Cell-type specific modification of PII is involved in the regulation of nitrogen metabolism in the cyanobacterium *Anabaena* PCC 7120

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Abstract In the heterocystous cyanobacterium *Anabaena* PCC 7120, the modification state of the signalling PII protein is regulated according to the nitrogen regime of the cells, as already observed in some unicellular cyanobacteria. However, during the adaptation to diazotrophic growth conditions, PII is phosphorylated in vegetative cells while unphosphorylated in heterocysts. Isolation of mutants affected on PII modification state and analysis of their phenotypes allow us to show the implication of PII in the regulation of molecular nitrogen assimilation and more specifically, the requirement of unmodified state of PII in the formation of polar nodules of cyanophycin in heterocysts.

Keywords: PII protein; Nitrogenase activity; Cyanophycin; *Anabaena* PCC 7120

1. Introduction

The signalling PII protein is widespread among bacteria, archaea and plants [1–3], where it plays central roles in the regulation of nitrogen assimilation. Depending on the organisms, PII proteins control different metabolic pathways and their regulatory functions involve various post-translational modifications (see below). Although there is no evidence for covalent modifications of plant PII protein [4], the eukaryotic protein exhibits [5] a property common with all studied prokaryotic PII, i.e., the formation of a complex with ATP and 2-oxoglutarate. In proteobacteria, under nitrogen-limiting conditions, PII is reversibly uridylylated [6] on a tyrosyl residue at position 51 [7]. In *Escherichia coli*, PII is uridylylated by GlnD, a bi-functional uridylyltransferase/removase. Glutamine inhibits the transferase reaction and activates uridylyl-removase activity, such that the uridylylation status of PII depends on the cellular glutamine level [8]. The uridylylation status of PII controls via the NtrB/NtrC-two component system the expression of *glnA* and via adenylyltransferase (GlnE), the activity of glutamine synthase (for a review, see [9]). In several diazotrophic proteobacteria, the PII protein GlnB or its various GlnK paralogues are implicated in the regulation of nitrogen fixation [10]. GlnK can regulate NifL [11] which mediates the expression of the *nif* genes by interacting with NifA, a specific transcription factor for *nif* genes. In *Rhodospirillum rubrum*, GlnB and GlnK are also involved in the reversible ADP-ribosylation of nitrogenase [12,13]. In unicellular cyanobacteria, except for the marine strain *Prochlorococcus marinus* PCC 9511 [14], protein PII is modified by phosphorylation on a seryl residue at position 49 [15]. The phosphorylation state of the protein is dependent on the intracellular concentration of 2-oxoglutarate reflecting the nitrogen/carbon status of the cells [15,16] and may respond to the redox level of the cells [17,18]. In *Synechocystis* PCC 6803, the binding of 2-oxoglutarate to PII strongly inhibits the dephosphorylation of the protein mediated by the PphA phosphatase [19], while it stimulates the phosphorylation of PII by a protein kinase that has still not been identified [20]. In *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803, PII is involved in the regulation of nitrate and bicarbonate uptake [21,17]. However, no interaction between PII and nitrate or bicarbonate transporters has been shown to date. Only recently, a direct interaction between PII and *N*-acetyl-L-glutamate kinase, the key enzyme of the arginine biosynthetic pathway, has been clearly demonstrated in *Synechococcus* PCC 7942 [22] and this interaction is conserved among the organisms performing oxygenic photosynthesis [23].

In filamentous cyanobacteria capable of heterocyst differentiation, the role of PII is not clearly defined up to now, mainly due to the failure to create PII deficient mutants [24]. In *Calothrix* PCC 7504 [25], PII modifications depend mainly on the growth phase of the cells. In this paper, we show for the first time differential modification of PII in heterocysts and vegetative cells under nitrogen-fixation conditions. Moreover, evidences are presented for the involvement of PII in the regulation of nitrogen metabolism, including nitrogenase activity and cyanophycin distribution between vegetative cells and heterocysts.

2. Materials and methods

2.1. Strains and culture conditions

The wild-type *Anabaena* PCC 7120 and its mutants were grown in BG11 medium as previously described [26], under three different

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nitrogen source conditions, 17.5 mM NO_3^- or 5 mM NH_4^+ or molecular nitrogen.

