Global carbon/nitrogen control by P₂ signal transduction in cyanobacteria: from signals to targets

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Abstract

P₂ signal transduction plays a pervasive role in microbial nitrogen control. Different phylogenetic lineages have developed various signal transduction schemes around the highly conserved core of the signalling system, which consists of the P₂ proteins. Among all various bacterial P₂ signalling systems, the one in cyanobacteria is so far unique: in unicellular strains, the mode of covalent modification is by serine phosphorylation and the interpretation of the cellular nitrogen status occurs by measuring the 2-oxoglutarate levels. Recent advances have been the identification of the phospho-P₂ phosphatase, the resolution of the crystal structure of P₂ proteins from Synechococcus and Synechocystis strains and the identification of novel functions of P₂ regulation in cyanobacteria, which highlight the central role of P₂ signalling for the acclimation to changing carbon–nitrogen regimes.

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1. Introduction: the regulatory potential of cyanobacteria

Cyanobacteria inhabit nearly all illuminated ecosystems in the biosphere, where they dominate the autotrophic microbial community. Their metabolism is based on oxygenic photosynthesis, using water as a ubiquitous source of reductant to assimilate inorganic nutrients from the environment. As free-living bacteria, they intimately interact with their environment and compete with other microbes for the utilization of the most favourable nutrients. Depending on the habitat in which the organisms have evolved, cyanobacteria may exhibit highly developed acclimation mechanisms to respond to changing nutritional conditions. The recent sequencing of several cyanobacterial genomes has revealed a remarkable abundance of genes encoding regulatory proteins, which correlates with the size of the genome and the complexity of the environment of these organisms. In the genome of the unicellular, fresh-water cyanobacterium *Synechocystis* sp. PCC 6803, 86 genes encoding putative histidine kinases and response regulators were identified, whereas the filamentous, nitrogen-fixing strain *Anabaena* sp. strain PCC 7120 was estimated to contain 211 two-component signalling elements. In the related strain *Nostoc punctiforme*, which is facultatively symbiotic, this number even amounts to 255 [1–3]. In addition to this large number of combined two-component signal transduction proteins, including complex hybrid two-component systems composed of multiple transmitter and receiver domains, the cyanobacterial genomes harbour a variety of genes whose products resemble protein serine/threonine or tyrosine kinases and phosphatases. The genome of *Synechocystis* PCC 6803 contains 13 putative protein-Ser/Thr and Tyr kinases, whereas *Anabaena* PCC 7120 and *N. punctiforme* contain 52 and 55, respectively (for a comprehensive review, see [2,3]). In most cases, the function and the substrates of these “eukaryotic-like” signal transducers are not known. During the last decade, a cyanobacterial phosphoprotein has been characterized which belongs to the family of the PII signalling proteins. Currently, this family is known to contain the most widely distributed signalling proteins in nature [4]. Members of the PII family play central roles in regulating various aspects of nitrogen metabolism and display great functional versatility. The PII system in cyanobacteria is unique among the known PII signalling systems, both with respect to its mode of modification and its novel targets of regulation. In the following, the current knowledge on PII signalling in cyanobacteria will be reviewed and discussed in the context of PII signal transduction schemes.

2. General properties of PII signal transduction

2.1. PII nomenclature

The family of PII signalling proteins has received increasing attention over the past few years, since it has become evident that these proteins play central roles in microbial nitrogen control. The advances in genome sequencing projects revealed that the PII proteins are represented in all three domains of life, being found in almost all free-living bacteria, archaea, eukaryotic algae and plants. Recent reviews have covered the biology of this signalling system in depth [4,5].

According to a recent suggestion concerning PII nomenclature, proteins of the PII family can be classified into three closely related subgroups, the products of the genes *glnB*, *glnK* and *nifI* [4]. The latter is only present in archaea and some strictly anaerobic bacteria, whereas *glnB* and *glnK* homologues are commonly found. The three PII types differ in the organization of their respective genes and are characterized by distinct sequence signatures [4]. The first PII protein to be characterized was the *glnB* product from *Escherichia coli*, which was discovered as a component of an enzyme system (present in a fraction termed PII [6]) that regulates the activity of glutamine synthetase reviewed in [7,8]. The name PII
is now used as a family name for this type of signal transduction proteins, but it is still used as a synonym for the first PII protein, GlnB. Over three decades of research on PII signalling in *E. coli* have revealed detailed insights into the function of this remarkable protein, which acts as a versatile signal processor and transducer and, therefore, has recently been proposed to be functionally equivalent to a central processor unit (CPU) of the cell [5].

2.2. *GlnB* and *GlnK* in *E. coli* and the paradigm of PII signalling

2.2.1. PII structure and binding of low molecular weight effectors

*Escherichia coli* possesses the two PII paralogues GlnB and GlnK, whose amino acid sequences are highly conserved and whose 12.5 kDa subunits form trimers in vivo. X-ray crystallographic analysis revealed that the GlnB and GlnK proteins are structurally nearly identical [9,10]. The three subunits of the trimer are arranged such that they form a short cylindrical structure with the core of the cylinder comprising antiparallel β-sheets. The lateral surface is composed of α-helices and three surface-exposed loops per subunit, termed the T-loop, B-loop and C-loop (see Fig. 1). The mechanism of PII signalling is based on a mutually dependent binding of ATP and 2-oxoglutarate [11,12] (see below). Co-crystals of GlnB and GlnK with ATP revealed three ATP-binding sites per trimer in the lateral clefts between the subunits [9,10]. The B- and the C-loops of two adjacent subunits are mainly involved in contacts with the nucleotide phosphates, whereas the adenosine moiety interacts with residues of the core structure and with the base of the T-loop. Although the 2-oxoglutarate-binding site within the PII structure has not been resolved by crystallographic analysis, Benelli et al. [13] suggested a model derived from structural comparison with other α-keto acid-binding proteins, according to which 2-oxoglutarate may bind in the cleft between the B- and C-loops from neighbouring subunits, in the vicinity of the γ-phosphate of ATP.

