PROTEIN PHOSPHATASE PHHA FROM SYNECHOCYSTIS SP. PCC 6803: THE PHYSIOLOGICAL FRAMEWORK OF PII-P DEPHOSPHORYLATION

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The phosphorylated signal transduction protein PII (PII-P) in the cyanobacterium Synechocystis sp. strain PCC 6803 is dephosphorylated by Phha, a protein phosphatase of the 2C family (PP2C). In this study, the physiological conditions of PII-P dephosphorylation were investigated with respect to the in vivo specificity of PII-P towards Phha and the cellular abundance of Phha in cells growing under different nitrogen regimes. Furthermore, the consequences of impaired PII-P dephosphorylation with respect to short-term inhibition of glutamine synthetase (GS) were studied. With a contribution of approximately 15% of total Mn2+-dependent p-nitrophenyl phosphate hydrolysis activity, Phha has only a minor impact on the total PP2C activity in Synechocystis extracts. Nevertheless, residual PII-P dephosphorylation in Phha-deficient cells could only be observed after prolonged incubation in the presence of ammonium. The abundance of Phha correlates with the phosphorylation state of PII under nitrogen-replete conditions and is specifically enhanced by nitrate. Regulation of phha expression operates at the post-transcriptional level. In the presence of nitrate/nitrite, Phha is present in molar excess over PII-P, enabling the cells to rapidly dephosphorylate PII-P in response to changing environmental conditions. A Phha-deficient mutant is not impaired in short-term inhibition of GS activity following ammonium treatment. Down-regulation of GS occurs by induction of gif genes (encoding GS inactivating factors 7 and 17), which is controlled by NtcA-mediated gene repression. Thus, impaired PII-P dephosphorylation does not affect ammonium-promoted inactivation of NtcA.

INTRODUCTION

Survival and proliferation of bacteria in natural habitats depends on the ability to rapidly acclimate to changes in the environment, which requires sensitive signal perception and signal transduction systems. Genomic sequences of bacteria, and in particular of cyanobacteria, have revealed a remarkably large number of genes which code for homologues of eukaryotic-type signalling proteins, such as protein-serine/threonine and protein-tyrosine kinases as well as various types of protein phosphatases (Ponting et al., 1999; Zhang et al., 1998). Among the protein phosphatases, members of the protein phosphatase 2C (PP2C) family are particularly abundant (Bork et al., 1996), but only a few of them have a known physiological function (reviewed by Shi, 2004). Recently, the function of a novel PP2C member could be identified in the cyanobacterium Synechocystis PCC 6803. The enzyme, termed Phha, catalyses the dephosphorylation of the phosphorylated signal transduction protein PII (Irmler & Forchhammer, 2001).

PPII signalling proteins are the most widely distributed signal transduction proteins in nature, and homologues are found in bacteria, archaea and plants. PII proteins play a central role in the regulation of anabolic nitrogen metabolism, but despite their high conservation at the structural level, the regulatory targets are quite diverse in different phylogenetic lineages (for reviews see Ninfa & Atkinson, 2000; Arcondeguy et al., 2001; Forchhammer 2004). The hallmark of PII signalling in the cyanobacteria Synechococcus and Synechocystis is reversible phosphorylation at a seryl residue (S49) (Forchhammer & Tandeau de Marsac, 1995a). The phosphorylation state of PII is an indicator of the cellular C/N status. The highest level of PII phosphorylation is observed in nitrogen-starved cells and intermediate degrees of PII phosphorylation are observed in the presence of nitrate. Carbon limitation and the presence of ammonium stimulate dephosphorylation of PII (reviewed by Forchhammer, 2004). PII regulates various cellular processes such as nitrate/nitrite and CO2 uptake (Lee et al., 1998; Hisbergues et al., 1999) or NtcA-dependent gene expression under conditions of nitrogen deprivation (Aldehni et al., 2003; Paz-Yepez et al., 2003). Recently, the first molecular target of PII regulation was identified in
Insights into the mechanism of the PII phosphorylation/deprophosphorylation cycle in _Synechococcus elongatus_ could be obtained by biochemical and physiological studies (reviewed by Forchhammer, 1999, 2004). The _S. elongatus_ PII protein binds the effector molecules 2-oxoglutarate and ATP in a synergistic manner (Forchhammer & Hedler, 1997). In the ATP and 2-oxoglutarate bound state, PII is phosphorylated by an ATP-dependent kinase activity (Forchhammer & Tandeau de Marsac, 1995a). Dephosphorylation of PII-P is catalysed by the PP2C homologue PphA. _In vitro_, PphA reactivity towards PII-P was strongly affected by the addition of ATP, ADP and 2-oxoglutarate. It was suggested that these effector molecules modulate the molecular recognition of PII-P by PphA through binding to PII (Ruppert et al., 2002). Inhibition of PII-P dephosphorylation by ATP was strongly enhanced by 2-oxoglutarate and to a lower extent by oxaloacetate. In the presence of physiological levels of the effector molecules, PII-P dephosphorylation mainly responded to the 2-oxoglutarate levels (Forchhammer et al., 2004).

