CcbP, a calcium-binding protein from *Anabaena* sp. PCC 7120, provides evidence that calcium ions regulate heterocyst differentiation

Yinhong Zhao*†, Yunming Shi*†, Weixing Zhao*, Xu Huang*, Donghui Wang*, Neil Brown‡, Jerry Brand‡, and Jindong Zhao*§

*State Key Laboratory of Protein and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China; and ‡Department of Molecular Cell and Developmental Biology, University of Texas, Austin, TX 78712

Communicated by Robert Haselkorn, University of Chicago, Chicago, IL, March 5, 2005 (received for review February 6, 2005)

Although it is known that calcium is a very important messenger involved in many eukaryotic cellular processes, much less is known about calcium's role in bacteria. CcbP, a Ca^{2+}-binding protein, was isolated from the heterocystous cyanobacterium *Anabaena* sp. PCC 7120, and the *ccbP* gene was cloned and inactivated. In the absence of combined nitrogen, inactivation of *ccbP* resulted in multiple contiguous heterocysts, whereas overexpression of *ccbP* suppressed heterocyst formation. Calmodulin, which is not present in *Anabaena* species, could also suppress heterocyst formation in both *Anabaena* sp. PCC 7120 and *Anabaena variabilis*. HetR induction upon nitrogen step-down was slow in the strain overexpressing *ccbP*. The Ca^{2+} reporter protein obelin was used to show that mature heterocysts had a high intracellular free Ca^{2+}concentration {$[Ca^{2+}]_i$}, and immunoblotting showed that CcbP was absent from heterocysts. A regular pattern of cells with higher $[Ca^{2+}]_i$ was established during heterocyst differentiation before the appearance of proheterocysts. A rapid increase of $[Ca^{2+}]_i$ could be detected 4 h after the removal of combined nitrogen, and this increase was suppressed by excessive CcbP. These results suggest that Ca^{2+} ions play very important roles in *hetR* induction and heterocyst differentiation.

cyanobacteria | hetR | pattern formation

In eukaryotes, Ca^{2+} ions play very important roles in cellular processes such as cell differentiation (1, 2). The intracellular free calcium ion concentration {$[Ca^{2+}]_i$} is tightly regulated through Ca^{2+} channels, Ca^{2+} pumps, and Ca^{2+}-binding proteins, and $[Ca^{2+}]_i$ is generally maintained in the nanomolar range. In bacteria, the role of Ca^{2+} in cellular activities is less clear (3–5). It has been demonstrated that $[Ca^{2+}]_i$ is tightly regulated in some bacteria (6), and there is evidence indicating that calcium is critical to some bacterial cellular processes such as sporulation of *Bacillus* (7), chemotaxis of *Escherichia coli* (8), and heterocyst differentiation of cyanobacteria (9). Heterocyst development may represent one of the earliest examples of pattern formation in evolution (10). Heterocysts are specialized cells for nitrogen fixation of some filamentous cyanobacteria (11–14). When heterocystous cyanobacteria are subjected to nitrogen step-down, some vegetative cells differentiate and become heterocysts. Many genes are specifically involved in heterocyst differentiation. The genes that are required for initiation of cell differentiation include *ntcA* (15, 16) and *hetR* (17). HetR is a serine-type protease that plays an important role in heterocyst formation (18).

Evidence obtained by manipulating extracellular calcium concentration and by using inhibitors of Ca^{2+}-binding proteins suggested that Ca^{2+} could be involved in heterocyst differentiation (9). Here we describe a Ca^{2+}-binding protein, CcbP, from *Anabaena* sp. PCC 7120. Our study shows that free calcium accumulates in differentiating cells and mature heterocysts, correlated with a drop in the level of the Ca^{2+}-binding protein. The free Ca^{2+}concentration appears to be critical for the differentiation process.

Materials and Methods

The following protocols can be found in *Supporting Text*, which is published as supporting information on the PNAS web site: growth of the strains of *Anabaena*; isolation of the Ca^{2+}-binding protein CcbP; isolation of the *ccbP* gene from total genomic DNA; expression of recombinant *ccbP* in *Escherichia coli*; construction of plasmids for inactivation of the *ccbP* gene, for copper-regulated expression of *ccbP*, for copper-regulated expression of rat calmodulin (CaM) gene, for *ccbP* promoter regulation of GFP expression, and for expression of obelin, a reporter for the level of free Ca^{2+}.

Assays for Ca^{2+}-Binding Proteins. $^{45}Ca^{2+}$ overlay assay was performed as follows: Proteins were first separated by SDS/PAGE and then transferred to a poly(vinylidene difluoride) membrane. It was then washed three times with buffer C (20 mM Tris·HCl, pH 7.2/5 mM $MgCl_2$/60 mM KCl/10 mM imidazole). The membrane was then soaked in 25 ml of buffer C containing 50 μCi $^{45}CaCl_2$ for 10 min before washing briefly with buffer C containing 5% ethanol and blotted to dry. The radioactive bands were detected with Kodak x-ray film. For the Ca^{2+}-dependent electrophoretic mobility-shift assay, Ca^{2+}-binding proteins were separated by SDS/PAGE (12%) in the presence of 2 mM $CaCl_2$ or 2 mM EGTA (19).

