). This fragment, 1200 bp long, contained the whole *glnB* gene (336 bp) [20,21].

A PII-null mutant, ΔPII, was constructed by deletion of an internal 26 bp *Hin*cII fragment and insertion, in its place, of the streptomycin/spectinomycin resistance interposon. The resulting *Hin*dIII-*Nco*I fragment carrying the interposon was excised from plasmid pUCBM20 and cloned into the pK19 vector which carries a kanamycin resistance gene. The latter construction allowed screening for a double recombination (selection for kanamycin sensitive, streptomycin/spectinomycin resistant transformed clones) in the *Synechocystis* WT *glnB* gene, replaced by the mutant allele. Replacement and complete segregation of the mutant allele were confirmed by Southern blot analysis.

### 2.3. Molecular genetic techniques

The standard techniques were performed according to Sambrook et al. [22] or the suppliers recommendations.

### 2.4. Nitrate uptake

Nitrate uptake assays were carried out according to Lee et al. [11].

### 2.5. Bicarbonate uptake

Bicarbonate uptake determination was performed as in Bédu et al. [12] with $H^{14}CO_3^-$ in a concentration range from 0.1 to 0.5 mM. Nitrocellulose filters (Millipore), 0.45 mm pore size, were used for cell filtration.

### 2.6. PII phosphorylation

The phosphorylation state of PII was analyzed as described by Forchhammer and Tandeau de Marsac [3], by non-denaturing polyacrylamide gel electrophoresis and immunoblotting with antibodies raised against the PII protein from *Synechococcus* PCC 7942. When indicated, cells were incubated, under HC conditions, with MV (100 μM) for 5 min, with or without further addition of duroquinol (500 μM) for 3 min, before collecting the samples.

### 2.7. ATP concentration

ATP concentration was determined by the luciferin-luciferase assay (Boehringer) as in Jeanjean et al. [18], in a Chem Glow photometer (Aminco).

### 2.8. Duroquinol preparation

Reduction of duroquinone was performed according to Rothery et al. [23].

## 3. Results

### 3.1. Involvement of protein PII in the adaptation to various nitrogen and Ci regimes

The involvement of PII in the adaptability of the cells to varying nitrogen and Ci regimes was tested by comparing the growth rates of a PII-null mutant, ΔPII, to those of the WT. Application of the LC or HC regimes in the presence of nitrate led to lower growth rates for the mutant than for the WT (Table 1). The ratio of the mutant/WT generation times increased with the Ci concentration in the medium. When cells were grown under HC conditions with additional bubbling of air with 5% $CO_2$, the ratio reached a value of 2.3.

When ammonium replaced nitrate, whether in LC or HC conditions, the mutant showed a 20 h lag phase before reaching a growth rate almost identical to that observed in the nitrate regime (not shown). This lag phase was not observed for the WT.

### 3.2. Regulation of nitrate and bicarbonate uptake by PII

In nitrate-grown WT cells, the nitrate uptake activity was reduced in LC-adapted cells as compared to HC-adapted ones (Fig. 1). When the assays were performed in the presence of ammonium, nitrate uptake was totally blocked. In the ΔPII mutant, the uptake activity displayed the same level in all conditions tested and no inhibition by ammonium was observed. The uptake activity was lower than that of WT cells grown under HC conditions, but higher than that of LC-grown WT cells.

Under LC growth conditions, the kinetics of bicarbonate uptake were identical for the ΔPII mutant and the WT, with both the high and low affinity systems detected (Table 2), whatever the nitrogen regime of the cells. However, a dramatic change was observed when the mutant cells were grown under HC conditions: the high affinity transport activity, with an apparent $K_m$ similar to that observed in WT cells adapted to LC conditions, was detected even when the cells were grown in HC. In other words, the ΔPII mutant permanently showed a high affinity transport activity, i.e. it seemed to react as if receiving an LC concentration signal whatever the actual Ci concentration in the growth medium.

### 3.3. Nitrogen/Ci regimes and PII phosphorylation

The phosphorylation pattern of the *Synechocystis* PCC

Table 1
Generation times (hours) for WT and ΔPII strains grown in nitrate under different Ci regimes

| Ci regime | WT | ΔPII | ΔPII/WT[a] |
|---|---|---|---|
| LC | 16 | 24 | 1.5 |
| HC | 10 | 20 | 2 |
| HC+5% $CO_2$ | 6 | 14 | 2.3 |

The Ci regimes are defined in Section 2.
[a]Ratios of mutant to WT generation times.

