Role of calcium in acclimation of the cyanobacterium *Synechococcus elongatus* PCC 7942 to nitrogen starvation

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A Ca^{2+} signal is required for the process of heterocyst differentiation in the filamentous diazotrophic cyanobacterium *Anabaena* sp. PCC 7120. This paper presents evidence that a transient increase in intracellular free Ca^{2+} is also involved in acclimation to nitrogen starvation in the unicellular non-diazotrophic cyanobacterium *Synechococcus elongatus* PCC 7942. The Ca^{2+} transient was triggered in response to nitrogen step-down or the addition of 2-oxoglutarate (2-OG), or its analogues 2,2-difluoropentanedioic acid (DFPA) and 2-methylenepentanedioic acid (2-MPA), to cells growing with combined nitrogen, suggesting that an increase in intracellular 2-OG levels precedes the Ca^{2+} transient. The signalling protein P_{II} and the transcriptional regulator NtcA appear to be needed to trigger the signal. Suppression of the Ca^{2+} transient by the intracellular Ca^{2+} chelator *N,N'*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-bis[(acetyloxy)methyl] ester (BAPTA-AM) inhibited expression of the *glnB* and *glnN* genes, which are involved in acclimation to nitrogen starvation and transcriptionally activated by NtcA. BAPTA-AM treatment partially inhibited expression of the *nblA* gene, which is involved in phycobiliprotein degradation following nutrient starvation and is regulated by NtcA and NblR; in close agreement, BAPTA-AM treatment partially inhibited bleaching following nitrogen starvation. Taken together, the results presented here strongly suggest an involvement of a defined Ca^{2+} transient in acclimation of *S. elongatus* to nitrogen starvation through NtcA-dependent regulation.

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INTRODUCTION

Cyanobacteria are a large group of photosynthetic oxygen-evolving prokaryotes. They originated during the Precambrian era (2.8×10^9 years ago) and are known to survive a wide spectrum of environmental stresses (Tandeau de Marsac & Houmard, 1993).

The ability to cope with nutrient limitation or starvation is a requirement for survival in nature. Diazotrophic cyanobacteria circumvent nitrogen starvation by fixing nitrogen; however, in non-diazotrophic strains, nitrogen starvation triggers an acclimation process known as chlorosis or bleaching (Allen & Smith, 1969; Schwarz & Forchhammer, 2005). During chlorosis the phycobilisomes, which account for up to 50 % of total cell protein,

are degraded to supply the cells with amino acids for the synthesis of proteins needed for acclimation and survival (Bhaya *et al.*, 2000).

The unicellular non-diazotrophic *Synechococcus elongatus* strain PCC 7942 (hereinafter termed *S. elongatus*) is a model organism for studying molecular and physiological mechanisms during the process of chlorosis (Schwarz & Forchhammer, 2005). Several non-bleaching (*nbl*) mutants have been isolated and genes cloned (Collier & Grossman, 1994; Grossman *et al.*, 2001; Li & Sherman, 2002). The *nblA* product, a small dimeric protein of 7 kDa helical subunits (Bienert *et al.*, 2006), triggers the initiation of phycobilisome degradation (Collier & Grossman, 1994; Luque *et al.*, 2001, 2003) by binding to the peripheral phycobilisome rods (Bienert *et al.*, 2006). The expression of *nblA* is controlled by two transcriptional regulators, NblR and NtcA. NblR acts as a general transcriptional regulator that integrates different stress signals that lead to phycobilisome degradation (Schwarz & Grossman, 1998). NtcA is a transcriptional activator (seldom a repressor) of a

Abbreviations: BAPTA-AM, *N,N'*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-bis[(acetyloxy)methyl] ester; $[\text{Ca}^{2+}]_{\text{i}}$, free intracellular calcium concentration; DFPA, difluoropentanedioic acid; 2-MPA, methylenepentanedioic acid; 2-OG, 2-oxoglutarate.

large number of genes mainly involved in nitrogen metabolism (reviewed by Herrero *et al.*, 2001, 2004). NtcA belongs to the cyclic AMP receptor protein family of transcriptional regulators (Vega-Palas *et al.*, 1992) and binds to a conserved palindromic sequence (GTA-N8-TAC) (Herrero *et al.*, 2001). 2-Oxoglutarate (2-OG), the signal of carbon/nitrogen status in cyanobacteria, directly stimulates NtcA–DNA binding as well as NtcA transcriptional activity *in vitro*; however, direct binding of 2-OG to NtcA has not yet been shown. Signalling protein P_{II}, encoded by *glnB*, was the first 2-OG-responsive factor identified in cyanobacteria. In *S. elongatus*, protein P_{II} is modified by phosphorylation on a seryl residue at position 49. The phosphorylation state of the protein is dependent on the intracellular concentration of 2-OG, reflecting the nitrogen/carbon status of the cell (Forchhammer, 2004). There is evidence of functional interaction of P_{II} and NtcA during nitrogen deprivation in *S. elongatus* (Aldehni *et al.*, 2003); recently, it has been reported that, depending on the cellular levels of 2-OG, protein PipX can form complexes with P_{II} or NtcA providing a physical/functional link between the two regulatory proteins (Espinosa *et al.*, 2006, 2007).