Detection of Intracellular Free Ca^{2+}. Ca^{2+}-dependent fluorescence emission by obelin was detected as follows. One milliliter of *Anabaena* sp. PCC 7120 (*Anabaena* 7120 from hereon) culture at an optical density (750 nm) of 0.3 was washed with BG11 or $BG11_0$ media before coelenterazine was added to a final concentration of 2 μM from a stock solution of 1 mM in methanol. The culture was incubated in darkness for 30 min at 28°C. The fluorescence images with UV light for excitation were recorded as described by Huang *et al.* (20). For measurement of the fluorescence spectra of *Anabaena* 7120 cells containing obelin, the cell cultures were prepared as above, and the spectra were recorded by a PTI (South Brunswick, NJ) fluorescence spectrophotometer. The excitation wavelength was 340 nm with a slit width of 1 nm. Changes of fluorescence emission at 460 nm were used to determine changes of $[Ca^{2+}]_i$ in *Anabaena* 7120.

Abbreviations: CaM, calmodulin; MCH, multiple contiguous heterocysts.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY919604).

†Y.Z. and Y.S. contributed equally to this work.

§To whom correspondence should be addressed. E-mail: jzhao@pku.edu.cn.

© 2005 by The National Academy of Sciences of the USA

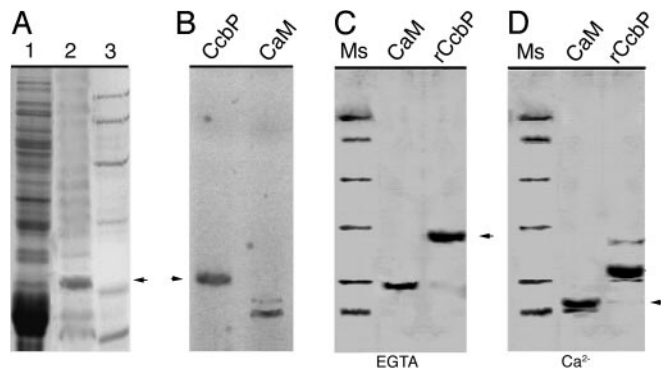

Fig. 1. Partial purification and characterization of CcbP from *Anabaena* 7120. (*A*) SDS/PAGE analysis of isolated CcbP. Lane 1, total cellular extract from *Anabaena* 7120; lane 2, partially purified CcbP after $(NH_4)_2SO_4$ precipitation, a DEAE column, and a gel filtration column; lane 3, protein molecular mass standards (in kDa, from top): 97, 64, 45, 31, 21.5, and 14. (*B*) $^{45}Ca^{2+}$ overlay. Partially purified CcbP (lane 1) and rat CaM (lane 2) were transferred to a poly(vinylidene difluoride) membrane after SDS/PAGE and labeled with $^{45}Ca^{2+}$ before exposure to x-ray film. (*C* and *D*) Characterization of the recombinant CcbP (rCcbP) by electrophoretic mobility in SDS/PAGE in the presence of 2 mM EGTA (*C*) or 2 mM $CaCl_2$ (*D*). The proteins were treated with 1 mM DTT before electrophoresis. The rat CaM was used as control. Molecular mass standards (Ms) are as described in *A*.

Other Methods. SDS/PAGE was performed according to Laemmli (21). Heterocyst isolation and immunoblotting for detection of HetR and CcbP were performed according to Zhou *et al.* (22). Protein concentration was determined as described (23). Localization of GFP on *Anabaena* 7120 filaments was carried out according to Yoon and Golden (24).

Results

Characterization of CcbP from *Anabaena* 7120. We tried several methods to isolate Ca^{2+}-binding proteins from *Anabaena* 7120. A method used for calsequestrin isolation (25) was found suitable, and we were able to partially purify a Ca^{2+}-binding protein from *Anabaena* 7120 (Fig. 1*A*). A protein band was labeled with Ca^{2+} in $^{45}Ca^{2+}$ overlay (Fig. 1*B*). The N-terminal sequence of the protein was determined (ASVERDETREHRIETEIIV). It matched a hypothetical protein encoded by an ORF (alr1010) in the genome sequence without the initial Met residue. The gene was named *ccbP* (cyanobacterial calcium-binding protein). The *ccbP* gene was overexpressed in *E. coli*, and the gene product was purified (Fig. 1*C*). A Ca^{2+}-dependent gel-shifting assay showed that CcbP had an apparent molecular mass of 31 kDa in the presence of EGTA, whereas it had an apparent molecular mass of 21 kDa in the presence of Ca^{2+}. This mobility shift was larger than that of rat CaM, which showed a shift of ≈4 kDa (Fig. 1 *C* and *D*).

CcbP from *Anabaena* 7120 has 126-aa residues (14.75 kDa) with a pI of 4.1. The CcbP contains no EF hand, and it does not have motifs or domains known to bind Ca^{2+}. There are two hydrophobic stretches flanking an Asp-rich area located in the middle of the protein.