2.2. Construction of a PII-S_{49}A mutant

Site-directed in vitro mutagenesis was done using the synthetic oligonucleotide 5′-CGCTATCGCGGCCTGAGTACACTGTG-3′ to mutate the seryl (TCT) 49 residue in *glnB* to an alanyl (GCT) residue. Mutated *glnB* was cloned into *Xho*1/*Pst*1 sites of the conjugative vector pRL271 [27]. Conjugation was carried out according to [27]. Replacement of wild-type *glnB* was controlled by sequencing of the gene, amplified by PCR, using chromosomal DNA of the recombinant as template, and analysing the modification state of the PII protein.

2.3. Cloning, insertional inactivation of the alr4516 gene and conjugation procedures

A genomic region including the *alr4516* gene (*prpS*) was obtained by PCR amplification and cloned into *Xho*I/*Spe*I sites of the conjugative vector pRL271 [27]. The coding region of *prpS* was interrupted by the insertion of the neomycin resistance gene cassette derived from the plasmid pRL278 [27] into the *Xmn*I site, at position 241 following the ATG start codon. The construct was then transferred into *Anabaena* PCC 7120 by conjugation to obtain the Δ*prpS* mutant through homologous recombination.

2.4. Immunoblot analysis

PII modifications were analysed using native gel and immunoblot, according to [28]. Nitrogenase modification was analysed according to [29], after separation of protein lysate on SDS–PAGE, using 18% acrylamide and 3 M urea, and immunoblot with antibodies raised against the NifH subunit of nitrogenase from *Anabaena variabilis*.

2.5. Other techniques

Heterocysts were isolated according to the methods of [30]. Electron microscopy techniques and cell preparations have been described in [31]. Nitrogenase activity was measured by the acetylene reduction assay, as described in [29]. Cyanophycin was isolated and arginine concentration determined according to [32,33].

3. Results

3.1. Differential modifications of PII in heterocysts and vegetative cells

The PII protein is known to be a homotrimer, and the modification state of PII namely the phosphorylation of one, two or three subunits can be revealed by non-denaturing gel electrophoresis and immunoblot analysis [28]. As shown in Fig. 1, PII from *Anabaena* PCC 7120 is apparently phosphorylated or unphosphorylated in cells adapted to nitrate or ammonium regime, respectively, as already observed in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942.

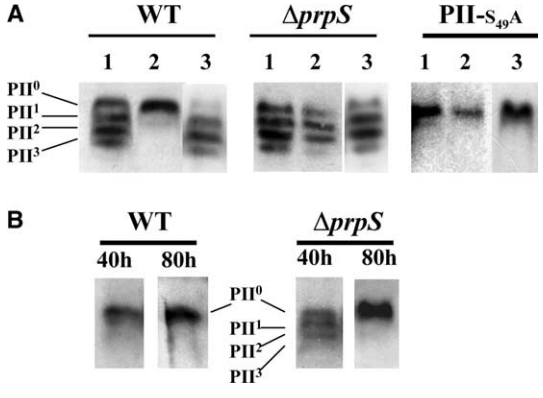

Fig. 1. PII modifications in wild-type strain (WT), Δ*prpS* and PII-S_{49}A mutants. PII^0 corresponds to the unphosphorylated form of PII; PII^1, PII^2 and PII^3 correspond to the number of phosphorylated monomers in the PII trimer. (A) Growth under nitrate (1), ammonium (2), or molecular nitrogen (3); (B) PII in heterocysts isolated after 40 and 80 h of molecular nitrogen regime.

After 24 h under combined nitrogen starvation, cell differentiation is effective and isolation of heterocysts away from vegetative cells is possible. Interestingly, analysis of the two cell type extracts shows that PII is phosphorylated in vegetative cells, while non-phosphorylated in the heterocysts (Fig. 1).

3.2. Isolation of Anabaena mutants affected on PII modifications

To understand the function of PII in *Anabaena*, we undertook the construction of several types of PII mutants. Attempts to isolate a PII-null mutant, constructed by insertional mutagenesis, failed despite the variety of selecting media tested (different concentrations of inorganic carbon, different nitrogen sources or different light intensities). The *glnB* gene encoding PII is likely to be essential as shown in another heterocystous cyanobacterium *Nostoc punctiforme* ATCC 29133 [24].