Despite the detailed knowledge about _in vitro_ PII dephosphorylation by PphA, the physiological context of PII dephosphorylation has been poorly investigated. Only the short-term response of PII-P dephosphorylation has been reported in a PphA-deficient mutant of _Synechocystis_ PCC 6803 (MPPhA) (Irmler & Forchhammer, 2001; Forchhammer et al., 2004). Within a period of 30 min, the mutant was unable to dephosphorylate PII-P in response to various external stimuli, suggesting that PphA was the major PII-P phosphatase in these cells. However, the contribution of PphA to total phosphatase activity in _Synechocystis_ cells was not known, nor has the long-term acclimatization of the PII-P phosphorylation state to various C/N conditions been investigated in the PphA-deficient mutant. Also, the abundance and cellular localization of PphA under various growth conditions was not known. The present investigation was performed to clarify these points, which are crucial to understanding the _in vivo_ specificity of PII-P-PphA recognition and to gain deeper insights into the _in vivo_ conditions of PphA-mediated PII dephosphorylation in _Synechocystis_ PCC 6803.

**METHODS**

**Culture conditions.** _Synechocystis_ sp. PCC 6803<sup>3</sup> (Grigorieva & Shestakov, 1982) and the derived PII-P-deficient mutant MPPhA (pphA<sup>Δ</sup>; Irmler & Forchhammer, 2001) were grown in liquid BG11 medium (Rippka, 1988) supplemented with 5 mM NaHCO<sub>3</sub>. Either 17·6 mM NaNO<sub>3</sub> (BG11<sup>β</sup>) or 5 mM NH<sub>4</sub>Cl (BG11<sup>α</sup>) buffered with 20 mM HEPES/NaOH pH 7·5 was used as nitrogen source. The cultures were incubated photoautotrophically at an illumination of 40 μmol photons m<sup>−2</sup> s<sup>−1</sup> at 25 °C and were constantly shaken at 150 r.p.m. The mutant strain was maintained with kanamycin (30 μg ml<sup>−1</sup>). For low-carbon conditions, mid-exponential-phase cultures were shifted to flasks filled to the top with bicarbonate-free BG11<sup>α</sup> medium and incubated without stirring at an irradiance of 20 μmol photons m<sup>−2</sup> s<sup>−1</sup>. Growth of the cultures was monitored by measuring the OD<sub>750</sub>.

**Determination of the modification state of PII.** The phosphorylation state of PII in _in vivo_ was analysed by non-denaturing PAGE followed by immunoblot analysis of PII as described by Forchhammer & Tandeau de Marsac (1994). In the figures, PII<sup>1</sup>, PII<sup>2</sup> and PII<sup>3</sup> represent isoforms of trimeric PII carrying no, one, two or three phosphorylated subunits, respectively.

**Enzyme assays**

_p-Nitrophenyl phosphate (pNPP) phosphatase activity._ Phosphatase activity with pNPP as substrate was assayed in cell-free extracts according to Mackintosh (1993). Cultures of _Synechocystis_ PCC 6803 wild-type and MPPhA were grown in BG11<sup>α</sup> medium to an OD<sub>750</sub> of 0·8. The cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris/HCl pH 7·4, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0·5 mM EDTA and 1 mM benzamidine) and broken by sonification. Cell debris and insoluble material was removed by two consecutive centrifugation steps (10 min at 10 000 × g and 1 h at 100 000 × g). The final supernatants were dialysed against a buffer without divalent cations (20 mM Tris/HCl pH 7·5, 50 mM NaCl, 1 mM DTT and 0·5 mM EDTA). In a 1 ml standard assay, 250 μg protein of the supernatants was reacted with 3 mM pNPP in a buffer (20 mM Tris/HCl pH 8·3, 10 mM NaCl and 0·2 mM DTT) in the presence or absence of 0·4 mM MnCl<sub>2</sub>. The increase in A<sub>405</sub> was recorded in an Ultrospec 3000 spectrophotometer (Amersham-Pharmacia) against a blank reaction in which pNPP was omitted.