CcbP Regulates Calcium Availability for Heterocyst Formation. To study the functions of CcbP, an insertion mutant of *ccbP* was constructed in *Anabaena* 7120 (CCBP-M). The mutant was confirmed by Southern hybridization and immunoblotting as shown in Fig. 8, which is published as supporting information on the PNAS web site. We also constructed a plasmid (pPpetE-ccbP) that had the *ccbP* gene under control of the *petE* promoter (26). This plasmid was used to transform *Anabaena* 7120 (CCBP-IE) so that the expression of *ccbP* was inducible with copper.

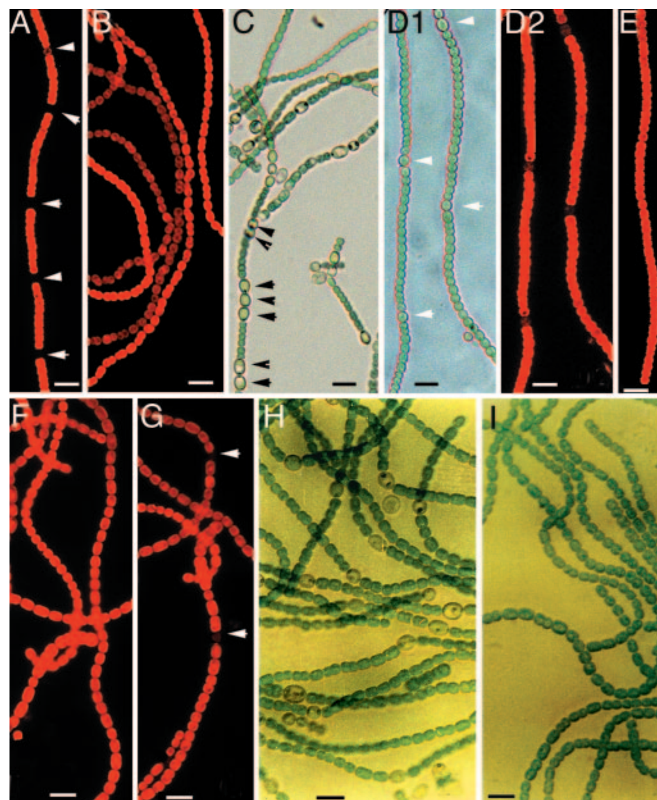

Fig. 2. The effects of inactivation of *ccbP* and overexpression of *ccbP* and *cam* on heterocyst differentiation in *Anabaena* 7120. (*A*) Fluorescence image of a wild-type filament. (*B*) Fluorescence image of CCBP-M grown in the presence of nitrate. (*C*) Bright-field image of CCBP-M in the absence of combined nitrogen. (*D*) Bright-field (*D1*) and fluorescence (*D2*) images of CCBP-IE grown in the absence of combined nitrogen without copper induction. (*E*) Fluorescence image of CCBP-IE grown in the absence of combined nitrogen with copper induction. (*F* and *G*) Fluorescence images of the wild type expressing a rat CaM gene (*cam*) and CCBP-M expressing *cam* in the absence of combined nitrogen with copper induction, respectively. (*H* and *I*) Bright-field images of *A. variabilis* carrying pP*petE-ccbP* grown in the absence of combined nitrogen without and with copper induction, respectively. Arrows indicate the positions of heterocysts. (Bar, 10 μm.)

When combined nitrogen was removed, wild-type filaments formed heterocysts within 24 h (Fig. 2*A*). CCBP-M was able to grow at a reduced rate (70%) and did not form heterocysts in BG11 (Fig. 2*B*). When CCBP-M was subjected to nitrogen step-down, its heterocyst frequency was higher than that of the wild type, and multiple contiguous heterocysts (MCH) were formed (Fig. 2*C*). The strain CCBP-IE was also able to grow in BG11. In the absence of added copper, CCBP-IE could form heterocysts with a slightly reduced heterocyst frequency under nitrogen-limiting condition (Fig. 2*D*). When copper was added to the growth medium, heterocyst formation was completely suppressed in CCBP-IE (Fig. 2*E*), suggesting that CcbP negatively regulates heterocyst differentiation.

Ca^{2+}-binding proteins might regulate gene expression either through Ca^{2+}-dependent protein–protein interactions or through regulation of $[Ca^{2+}]_i$ (calcium homeostasis). To understand the mechanism by which CcbP regulates heterocyst differentiation, we studied the effect of expressing a rat CaM gene (*cam*) in *Anabaena*. When the wild type carrying pPpetE-cam was induced to express *cam* with copper, it failed to form heterocysts 48 h after nitrogen step-down (Fig. 2*F*). Expression of *cam* in CCBP-M by copper induction suppressed MCH in the absence of combined nitrogen (Fig. 2*G*). A few heterocysts could