There are two possibilities to obtain mutants that are affected in PII modification: either by directed mutagenesis on the phosphorylation site, the seryl 49 residue, or by mutagenesis of the enzymes responsible for PII phosphorylation/dephosphorylation. Mutants in which the serine 49, putative site of phosphorylation, is changed into alanine or glutamate has been undertaken. Such mutants have been shown to mimic unphosphorylated and phosphorylated forms of PII with respect to the regulation of nitrate uptake in *Synechococcus* PCC 7942 [21].

A PII-S_{49}A mutant was isolated with complete segregation as controlled by DNA sequencing of the corresponding *glnB* gene, after PCR amplification, using total DNA from the exconjugant as template. As expected, PII is permanently unmodified in this mutant, whatever the nitrogen regime of the cells be (Fig. 1). The same approach was used to construct a PII-S_{49}E mutant, but no complete segregation has been obtained.

A mutant inactivating a putative phospho-PII phosphatase and in which PII remains phosphorylated was successfully obtained. In the genome of *Anabaena* PCC 7120 [34], the open reading frame *alr4516*, hereafter designated as *prpS*, encodes a protein presenting 60% identity with PphA (*sll1771*), the PP2C type protein phosphatase from the unicellular strain *Synechocystis* PCC 6803 responsible for PII dephosphorylation [19]. *prpS* is part of a cluster including three genes with the same organisation as the gene cluster of *sll1771* in *Synechocystis* PCC 6803. A high sequence similarity between the corresponding genes in the two clusters is observed. Altogether, these observations are good arguments to consider *alr4516* as the gene coding the putative phosphatase of PII in *Anabaena* PCC 7120. A null mutant, named Δ*prpS* in which *prpS* was inactivated, was isolated. Segregation of the Δ*prpS* mutant was controlled by PCR, showing a unique amplified fragment, 1.9 kb long, which corresponds to the *alr4516* gene interrupted by a neomycin resistant cassette (data not shown).

In the Δ*prpS* mutant, PII is phosphorylated even in cells grown under ammonium regime as well as in heterocysts (Fig. 1). These observations strengthen the hypothesis of PrpS being the phosphatase responsible for PII dephosphorylation. However, after 3 days under diazotrophic growth, PII becomes dephosphorylated in the heterocysts as shown in Fig. 1B. This

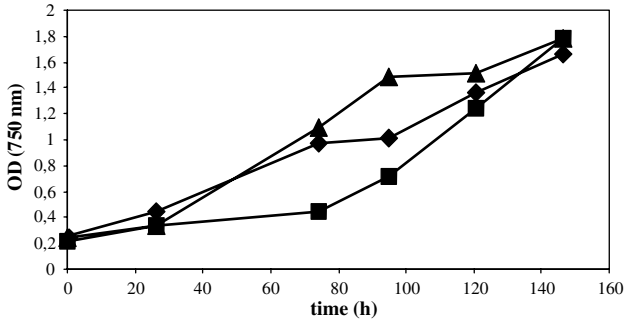

Fig. 2. Growth rate under N_2 regime, (◆) wild type strain, (■) Δ*prpS* and (▲) PII-S_{49}A mutants, measured by spectrometry at 750 nm.

delayed demodification of PII may be attributed to the activation of another protein phosphatase and/or the inactivation of the PII kinase activity.

3.3. Mutant growth rate according to the nitrogen source

Growth rates of Δ*prpS* and PII-S_{49}A mutants and wild-type strain were compared under different nitrogen regimes, with nitrate or ammonium or without any combined nitrogen source. No difference was observed between the three strains under combined nitrogen growth conditions (data not shown). When molecular nitrogen is the sole nitrogen source, growth Δ*prpS* of Δ*prpS* mutant exhibits a lag phase during at least 3 days (Fig. 2). However, during this period of time, we observed the formation of heterocysts along the filaments, presenting a pattern similar to what was observed in the wild type. These results suggest that *prpS* or the unmodified state of PII is required for adaptation of the filaments to diazotrophic growth conditions but not for initiation of the differentiation process.

3.4. Characteristics of nitrogenase activity in the mutants and the wild-type strain

The level of nitrogenase activity from the Δ*prpS* mutant (Fig. 3A), measured during the first days of adaptation to diazotrophic growth conditions, is only 15–20% of the enzyme activity measured on the wild-type strain and the PII-S_{49}A mutant. To test whether an impaired heterocyst cell wall is responsible for the decreased nitrogenase activity, mutant cells were incubated in a microaerobic environment. However, this treatment did not restore nitrogenase activity, arguing against an involvement of oxygen damage in decreased nitrogenase activity.