2.2.2. Uridylylation of PII proteins

In addition to binding low-molecular weight effectors, the *E. coli* GlnB and GlnK proteins are covalently modified by uridylylation at tyrosyl-residue 51, located at the apex of the T-loop [14–16]. Uridylation and deuridylylation are catalysed by a bifunctional enzyme, the glnD product, whose activity responds to glutamine levels [12,17]. At low intracellular glutamine levels, characteristic of nitrogen-poor conditions, the uridylyltransferase activity of GlnD is stimulated and the PII proteins are converted to the uridylylated state. Under nitrogen-excess conditions, the increasing glutamine levels favour the uridylyl-“removase” activity of GlnD, and therefore, the uridylylated PII proteins return to the unmodified form. While GlnB is constitutively expressed [18], the synthesis of GlnK is subject to nitrogen control, depending on GlnB [19]. Consequently, when cells grow under nitrogen excess conditions, the main PII protein in the cell is non-uridylylated GlnB, whereas under nitrogen-poor conditions, both GlnB-UMP and GlnK-UMP accumulate [20].

2.2.3. Receptors of PII signalling

Depending on their uridylylation status and their ligand-binding status, the PII proteins can interact with various receptors. A main receptor for PII signalling is NtrB, the histidine kinase of the NtrB/NtrC two-component regulatory system [21,22]. NtrC is the major transcription factor required for the expression of numerous nitrogen-regulated genes, among which the genes *glnA* (encoding glutamine synthetase, GS) and *glnK*. NtrB forms a complex with unmodified PII, in which it dephosphorylates and thereby inactivates the phosphorylated response regulator NtrC-P [23]. Uridylation of PII prevents complex formation with NtrB. In the absence of unmodified PII, NtrB phosphorylates NtrC to turn on the expression of nitrogen-regulated genes. Consistent with this, GlnB-deficient mutants show elevated *glnA* expression, whereas in GlnD-deficient cells, which are unable to uridylylate GlnB, *glnA* is constitutively repressed [24]. Another target of PII regulation is the *glnE* product, a bifunctional enzyme that modifies the enzymatic activity of GS by covalent modification through adenylylation/deadenylylation [25,26]. GlnE forms complexes with both modified and unmodified PII [27]. In complex with non-modified PII, GlnE exhibits its adenylyltransferase activity, whereas binding of PII-UMP to GlnE stimulates its adenylyl-“removase”
activity. Thus, when PII becomes uridylylated under nitrogen-poor conditions, PII-UMP will mediate deadeudidylylation, and consequently activation of GS.

A recently discovered target of PII interactions is the high affinity ammonium transporter AmtB, whose gene (amtB) is co-transcribed with glnK in a NtrC-dependent manner [28]. Under nitrogen-poor conditions, AmtB accumulates and, following the addition of ammonium, it sequesters GlnK and GlnB to the membrane [28]. In the absence of GlnK, excess AmtB inactivates GlnB signalling, probably by forming a non-functional complex [29].

2.2.4. PII signal integration

PII signalling has the potential to respond to three central metabolic signals: the energy signal ATP, the carbon signal 2-oxoglutarate and the nitrogen signal glutamine through glutamine-dependent uridylylation/deuridylylation. In vitro studies suggested that PII might indeed act as a central processor unit to integrate these different signals; high 2-oxoglutarate concentrations overcome the glutamine signal “non-uridylylated PII” by preventing complex formation of non-modified PII with NtrB [22,23] and thus allow the expression of NtrC-dependent genes even in the presence of elevated glutamine levels. In vivo, the E. coli PII proteins transduce signals of the glutamine status of the cells through uridylylation, but whether under physiological conditions ATP and 2-oxoglutarate contribute to PII signalling remains to be established. Effects of glucose on the PII signalling system, which were regarded as evidence for the contribution of a carbon signal [30], could be antagonized by cAMP and were shown to be caused by catabolite repression of glutamine uptake [20]. Thus, glucose affects the cellular nitrogen status and the PII proteins respond to a glutamine signal under these conditions.

2.3. Versatility of PII signalling in bacteria

Modification of the two PII homologues GlnB and GlnK in proteobacteria occurs through uridylylation, in those systems investigated to date (summarized in [4]). Although this type of modification is widespread, it is not universal. In the Gram-positive actinomycetes, modification of a GlnK homologue occurs through phosphorylation at Ser49 as the site of phosphorylation in the S. elongatus strain PCC 7942 [41]. In contrast to initial studies, which indicated that this protein might be uridylylated-like E. coli GlnB, subsequent studies revealed that the protein was phosphorylated at a seryl residue [42]. Sequencing of the isolated phosphopeptide from a tryptic digest revealed Ser49 as the site of phosphorylation in the S. elongatus PII homologue [43]. In the sequences of GlnB and GlnK proteins from proteobacteria, position 49 is occupied by alanine.

3. PII signalling proteins in cyanobacteria

3.1. The discovery of PII in cyanobacteria

In the late 1980s, the group of J.F. Allen [39] performed 32P-labelling experiments in a unicellular cyanobacterium, which today is termed Synechococcus elongatus strain PCC 6301, to study the effect of different light qualities on protein phosphorylation. One of the prominently labelled proteins, appearing under orange light illumination (primarily captured by the phycobilisome light harvesting antennae), was a 12.5 kDa protein. N-terminal sequencing of the purified labelled protein revealed homology to the N-terminus of GlnB from E. coli [40]. Subsequently, a glmB homologue was identified in the related strain S. elongatus strain PCC 7942 [41]. The deduced amino acid sequence revealed high similarity to the GlnB protein from E. coli and other proteobacteria (62–66% identity). According to signature sequences and its monocistronic gene organization it is classified in the GlnB subfamily of PII signal transducers [4]. In contrast to initial studies, which indicated that this protein might be uridylylated-like E. coli GlnB, subsequent studies revealed that the protein was phosphorylated at a seryl residue [42]. Sequencing of the isolated phosphopeptide from a tryptic digest revealed Ser49 as the site of phosphorylation in the S. elongatus PII homologue [43]. In the sequences of GlnB and GlnK proteins from cyanobacteria, position 49 is occupied by alanine.