In the diazotrophic filamentous cyanobacterium *Anabaena* sp. PCC 7120, nitrogen starvation not only leads to increased 2-OG levels (Laurent *et al.*, 2005), but also to increased concentrations of free Ca²⁺ ions, which are found to be required for heterocyst differentiation (Torrecilla *et al.*, 2004; Zhao *et al.*, 2005). By using a recombinant strain expressing the Ca²⁺-binding photo-protein apoequorin (Torrecilla *et al.*, 2000), we have found that a transient increase in intracellular free Ca²⁺ (hereinafter, a Ca²⁺ transient) is also triggered following nitrogen deprivation in the unicellular non-diazotrophic *S. elongatus*. The addition of 2-OG or its nonmetabolizable analogues 2,2-difluoropentanedioic acid (DFPA) or 2-methylenepentanedioic acid (2-MPA) (Laurent *et al.*, 2005; Chen *et al.*, 2006) also induced the Ca²⁺ transient. We present evidence to suggest a role for Ca²⁺ in the acclimation of *S. elongatus* to nitrogen starvation through regulation of NtcA-dependent promoters.

METHODS

Cyanobacterial strains and culture conditions. The following cyanobacterial strains were used in this study: *S. elongatus* PCC 7942; *S. elongatus* mutants MP2, lacking the P_{II} signalling protein (Forchhammer & Tandeau de Marsac, 1995), and MNtcA, lacking the NtcA protein (Sauer *et al.*, 1999); the *S. elongatus luxAB* reporter strains FAM1, carrying a full-length *glnB* promoter fused to *luxAB*, FAM2, carrying a truncated *glnB* promoter containing only the NtcA-dependent part (Aldehni *et al.*, 2003), FAM-431, with a full-length *glnN* promoter, and FAM-84, with a truncated *glnN* promoter containing only the NtcA-binding site (Aldehni & Forchhammer, 2006); and *S. elongatus* WT-103, with the full-length *nblA* promoter fused to *luxAB* (Espinosa *et al.*, 2007). Cells were grown in liquid BG11 medium (Rippka, 1988), except for MNtcA and derivatives expressing apoequorin, which were grown in BG11 in which the nitrate salt was replaced by 5 mM NH₄Cl (BG11-NH₄), buffered with

5 mM HEPES, pH 7.8, on a rotary shaker at 28 °C under 65 µE m⁻² s⁻¹ fluorescent white light. When ammonium was used as the nitrogen source, BG11-NH₄ buffered with 5 mM HEPES, pH 7.8, was used. For nitrogen step-down experiments, BG11₀ (medium deprived of combined nitrogen) buffered with 5 mM HEPES, pH 7.8, was used. Cultures of mutants MP2 and MNtcA were maintained in the presence of 25 µg kanamycin ml⁻¹. Strains expressing apoequorin were treated with 5 µg gentamicin ml⁻¹. Strains containing the *luxAB* reporter constructs were grown with 5 µg chloramphenicol ml⁻¹.

Construction of apoequorin-expressing *S. elongatus* strains.

A fragment of 0.78 kb containing the apoequorin gene was PCR-amplified from template pBG2000 (Torrecilla *et al.*, 2000) using primers aequofor (5'-GCTCTAGAGGAGAAGCAAACATGACAA-GCAAAC-3'), containing an *Xba*I restriction site, and aequorev (5'-TCCCCCGGGTGCATCACCACCGTA-3'), containing an *Sma*I site. Following appropriate restriction, the fragment was cloned into the *Xba*I–*Sma*I sites of pHCN2, a derivative of pCB4 (Gendel *et al.*, 1983). Briefly, the gentamicin-resistance gene from pVZ322 (Zinchenko *et al.*, 1999) with a modified 3' terminal part (5'-CCCCGGGTCGACG-AGCTCTAGAATTC-3') to generate additional restriction sites (*Eco*RI, *Xba*I, *Sac*I, *Sal*I and *Sma*I) was cloned into the *Bam*HI restriction site of pCB4; into the *Eco*RI site of the resulting pCB-Gen plasmid, the entire promoter fragment of the PCC6803 *petJ* gene was cloned. Ligation of the PCR-amplified aequorin fragment into pHCN2, such that the apoequorin gene is transcribed from the *petJ* promoter (Tous *et al.*, 2001), resulted in pHCN-aeq. This plasmid was introduced into *S. elongatus* wild-type and mutant MP2 strains by transformation (Golden *et al.*, 1987). pHCN-aeq was introduced into mutant strain MNtcA by conjugation (Wolk *et al.*, 1984; Elhai & Wolk, 1988). Transformants were selected on BG11 or BG11-NH₄ plates containing 5 µg gentamicin ml⁻¹.

In vivo aequorin reconstitution and luminescence measurements.

For aequorin luminescence measurements, *in vivo* reconstitution of aequorin was performed by the addition of 5 µM coelenterazine (FluoProbes) to cell suspensions of *S. elongatus* wild-type, MNtcA and MP2 strains expressing apoequorin, followed by incubation for 4 h in darkness with shaking, as previously described (Torrecilla *et al.*, 2000). Before Ca²⁺ measurements were made, cells were washed twice in BG11 medium buffered with 10 mM HEPES, pH 7.5, containing 0.5 mM EGTA, to remove excess coelenterazine.

For luminescence measurements, 150 µl reconstituted cell suspension was transferred to wells of an opaque white 96-well microtitre plate (Porvair Sciences). Luminescence was recorded every 1 s for the duration of the experiment in a Centro LB 960 luminometer (Berthold Technologies).