Expression level of the iron protein NifH and its modification state were analysed by immunoblot using antibodies against NifH from *Anabaena variabilis*. In both the wild-type and the Δ*prpS* mutant strains, the presence of the protein is detected after less than 16 h under combined nitrogen starvation. Moreover, the profile of the post-translational modification of the NifH protein is similar in the two strains, as shown in Fig. 3B. After 16 h of nitrogen starvation, the active form of nitrogenase, i.e., the lower band on the blot according to [35], is observed in both strains.

3.5. Cyanophycin distribution between vegetative cells and heterocysts

Mature heterocysts in the wild-type strain present polar nodules, corresponding to the accumulation of cyanophycin, a nitrogen reserve [36], in the regions adjacent to vegetative cells,

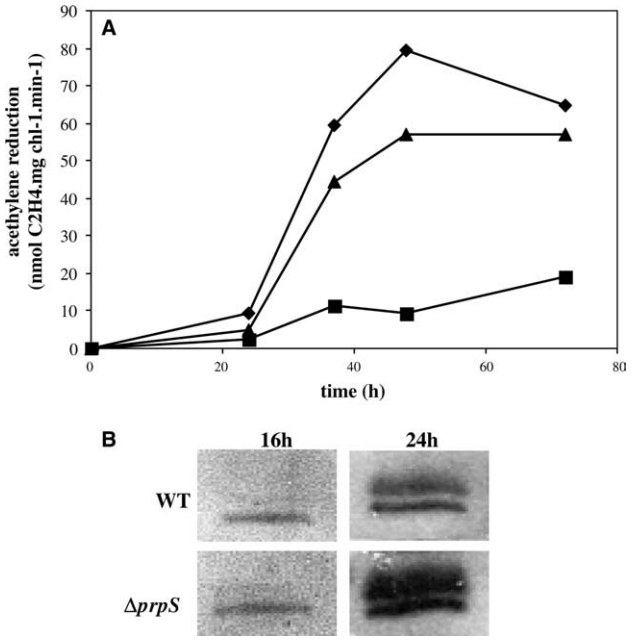

Fig. 3. (A) Nitrogenase activity on wild type (◆) and Δ*prpS* (■) and (▲) PII-S_{49}A cells shifted from nitrate (time 0) to N_2 regime. Data are representatives of 3 independent experiments. (B) Expression of the nitrogenase Fe-subunit (NifH) after 16 and 24 h of combined nitrogen starvation on cell extracts from wild type strain and Δ*prpS* mutant; immunoblot with antibodies raised against the Fe-subunit from *Anabaena variabilis*, after SDS-PAGE.

as observed by transmission electron microscopy (Fig. 4A). In contrast, these polar nodules are lacking in heterocysts of the Δ*prpS* mutant. Moreover, an accumulation of granules appears in the vegetative cells of the mutant Δ*prpS* (Fig. 4B). Under identical growth conditions, the amount of granules in the wild type strain is weak and their distribution is uniform between the two types of cells (Fig. 4A). These cellular inclusions present a classical aspect of cyanophycin and were isolated, according to [32], and analysed for their arginine content [33]. The results presented in Table 1 show an increase of

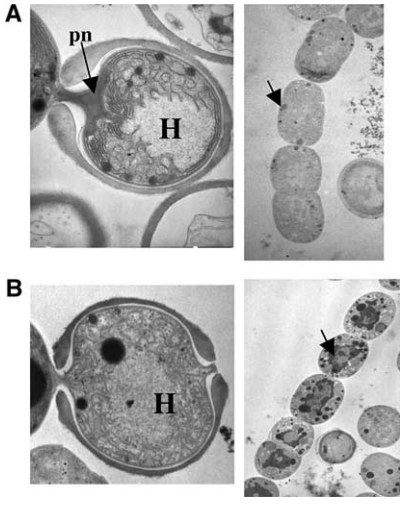

Fig. 4. Ultrastructure of wild type strain (A) and Δ*prpS* (B) cell filaments after 48h under diazotrophic growth conditions. H, heterocysts; arrows indicate the cyanophycin granules. pn, polar nodules.