3.2. PII homologues in cyanobacteria and plants

Following the discovery of the glmB gene in S. elongatus, the occurrence of glmB homologues in other cyanobacterial strains was screened by Southern blot hybridisation. In all cases analysed – unicellular and filamentous strains – a hybridization signal corresponding to one homologue was detected [41]. Genes encoding GlnB were subsequently cloned from the
unicellular strain *Synechocystis* sp. PCC 6803 [44] and from the filamentous heterocystous strains *N. punctiforme* [45] and *Anabaena* sp. PCC 7120 [46]. Meanwhile, genome sequencing of numerous cyanobacterial species revealed, without exception, one *glnB* gene per genome and the absence of a *glnK* homologue (www.kazusa.or.jp/cyano/; http://www.jgi.doe.gov/JGImicrobial/html/index.html; http://genolist.pasteur.fr/CyanoList, http://pedant.gsf.de/#Complete.) This also holds for prochlorophytes, marine cyanobacteria, which instead of phycobilisomes have chlorophyll b-containing light harvesting pigments [47]. The species *Prochlorococcus marinus* exhibits the smallest genome of oxygenic photosynthetic organisms known to date [48]. It lacks many genes involved in the photosynthetic electron transport chain. The P II phosphorylation responds, but rather its assimilation via the glutamine synthetase–glutamate synthase pathway (GS–GOGAT). Inhibition of one of the enzymes of this pathway leads to a strong increase in P II phosphorylation, even in the presence of ammonium [55]. This implies that a metabolite of this pathway is involved in P II phosphorylation, P II phosphorylation and dephosphorylation in vivo do not only respond to the cellular nitrogen status. A similar effect to that of the addition of ammonium on eliciting P II dephosphorylation can be achieved by inhibiting CO₂ fixation, or by limiting the CO₂ supply to the cells [54,55]. In nitrate-grown cells, the actual state of P II phosphorylation increases with increasing CO₂ concentrations. Different illumination conditions and dark–light transitions also affect the P II phosphorylation state; however, these effects are subordinate to the nitrogen conditions [42]. Similar studies in *Synechocystis* sp. PCC 6803 [56] indicated that its GlnB protein might also respond to the redox-state of the photosynthetic electron transport chain. The phosphorylation state of P II immediately responded to oxidation of the electron acceptor of photosystem I, an effect that could be reversed by the addition of excess reductant [56].

In *in vitro* phosphorylation studies were carried out with purified *S. elongatus* P II protein as a substrate, using cell extracts from a P II-deficient mutant of *S. elongatus* as a source of P II kinase activity [43]. Phosphorylation of P II could be achieved only in the presence of 2-oxoglutarate and ATP. No other compound could replace the requirement for these metabolites, nor were any other metabolites able to interfere with the kinase reaction. A phosphatase assay was established with purified phospho-P II (P II-P) as a substrate. Extracts of P II-deficient cells contained a P II-P dephosphorylating activity, which was stimulated by Mg²⁺ and which, in contrast to the kinase activity, was inhibited by ATP and 2-oxoglutarate [55]. Attempts to purify the P II modifying activities revealed that kinase and phosphatase activities could be separated by gel filtration chromatography. Whereas the kinase activity could be separated into several fractions, which rapidly lost activity after further purification, a partially purified, stable phosphatase activity could be obtained. It exhibited typical biochemical features of protein phosphatases of the 2C family (PP2C) [55].
4.2. Binding of ATP and 2-oxoglutarate by S. elongatus PII

The ligand-binding properties of *S. elongatus* PII protein show remarkable similarities to those of *E. coli* GlnB, although some differences are quite obvious and may have important physiological consequences [57,58]. Both proteins bind ATP and 2-oxoglutarate in a mutually dependent manner. Only one 2-oxoglutarate molecule binds to *E. coli* GlnB with high affinity. This binding event exerts strong negative co-operativity on the second and third putative-binding sites of the trimeric protein. The presence of ATP increases the affinity for the first binding site, but no effect of ATP on the stoichiometry of 2-oxoglutarate binding has been reported [12]. Uridylylation of GlnB is required to increase the stoichiometry of 2-oxoglutarate binding. In *S. elongatus* PII, 2-oxoglutarate binding can only be measured in the presence of ATP but not in its absence. At low ATP concentrations (25 μM), one 2-oxoglutarate molecule binds to PII (at a Kd of 6 μM), and with increasing ATP concentrations both binding affinity and stoichiometry increase, suggesting that ATP not only affects the affinity of 2-oxoglutarate binding but also decreases the anti-cooperativity of the other 2-oxoglutarate-binding sites in the trimeric protein [57].

ATP binding to *E. coli* GlnB is enhanced by 2-oxoglutarate, with the Kd dropping from about 30 μM in the presence of 33 μM 2-oxoglutarate to about 2 μM in the presence of 2 mM 2-oxoglutarate. However, no effect on the stoichiometry of ATP binding has been observed; three molecules of ATP are bound to GlnB, regardless of the 2-oxoglutarate concentration [12]. Binding of ATP to *S. elongatus* PII has a similar affinity; in the absence of 2-oxoglutarate, an apparent Kd of ATP binding of 37 μM was calculated. In contrast to *E. coli* GlnB, the stoichiometry is only about 1 ATP bound per PII trimer. In the presence of 2 mM 2-oxoglutarate, the affinity for ATP increases by nearly two orders of magnitude and the stoichiometry of the high-affinity ATP-binding sites increases to about 2 per PII trimer [57]. The binding data indicate that a third site is occupied with lower affinity (K. Forchhammer, unpublished results). This shows that in the cyanobacterial PII protein, ATP and 2-oxoglutarate not only affect the binding affinity of the partner ligand, but also reduce the anti-cooperativity of the partner ligand-binding sites in a mutually dependent manner. Similar properties have recently been described for the GlnB protein of *Arabidopsis thaliana* [53].