Calibration of changes in the free intracellular Ca²⁺ concentration ([Ca²⁺]_i) requires knowledge of the total available amount of reconstituted aequorin in cell suspensions (*L*_{max}) at any one point in time during the experiment as well as the running luminescence (*L*₀). For estimation of total aequorin luminescence, the remaining aequorin was discharged at the end of the experiment by the addition of 150 µl 1 M CaCl₂ and 5% (v/v) Triton X-100. Rate constants of luminescence (*L*₀/*L*_{max}) were determined for each point during the experiment, and [Ca²⁺]_i was calculated as described by Torrecilla *et al.* (2000).

Nitrogen and sulphur step-down experiments. Nitrogen or sulphur step-down was initiated by washing cells, grown with either ammonium or nitrate until they reached the mid-exponential phase of growth (OD₇₅₀=0.5), three times with BG11₀ medium or BG11 deprived of a sulphur source, buffered with 5 mM HEPES (pH 7.8), and final resuspension in the same combined nitrogen- or sulphur-free medium. For sulphur deprivation experiments, MgSO₄, CuSO₄

and ZnSO_4 of medium BG11 were replaced by MgCl_2 , CuCl_2 and ZnCl_2 , respectively.

To facilitate cell loading of the intracellular Ca^{2+} chelator *N,N'*-[1, 2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-bis[(acetyloxy)methyl] ester (BAPTA-AM; FluoProbes), cells were preincubated with the chelator at a final concentration of 300 μM for 2 h in darkness with shaking prior to nitrogen or sulphur step-down; immediately after nitrogen or sulphur step-down, the chelator at the same final concentration was again added. Experiments with BAPTA-AM were made with cells resuspended in Ca^{2+} -free BG11, BG11- NH_4 or BG11₀ medium.

Analytical methods. For chlorophyll determinations, samples were extracted in methanol at 4 °C for 24 h in darkness. The chlorophyll content of the extract was estimated according to the spectrophotometric method of Marker (1972).

Phycobiliproteins were extracted after osmotic shock (Wyman & Fay, 1986); 1 ml aliquots were centrifuged and the pellets homogenized in 60–150 μl glycerol and incubated in the dark at 4 °C for 24 h. Water was then added to osmotically lyse the cells. The samples were centrifuged and the phycocyanin content was estimated according to Bennet & Bogorad (1973).

Determination of luciferase activity. To determine luciferase activity from the various *luxAB* reporter strains, 0.5 ml cell culture was supplemented with 0.5 ml 0.1 % (v/v) emulsion of *n*-decanal in 0.1 % (v/v) Triton X-100. Luminescence was measured using a digital luminometer (Bio-Orbit 150 luminometer). The luminometer was calibrated by setting the background counts to zero and the built-in standard photon source [a sealed ampoule of ^{14}C with an activity of 0.26 μCi (9.62 kBq)] to 10 mV. Calculations made according to the method described by Hastings & Weber (1963) indicated that 1 U (1 mV) corresponded to a light emission of 6.7×10^5 quanta s^{-1} from the vial.

RESULTS

$[\text{Ca}^{2+}]_i$ changes in *S. elongatus* wild-type and mutant strains MNtcA and MP2 in response to nitrogen starvation

Cell suspensions of *S. elongatus* wild-type, expressing apoaquorin, grown with a source of combined nitrogen, either nitrate or ammonium, were deprived of combined nitrogen as described in Methods, and any change in $[\text{Ca}^{2+}]_i$ was monitored for at least 20 h. In the wild-type strain, a specific and reproducible Ca^{2+} transient could be detected in response to nitrogen starvation (Fig. 1); the transient began around 30 min to 1 h after nitrogen step-down, gradually increased in magnitude, reaching a maximum $[\text{Ca}^{2+}]_i$ value of $0.376 \pm 0.12 \mu\text{M}$ ($n=5$) after 6 h; a slow decrease followed afterwards until the $[\text{Ca}^{2+}]_i$ approached the basal level (around 150–200 nM in this strain). The total length of the Ca^{2+} transient was nearly 15 h. As a control, cell suspensions were washed three times with BG11 medium containing the standard nitrate/ammonium concentration and finally resuspended in the combined nitrogen medium; these cell suspensions did not show any significant change in $[\text{Ca}^{2+}]_i$ (Fig. 1), which indicated that the observed Ca^{2+} transient was not due to the manipulation of the sample or the experimental

Fig. 1. Effect of combined-nitrogen deprivation on the $[\text{Ca}^{2+}]_i$ of *S. elongatus* wild-type, MNtcA and MP2 strains expressing apoaquorin. Cells were grown in BG11- NH_4 medium until they reached the mid-exponential phase of growth ($\text{OD}_{750}=0.5$). After loading the cells with coelenterazine, they were washed three times with BG11₀ medium and luminescence was continuously recorded in a luminometer over 24 h. As a control, cell suspensions were washed three times with BG11- NH_4 medium and resuspended in the same medium. The intracellular Ca^{2+} chelator BAPTA-AM, at a final concentration of 300 μM , was added to cells 2 h prior to reconstitution of apoaquorin with coelenterazine and again immediately after nitrogen deprivation. Experiments were repeated at least five times; the traces shown have been chosen to best represent the average results; the maximum coefficient of variation of the different experiments was 8%. Solid line, shift of cells from BG11- NH_4 to BG11- NH_4 ; short dashes, shift of wild-type cells from BG11- NH_4 to BG11₀; dotted line, shift of MNtcA cells from BG11- NH_4 to BG11₀; dots and dashes, shift of MP2 cells from BG11- NH_4 to BG11₀; long dashes, treatment of wild-type cells with BAPTA-AM.

conditions of measurement; the above data strongly suggest that the observed Ca^{2+} transient is triggered by the withdrawal of the source of combined nitrogen from the external medium. Furthermore, the intracellular Ca^{2+} chelator BAPTA-AM suppressed the observed transient (Fig. 1), indicating that the recorded transient was a true Ca^{2+} response.