Table 1
Cyanophycin content (mg arginine. mg chl^{-1}) in *Anabaena* PCC 7120 and mutants during the adaptation to diazotrophic regime

Strains	0 h	24 h	48 h	72 h
Wild-type	0.32	0.56	0.49	0.41
Δ*prpS*	0.32	0.96	0.85	0.75
PIIS$_{49}$A	0.34	0.57	0.40	0.35

cyanophycin after 24 h under diazotrophic regime, higher in the Δ*prpS* mutant.

4. Discussion

Anabaena PCC 7120 is extensively studied for its capability to fix molecular nitrogen under conditions of nitrogen starvation, through the differentiation of heterocysts. We present here evidences for the involvement of the regulatory protein PII in this adaptation process. Isolation of a rather pure fraction of heterocysts is possible after 24 h of nitrogen starvation. At this growth stage, the PII protein is phosphorylated in vegetative cells and unphosphorylated in heterocysts. This observation can be correlated with the physiological activities of the two types of cells. Photosynthetic activity and carbon fixation in the vegetative cells drive the production of 2-oxoglutarate; high level of 2-oxoglutarate is known to favour a phosphorylated state of PII [15]. In the heterocysts, the intracellular concentration of 2-oxoglutarate depends on two opposite fluxes, the import of carbohydrates from the vegetative cells and, according to [37], the export of the metabolite towards these cells. 2-oxoglutarate, as well as glutamine synthesised in the heterocysts, has to be exported towards vegetative cells to provide substrate for the GOGAT activity, present only in these cells. Then, the glutamate flux is directed towards the heterocysts and used for ammonium fixation by GS activity. The asymmetry of the PII modification state, phosphorylated in vegetative cells and unphosphorylated in heterocysts, reflects the different metabolism in the two types of cells and raises the question of the possible involvement of PII in the regulation of the complex metabolic fluxes between the two types of cells.

Major perturbations in nitrogen metabolism are observed in the Δ*prpS* mutant when the strain is shifted from combined to molecular nitrogen regime. In this mutant, the gene coding for the putative PII phosphatase is interrupted and the PII protein remains phosphorylated in the heterocysts, in contrast to the PII protein in the wild-type strain which is rapidly dephosphorylated during the differentiation process. Only three days after the limitation of combined nitrogen in the growth medium, PII becomes unmodified in the Δ*prpS* mutant (see Section 3.2). The delay for PII demodification in the mutant coincides with the lag phase observed on cell growth after a shift from combined to molecular nitrogen regime. During this period of time, nitrogenase activity is weak and heterocysts fail to build up the polar cyanophycin nodules. Instead, cyanophycin is largely accumulated in the vegetative cells, reflecting a disorder in the metabolic activities between the 2 types of cells. It is remarkable that in the mutant PII-S$_{49}$A, where PII is permanently under unmodified state, no such metabolic perturbation is observed and the mutant growth rate under molecular nitrogen regime and the level of nitrogenase activity are similar to what were observed for the wild type strain.

In Δ*prpS*, the impairment of nitrogenase activity is surprising, since neither *nif*-gene expression nor NifH protein modification state seems to be affected. In some proteobacteria, the involvement of PII in the regulation of nitrogenase has been observed, at the transcriptional or post-transcriptional level, depending on the organisms [13]. None of these levels of regulation is modified in the Δ*prpS* mutant, which could indicate that the impairment of the nitrogenase activity could be due to perturbation in metabolite and reductant fluxes and/or be related to the impaired formation of polar nodules in maturating heterocysts.

The distribution of cyanophycin between the two types of cells along the filaments is affected in Δ*prpS*. Involvement of PII in the regulation of cyanophycin metabolism has been strongly suggested [22], since a direct interaction between PII and *N*-acetyl glutamate kinase was observed in *Synechococcus* PCC 7942. This enzyme controls flux in the arginine biosynthesis pathway [38] and arginine is a major compound of cyanophycin. According to the results presented here, the PII protein appears essential for the regulation of cyanophycin distribution along the filaments. Up to now, lack of polar cyanophycin has been observed in mutant impaired in genes encoding cyanophycin synthase [39,40]. We show here that the unmodified state of PII in the heterocysts of the wild type cells contributes to the formation of polar nodules. This agrees with the activation of *N*-acetyl glutamate kinase activity by the non-phosphorylated form of PII, although an interaction between PII and NAGK awaits formal demonstration in *Anabaena* PCC 7120. By contrast, cyanophycin synthesis in vegetative cells occurs in the presence of phosphorylated PII and the different cyanophycin accumulation in wild-type and mutant cells may depend on additional factors.

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