The biochemical properties of *S. elongatus* PII, together with the responses of PII to phosphorylation, imply that PII is a sensor of the cellular 2-oxoglutarate level under ATP-deplete conditions. Thus, 2-oxoglutarate has been suggested to act as a central signalling metabolite of carbon/nitrogen balance in cyanobacteria [58].

4.3. PphA, the PII-P-specific protein phosphatase

From the genome sequence of *Synechocystis* sp. PCC 6803, a variety of putative protein serine/threonine kinases and phosphatases could be identified [59,60]. Among these are eight putative protein phosphatase 2C (PP2C) homologues. Mutants in the corresponding genes were created and analysed with respect to their ability to dephosphorylate PII-P after ammonium treatment [61]. One of the mutants (mutation in ORF sll1771 [62]) showed a complete inability to dephosphorylate PII-P and, under all growth conditions, the phosphorylation status of PII was markedly increased compared to the wild-type. All other mutants were not impaired in dephosphorylating PII-P upon ammonium treatment. This implied that the sll1771 gene, which was subsequently designated *pphA*, encodes the cellular PII-P phosphatase. PphA consists of 254 amino acids (28.4 kDa) [61], which encompass the catalytic core of PP2C enzymes, characterized by 11 conserved motifs distributed over the sequence [59]. Sequence alignments show that PphA is closely related to a group of bacterial PP2C homologues with a so far unknown function. Among these, a protein phosphatase from *Myxococcus xanthus* was identified, involved in fruiting body formation [63]. The bacterial PP2C homologues are characterized by their small size, which corresponds to the catalytic core of type 2C protein phosphatases, and lack putative regulatory domains. These proteins are only distantly related to eukaryotic PP2C members or the previously characterized PP2C homologues of the SpoIIE type from *Bacillus subtilis*, which contain regulatory or substrate-binding domains [59]. This raises questions regarding the specificity of PphA towards PII-P and the regulation of its activity.

The biochemical properties of purified PphA are typical for PP2C enzymes with respect to Mn2+/Mg2+-dependent activity and sensitivity towards protein phosphatase inhibitors. PphA is monomeric and soluble in aqueous buffers. Depending on the assay conditions, it dephosphorylates a wide range of phosphorylated substrates [64]. PphA reacts with artificial substrates such as *p*-nitrophenyl phosphate or synthetic peptides containing phosphoseryl-, phosphothreonyl- or phosphothryosyl-residues, albeit with a catalytic activity that is three orders of magnitude lower than towards serine/threonine-phosphorylated proteins. The reactivity towards these low molecular weight substrates is stimulated by Mn2+ ions and alkaline pH. At physiological pH and in the presence of Mg2+ ions, PphA specifically reacts with serine/threonine-phosphorylated proteins such as phosphocasein or phosphohistones and with its physiological substrate, PII-P. In the absence of effector molecules, the reactivity towards the non-physiological phosphoproteins was estimated to be similar as the reactivity towards PII-P, and with phosphocasein as...
PphA-catalysed PII-P dephosphorylation.

In the presence of certain effector molecules, notably ATP and 2-oxoglutarate, the reactivity of PphA towards PII-P is strongly inhibited, whereas the reactivity towards phosphocasein is unaffected [64]. In agreement with the lack of regulatory domains in PphA and with the biochemical properties of \(S.\) elongatus \(P_{II}\), this suggests that the effector molecules specifically affect the molecular recognition between PphA and PII-P, presumably through their binding to PII-P. ATP, GTP or ADP reduce the rate of PII-P dephosphorylation in a concentration-dependent manner. In the presence of 1 mM of these nucleotides, PII-P dephosphorylation is decreased by 25–40\%. In the absence of nucleotides, 2-oxoglutarate has no effect on PII-P dephosphorylation. In combination with ATP, however, 2-oxoglutarate strongly enhances the inhibition of PII-P dephosphorylation. In contrast, no synergy between 2-oxoglutarate and GTP and only a weak synergy with ADP was detected (Fig. 2). In the presence of ATP concentrations in the physiological range (1–2.5 mM), PII-P dephosphorylation is very sensitive to low 2-oxoglutarate concentrations: 10 \(\mu\)M of this metabolite has already a significant effect and 100 \(\mu\)M is sufficient to achieve nearly maximal inhibition of PII-P dephosphorylation. Besides 2-oxoglutarate, oxaloacetate is also able to inhibit PII-P dephosphorylation in synergy with ATP, although 20-fold higher concentrations are required to yield similar effects. Other glycolytic and TCA metabolites, as well as various amino acids, do not affect PphA-catalysed PII-P dephosphorylation.

At non-saturating 2-oxoglutarate concentrations (in the low \(\mu\)M range), inhibition of PII-P dephosphorylation can be enhanced by raising the ATP concentration. This suggests that under physiological conditions of low cellular 2-oxoglutarate levels, PII-P dephosphorylation may be sensitive to the cellular concentration of ATP. However, ADP also partially inhibits the dephosphorylation reaction. To demonstrate the combined effect of both nucleotides, dephosphorylation assays were carried out with various ATP:ADP ratios (at a total adenylate concentration of 3 \(\mu\)M) in the presence of 10 \(\mu\)M 2-oxoglutarate. Under these conditions, the level of PII-P dephosphorylation did not change in response to various ATP:ADP ratios. Apparently, the decrease in the ATP level is compensated by a reciprocal increase in the ADP concentration [65]. These data argue against the PII phosphorylation state signalling the energy status of the cyanobacterial cell.

4.4. \(PphA\)-catalysed dephosphorylation of PII-P

In vivo, the phosphorylation state of PII responds to a variety of stimuli. In addition to various nitrogen and carbon conditions, treatments that affect photosynthetic electron transport cause a PII response. For example, treatment of \(Synechocystis\) cells with methylviologen, a compound which oxidizes photosystem I-reduced ferredoxin, results in an immediate dephosphorylation of PII [56]. This reaction can be prevented by the addition of duroquinol, a reducing agent which feeds electrons into the photosynthetic electron transport chain. Therefore, it has been suggested that PII might integrate signals of the C and N status, but also of the redox status of the photosynthetic electron transport.