Due to the central role played by both NtcA and P_{II} in global nitrogen control in cyanobacteria, we considered it interesting to check whether the observed Ca^{2+} transient was triggered in a *glnB* or *ntcA* mutant background. Cell suspensions of mutant strains MNtcA and MP2 expressing apoaquorin were nitrogen-deprived, as explained above. As shown in Fig. 1, nitrogen starvation did not trigger any significant Ca^{2+} transient in mutant strains MNtcA and MP2; their $[\text{Ca}^{2+}]_i$ basal levels did not stay constant and showed some fluctuations around a value of 200 nM during the time of measurement (Fig. 1).

It has been shown that 2-OG is an intracellular signal for nitrogen status in cyanobacteria. Artificially increased levels of 2-OG mimic, to some extent, nitrogen starvation in both *S. elongatus* and the heterocystous *Anabaena* sp. PCC 7120 (Li *et al.*, 2003; Vázquez-Bermúdez *et al.*, 2003). The non-

Fig. 2. Effect of the addition of 25 mM 2-OG (a), 2 mM DFPA (b) or 2 mM 2-MPA (c) on the $[Ca^{2+}]_i$ of *S. elongatus* wild-type, MNtcA and MP2 strains expressing apoaequorin. Cells were grown in BG11-NH₄ medium until they reached the mid-exponential phase of growth ($OD_{750}=0.5$). After loading the cells with coelenterazine, they were resuspended in BG11-NH₄, and 2-OG (a), DFPA (b) or 2-MPA (c) was immediately added; luminescence was continuously recorded in the luminometer over 24 h. As a control, cell suspensions were washed three times with BG11-NH₄ medium and resuspended in the same medium. The intracellular Ca^{2+} chelator BAPTA-AM, at a final concentration of 300 μ M, was added to cells 2 h prior to reconstitution of apoaequorin with coelenterazine and again immediately after the addition of 2-OG, DFPA or 2-MPA to cells resuspended in BG11-NH₄. Experiments were repeated at least five times; the traces shown have been chosen to best represent the average results; the maximum coefficient of variation of the different experiments was 8.5% for 2-OG and 2-MPA experiments and 2% for DFPA experiments. Solid lines, shift of cells from BG11-NH₄ to BG11-NH₄; short dashes, shift of wild-type cells from BG11-NH₄ to BG11-NH₄ plus 25 mM 2-OG (a), 2 mM DFPA (b) or 2 mM 2-MPA (c); dotted lines, shift of MNtcA cells from BG11-NH₄ to BG11-NH₄ plus 25 mM 2-OG (a), 2 mM DFPA (b) or 2 mM 2-MPA (c); dots and dashes, shift of MP2 cells from BG11-NH₄ to BG11-NH₄ plus 25 mM 2-OG (a), 2 mM DFPA (b) or 2 mM 2-MPA (c); long dashes, treatment of wild-type cells with BAPTA-AM.

metabolizable 2-OG analogues DFPA and 2-MPA triggered heterocyst differentiation in the presence of ammonium in the *Anabaena* strain (Laurent *et al.*, 2005; Chen *et al.*, 2006). In *Anabaena*, nitrogen starvation leads not only to increased 2-OG levels but also to an increased concentration of free Ca^{2+} that is necessary for heterocyst differentiation (Torrecilla *et al.*, 2004; Zhao *et al.*, 2005). The fact that in *Anabaena* free Ca^{2+} ions and 2-OG are early signals following nitrogen starvation raises many questions about their role in the process of acclimation to this stress. As described, we have also found that a defined Ca^{2+} transient is triggered following nitrogen step-down in *S. elongatus*; in order to shed light on a possible relationship between the two signals, 2-OG and Ca^{2+} , we investigated whether exogenous addition of 2-OG or either of its two analogues, 2-MPA or DFPA, triggered Ca^{2+} transients in the presence of a source of combined nitrogen (nitrate/ammonium) in *S. elongatus*. In the wild-type strain, 25 mM 2-OG elicited a wide Ca^{2+} transient that started around 1 h after the addition of 2-OG; the recorded Ca^{2+} transient had a shoulder at 5 h 30 min reaching a $[Ca^{2+}]_i$ value of $0.36 \pm 0.01 \mu$ M ($n=5$), and peaked at around 15 h reaching a $[Ca^{2+}]_i$ maximum of $0.47 \pm 0.04 \mu$ M ($n=5$); the transient lasted more than 20 h (Fig. 2a). Strain MP2 showed a slight increase in its $[Ca^{2+}]_i$ basal levels; strain MNtcA also showed some fluctuations of its $[Ca^{2+}]_i$ basal levels but no significant Ca^{2+} transient was recorded in any of the two mutant strains in any of the experiments that were carried out (Fig. 2a). As expected, treatment with BAPTA-AM suppressed the observed Ca^{2+} transient in the wild-type strain. The 2-

Fig. 3. Time-course of *glnB::luxAB* induction following nitrogen step-down (a), the addition of 25 mM 2-OG (b), or the addition of 2 mM 2-MPA (c) in reporter strains FAM1 and FAM2 of *S. elongatus* wild-type. After shifting the cells from BG11 to BG11₀ (a), from BG11 to BG11 plus 25 mM 2-OG (b), or from BG11 to BG11 plus 2 mM 2-MPA (c), bioluminescence from the reporter strains was monitored over 24 h; the intracellular Ca^{2+} chelator BAPTA-AM, at a final concentration of 300 μM , was added 2 h prior to nitrogen step-down (a), the addition of 2-OG (b), or 2-MPA (c), and immediately afterwards. Three independent experiments with duplicate samples yielded similar results; for each case, results from a representative time-course experiment are shown. (●) FAM1 reporter strain, (▼) FAM2 reporter strain, (○) FAM1 reporter strain treated with BAPTA-AM, (▽) FAM2 reporter strain treated with BAPTA-AM.