**In situ glutamine synthetase (GS) assay.** GS activity was determined by the formation of γ-glutamylhydroxamate (transferase assay) as described previously (Forchhammer & Tandeau de Marsac, 1995b).

**In vitro PII dephosphorylation in cell extracts.** Cultures of the wild-type and MPPhA strain of _Synechocystis_ PCC 6803, grown in liquid BG11<sup>α</sup> medium to mid-exponential phase, were treated with 0·5 mM DON (6-diazo-5-oxo-1-norleucine) for 1 h prior to harvest, to maximize the phosphorylation state of PII. Extracts of these cells were prepared in a Tris/HCl pH 7·4, 4 mM EDTA buffer by grinding with glass beads in a RiboLyser (Hybaid) apparatus as described by Heinrich et al. (2004). After removal of small effector molecules through a protein-desalting spin column (Pierce) the extracts were concentrated by ultrafiltration using a 5 kDa cut-off membrane (Nanosept, Pall Life Science). The extracts (approx. 8 μg protein μl<sup>−1</sup>) were diluted in reaction buffer (10 mM Tris/HCl pH 7·4, 1 mM DTT, 50 mM NaCl and 5 mM benzamidine) with or without 20 mM MgCl<sub>2</sub> to a final concentration of 1 μg μl<sup>−1</sup> and incubated at 37 °C. After different times, samples (each 15 μg protein) were removed and the phosphorylation state of PII was analysed by non-denaturing PAGE followed by immunoblot analysis.

**PphA expression under different growth conditions.** To analyse nitrogen-dependent expression of PphA in _Synechocystis_ sp. PCC 6803, a pre-culture was grown in BG11<sup>α</sup> or in modified BG11<sup>α</sup> medium, in which molybdenum was replaced by tungsten (4·8 μM), as indicated. At the mid-exponential phase of growth, the cells were harvested by centrifugation and washed in medium free of combined nitrogen (BG11<sup>β</sup>). The cells were resuspended in BG11<sup>β</sup> or in modified BG11<sup>α</sup> medium (containing tungsten) to an OD<sub>750</sub> of approx.
0.4 and distributed to culture flasks. NH4Cl (5 mM final concentration), NaNO3 or NaN3O3 (concentrations as indicated) were added, the cultures were incubated for 24 h as described above and then samples were removed for PphA analysis. For the analysis of PphA expression in the presence of NaNO3 and MSX (L-methionine-DL-sulfoximine), BG11S-grown cells were washed in BG11S medium and resuspended in nitrogen-free medium to an OD750 of 0.5. One half of the culture was treated with MSX (0.2 mM final concentration) as a control, while the other half was treated with MSX and NaNO3 (10 mM final concentration). Samples were removed after 4 h and 8 h incubation.

To determine PphA abundance in the various samples as described above, the cells from a sample equivalent to 1 ml with an OD750 of 1 were harvested by centrifugation. Cell pellets were resuspended in 80 μl SDS sample buffer (75 mM Tris/HCl pH 6-8, 100 mM DTT, 70 mM SDS, 10%, v/v, glycerol and bromophenol blue) and were lysed by heating for 5 min at 95°C. After removing the cell debris by centrifugation, 20 μl aliquots were subjected to electrophoresis in a 12.5% SDS-polyacrylamide gel. Following electrophoresis, PphA was revealed by immunoblot analysis, using polyclonal antibodies (Pineda, Berlin) raised against purified PphA (Irmler & Forchhammer, 2001). The amount of PphA was quantified using a Bio-Rad FluorS Imager together with the Quantity One software (Bio-Rad).