Two alternative mechanisms could explain the observation. The first involves a direct redox-sensing mechanism resulting in PII-P dephosphorylation. The second mechanism is an indirect effect due to the diverse treatments interfering with CO\(_2\)-fixation, thus altering the cellular 2-oxoglutarate level, with the latter being perceived directly by PII. PII phosphorylation in the PphA-deficient mutant does not respond to various treatments affecting the redox state [65]. Therefore, the response of wild-type cells has to be transduced via PphA. Under in vitro assay conditions, different treatments that influence redox-conditions did not affect PphA activity, thus PphA is unlikely to be redox-sensitive. More likely, the various effects on PII dephosphorylation are mediated by changes in the cellular 2-oxoglutarate levels.

4.5. Redox and PII signalling

Fig. 2. Inhibition of PphA-catalysed dephosphorylation of PII-P by effector molecules. The effect of various nucleotides or ATP analogues (1 mM) was assayed in the presence or absence of 0.5 mM 2-oxoglutarate, as indicated. For details see [64].

Recently, the crystal structures of the PII proteins from \(Synechococcus\) PCC 6803 and \(S.\) elongatus were determined [66]. Whereas the core of the two cyanobacterial PII proteins is similar to the core of GlnB and GlnK from \(E.\) coli, the structures of the T- and the C-loops differ. Generally, the T-loop seems to be flexible in solution, since in various forms of PII crystals this loop is disordered. In some PII crystals, a T-loop structure is...
stabilized by crystal contacts, allowing the resolution of a structure. In the case of *Synechocystis* PII, the T-loop is disordered, whereas the T-loop of *S. elongatus* PII is not. Its conformation is elongated compared to the T-loop of *E. coli* GlnB, due to an extension of the β2 and β3 strands (see Fig. 1). The second remarkable feature of the two cyanobacterial PII proteins concerns the C-terminal peptide, which forms the C-loop and is involved in ATP binding. It appears that this part of the molecule is also capable of conformational changes. In the crystal structure of *Synechocystis* PII, the C-terminus points towards the N-terminus of the same subunit and is stabilized by coordination of a metal ion, most likely Ni²⁺, which was added during crystallization. This conformation facilitates crystallization, but it seems unlikely to have physiological significance. These observations are compatible with the idea that the C-loop as well as the T-loop is flexible and capable of adopting several conformations. Upon crystallization, they may loose flexibility if side chains are involved in crystal contacts. In earlier studies, it was observed that *S. elongatus* PII is able to form heterotrimers with *E. coli* GlnB and GlnK [67]. This finding is in accordance with the highly conserved core structures of these proteins, allowing hetero-oligomer formation. However, the *E. coli* GlnB and GlnK subunits were functionally “poisoned” in heterotrimers by the *S. elongatus* PII. In these heterotrimers, uridylylation of the T-loop of the *E. coli* PII subunits was severely impaired and stimulation of regulated NtrB phosphatase activity was almost completely defective. This implies that the subunits cooperate to adopt the conformation that is required to interact with the receptors.

Recently, co-crystals of *E. coli* GlnB with ATP were resolved at high resolution [10]. Since the structure of the ATP-binding pocket of the cyanobacterial PII proteins is well conserved compared to the proteobacterial GlnB (compare Fig. 1), similar side-chain contacts with ATP and presumably 2-oxoglutarate can be assumed. The suggestion that the binding pocket for 2-oxoglutarate is in the vicinity of the γ-phosphate group of ATP [13] agrees well with biochemical data obtained from dephosphorylation studies of cyanobacterial PII-P [64]. As outlined above, dephosphorylation of PII-P by PphA is highly sensitive to the ligand-bound status of PII. Replacing ATP by the non-hydrolyzable analogues AMPPCP (adenosine-5′-[β,γ-methylene]-triphosphate) or ATP-γ-S (adenosine-5′-[γ-thio]-triphosphate) reveals that these analogues have quite different effects on PII-P dephosphorylation (Fig. 2). AMPPCP is very efficient in inhibiting PII-P dephosphorylation in the absence of 2-oxoglutarate, but in its presence, no additional inhibition occurs. The opposite is observed with ATP-γ-S. In the absence of 2-oxoglutarate, ATP-γ-S is almost completely inactive, but in its presence, it is as efficient as ATP. These data suggest that the synergy between 2-oxoglutarate and ATP involves the γ-phosphate, since this is the only part in which the analogues differ. The subtle conformational changes in the γ-phosphate moiety of the analogues are apparently sufficient either to increase or to decrease the synergy. These data are consistent with the assumption that an important function of 2-oxoglutarate is to reduce the anti-cooperativity between the three ATP-binding sites. At increasing 2-oxoglutarate concentrations, the second and third ATP-binding site may be occupied more easily, resulting in a PII conformation that is protected from dephosphorylation. This model is also in accord with the 2-oxoglutarate-independent ADP effect on PII-P dephosphorylation, since in the absence of a γ-phosphate group, no synergistic interaction between ADP and 2-oxoglutarate can occur [64].

5. The physiological functions of PII in cyanobacteria

5.1. General phenotype of PII-deficient mutants

PII-deficient mutants of *S. elongatus* are unable to acclimate rapidly to changing environmental conditions, in particular to shifts from nitrate- to ammonia-supplemented medium, to increasing CO₂ concentrations or to sudden decreases in light intensity [54]. However, following a prolonged lag-phase, the mutant cells finally grow with similar rates to those of the wild-type. The mutants accumulate more glycogen than the wild-type, while the total protein content decreases, indicating a rise in the cellular C/N ratio. In nitrate-grown PII mutants, the activities of enzymes required for nitrate utilization and ammonium assimilation (nitrate/nitrite transporter, nitrate and nitrite reductases and glutamine synthetase) are increased. However, when they are acclimated to ammonium, these activities are depressed as in wild-type cells.