$0.48 \pm 0.01 \mu\text{M}$ ($n=5$), and decreased until it reached the basal level of 200 nM. The transient lasted around 13 h (Fig. 2b). The Ca^{2+} transient induced by 2-MPA started later, around 1.5–2 h after the addition of the analogue, peaked at around 12 h with a maximum $[\text{Ca}^{2+}]_i$ value of $0.58 \pm 0.05 \mu\text{M}$ ($n=5$), and decreased to basal levels; the transient lasted almost 20 h (Fig. 2c). As with 2-OG, no significant Ca^{2+} transient was recorded in mutant strains MNtCA and MP2 after the addition of the analogues, although slight fluctuations of $[\text{Ca}^{2+}]_i$ basal levels were also observed (Fig. 2b, c). Treatment with BAPTA-AM suppressed the observed Ca^{2+} transients triggered by the analogues in the wild-type strain (Fig. 2). Thus, artificial conditions mimicking nitrogen starvation (addition of 2-OG or its analogues 2-MPA and DFPA to *S. elongatus* cells growing with a source of combined nitrogen) triggered Ca^{2+} transients similar to that obtained following nitrogen step-down, suggesting that an increase in intracellular 2-OG is needed to induce the Ca^{2+} transients. As the MNtCA and MP2 strains do not show the Ca^{2+} transients, both proteins might also be needed to trigger the observed Ca^{2+} transients.

Effect of suppression of the Ca^{2+} transient on the expression of NtcA-regulated genes involved in acclimation to nitrogen starvation in *S. elongatus*

Does the observed Ca^{2+} transient have a role in acclimation to nitrogen starvation in *S. elongatus*? To try to answer this question, we first checked the effect of suppression of the Ca^{2+} transient on the expression of genes involved in acclimation to nitrogen starvation.

We first studied the *glnB* gene, whose expression is controlled by NtcA under nitrogen-deprivation conditions (Aldehni *et al.*, 2003), and studied the effect of the intracellular Ca^{2+} chelator BAPTA-AM, which completely suppressed the Ca^{2+} transient, on its expression under conditions of actual or mimicked nitrogen starvation (induced by either nitrogen step-down or the addition of 2-OG or its analogues in the presence of combined

OG analogues DFPA and MPA at a final concentration of 2 mM triggered bell-shaped Ca^{2+} transients with some differences in their temporal pattern (Fig. 2b, c, respectively). The Ca^{2+} transient induced by DFPA started shortly (30–45 min) after the addition of the analogue, peaked at around 7 h with a maximum $[\text{Ca}^{2+}]_i$ value of

nitrogen) (Fig. 3). Two reporter strains were used to study *glnB* expression, FAM1 and FAM2 (Aldehni *et al.*, 2003); FAM1 contains the entire upstream region of *glnB* with two promoters, constitutive promoter P1 and the NtcA-regulated promoter P2, fused to *luxAB*; FAM2 contains only the NtcA-dependent promoter P2. As shown in Fig. 3(a), nitrogen depletion initiated from nitrate-supplemented cultures triggered a rapid increase in luminescence in both strains: peak values 8 h after nitrogen step-down corresponded to an approximately 10- or 150-fold increase in induction for FAM1 [luminescence value of $3.23 \times 10^8 \pm 0.5$ quanta (mg Chl) $^{-1}$ min $^{-1}$ at time 0, where Chl stands for chlorophyll] or FAM2 [luminescence value of $0.32 \times 10^8 \pm 0.06$ quanta (mg Chl) $^{-1}$ min $^{-1}$ at time 0], respectively, compared with that for nitrate-grown cells; after 24 h, peak values corresponded to an 18- or 300-fold increase in luminescence for FAM1 and FAM2, respectively, indicating, as pointed out by Aldehni *et al.* (2003), that transcription from the NtcA-dependent promoter P2 is sufficient to yield the high expression of *glnB* under conditions of combined-nitrogen starvation. As also shown in the figure, treatment with BAPTA-AM significantly inhibited the induction of luminescence, although after 24–30 h, a 10-fold increase in luminescence in both strains treated with the chelator was observed (Fig. 3a). Similar results were obtained when cells were shifted from ammonium to combined nitrogen-free medium (not shown). The addition of 25 mM 2-OG to cell suspensions grown with nitrate induced the expression of the *glnB* gene in both strains, although luminescence induction was not as strong as that observed after nitrogen step-down; for strain FAM1, with a luminescence value of $25 \times 10^8 \pm 1.17$ quanta (mg Chl) $^{-1}$ min $^{-1}$ at time 0, a twofold increase in induction was seen 8 h after addition of 2-OG, while at the same time point, a 10-fold increase in induction was observed for FAM2 [luminescence value $3.3 \times 10^7 \pm 0.30$ quanta (mg Chl) $^{-1}$ min $^{-1}$ at time 0] (Fig. 3b). Treatment with BAPTA-AM triggered slightly different induction patterns in the two strains: while it completely inhibited induction of gene expression in FAM2, it did not inhibit induction of FAM1 at the earliest times assayed, although 5 h after addition of 2-OG there was a significant decrease of luminescence in FAM1 (Fig. 3b). The addition of 2 mM of the 2-OG analogue 2-MPA triggered a rapid induction of the *glnB* gene: after 7 h, peak values corresponded to a 320-fold increase in induction for FAM1 [luminescence value $0.17 \times 10^8 \pm 0.06$ quanta (mg Chl) $^{-1}$ min $^{-1}$ at time 0] and a 380-fold increase in induction for FAM2 [luminescence value $0.08 \times 10^8 \pm 0.01$ quanta (mg Chl) $^{-1}$ min $^{-1}$ at time 0]. BAPTA-AM completely inhibited the observed induction of *glnB* in both strains (Fig. 3c).