Table 1. Primers used for RT-PCR analysis or to generate probes for RNA–DNA hybridization

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primer*</th>
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<tbody>
<tr>
<td>IF7</td>
<td>F: 5’-CGCCACCACACAAATTCCATC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-AGTTTGGCAGGAAACTGGAAG-3’</td>
</tr>
<tr>
<td>IF17</td>
<td>F: 5’-GCTCCAGCCAAAATTTCC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ACGCCCTTGACTGGCATA-3’</td>
</tr>
<tr>
<td>rnpB</td>
<td>F: 5’-GGAGGAGCCAGGCAAGA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GCCAGGAAAAGGAGCAACCT-3’</td>
</tr>
<tr>
<td>pphA</td>
<td>F: 5’-AGCCGCATTCGCGGCTTATG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TCCGAGGTAATCTCGGATTG-3’</td>
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*F, forward; R, reverse.

RESULTS AND DISCUSSION

Characterization of pNPP phosphatase activity in a PphA-deficient mutant of Synechocystis sp. PCC 6803

The Synechocystis sp. PCC 6803 genome contains eight putative PP2C homologues and several other putative phosphatases (Kaneko et al., 1996; Zhang et al., 1998; Shi, 2004). To estimate the contribution of PphA to the total phosphatase activity in Synechocystis PCC 6803, we used pNPP, a broad range substrate for various phosphatases (Mackintosh, 1993), to determine total phosphatase activity in cell-free extracts from wild-type and PphA-deficient cells (Table 2). As a typical member of the PP2C family, PphA requires Mg2+ or Mn2+ ions for catalytic activity; reactivity towards the artificial substrate pNPP is only observed in the presence of Mn2+ ions at pH values above 8 (Ruppert et al., 2002). Therefore, we determined pNPP hydrolysis activity in cell-free extracts at pH 8.3 in the presence or absence of 0.4 mM MnCl2. In the absence of divalent cations, pNPP hydrolysis was almost identical in extracts from wild-type and MPpHα cells (Table 2), indicating the activity of phosphatases not belonging to the 2C family. In the presence of Mn2+ ions, pNPP hydrolysis increased strongly due to the contribution of PP2C phosphatases. Under these assay conditions, extracts from wild-type cells displayed approximately 15% higher Mn2+ specific activity than extracts from MPpHα cells, revealing

Table 2. Reactivity of cell extracts from the wild-type (WT) and the PphA-deficient mutant (MPpHα) towards pNPP

<table>
<thead>
<tr>
<th>Buffer conditions</th>
<th>WT</th>
<th>MPpHα</th>
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<tbody>
<tr>
<td>Divalent cation-free</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>0–4 mM MnCl2</td>
<td>5.9 ± 1.3</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>Mn-specific activity</td>
<td>5.2 ± 1.5</td>
<td>4.4 ± 1.3</td>
</tr>
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Activities are given in nmol pNPP hydrolysed min⁻¹ (mg protein)⁻¹. Means ± SD from three independent experiments are given.
the contribution of PphA to the Mn\(^{2+}\)-specific pNPP hydrolysis activity.

**P\(_{II}\)-P dephosphorylation in PphA-deficient cells**

The PphA-deficient mutant is characterized by its inability to rapidly dephosphorylate P\(_{II}\)-P in response to various stimuli, such as addition of ammonium, CO\(_2\) shortage, inhibition of CO\(_2\) fixation or any treatments which inhibit photosynthetic electron flow (Irmler & Forchhammer, 2001; Forchhammer et al., 2004). Whereas in wild-type cells P\(_{II}\)-P is rapidly dephosphorylated within minutes after an appropriate treatment like the addition of 5 mM ammonium, P\(_{II}\) remains phosphorylated in MPphA cells, indicating that PphA is the only specific P\(_{II}\)-P phosphatase in *Synechocystis* PCC 6803. In contrast to the impaired fast P\(_{II}\)-P dephosphorylation, we observed that 1 day after exposure to ammonium, P\(_{II}\) accumulated in its non-phosphorylated form in MPphA cells (Fig. 1A). When, however, cells were maintained in the presence of nitrate under C\(_{i}\)-limiting conditions (non-aerated, standing culture) the phosphorylation state of P\(_{II}\) remained high in MPphA cells whereas P\(_{II}\) was almost completely dephosphorylated in the wild-type (Fig. 1B). Compared to ammonium-treated cells, this is an intriguing difference. Complete P\(_{II}\) dephosphorylation in wild-type cells indicates that the 2-oxoglutarate level, which was shown to be the major signal for P\(_{II}\) dephosphorylation (Forchhammer et al., 2004), is low under both conditions, in agreement with measurements of cellular 2-oxoglutarate levels (Muro-Pastor et al., 2001). A possible explanation could be a lower activity of P\(_{II}\) kinase activity in ammonium-grown cells, which only becomes evident in the PphA-deficient background. Indeed, it has been reported that P\(_{II}\) kinase activity is impaired in a NtcA-deficient mutant of *S. elongatus* (Lee et al., 1999; Sauer et al., 1999), indicating that this activity is under nitrogen control. This is further supported by *in vitro* measurements of P\(_{II}\) kinase activity in *S. elongatus* extracts: in extracts from ammonium-grown cells, kinase activity was much lower than in extracts from nitrate-grown cells (H. Dierks, Diplomarbeit, Lehrstuhl für Mikrobiologie der LMU München, and K. Forchhammer, unpublished data).