DEVELOPMENTAL BIOLOGY

PNAS PNAS PNAS

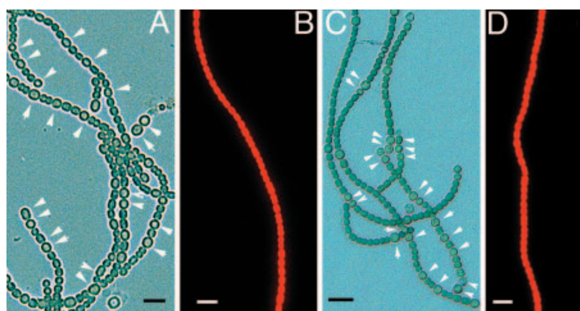

Fig. 3. The effect of expression of *ccbP* in *patS*$^-$ and the wild-type strain carrying pRL25C-*hetR*. (*A*) *patS*$^-$ formed MCH in the absence of combined nitrogen. (*B*) *patS*$^-$ with pP*petE-ccbP* did not form heterocysts in the absence of combined nitrogen when induced with copper. (*C*) The wild type with pRL25C-*hetR* has MCH in the absence of combined nitrogen. (*D*) The MCH phenotype shown in *C* was suppressed when the strain also carried pP*petE-ccbP* and induced with copper. Arrows indicate the positions of heterocysts. (Bar, 10 μm.)

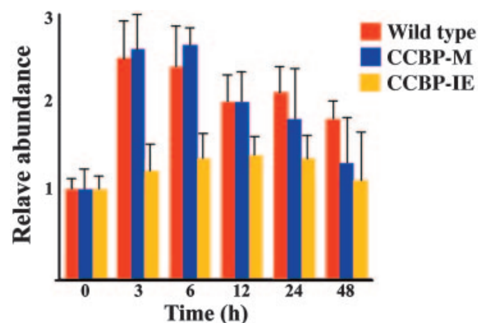

Fig. 4. Determination of HetR level by immunoblotting during the process of heterocyst differentiation. Cells of the wild type, CCBP-M, and CCBP-IE were collected at the times indicated. Total proteins were separated by SDS/PAGE and transferred to a poly(vinylidene difluoride) membrane. The amount of HetR was determined with antibodies against HetR. The values are the average of triplicate measurements.

be observed, but the frequency was <4%. Transformation of CCBP-M with the plasmid pPpetE-ccbP(*ery*^{R}) could also suppress the MCH phenotype (data not shown). *Anabaena variabilis* is another species that also forms heterocysts under nitrogen-limiting conditions. When pPpetE-cam was transformed into *A. variabilis*, it suppressed heterocyst formation completely in the absence of combined nitrogen with copper induction (Fig. 2*I*). However, the strain could grow under this condition, probably because of the presence of the alternative nitrogenase present in vegetative cells (27). Thus, the inhibition of heterocyst formation in *Anabaena* by CcbP and CaM was likely to result from Ca^{2+} sequestration. These results strongly indicate that Ca^{2+} is required for heterocyst differentiation and predict that $[Ca^{2+}]_i$ would increase in differentiating cells and mature heterocysts of *Anabaena*.

patS and *hetR* are critical to heterocyst formation in *Anabaena* (17, 18, 26, 28). The strain *patS*$^-$ formed MCH in the absence of combined nitrogen (Fig. 3*A*). However, when *patS*$^-$ was transformed with pPpetE-ccbP(*ery*^{R}), it did not form heterocysts when induced with copper (Fig. 3*B*). When the *hetR* gene was present on a multicopy plasmid (pRL25C-hetR), *Anabaena* 7120 formed MCH in $BG11_0$ (Fig. 3*C*). When the strain with pRL25C-hetR also contained the pPpetE-ccbP(*ery*^{R}) and was induced with copper, it did not form heterocysts in the absence of combined nitrogen (Fig. 3*D*). This result suggests that CcbP could act at an early stage of heterocyst differentiation, possibly by preventing the HetR activation of transcription.

The expression of *hetR* was investigated by immunoblotting with the results shown in Fig. 4. The amount of HetR reached its maximum level within 3 h after nitrogen step-down in the wild type. The expression of *ccbP* induced by copper prevented the fast induction of *hetR* in CCBP-IE. The HetR level in this strain increased gradually and reached a low plateau 12 h after the removal of combined nitrogen (Fig. 4). The induction of *hetR* in CCBP-M was similar to that of the wild type within 24 h after nitrogen step-down. The HetR level in CCBP-M declined faster after 24 h of nitrogen step-down than that of the wild type, probably because the cells were under a more stressed condition.

Localization of CcbP. The spatial pattern of *ccbP* expression in *Anabaena* 7120 was studied by using *gfp* as a reporter gene under control of the *ccbP* promoter (800-bp fragment upstream of *ccbP*). Fig. 5 *A1* and *A2* show that, in the strain carrying pPccbP-gfp, the GFP was mostly located in vegetative cells, whereas the green fluorescence was much weaker in heterocysts, suggesting that transcription of *ccbP* was down-regulated in heterocysts. As a control, the green fluorescence in the strain expressing *gfp* under control of the *patS* promoter (24) was almost exclusively located in heterocysts (Fig. 5*A3*). Immunoblotting the proteins from isolated heterocysts (Fig. 5*B*) showed that, whereas HetR was more abundant in heterocysts, CcbP could not be detected in heterocysts, suggesting that the weak green fluorescence observed in heterocysts in Fig. 5 *A1* and *A2* could be due to incomplete degradation of GFP during differentiation. We also studied the level of CcbP from entire filaments during the process of heterocyst differentiation and found that it was largely unchanged (data not shown).