5.2. PII-dependent regulation of nitratelnitrite transport

Measurement of the short-term response of nitrate/nitrite transport to inhibitory conditions revealed that PII-deficient mutants were unable to depress this activity upon ammonium-treatment [56,68] or inhibition of CO₂-fixation [54]. Both conditions are known to immediately depress nitrate/nitrite transport in the wild-type. Since these inhibitory conditions are correlated with PII dephosphorylation in the wild-type, these experiments suggested that the non-phosphorylated form of PII may be inhibitory for the permease, whereas in the absence of the non-phosphorylated form, the permease is active. To further investigate this, PII mutants were created in which the phosphorylated Ser 49 residue was either replaced by alanine (termed MP2-A), creating a PII mutant which is permanently unmodified, or by...
suggesting that this site may be involved in PII-mediated

ders the mutant less sensitive to ammonium inhibition,

conserved in other ABC-transporters. Its deletion ren-

NrtC has a distinct C-terminal domain, which is not

still bound the ligands ATP and 2-oxoglutarate [69].

From these data, it was concluded that phosphorylation

of PII alone is not sufficient to relieve the inhibitory ef-

fect of PII on the nitrate/nitrite permease, but that

binding of effector molecules may be required. In other

words, the phosphorylated form of PII may switch be-

tween two states; one being inhibitory for the permease

and another (presumably the 2-oxoglutarate- and ATP-

liganded form), which relieves inhibition. By contrast,

the unmodified form is inhibitory, regardless of the

presence of effector molecules (Fig. 3).

Nitrate transport in S. elongatus occurs through an

ATP-binding cassette (ABC) transporter, a nitrate–

nitrite bispecific permease, encoded by the genes nrtA,

nrtB, nrtC and nrtD [70]. The ATP-binding subunit

NrtC has a distinct C-terminal domain, which is not

conserved in other ABC-transporters. Its deletion ren-

ders the mutant less sensitive to ammonium inhibition,

suggesting that this site may be involved in PII-mediated

control. However, regulation by PII may be more com-

plicated, since the NrtC C-terminal domain deletion

mutant still responds to inhibition of CO2-fixation by

D,L-glyceraldehyde [71], indicating that C- and N-con-

trol of nitrate transport may be distinct, with PII being

involved in both processes. Up to now, the mode of

interaction between PII and the nitrate/nitrite permease,

in particular NrtC, has not been defined biochemically.

5.3. PII-dependent control of CO2 uptake and methy-

l ammonium transport

High affinity transport of ammonium in S. elongatus is

achieved by an AmtB-like permease, encoded by the

amt1 gene [72]. Expression of the amt1 gene is NtcA-

dependent (see below) and therefore is induced in the

absence of ammonium. In wild-type cells, transport of

[14C]methylammonium, which is used as a radioactive

analogue of ammonium, is similar in nitrate-grown and

in nitrogen-starved cells [54,72]. However, in the PII-

deficient S. elongatus strain, no methylammonium up-
take can be measured in nitrate-grown cells, whereas

when the cells are starved for combined nitrogen, a ra-
pid increase in this activity can be detected, surpassing

the wild-type level 5-fold [54]. Thus, the Amt transporter

may be a receptor for PII regulation, as already shown

for various proteobacteria [28,73].

Cyanobacteria possess several systems for the uptake

of CO2 and bicarbonate [74]. Under conditions of lim-
inating inorganic carbon, the cells express high affinity

carbonate transporters, whose activity is down-regu-
lated by elevated CO2 concentrations. In a PII-deficient

mutant of Synechocystis sp. PCC 6803, this CO2-

dependent inhibition of the high affinity bicarbonate

transporter is impaired, irrespective of the nitrogen

source [56] and, thus, irrespective of the phosphoryla-
tion status of PII. This suggests a phosphorylation-

independent mode of PII signalling for the regulation of

bicarbonate transport.

5.4. Relation between PII and the global nitrogen control

factor NtcA

In all cyanobacteria investigated so far, nitrogen

control is exerted at the transcriptional level, which by a

transcription factor termed NtcA, which belongs to the

CRP/FNR family of bacterial transcription factors [75],

whereas a NtrB/NtcC two-component system, known

from proteobacteria, is not present. NtcA was shown to

be required for the induced expression of nitrogen-regu-

lated genes in the absence of ammonium. In cyano-
bacteria of the order Nostocales, NtcA is the key control

factor for the differentiation of heterocysts [76], spe-
cialized cells for nitrogen fixation for recent reviews see

[77,78]. Details of the properties of NtcA have been

reviewed recently [79].
Expression of the *glnB* gene in *S. elongatus* and *Synechocystis* sp. PCC 6803 is increased by nitrogen-poor conditions [44,80]. The *glnB* genes of both organisms have two transcriptional starter sites. A weak constitutive promoter drives transcription in the presence of ammonium, whereas under nitrogen-poor conditions, most transcripts originate from an NtcA-dependent promoter, characterized by a perfect NtcA-binding motif. In *S. elongatus glnB*, the NtcA-dependent promoter is located downstream of a constitutive promoter [80], whereas in the *Synechocystis* PCC 6803 *glnB* gene, it is located upstream [44].

In an NtcA-deficient mutant, the phosphorylation state of PII is significantly lower than in the wild-type, and upon a shift to nitrogen-deprived conditions, the NtcA mutant is unable to phosphorylate PII efficiently [80,81]. Even after 20 h of nitrogen starvation, PII was mainly present in its non-phosphorylated form [81]. These results suggest that the PII kinase may be subjected to NtcA control.