Accumulation of non-phosphorylated P\(_{II}\) in ammonium-treated MPphA cells could be caused by a residual P\(_{II}\)-P dephosphorylation activity catalysed by other cellular phosphatases or may result from degradation of P\(_{II}\)-P concomitantly with *de novo* synthesis of non-phosphorylated P\(_{II}\). To distinguish between these possibilities, the long-term effect of ammonium was analysed in the presence of the protein synthesis inhibitor chloramphenicol, to inhibit *de novo* protein synthesis (Fig. 1C). Following the addition of antibiotic and ammonium, P\(_{II}\) was completely dephosphorylated in the wild-type after 2.5 h, whereas only a minor change in the phosphorylation status of P\(_{II}\) occurred in MPphA during this time period, as expected. However, during prolonged incubation of the PphA-deficient mutant, P\(_{II}\)-P was completely substituted by non-phosphorylated P\(_{II}\). The concomitant arrest of cell growth revealed that chloramphenicol was biologically active and *de novo* protein synthesis was in fact inhibited. Therefore, the apparent P\(_{II}\)-P dephosphorylation cannot be attributed to protein turnover, but rather results from a slow, PphA-independent, P\(_{II}\)-P dephosphorylation activity catalysed by phosphatases which are already present in nitrate-grown cells.

To gain further insight into the residual P\(_{II}\)-P dephosphorylating activity, crude extracts from wild-type and PphA-deficient cells were tested for their ability to dephosphorylate endogenous P\(_{II}\)-P protein. As shown in Fig. 2, P\(_{II}\)-P was rapidly dephosphorylated in wild-type extracts in a Mg\(^{2+}\)-dependent manner, whereas no dephosphorylation was detected in the absence of divalent cations. By contrast, Mg\(^{2+}\)-dependent dephosphorylation of P\(_{II}\) was almost completely absent in MPphA extracts. Although PphA contributes only a minor proportion of total PP2C activity (see above), *in vitro* and *in vivo* dephosphorylation of P\(_{II}\)-P...
is strongly impaired in the PphA-deficient mutant, implying that PII-P is a poor substrate for the other protein phosphatases. The mechanistic basis of its specificity towards PphA deserves further investigation.