Measurement of Intracellular Free Calcium with Obelin. It was reported that a transient increase of $[Ca^{2+}]_i$ occurred after nitrogen step-down in *Anabaena* 7120 based on whole-filament measurements (29). The level of $[Ca^{2+}]_i$ in individual cells in *Anabaena* has not been reported. To monitor $[Ca^{2+}]_i$ in individual cells, we used recombinant obelin as an indicator of $[Ca^{2+}]_i$ in *Anabaena* 7120. In the presence of coelenterazine, the intensities of luminescence and fluorescence generated by obelin are linearly related to Ca^{2+} concentration (30). The blue fluorescence of obelin-generated coelenteramide is excellent for studying cyanobacterial $[Ca^{2+}]_i$, because the fluorescence from

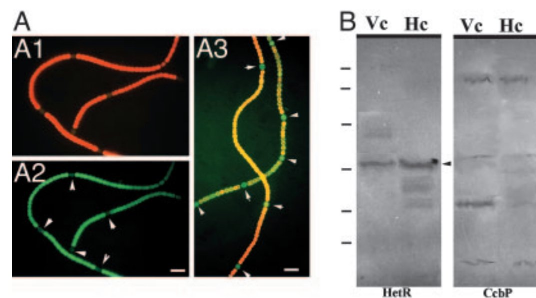

Fig. 5. Analysis of the *ccbP* promoter in *Anabaena* 7120 with *gfp* as reporter gene (*A*) and localization of CcbP by immunoblotting (*B*). (*A1* and *A2*) *Anabaena* 7120 carrying pP*ccbP-gfp* was subjected to nitrogen step-down for 24 h. The fluorescence image was obtained with blue light excitation without (*A1*) and with (*A2*) the red fluorescence blocked. (*A3*) GFP fluorescence from *Anabaena* 7120 carrying plasmid pAM1951 that contained a P*patS-gfp* fusion (24) after nitrogen step-down for 24 h. Arrows indicate the positions of heterocysts. (*B*) Immunoblotting analysis of CcbP in vegetative cells (Vc) and isolated heterocysts (Hc). Antibodies against HetR and CcbP were used as primary antibodies in these reactions. Arrows indicate the positions of HetR or CcbP. The molecular mass standards shown on the left side of the blots are the same as in Fig. 1. (Bar, 10 μm.)

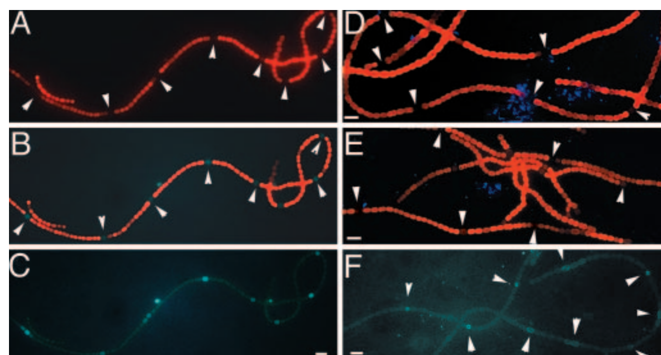

Fig. 6. Increase of intracellular free Ca^{2+} concentration as revealed by recombinant obelin. *Anabaena* 7120 carrying pAM505-ob (*A–D*, *F*) or without pAM505-ob (*E*) was subjected to nitrogen step-down for 24 h (*A–E*) or for 12 h (*F*). Except for *D*, coelenterazine was added to 1 ml of cultures of *Anabaena* 7120 at an optical density (750 nm) of 0.3 to a final concentration of 2 μM and incubated for 30 min before they were observed with the fluorescence microscope. (*A*) Fluorescence image with green-light excitation that excited phycobiliproteins (PBP). (*B* and *C*) Fluorescence images with near-UV light excitation with the red fluorescence from chlorophyll and PBP unblocked (*B*) and blocked (*C*). (*D*) Fluorescence image in the absence of coelenterazine when excited with near-UV light. (*E*) Fluorescence image of the wild-type *Anabaena* 7120 without obelin in the presence of coelenterazine with near-UV light excitation. (*F*) Fluorescence image of *Anabaena* 7120 carrying pAM505-ob 12 h after the removal of combined nitrogen. The filament was excited with near-UV light, and the image was obtained as in *C*. (Bar, 10 μm.)

phycobiliproteins (PBP) and chlorophyll in this wavelength range is much lower. The *obe* gene on pAM505-ob was controlled by an *E. coli* promoter, Ptac, and the blue fluorescence generated by obelin was observed in *Anabaena* 7120 (Fig. 6). When the filaments were excited by PBP-absorbing light, red fluorescence was observed only from vegetative cells (Fig. 6*A*). When the filaments were excited with near-UV light, vegetative cells showed red fluorescence, whereas heterocysts were blue (Fig. 6*B*). Fig. 6*C* shows the same filament with the red fluorescence blocked. The level of blue fluorescence in vegetative cells was comparable in BG11 and $BG11_0$ (not shown). The blue fluorescence depended upon coelenterazine, because none was observed in its absence (Fig. 6*D*). The heterocysts from the wild type without obelin showed no blue fluorescence even when coelenterazine was added (Fig. 6*E*). Fig. 6*F* shows the pattern of blue-fluorescent cells along the filaments 12 h after the removal of combined nitrogen.