Initial studies of gene expression indicated that regulation of NtcA activity is not impaired in PII-deficient mutants, since the NtcA-dependent genes were repressed by ammonium and showed induced expression in the presence of nitrate, although at elevated levels compared to the wild-type [54,69]. Together with the NtcA-dependent synthesis and phosphorylation of PII, this implied that PII is subordinate to NtcA rather than PII transferring signals to NtcA. The expression of an alternative glutamine synthetase gene, termed *glnN* (encoding a GS of type III), in PII-deficient mutants contradicted this view. GSIII is present in all non-diazotrophic cyanobacteria investigated so far [82–84] and is specifically induced under conditions of nitrogen starvation [83,84], but in the PII-deficient mutant, this induction is impaired [83]. Since the putative *glnN* promoter region contains a non-canonical NtcA-binding site, it is conceivable that PII affected transcription by NtcA. To further investigate PII- and NtcA-dependent gene expression under conditions of nitrogen deprivation, de novo protein synthesis in wild-type, PII- and NtcA-deficient *S. elongatus* cells was analysed by proteome analysis [85]. PII-deficient and NtcA-deficient mutants were both unable to induce or repress synthesis of those proteins, which in the wild-type were induced or repressed in response to nitrogen depletion. The effect of PII on NtcA-induced gene expression was investigated by constructing *luxAB* fusions to the NtcA-dependent *glnB* promoter. When the cells were shifted to nitrogen-deprived conditions, a strong NtcA-dependent activation of the NtcA-dependent *glnB* promoter occurred in wild-type cells, whereas no activation was observed in the PII-deficient mutant [85]. Another study performed on different NtcA-induced promoters yielded similar results; PII-deficient cells were unable to increase the level of these genes upon a shift to conditions of nitrogen deprivation [86]. The permanently non-phosphorylated mutant PII-A was less efficient in inducing gene expression than the wild-type, whereas the mutants PII-D and PII-E showed higher levels of gene expression. These results indicate that the phosphorylated PII protein signals nitrogen deficiency to NtcA under conditions of nitrogen deprivation. Since the molecular basis for this effect has not been elucidated so far, it can only be assumed that PII might interact with NtcA directly, or the PII effect on NtcA might be transmitted by a so far unidentified factor, which acts on NtcA.

5.5. Functions of PII in prochlorophytes and in heterocystous cyanobacteria

PII signalling in a prochlorophyte has been investigated in strains of the species *P. marinus* [49]. Although the *P. marinus* PII protein is highly conserved as compared to *S. elongatus* PII, including the phosphorylation site Ser49, no phosphorylation or any indication of modification could be detected under various nitrogen and carbon regimes. Absence of PII phosphorylation correlates with an absence of a gene encoding a PphA homologue. The lack of PII phosphorylation might be related to the inability of *P. marinus* to utilize nitrate as a nitrogen-source. In accord with this, the enzymes for nitrate uptake and reduction, putative targets of PII regulation, are missing. By contrast, other possible PII targets, NtcA, Amt or transport systems for the uptake of inorganic carbon are present, suggesting that PII signalling is performed by the unphosphorylated protein. In cyanobacteria of the order *Nostocales*, filamentous organisms with cellular differentiation, PII signalling might be particularly interesting, since these organisms respond to nitrogen starvation by differentiating specialized cells, the heterocysts, to perform nitrogen fixation in an oxygen-producing environment. Studies were performed in strains of the genera *Calothrix*, *Nostoc* and *Anabaena*. Investigation of PII phosphorylation in the strains *Calothrix* PCC 7504 and PCC 7601 [87] yielded ambiguous results. In non-denaturing gels, modified forms of PII-trimers could be observed, which were similar to those that occur upon PII phosphorylation in *S. elongatus*. However, the emergence of faster migrating PII forms did not strictly correlate with the nitrogen status of the cells and PII did not respond to inhibition of nitrogen assimilation. Furthermore, these forms were insensitive to treatment with alkaline phosphatase, which in the case of *S. elongatus* PII led to a demodification of PII, visible as disappearance of the faster migrating PII forms. Thus, in spite of the conserved phosphorylation site, the modification type of PII in this strain remains to be established. Similar results were obtained in *N. punctiforme* strain ATCC 29133 [45], a strain with one of the largest bacterial genomes, which is frequently involved in symbiotic associations.
with plants. Although a smear of faster migrating forms of \( \text{P}_{II} \) in non-denaturing gels indicated modification, evidence for phosphorylation was not found. In vitro phosphorylation of purified \( \text{N. punctiforme} \) \( \text{P}_{II} \) could be achieved using kinase-containing extracts from \( \text{S. elongatus} \) [45]. This demonstrates that the protein has retained the capacity to become phosphorylated. The genome of \( \text{N. punctiforme} \) (access: http://genome.jgi-psf.org/draft_microbes/nospu/nospu.home.html) contains a close homologue to \( \text{PphA} \), which is a further indication that \( \text{P}_{II} \) phosphorylation might occur under certain circumstances. Attempts to create a knock-out mutant of the \( \text{glnB} \) gene in \( \text{N. punctiforme} \) were not successful, unless \( \text{glnB} \) was provided in trans [45]. This implies that the \( \text{glnB} \) gene is essential in this organism, thus making it difficult to take a genetic approach to elucidate the function of \( \text{P}_{II} \). Similar attempts to create \( \text{glnB} \) knock-out mutants also failed in the related strain \( \text{Anabaena sp. PCC 7120} \). In this strain, \( \text{P}_{II} \) responds to decreasing N/C balance by becoming modified. Whether this modification is phosphorylation or not remains unclear, since attempts to label \( \text{P}_{II} \) with \( ^{32}\text{P} \) in vivo were unsuccessful [C.-C. Zhang, personal communication]. Several Ser/Thr kinase homologues were investigated in \( \text{Anabaena sp. PCC 7120} \), and among them one kinase (PknC) was able to phosphorylate a recombinant \( \text{P}_{II} \) protein. However, this phosphorylation did not occur on Ser 49 but on a seryl-residue located at the thrombin cleavage site of the His tag [88]. Therefore, the nature of \( \text{P}_{II} \) modification in the filamentous strains awaits further investigation.