**Nitrogen-source-dependent accumulation of PphA**

To reveal the cellular localization of PphA, extracts of *Synechocystis* PCC 6803 cells were fractionated into soluble and particulate (membrane-containing) fractions, and the presence of PphA was analysed by immunoblotting with an antiserum raised against purified PphA. The analysis was performed with nitrate-grown cells and with cells that had been treated with 5 mM ammonium for 15 min. As shown in Fig. 3, under both conditions, PphA was only recovered in the soluble fraction, with no traces detectable in the particulate fraction, confirming the assumption from primary structure analysis that PphA is a soluble, cytoplasmic protein. The abundance of PphA in extracts from cells that were grown in standard BG11N medium was estimated by densitometric quantification of the PphA reactive band compared to PphA standards. Approximately 40–50 ng PphA was detected in 15 μg total cellular protein, corresponding to about 0·3 % of total cellular protein (data not shown). To elucidate whether PphA is differentially expressed under conditions corresponding to different degrees of PII phosphorylation, extracts from cells which had been grown with different nitrogen supplies were subjected to immunoblot analysis (Fig. 4). In fact, the amount of PphA varied considerably under those conditions. In cells that were starved for combined nitrogen or were grown in the presence of ammonium (each for 24 h), only low levels of PphA could be detected. The cellular PphA abundance was higher with increasing nitrate or nitrite concentrations in the medium. Maximal levels were obtained with approximately 10 mM nitrate or 5 mM nitrite (Fig. 4A). To reveal whether the reduced amount of PphA in ammonium-grown cultures was due to ammonium repression or was caused by the absence of nitrate/nitrite, cells grown in nitrate-supplemented medium were either challenged with 5 mM ammonium (without removal of nitrate, Fig. 4B) or shifted to nitrate-free, ammonium-supplemented medium (Fig. 4C). The amount of PphA rapidly decreased in the culture in which nitrate was substituted by ammonium, whereas the addition of ammonium in the presence of nitrate did not affect the abundance of PphA. This indicated that the removal of nitrate rather than ammonium is the cause of the reduced abundance of PphA.
than the addition of ammonium led to reduced PphA levels. The differential accumulation of PphA at various concentrations of nitrate and nitrite was intriguing and raised the question of a specific effect of these molecules. To clarify this point, nitrate reductase was poisoned by growing cells in a medium in which molybdenum was replaced by tungsten. Under this condition, the cells expressed a non-active nitrate reductase. After verification of the absence of nitrate reductase activity in these cells, the response of PphA accumulation to the nitrogen source was re-examined (Fig. 5A). In the absence of functional nitrate reductase activity, nitrate could not enhance the PphA level, whereas the positive effect of nitrite was not impaired. This implied that nitrite (an intermediate of nitrate reduction) was directly involved in the control of the PphA abundance. To distinguish between an anabolic effect and a direct impact of nitrite on PphA accumulation, an experiment was performed in the presence of the GS inhibitor MSX. After shifting cells to nitrogen-depleted medium containing MSX, the culture was divided into two and nitrite was added to one of the samples. Aliquots were withdrawn and PphA abundance was analysed (Fig. 5B). PphA levels increased in the presence of nitrite whereas they decreased in its absence, suggesting that nitrite assimilation is not required to promote PphA accumulation. This strongly suggests a direct effect of nitrite on the level of PphA.

The nitrite dependence of PphA accumulation raised the question of whether the nitrite-specific gene activator NtcB (Aichi et al., 2001) was involved in this response. However, almost identical levels of PphA were detected in a NtcB-deficient mutant compared to the wild-type under the various nitrogen conditions (data not shown), implying that pphA expression is not under NtcB control. To reveal whether the abundance of PphA is regulated at the transcriptional level, the amount of pphA mRNA was examined by Northern blot analysis (Fig. 6A). As noted previously (Irmler & Forchhammer, 2001), pphA mRNA appears as a rather unstable transcript, since a broad band with considerable amount of degradation was observed with no apparent difference between preparations from different nitrogen regimes. Hence quantitative RT-PCR was carried out to clarify this expression pattern (Fig. 6B, C). Based on cycles required for exponential PCR amplification of pphA (Fig. 6B), RNA preparations from cells grown under various nitrogen regimes were amplified with 22 cycles. Regardless of the nitrogen conditions, the abundance of the pphA transcript was not significantly altered, suggesting a low constitutive expression of pphA (Fig. 6C). The apparent lack of transcriptional control agrees with the NtcB independence of PphA, suggesting that its accumulation is

![Fig. 5. Effect of nitrite on the accumulation of PphA in Synechocystis PCC 6803. Cell-free extracts were prepared and PphA was revealed by immunoblotting. (A) Cells were incubated for 24 h in the absence of combined nitrogen source (−) or were grown in the presence of 5 mM NH₄Cl or in the presence of 20 or 10 mM nitrate or nitrite, as indicated. In the medium, molybdenum was replaced with tungsten, so that the cells expressed a non-active nitrate reductase. (B) Nitrate-grown cells (NO₃⁻) were transferred to a medium containing 0.2 mM MSX (inhibitor of GS) in the presence or absence of nitrite (10 mM) as described in Methods. The abundance of PphA was analysed at the times indicated.](image-url)
rather controlled post-transcriptionally. Possible explanations are nitrite-dependent control at the level of pphA translation or PphA protein stability; this awaits further investigation.