We have shown that the *glnB* gene is induced not only after shifting nitrate- or ammonium-grown cells to combined nitrogen-free medium (nitrogen step-down experiments) but also when nitrogen-starvation conditions are mimicked by the addition of 2-OG or its analogue 2-MPA to cells growing with a source of combined nitrogen. The

expression of *glnB* under nitrogen-starvation conditions seemed to be regulated by $[Ca^{2+}]_i$, as the Ca^{2+} chelator BAPTA-AM, which suppressed the observed Ca^{2+} transients (Figs 1 and 2), significantly inhibited the induction of expression of the gene. We wished to check whether other genes involved in acclimation to nitrogen starvation whose promoters were also regulated by NtcA could also be regulated by Ca^{2+} . We chose *glnN* and *nblA* for this purpose. *glnN* encodes a nitrogen starvation-specific glutamine synthetase in *S. elongatus*. The promoter of *S. elongatus glnN* displays an imperfect NtcA-binding site (Aldehni & Forchhammer, 2006). We used two *luxAB* reporter strains, strain FAM431, which contains the full-length *glnN* promoter fused to *luxAB*, and FAM84, which only retains the NtcA-binding site in its promoter fused to *luxAB* (Aldehni & Forchhammer, 2006). As shown in Fig. 4, nitrogen starvation induced *glnN* expression in both strains, although the luminescence induction was moderate in both strains as compared with the level of induction of *glnB* (Fig. 3). The FAM84 reporter strain displayed more activity [a 30-fold increase in induction after 24 h of starvation from an initial luminescence value of $0.8 \times 10^7 \pm 0.08$ quanta (mg Chl) $^{-1}$ min $^{-1}$] than FAM431 [a 19-fold increase in induction after 24 h of starvation from an initial luminescence value of $0.7 \times 10^7 \pm 0.09$ quanta (mg Chl) $^{-1}$ min $^{-1}$], as already observed by Aldehni & Forchhammer (2006), who have suggested that

Fig. 4. Time-course of *glnN*::*luxAB* induction following nitrogen step-down in reporter strains FAM84 and FAM431 of *S. elongatus* wild-type. After shifting the cells from BG11 medium to BG11₀, bioluminescence from the reporter strains was monitored over 24 h. The intracellular Ca^{2+} chelator BAPTA-AM, at a final concentration of 300 μ M, was added 2 h prior to nitrogen step-down and immediately afterwards. Three independent experiments with duplicate samples yielded similar results; for each case, results from a representative time-course experiment are shown. (●) FAM84 reporter strain, (▼) FAM431 reporter strain, (○) FAM84 reporter strain treated with BAPTA-AM, (▽) FAM431 reporter strain treated with BAPTA-AM.

this effect is due to a negatively acting *cis*-element in the upstream region of the *glnN* promoter. Intracellular Ca^{2+} depletion by BAPTA-AM significantly inhibited *glnN* expression, particularly in the FAM84 reporter strain, which has a shorter promoter containing the imperfect NtcA-binding site (Fig. 4), again confirming control by Ca^{2+} of an NtcA-regulated promoter. We could not detect any clear induction of the *glnN* gene 48 h after the addition of 2-OG or its analogue 2-MPA to cells growing with either nitrate or ammonium. Expression of the *nblA* gene is controlled by two regulators, NtcA and NblR; *nblA* dependency on NtcA is nitrogen-specific, while the response-regulator NblR acts as the general regulator of *nblA* transcription in response to a variety of stresses; both regulators seem to stimulate transcription from the major *nblA* promoter *PnblA-2* (Luque *et al.*, 2001). As shown in Fig. 5, nitrogen starvation induced *nblA* expression, as expected [40-fold increase in expression after 8 h of starvation from an initial luminescence value of $0.37 \times 10^8 \pm 0.01$ quanta (mg Chl) $^{-1}$ min $^{-1}$]; $[\text{Ca}^{2+}]_i$ depletion by BAPTA-AM partially inhibited *nblA* expression at shorter times (i.e. after 8 h, a 40-fold increase in induction in the control vs a 25-fold induction in cultures treated with BAPTA-AM); in the longer term (24 h) there was no difference in expression levels between control cultures and those treated with BAPTA-AM (Fig. 5). We