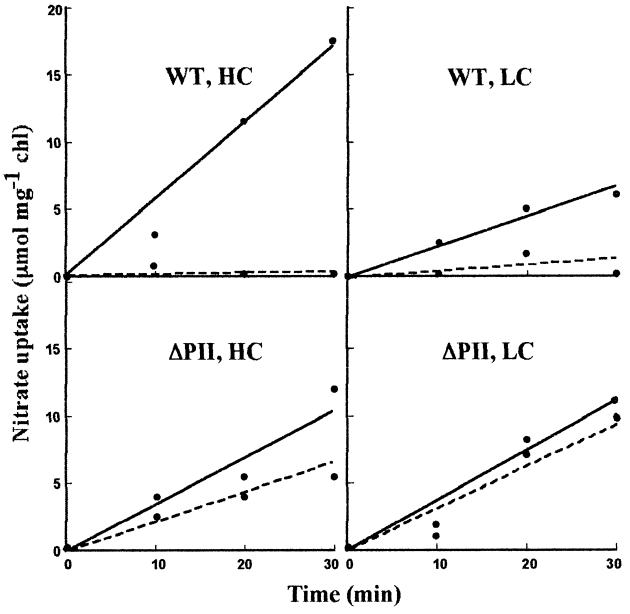


Fig. 1. Nitrate uptake by WT and ΔPII cells adapted to nitrate and to HC or LC. Assays were performed in the presence (—) or absence (- - -) of 0.5 mM ammonium. Data are representatives of three separate experiments.

6803 PII protein followed that of its homologue in *Synechococcus* PCC 7942. The native trimeric PII protein was phosphorylated in nitrate-grown cells while no phosphorylation occurred in cells grown with ammonium as nitrogen source (Fig. 2). LC growth conditions also induced a dephosphorylation of protein PII whatever the nitrogen source available.

### 3.4. Cellular redox state and PII phosphorylation

Because the internal redox state could play a role as signal for the phosphorylation of PII, modification of the protein was also analyzed in cells exposed to conditions that impair the photosynthetic electron flow. In a first approach, incubation of the cells with the artificial electron acceptor MV induced PII to remain unphosphorylated even when the cells were grown in nitrate and HC (Fig. 2). Subsequent incubation with duroquinol partially restored phosphorylation of PII. Treatment of cells with only duroquinol did not change the phosphorylation pattern of PII (data not shown). To distinguish between possible effects of changes in ATP levels or in redox state of the interchain carriers on PII phosphorylation, the ATP content was determined after either MV or MV+duroquinol treatments. No significant variations of the intracellular ATP concentrations occurred after either treatment (Table 3A).

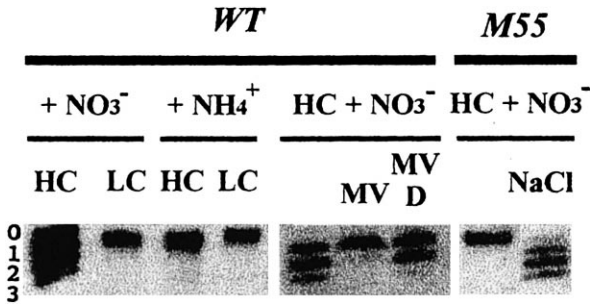


Fig. 2. Dependence on various metabolic conditions of protein PII phosphorylation level in WT and M55 mutant cells. WT cells were grown with either nitrate or ammonium, under HC or LC regimes. 0 corresponds to the unphosphorylated form of PII; 1, 2, 3 correspond to one, two or three phosphorylated subunits of the PII trimer. Samples grown under HC conditions were incubated with MV (100 μM) for 5 min (lane MV), followed by incubation with duroquinol (D) (500 μM) for 3 min (lane MV+D). M55 cells were grown in HC/nitrate, in standard or high (lane NaCl) NaCl concentrations.

In a second approach, the PII modification level was analyzed in the NDH1-deficient mutant M55, grown in the presence of standard or high salt concentrations. When the cells were grown in HC/nitrate conditions with a standard salt concentration, PII was not phosphorylated in the M55 cells (Fig. 2), whereas the protein was phosphorylated in the WT strain cultivated under the same conditions. Adaptation of the mutant cells to the high salinity, conditions that restore their energetic capacity, restored a level of PII phosphorylation similar to that of the WT (Fig. 2). Again, there were no significant differences in ATP content between WT and M55 cells grown in HC/nitrate adapted to either standard or high salinity (Table 3B).

## 4. Discussion

Our results provide evidence for the involvement of PII in the coordination of carbon and nitrogen metabolism through the control of both nitrate and bicarbonate uptake processes. However, the mechanisms of regulation by this protein are different for each anion. The nitrate uptake activity is correlated with the level of phosphorylation of PII, as was established in *Synechococcus* PCC 7942 [11]. In cells incubated in nitrate/HC, PII phosphorylation was correlated with active nitrate uptake and this activity was inhibited when the protein was non-phosphorylated, i.e. when the cells were grown in ammonium. However, the level of PII phosphorylation cannot be the only factor controlling nitrate uptake, as shown by the

Table 2
Apparent affinity constants ($K_{m,app}$, μM) for bicarbonate transport in WT and ΔPII strains grown in HC or LC with nitrate or ammonium

| Strain | Ci regime | HC | | LC |
|---|---|---|---|---|
| | Nitrogen source | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| WT | Low affinity | 300 ± 60 | 320 ± 55 | 350 ± 60 |
| | High affinity | ND[a] | ND | 60 ± 12 |
| ΔPII | Low affinity | 337 ± 20 | 264 ± 50 | 330 ± 40 |
| | High affinity | 49 ± 12 | 59 ± 12 | 62 ± 10 |

Data are mean values of five separate experiments.
[a]ND: not detectable.