Whatever the molecular mechanism for pphA expression, PphA abundance seems to be optimized to allow rapid dephosphorylation of PII-P in nitrate- and nitrite-grown cells. In the presence of ammonium, PII is barely phosphorylated, due to the low PII kinase activity. Under these conditions, low amounts of PphA are sufficient to maintain PII in the dephosphorylated state. In nitrate- or nitrite-grown cells, however, the amount of PphA increases as PII is phosphorylated. The elevated levels of PphA might be required to rapidly dephosphorylate PII-P under appropriate conditions. Considering the size and the estimated abundance of PphA (M_r 28 472; approx. 0.3 % of total protein) and of PII (M_r of monomer 12 397; 0.1–0.2 % of soluble protein, Forchhammer & Tandeau de Marsac, 1994; K. Forchhammer, unpublished), PphA seems to be in nearly stoichiometric amounts or slightly in excess over its substrate PII-P. In vitro studies suggest that, as long as the cellular concentrations of ATP and 2-oxoglutarate are high, PphA is not able to dephosphorylate PII-P (Ruppert et al., 2002). When, however, the cellular 2-oxoglutarate level decreases, PphA can rapidly dephosphorylate PII-P without the need for multiple substrate turnover. This enables the cells to rapidly respond to changing 2-oxoglutarate concentrations.

Effect of PphA deficiency on short-term inhibition of GS

The PphA-deficient strain was not impaired in acclimatizing to different nitrogen sources, as far as cell growth was concerned. Since it was shown recently that NtcA-dependent gene expression depends on PII signalling under conditions of nitrogen deprivation (Aldehni et al., 2003; Paz-Yepez et al., 2003), we wanted to investigate whether impaired PII-P dephosphorylation affects an NtcA-dependent response. In Synechocystis PCC 6803, GS activity is down-regulated by the inactivating factors IF7 and IF17, whose expression depends on NtcA (Garcia-Dominguez et al., 2000). In the absence of ammonium, active NtcA represses transcription of the IF-encoding gif genes. The addition of ammonium leads to inactivation of NtcA (Herrero et al., 2001), and thereby to the loss of gif repression. Synthesis of IF7/IF17 then depresses GS activity. Down-regulation of GS activity can therefore be used as a means to determine in vivo inactivation of NtcA following ammonium addition, a strategy which has been used successfully in previous studies (Muro-Pastor et al., 2001). To investigate whether the PphA-deficient mutant was able to rapidly acclimatize to ammonium with respect to the regulation of GS activity, nitrate-grown cells of the wild-type and MPphA strain were challenged with 5 mM NH_4Cl, and after different times, aliquots were removed and the in situ GS (transferase) activity was determined. As shown in Fig. 7(A), GS activity declined in the mutant even faster than in the wild-type, indicating that the lack of PphA (and thereby the lack of PII-P dephosphorylation) does not affect the rapid in vivo inactivation of GS. The mechanistic basis of the accelerated GS inactivation in MPphA requires further investigation. To confirm that GS inactivation was indeed due to ammonium-prompted gif gene expression, the levels of gifA and gifB mRNA from cells that were challenged with ammonium was analysed by Northern blotting (Fig. 7B). The same expression pattern as reported in previous studies of ammonium-treated Synechocystis wild-type cells was observed. The transcript of the tightly controlled gifB gene increased strongly and that of gifA partially (Muro-Pastor et al., 2001) in both the wild-type and MPphA strains, confirming that gif induction is independent of PII dephosphorylation.

It was previously known that NtcA-dependent gene expression is down-regulated in PII-deficient mutants acclimatized to ammonium (Lee et al., 2000; Aldehni et al., 2003; Paz-Yepez et al., 2003). This investigation shows, in addition, that the presence of PII-P does not impair the response of NtcA to ammonium, at least as far as gif gene repression is concerned. This conclusion is corroborated by the observation that in PII S49D/E mutants, which potentially mimic phosphorylated PII, NtcA-dependent gene expression still responds to the nitrogen status (Paz-Yepez et al., 2003).

**Fig. 7.** GS activity and gif expression in Synechocystis PCC 6803 wild-type and MPphA cells. (A) Short-term response of GS (transferase) in wild-type (○) and MPphA-deficient cells (○) to the addition of 5 mM ammonium. GS activity prior to the addition of ammonium was taken as 100 %. (B) Northern blot analysis of the expression of gifA and gifB (encoding IF7 and IF17, respectively) in wild-type and the mutant MPphA in nitrate-grown cells and 15 min after addition of ammonium (5 mM).
Previous in vitro studies showed direct regulation of NtcA by 2-oxoglutarate levels (Vazquez-Bermudez et al., 2002; Tanigawa et al., 2002). Whether this sufficiently explains the rapid NtcA response towards ammonium remains to be demonstrated; however, PII dephosphorylation seems not to be involved in this process.

ACKNOWLEDGEMENTS

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