The emission spectrum generated by Ca^{2+}–obelin during heterocyst differentiation was measured (Fig. 7). The spectrum showed an emission peak at 460 nm that depended upon the presence of coelenterazine (Fig. 7*A*). The change of fluorescence intensity at 460 nm (Fig. 7*B*) showed a fast increase of $[Ca^{2+}]_i$ 4 h after nitrogen step-down, and a higher $[Ca^{2+}]_i$ was maintained in the following period of heterocyst differentiation. Expression of *ccbP* with copper induction in CCBP-IE completely suppressed the increase of $[Ca^{2+}]_i$ after nitrogen step-down. Because the fluorescence levels in vegetative cells were similar, a doubling of fluorescence intensity in whole filaments (Fig. 7*B*) suggests that $[Ca^{2+}]_i$ in heterocysts or differentiating cells would be ≈10 times higher than in vegetative cells, assuming that their frequency is ≈10%.

Discussion

A low $[Ca^{2+}]_i$ is normally maintained in *Anabaena* 7120 (31). The detection of a transient increase of $[Ca^{2+}]_i$ during heterocyst differentiation (29) and the results obtained with manipulation of extracellular Ca^{2+} concentrations (9) suggested that Ca^{2+} might be a signal for the differentiation process. In this report, we describe a Ca^{2+}-binding protein, CcbP, from *Anabaena* 7120 and suggest a role of CcbP in regulation of heterocyst differentiation. CcbP was isolated by using a procedure similar to that for calsequestrin (25). CcbP is rich in acidic amino acids and lacks an EF hand. So it is possible that CcbP binds Ca^{2+} similarly to calsequestrin, which also lacks an EF hand and binds Ca^{2+} by protein surface charge (32). The binding of Ca^{2+} to calsequestrin generates a more compact structure of the protein (33). The mobility shift of CcbP in SDS/PAGE induced by Ca^{2+} (Fig. 1 *C* and *D*) suggests that CcbP could at least partially maintain its structure when treated with SDS, as does calsequestrin (33). It also suggests that CcbP has a more compact structure with bound

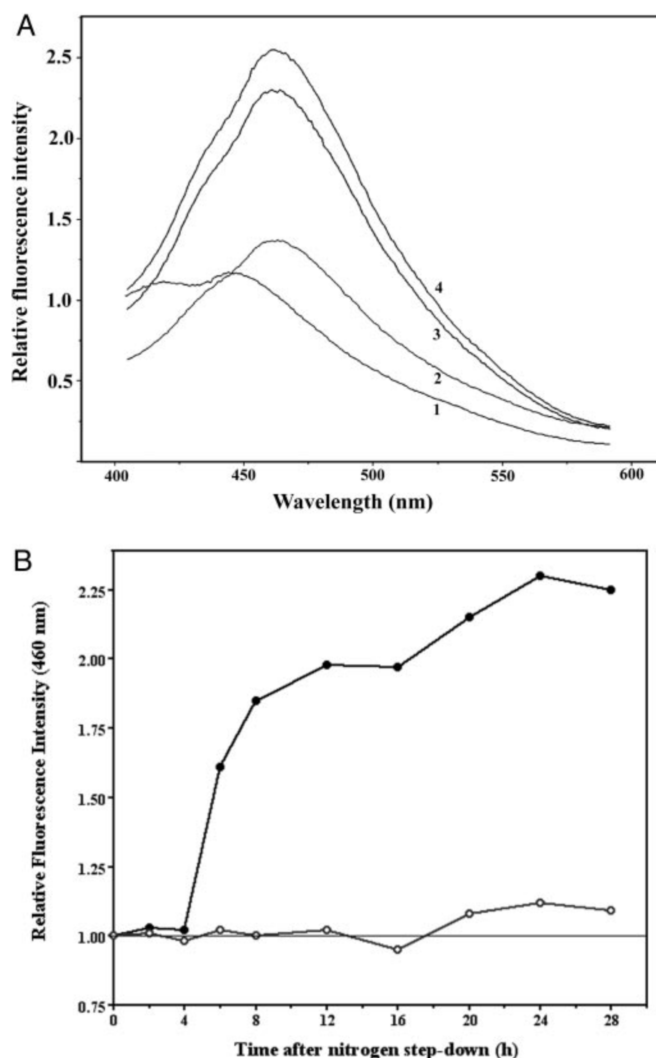

Fig. 7. Fluorescence emission spectra *in vivo* and time course of the increase of emission at 460 nm during heterocyst differentiation. (*A*) Selected fluorescence spectra from coelenteramide-obelin in *Anabaena* 7120 carrying pAM505-ob during heterocyst differentiation. Cells were collected, and coelenterazine was added as described in Fig. 6. The cultures were then excited with UV light at 340 nm, and four emission spectra were obtained and averaged. Spectrum 1, emission spectrum without coelenterazine; spectra 2–4, emission spectra obtained at 0, 12, and 24 h after the removal of combined nitrogen, with coelenterazine. (*B*) Increase of fluorescence emission at 460 nm of *Anabaena* 7120 carrying pAM505-ob during heterocyst differentiation. Cells were collected at the times indicated and incubated with coelenterazine before emission spectra were obtained as in *A*. Each point represents an average of four independent measurements. Solid circles: *Anabaena* 7120 carrying pAM505-ob. Open circles: CCBP-IE(*eryR*) carrying pAM505-ob in the presence of 2.5 μM $CuCl_2$.