Table 3
Intracellular ATP contents (nmol/mg chlorophyll)

| A | | B | | |
|---|---|---|---|---|
| Control | 244 ± 26 | Salinity | WT | M55 |
| MV | 212 ± 20 | Standard | 250 ± 23 | 230 ± 17 |
| MV+D | 270 ± 24 | High | 240 ± 29 | 210 ± 13 |

(A) WT cells grown in HC/nitrate medium, after 5 min incubation with MV (100 μM), followed by 3 min incubation with duroquinol (D) (500 μM). (B) WT and M55 cells grown under HC/nitrate conditions in standard (50 mM NaCl) or high (500 mM NaCl) salinity. All data are mean values of at least four separate experiments.

results obtained for LC-grown cells: though PII was not phosphorylated in these conditions, nitrate uptake was only partly (50%) inhibited. The implication of protein PII in the regulation of nitrate uptake was confirmed by the phenotype of the ΔPII mutant, in which the uptake activity is maintained whatever the growth conditions of the cells, in particular under the LC or ammonium regimes. These results indicate that control of nitrogen metabolism, at least at the level of nitrate uptake, involves PII in a process responding to both nitrogen and Ci metabolism.

PII is also crucial for the control of Ci transport since the high affinity system became constitutive in the ΔPII mutant. The protein could be implicated in a negative regulation of this transport system. However, in contrast to the control of nitrate uptake, regulation of the bicarbonate high affinity transport system was not at all affected by the level of phosphorylation of PII. This system was active in WT cells under LC/nitrate conditions, in which PII is unphosphorylated, but it was inactive in HC/ammonium-grown cells, which also maintain PII in its unphosphorylated form. Irmler et al. [24] have shown that PII binds 2-oxoglutarate and ATP. While the cellular ATP content remains stable, the 2-oxoglutarate intracellular concentration is expected to fluctuate with the growth conditions. In particular, it should be low under the LC condition. A function of PII as 2-oxoglutarate sensor was therefore proposed. The 2-oxoglutarate-PII complex could be responsible for the regulation of the high affinity Ci transport system. In the ΔPII mutant, the absence of this complex, and thus of the 2-oxoglutarate signal, would mimic a LC situation and consequently activate or derepress the high affinity transport system even in cells submitted to a HC regime. This LC-like signal in ΔPII mutant cells could also explain the disturbance of their growth rate when provided with a high concentration of Ci.

It has previously been shown that the redox state of the cells affected the level of *glnB* gene expression in *Synechocystis* PCC 6803 [20]. The hypothesis of a correlation between the level of PII phosphorylation and the redox state of the interchain carriers was supported by the analysis of the state of PII when photosynthetic and/or respiratory electron flows were impaired. Two conditions led to the unphosphorylated form of PII, unexpected in HC/nitrate-grown cells: (1) the establishment of an artificial routing of the electrons to MV or (2) the decrease of the respiratory and PS1 cyclic electron transfer activities resulting from the NDH1 deficiency in the mutant M55 cells [16]. Both conditions decrease the extent of reduction of the interchain electron carriers. Restoration of reduction of these carriers by concomitant addition of MV and duroquinol or by growth of M55 cells in high salinity [20] also restored the expected modification of PII. The intracellular ATP concentration is not significantly modified under these different experimental conditions.

The terminal electron acceptor MV short-circuits the reduction of NADP and consequently prevents Ci assimilation. The non-phosphorylation of PII in cells incubated with this acceptor might be a consequence of a lack of 2-oxoglutarate, as proposed by Irmler et al. [24]. Under this treatment, the addition of duroquinol partly restores PII phosphorylation. On the base of these results, we postulate that the modification of PII is driven by a signal related to the changes produced on the redox state.

The hypothesis of a redox signal implicated in the control of PII modification is not exclusive of the role of 2-oxoglutarate. On the contrary, our results light up the interdependence of both effectors on PII modification. Partial phosphorylation of PII when duroquinol is added with MV may be explained by a strong redox effect of duroquinol under conditions of low concentration of 2-oxoglutarate due to the inhibition of Ci assimilation by MV. Dephosphorylation of PII under limiting Ci may also be explained if the lack of 2-oxoglutarate has a dominant effect. However, the dephosphorylation of PII in cells under ammonium/HC regime is difficult to understand solely with regard to the intracellular redox level and the 2-oxoglutarate concentration.

Modifications of PII via phosphorylation are catalyzed by protein kinase and phosphatase still not identified [3,24]. Redox-dependent activity of protein kinases has already been proposed for enzymes located in the thylakoids of cyanobacteria [25] or of chloroplasts [26].

The present results suggest the following hypothesis: the 2-oxoglutarate-PII complex would be involved in the regulation of Ci uptake; this complex is determinant in the control of PII phosphorylation [8] involved in the regulation of nitrate uptake; either the PII kinase or the phosphatase could act as sensor in the control of PII modification by a redox signal.

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