Fig. 5. Time-course of *nblA::luxAB* induction following nitrogen (●, ○) or sulphur (▼, ▽) step-down in reporter strain *S. elongatus* WT-C103. After shifting the cells from BG11 medium to BG11₀, bioluminescence from the reporter strains was monitored over a time period of 24 h. The intracellular Ca^{2+} chelator BAPTA-AM, at a final concentration of 300 μM , was added 2 h prior to nitrogen step-down and immediately afterwards. Three independent experiments with duplicate samples yielded similar results; for each case, results from a representative time-course experiment are shown. (●) *S. elongatus* WT-C103 reporter strain, (○) *S. elongatus* WT-C103 reporter strain treated with BAPTA-AM, (▼) *S. elongatus* WT-C103 reporter strain, (▽) *S. elongatus* WT-C103 reporter strain treated with BAPTA-AM.

could not detect any clear induction of the *nblA* gene 48 h after the addition of 2-OG or its analogue 2-MPA to cells growing with either nitrate or ammonium. If Ca^{2+} is regulating NtcA-dependent promoters, the observed partial effect of Ca^{2+} depletion on expression of *nblA* in the short term might indicate that NtcA and Ca^{2+} are needed to rapidly upregulate *nblA* expression under nitrogen-starvation conditions. The residual response of *nblA* expression indicates an NtcA-independent regulatory mechanism, probably involving the NblR factor, which is also required for *nblA* induction following sulphur deprivation (Schwarz and Grossman, 1998). The Ca^{2+} chelator itself did not have any negative effect on growth in the presence of ammonium (results not shown), suggesting that it did not cause an intracellular Ca^{2+} depletion that could compromise the viability of the cells and have a negative effect on gene expression. To reinforce this, we checked the effect of BAPTA-AM on *nblA* activation under sulphur deprivation; *nblA* is activated not only by nitrogen deprivation but also by sulphur and phosphate deprivation (Collier & Grossman, 1994; Schwarz & Forchhammer, 2005); *nblA* dependency on NtcA has been shown to be nitrogen-specific (Luque *et al.*, 2001), while NblR acts as the general regulator of *nblA* transcription in response to most stresses. We did not find any significant Ca^{2+} transient following sulphur step-down (not shown); as also shown in Fig. 5, *nblA* expression was induced within a few hours of sulphur deprivation [fivefold increase in luminescence 6 h after sulphur stepdown from an initial luminescence value of $0.24 \times 10^8 \pm 0.05$ quanta (mg Chl) $^{-1}$ min $^{-1}$]; as shown, the chelator did not have any effect on *nblA* expression under sulphur-starvation conditions.

Effect of suppression of the Ca^{2+} transient on bleaching caused by nitrogen starvation

nblA is a key gene controlling phycobilisome degradation in response to environmental stress conditions such as nutrient deprivation. As Ca^{2+} seems to some extent to control *nblA* expression, we checked the effect of BAPTA-AM treatment on the bleaching process in the wild-type, MNtcA and MP2 strains. As shown in Fig. 6, after 3 days of nitrogen starvation, BAPTA-AM partially protected the wild-type strain from bleaching (Fig. 6a); the phycocyanin:chlorophyll a ratio decreased fourfold in the control wild-type versus 1.7-fold in the wild-type treated with BAPTA-AM (Fig. 6b); BAPTA-AM-treated cells retained the red phycobiliprotein autofluorescence that the non-treated cells had almost completely lost by this time of nitrogen starvation (not shown). As mutants MNtcA and MP2 did not show the Ca^{2+} transient (Figs 1 and 2), treatment with BAPTA-AM did not have any effect on their bleaching pattern. MNtcA, as already described (Sauer *et al.*, 1999; Espinosa *et al.*, 2007), showed a delay in phycocyanin degradation compared with the wild-type; after 3 days, the phycocyanin:chlorophyll a ratio decreased twofold after nitrogen step-down both in cultures treated and in those not treated with BAPTA-AM (Fig. 6a, b); the

Fig. 6. Effect of BAPTA-AM on bleaching of *S. elongatus* wild-type (WT), MNtCA and MP2 mutant strains. (a) Cultures of WT, MNtCA and MP2 grown in BG11-NH₄ (+N), incubated in BG11₀ (-N) or BG11₀ plus 300 μ M BAPTA-AM (-N+BAPTA) for 72 h. (b) Phycocyanin : chlorophyll a ratios of cultures of WT, MNtCA and MP2 incubated as indicated in (a). PC, phycocyanin; Chla, chlorophyll a. At least five independent experiments were performed; results from a representative experiment are shown.

MP2 mutant showed the same pattern of bleaching as the wild-type, as already described (Sauer *et al.*, 1999), but no protection by BAPTA-AM was observed. BAPTA-AM partially protected the wild-type from bleaching for at least 9 days (results not shown).

DISCUSSION

In this study, we report an early Ca²⁺ transient that is triggered under conditions of nitrogen starvation in *S. elongatus* (Fig. 1). The Ca²⁺ transient is very similar to the one that we recorded in the heterocystous *Anabaena* sp. PCC 7120 in shape, time of appearance (45 min to 1 h) and magnitude (0.37 μ M vs 0.39 μ M in *Anabaena*), although it has a longer duration (15 h vs 4 h in *Anabaena*) (Torrecilla *et al.*, 2004). The Ca²⁺ transient occurred not only after removing nitrate or ammonium from the growth medium (nitrogen step-down) but also after the addition of 2-OG or its analogues DFPA or 2-MPA to cells growing with nitrate or ammonium (Fig. 2). The latter conditions mimic, to some extent, nitrogen starvation in *Anabaena* sp. PCC 7120 (Li *et al.*, 2003; Laurent *et al.*, 2005; Chen *et al.*, 2006). Interestingly, 2-OG and both its analogues also

trigger Ca²⁺ transients in *Anabaena* similar to the ones observed in *S. elongatus* (F. Leganés and F. Fernández-Piñas, unpublished results).