Ca^{2+} as calsequestrin and CaM, although the possibility that the band of CcbP in SDS/PAGE in the presence of EGTA is a stable noncovalent dimer could not be completely ruled out.

Our study shows that lack of *ccbP* results in a MCH phenotype, and overexpression of *ccbP* suppresses heterocyst formation (Fig. 2). Overexpression of *cam* in both *Anabaena* 7120 and *A. variabilis* also suppressed heterocyst formation (Fig. 2). Because CaM is absent from both *Anabaena* strains, the effect we observed in the strains overexpressing *cam* is most likely a result of Ca^{2+} sequestration that reduces $[Ca^{2+}]_i$ rather than a result of protein–protein interactions. That excessive CcbP suppresses the increase of $[Ca^{2+}]_i$ during heterocyst differentiation (Fig. 7*B*) supports this suggestion. This suggestion is also supported by the results of CcbP localization, which showed that it was present in vegetative cells but absent from heterocysts (Fig. 5). These results indicate that an increase of $[Ca^{2+}]_i$ is required for cells to differentiate.

To measure $[Ca^{2+}]_i$, we expressed the obelin gene from *Obelia geniculata* (30) in *Anabaena* 7120. Obelin is an excellent indicator for Ca^{2+} and is less sensitive to Mg^{2+} (30). Although the maximum fluorescence emission of recombinant obelin–Ca^{2+} *in vitro* is at 517 nm (30), the peak of obelin-Ca^{2+} fluorescence emission is at 460 nm in *Anabaena* cells (Fig. 7). This result suggests that the emission is contributed mostly by an ion pair state and the amide anion of coelenteramide (30). The total cellular-free Ca^{2+} concentration increases rapidly 4 h after nitrogen step-down (Fig. 7*B*), and this increase is due to the increase of $[Ca^{2+}]_i$ in mature heterocysts and differentiating cells (Fig. 6). The results in Fig. 6 suggest that Ca^{2+} plays an important role early in heterocyst differentiation, because a pattern of cells with high blue fluorescence was established earlier than the appearance of proheterocysts. That overexpression of *ccbP* prevents heterocyst formation in the strain $patS^-$ or the strain with pRL25C-hetR (Fig. 3) also suggests that Ca^{2+} functions early in the process of differentiation. HetR is an important protein in regulation of heterocyst differentiation and pattern formation (17, 18, 20, 26, 34, 35), and *hetR* is up-regulated early in the process of heterocyst differentiation (17, 22). Sequestration of Ca^{2+} with CcbP prevented the initial *hetR* induction (Fig. 4), indicating that *hetR* up-regulation could not be accomplished when $[Ca^{2+}]_i$ is too low. Because HetR is possibly a Ca^{2+}-dependent protease (18), the increased $[Ca^{2+}]_i$ is likely to be important to the functions of HetR in the differentiating cells.

The results shown in Figs. 6 and 7 demonstrate that $[Ca^{2+}]_i$ in mature heterocysts is 10 times higher than in the vegetative cells. The increase of $[Ca^{2+}]_i$ in mature heterocysts and differentiating cells could result from specific degradation of CcbP and the release of the bound Ca^{2+}, which could occur 4 h after nitrogen step-down (Fig. 7*B*). The phase of fast $[Ca^{2+}]_i$ increase (Fig. 7*B*) was ≈1–2 h behind the phase of HetR increase (Fig. 4). The timing and kinetics of HetR induction (Fig. 4) and $[Ca^{2+}]_i$ increase (Fig. 7) after nitrogen step-down are suggestive that HetR can be a candidate responsible for CcbP degradation, although experimental data are lacking at the moment. *Anabaena* 7120 has at least one potential P-type calcium-pump (all3245) based on surveys of its genome. It is possible that the activity of the pump is down-regulated in differentiating cells and mature heterocysts.

The increase of $[Ca^{2+}]_i$ in heterocysts strongly suggests that Ca^{2+} is important for heterocyst functions. It has been shown that GlnB modification is inhibited by Ca^{2+} (K. Forchhammer, personal communication). Laurent *et al.* (36) have shown that GlnB in heterocysts is present in an unmodified form, which is required for the activation of *N*-acetyl glutamate kinase (37, 38), a key enzyme involved in arginine synthesis. Thus, a higher $[Ca^{2+}]_i$ might be critical for nitrogen assimilation in heterocysts.