6. Conclusion

6.1. \( \text{P}_{II} \) phosphorylation and 2-oxoglutarate signalling

The \( \text{P}_{II} \) signalling proteins in cyanobacteria recognize the cellular nitrogen status by measuring the levels of 2-oxoglutarate. According to the interpretation of the data presented here, kinase and phosphatase activities respond to the conformational states of \( \text{P}_{II} \), determined by 2-oxoglutarate-dependent ATP binding, but they are not the sensors of the nitrogen status per se (see Fig. 3). This contrasts with the situation in proteobacteria, where the nitrogen status is sensed by the glutamine-dependent activity of a \( \text{P}_{II} \)-modifying enzyme. Why do cyanobacteria measure the nitrogen status by sensing 2-oxoglutarate instead of glutamine? 2-Oxoglutarate is produced in the oxidative branch of the TCA-cycle, which in cyanobacteria has solely an anabolic function, delivering precursors for various biosynthetic reactions (for review see [89]). One of the main routes of newly fixed \( \text{CO}_2 \) is the synthesis of 2-oxoglutarate to produce glutamate via the GS-GOGAT cycle. Thus, 2-oxoglutarate is the primary carbon-skeleton for the incorporation of ammonium and represents the intersection between the carbon and nitrogen metabolic routes. Changes of the ammonium supply, the substrate of the GS reaction, are inversely correlated with changes of 2-oxoglutarate levels [90]. 2-Oxoglutarate levels are low in ammonium-grown cells and they increase when the cells experience ammonium deficiency. Isocitrate dehydrogenase (IDH), the 2-oxoglutarate producing enzyme, is induced by nitrogen starvation [91]. Therefore, under nitrogen-limited conditions, a positive feedback loop will sharply increase the 2-oxoglutarate levels: its consumption is reduced due to ammonium limitation of the GS-GOGAT cycle, whereas its synthesis increases due to increased IDH activity. In addition to nitrogen assimilation, 2-oxoglutarate levels also depend on \( \text{CO}_2 \) fixation. For example, when \( \text{CO}_2 \) supply becomes limiting, synthesis of 2-oxoglutarate will decrease, causing a drop in its level, which is paralleled by \( \text{P}_{II} \) dephosphorylation. Measuring the nitrogen status by monitoring the 2-oxoglutarate levels instead of glutamine allows the cell to buffer the glutamine pool, which can be replenished by protein turnover, without interfering with nitrogen-control.

Recent biochemical studies revealed that 2-oxoglutarate not only acts on \( \text{P}_{II} \), but directly regulates the activity of NtcA. This metabolite increases both the specific DNA-binding affinity of NtcA [92] and the transcriptional initiation at NtcA-dependent promoters [93]. These biochemical data were corroborated by an in vivo study, in which uptake of 2-oxoglutarate by \( \text{S. elongatus} \) cells was shown to enhance the expression of NtcA-dependent genes [94]. The regulation of NtcA by 2-oxoglutarate provides an explanation for \( \text{P}_{II} \)-independent NtcA deactivation in response to ammonium. When 2-oxoglutarate levels are as low as they are in ammonium-grown cells, NtcA is inactive, regardless of \( \text{P}_{II} \) regulation. When the 2-oxoglutarate levels increase, NtcA is directly activated by 2-oxoglutarate in a \( \text{P}_{II} \)-independent manner. However, without \( \text{P}_{II} \), a further increase of 2-oxoglutarate, as it occurs in response to nitrogen starvation, does not further enhance NtcA activity. Only in presence of \( \text{P}_{II} \) a further stimulation of the NtcA activity occurs, resulting in the high levels of activity found under nitrogen-deprived conditions. According to this interpretation, 2-oxoglutarate acts on two levels: first, in its basic mode, it switches on and off the NtcA and on a second level, it acts via \( \text{P}_{II} \) signalling (see Fig. 3). However, the mechanistic basis of the \( \text{P}_{II} \)-aNtcA interaction, whether direct or indirect, remains to be elucidated.

6.2. The rationale of \( \text{P}_{II} \) phosphorylation

The biological necessity of the \( \text{P}_{II} \) phosphorylation cycle is not initially evident, since \( \text{P}_{II} \) could function also in its absence. With respect to the regulation of nitrate
transport and NtcA activity, the way in which the mutant forms wherein Ser 49 was replaced by Asp or Glu respond to the nitrogen status of the cells is similar as compared to the wild-type protein. Furthermore, the non-phosphorylated form of P II may be sufficient to regulate high affinity bicarbonate transport. Presumably, conformational changes induced by the effector molecules are sufficient to modulate the P II receptor interactions. What then is the benefit of P II phosphorylation? Phosphorylation may be required to regulate additional targets, or may represent a means of fine-tuning the signal. The actual phosphorylation status is a consequence of both phosphorylation and dephosphorylation reactions, and thus temporally integrates the 2-oxoglutarate signal. Only when the 2-oxoglutarate levels accumulate over a certain period of time, phosphorylated P II-protein accumulates with concomitant activation of NtcA and release of nitrate/nitrite transport inhibition, as depicted in Fig. 3. In accord with this model, the mutants MP2-D and MP2-E, which are always present in a pseudo-phosphorylated form, show an enhanced NtcA activation following nitrogen limitation [86]. In prochlorophytes, which lack P II phosphorylation, the simple mode of P II regulation by allosteric interaction with the effector molecules may be sufficient. They live in a stable environment and are not faced with sudden fluctuations in the carbon and nitrogen supply.

6.3. Outlook

Although recent studies have yielded deeper insights into the function of P II in unicellular cyanobacteria, important questions still remain to be resolved. The most intriguing question concerns P II phosphorylation. Despite considerable efforts in a number of laboratories, a P II kinase has not been identified so far. None of the protein serine/threonine kinase knock-out mutants in *Synechocystis* PCC 6803 has lost the ability of P II phosphorylation (S. Bedu, personal communication), suggesting that there is redundancy through multiple kinases or that P II is phosphorylated by an unique kinase, which has not been identified by homology searches. A further important problem relates to the interaction of P II with regulation targets. The molecular basis of this interaction must be established and, upon defining this, new targets of P II regulation will be discovered. In addition, even more challenging problems exist in filamentous cyanobacteria. The mode of P II modification is not clear and functional analysis of P II has been hampered by the fact that the P II protein is essential in these organisms. Since NtcA is now known to be a global regulatory factor required for several differentiation processes in these species, it is likely that P II will also play a central role in these highly evolved prokaryotes. The application of the powerful tools of molecular biology, biochemistry and physiology in combination with the access of the genome data will help to gain novel insights into this fascinating story of signal transduction.

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