Therefore, nitrogen starvation leads not only to increased 2-OG levels but also to increased concentrations of free Ca²⁺ ions both in *Anabaena* and *S. elongatus*: intracellular 2-OG levels increase immediately in response to nitrogen limitation in both strains (Laurent *et al.*, 2005; Schwarz & Forchhammer, 2005), while Ca²⁺ ions start to accumulate 45 min–1 h after starvation (this work; Torrecilla *et al.*, 2004); the fact that artificially increased 2-OG or analogue levels also trigger the Ca²⁺ transients in both strains suggests that an increase in intracellular 2-OG precedes the Ca²⁺ transient.

Also, the fact that the MNtCA and MP2 mutant strains of *Synechococcus* did not show the calcium signal implies that both proteins might be needed in conjunction with high levels of intracellular 2-OG to trigger the Ca²⁺ transient; Shi *et al.* (2006) have already found that the regulation of [Ca²⁺]_i in *Anabaena* depends on NtcA.

Our results also showed that *glnB* expression can be induced by the addition of 2-OG or its analogue 2-MPA in the presence of combined nitrogen, while that of *nblA* and *glnN* cannot. *glnB* has a canonical NtcA-binding site (Aldehni *et al.*, 2003), *glnN* displays an imperfect NtcA-binding motif and a negatively acting *cis*-element in the upstream region of its promoter (Aldehni & Forchhammer, 2006), and *nblA* has a complex regulatory region containing multiple promoters: under nitrogen deprivation, its major promoter (*pnblA-2*) requires both NtcA and NblR for maximal activation, although the structure of this promoter does not match that of a typical NtcA-activated promoter (Luque *et al.*, 2001). The lack of a canonical NtcA-activated promoter and the presence of other factors or regulators may hamper induction of *glnN* and *nblA* expression by 2-OG under nitrogen-replete conditions.

Anabaena responds to the removal of a combined nitrogen source by differentiating heterocysts, specialized cells that fix molecular nitrogen. Our group and that of Zhao (Torrecilla *et al.*, 2004; Zhao *et al.*, 2005) have demonstrated that the Ca²⁺ transient in *Anabaena* is necessary for heterocyst development; non-diazotrophic strains such as *S. elongatus* respond to nitrogen deprivation by an acclimation process that begins with pigment degradation (phycobiliprotein proteolysis followed by chlorophyll a loss) that leads to chlorosis before differentiating into non-pigmented cells able to endure long periods of starvation (Görl *et al.*, 1998). A relevant question is whether the observed Ca²⁺ transient in response to nitrogen starvation in *S. elongatus* has a role in this process of acclimation; we have found that suppression of the Ca²⁺ transient by the intracellular Ca²⁺ chelator BAPTA-AM inhibits induction of *glnB* and *glnN* expression under nitrogen-starvation conditions (Figs 3 and 4); both genes are involved in acclimation to nitrogen limitation in *S. elongatus* and both are transcriptionally activated by NtcA (Aldehni *et al.*,

2003; Aldehni & Forchhammer, 2006; Schwarz & Forchhammer, 2005). Furthermore, BAPTA-AM partially inhibited induction of expression of *nblA* (Fig. 5), a crucial gene involved in the process of phycobiliprotein degradation that is controlled by two transcriptional regulators, NtcA (specifically under conditions of nitrogen starvation) and NblR. The inhibitory effect of BAPTA-AM occurred only in the short term after starvation; in the longer term (24 h), BAPTA-AM did not have any effect. This partial inhibitory effect might indicate that Ca^{2+} is acting through NtcA but not NblR in the induction of expression of *nblA* under nitrogen limitation; if that is true, it also might imply that NtcA is an early activator of transcription of *nblA*, while the main activator at longer times of starvation is NblR. This partial inhibitory effect of the chelator on *nblA* expression correlated with the observed partial inhibition of phycobiliprotein degradation (Fig. 6). In *Anabaena*, we have also found that suppression of the Ca^{2+} transient by treatment with the chelator arrests heterocyst differentiation at an early stage, as evidenced by the absence of patterned loss of phycobiliprotein fluorescence, suggesting a role of the Ca^{2+} transient in proteolysis of the phycobiliproteins (Torrecilla *et al.*, 2004). The results presented here strongly suggest that Ca^{2+} ions are involved in the induction of NtcA-regulated genes by nitrogen starvation; Ca^{2+} might act at different levels: it may physically interact with NtcA and facilitate DNA binding and/or stabilize PipX–NtcA complexes that have been proposed to activate NtcA-dependent promoters (Espinosa *et al.*, 2006, 2007). *In vivo*, the PipX–NtcA complexes are thought to be formed under high intracellular levels of 2-OG and, as we have reported, increased intracellular 2-OG triggers the Ca^{2+} transient. A stabilizing role of Ca^{2+} has already been proposed for the NAGK–P_{II} complex in *S. elongatus* (Maheswaran *et al.*, 2004), although in this case, Ca^{2+} and 2-OG seem to act antagonistically. Alternatively, the Ca^{2+} transient, probably acting synergistically with increased intracellular 2-OG levels, could sense C/N imbalance and transduce it, by a yet unknown signalling mechanism, to some other factor(s) (perhaps P_{II} and/or PipX) that may facilitate NtcA transcriptional activation.

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