We thank Dr. J. Golden (Texas A&M University, College Station, TX) for providing useful plasmids, Dr. X. Xu (Institute of Hydrobiology, Wuhan, China) for providing the $patS^-$ strain, and Dr. J. Brosius (Mount Sinai School of Medicine, New York) for providing the rat CaM gene. We also thank Dr. K. Forchhammer (University of Giessen, Giessen, Germany) for communicating his experimental results before publication. This research is supported by the National Natural Science Federation of China (30230040) and by grants from the Ministry of Science of Technology of China (01CB108903 and 2004AA626021).

1. Campbell, A. K. (1983) *Intracellular Calcium: Its Universal Role as Regulator* (Wiley, New York).
2. Clapham, D. E. (1995) *Cell* **80,** 259–268.
3. Youatt, J. (1993) *Crit. Rev. Microbiol.* **19,** 87–93.
4. Michiels, J., Xi, C., Verhaert, J. & Vanderleyden, J. (2002) *Trends Microbiol.* **10,** 83–97.
5. Dominguez, D. C. (2004) *Mol. Microbiol.* **54,** 291–297.
6. Gangola, P. & Rosen, B. P. (1987) *J. Biol. Chem.* **262,** 12570–12574.
7. Herbaud, M. L., Guiseppi, A., Denizot, F., Haiech, J. & Kihoffer, M. C. (1998) *Biochim. Biophys. Acta* **1448,** 212–226.
8. Tisa, L. S., Olivera, B. M. & Adier, J. (1993) *J. Bacteriol.* **175,** 1235–1238.
9. Smith, R. L., Hobson, S. & Ellis, I. R. (1987) *New Phytol.* **105,** 531–541.
10. Cloud, P., Moorman, M. & Pierce, D. (1975) *Q. Rev. Biol.* **50,** 131–150.
11. Buikema W. J. & Haselkorn, R. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 33–52.
12. Wolk, C. P. (2000) in *Prokaryotic Development*, eds. Brun, Y. V. & Shimkets, L. J. (Academic, Washington, DC), pp. 83–104.
13. Meeks, J. C. & Elhai, J. (2002) *Microbiol. Mol. Biol. Rev.* **66,** 94–121.
14. Golden, J. W. & Yoon, H. S. (2003) *Curr. Opin. Microbiol.* **6,** 557–563.
15. Frias, J. E., Flores, E. & Herrero, A. (1994) *Mol. Microbiol.* **14,** 823–832.
16. Wei, T.-F., Ramasubramanian, T. S. & Golden, J. W. (1994) *J. Bacteriol.* **176,** 4473–4482.
17. Buikema, W. J. & Haselkorn, R. (1991) *Genes Dev.* **5,** 321–330.
18. Zhou, R., Wei, X., Jiang, N., Li, H., Dong, Y., Hsi, K. L. & Zhao, J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4959–4963.
19. Burgess, W. H., Jemiolo, D. K. & Kretsinger, R. H. (1980) *Biochim. Biophys. Acta* **623,** 257–270.
20. Huang, X., Dong, X. & Zhao, J. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 4848–4853.
21. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
22. Zhou, R., Cao, Z. & Zhao, J. (1998) *Arch. Microbiol.* **169,** 417–423.
23. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356.
24. Yoon, H. S & Golden, J. W. (1998) *Science* **282,** 935–938.
25. Campbell, K. P., McLennan, D. H., Jorgensen, A. O. J. & Mintzer, M. C. (1983) *J. Biol. Chem.* **258,** 1197–1204.
26. Buikema, W. J. & Haselkorn, R. (2001) *Proc. Natl. Acad. Sci. USA.* **98,** 2729–2734.
27. Thiel, T., Lyons, E. M. & Erker, J. C. (1997) *J. Bacteriol.* **179,** 5222–5225.
28. Yoon, H. S. & Golden, J. W. (2001) *J. Bacteriol.* **183,** 2605–2613.
29. Torrecilla, I., Leganes, F., Bonilla, I. & Femandez-Pinas, F. (2004) *Microbiology* **150,** 3731–3739.
30. Markova, S. V., Vysotski, E. S., Blinks, J. R., Burakova, L. P., Wang, B. C. & Lee, J. (2002) *Biochemistry* **41,** 2227–2236.
31. Torrecilla, I., Leganes, F., Bonilla, I. & Femandez-Pinas, F. (2000) *Plant Physiol.* **123,** 161–175.
32. Maclennan, D. H., Abu-Abed, M. & Kang, C. (2002) *J. Mol. Cell Cardiol.* **34,** 897–918.
33. Cozens, B. & Reithmeier, R. A. F. (1984) *J. Biol. Chem.* **259,** 6248–6252.
34. Black, T. A. & Wolk, C. P. (1994) *J. Bacteriol.* **176,** 2282–2292.
35. Khudyakov, I. & Golden, J. W. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 16040–16045.
36. Laurent, S., Forchhammer, K., Gonzalez, L., Heulin, T., Zhang, C. C. & Bedu, S. (2004) *FEBS Lett.* **576,** 261–265.
37. Heinrich, A., Maheswaran, M., Ruppert, U. & Forchhammer, K. (2004) *Mol. Microbiol.* **52,** 1225–1228.
38. Burillo, S., Luque, I., Fuentes, I. & Contreras, A. (2004) *J. Bacteriol.* **186,** 3346–3354.