Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NtcA in *Anabaena* sp. PCC 7120

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Calcium ions are important to some prokaryotic cellular processes, such as heterocyst differentiation of cyanobacteria. Intracellular free Ca^{2+}concentration, $[Ca^{2+}]_i$, increases several fold in heterocysts and is regulated by CcbP, a Ca^{2+}-binding protein found in heterocyst-forming cyanobacteria. We demonstrate here that CcbP is degraded by HetR, a serine-type protease that controls heterocyst differentiation. The degradation depends on Ca^{2+} and appears to be specific because HetR did not digest other tested proteins. CcbP was found to bind two Ca^{2+} per molecule with K_D values of 200 nM and 12.8 μM. Degradation of CcbP releases bound Ca^{2+} that contributes significantly to the increase of $[Ca^{2+}]_i$ during the process of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. We suggest that degradation of CcbP is a mechanism of positive autoregulation of HetR. The down-regulation of *ccbP* in differentiating cells and mature heterocysts, which also is critical to the regulation of $[Ca^{2+}]_i$, depends on NtcA. Coexpression of *ntcA* and a *ccbP* promoter-controlled *gfp* in *Escherichia coli* diminished production of GFP, and the decrease is enhanced by α-ketoglutarate. It was also found that NtcA could bind a fragment of the *ccbP* promoter containing an NtcA-binding sequence in a α-ketoglutarate-dependent fashion. Therefore, $[Ca^{2+}]_i$ is regulated by a collaboration of HetR and NtcA in heterocyst differentiation in *Anabaena* sp. strain PCC 7120.

cyanobacteria | protease

Cyanobacteria appeared on Earth ≈2.5–3 billion years ago (1). The release of oxygen as a by-product of photosynthetic electron transfer by the cyanobacteria led to a fundamental change of the biosphere. The accumulation of oxygen in the environment greatly stressed many organisms because oxygen is highly toxic to many biochemical reactions that could only be carried out under anaerobic conditions. Nitrogenase, for example, is an enzyme that is sensitive to oxygen, and biological nitrogen fixation could only take place in the absence of oxygen molecules. One of the mechanisms by which cyanobacterial nitrogen fixation adapted to an oxidizing environment was the restriction of nitrogenase to specialized cells called heterocysts (2–6). Heterocysts have several means for protection of nitrogenase from oxygen molecules: a thick envelope to limit oxygen penetration, the absence of photosystem II so that no oxygen is evolved, and a high respiratory rate to consume oxygen. In some cyanobacteria, heterocysts are distributed in a semiregular pattern along the filaments.

One of the signals that triggers the differentiation from a vegetative cell to a heterocyst in response to nitrogen deprivation is the increase of the intracellular concentration of α-ketoglutarate (2-OG) (7, 8). Another important signal in heterocyst differentiation is the intracellular concentration of free calcium, $[Ca^{2+}]_i$ (9, 10). It is known that Ca^{2+} ions play very important roles in cellular processes in eukaryotes and that eukaryotic $[Ca^{2+}]_i$ is tightly regulated and maintained in the nanomolar range. Although the role of Ca^{2+} in prokaryotic cellular activities is less clear (11–13), current evidence also shows that $[Ca^{2+}]_i$ is also tightly regulated in bacteria (14, 15) and that Ca^{2+} plays important roles in bacterial cell differentiation such as sporulation of *Bacillus* (16) and heterocyst formation of cyanobacteria (10). It was recently shown that $[Ca^{2+}]_i$ increases in differentiating cells after transfer from a nitrogen-replete condition to a nitrogen-deprived condition (10). CcbP, a calcium-binding protein in heterocyst-forming cyanobacteria, plays an important role in the regulation of $[Ca^{2+}]_i$ and is absent in mature heterocysts. The expression of *ccbP* is also down-regulated in heterocysts (10). However, it is not known at present how Ca^{2+} is released from CcbP and how the expression of *ccbP* is regulated.

The initiation of heterocyst differentiation is controlled by key genes *hetR* (17) and *ntcA* (18, 19). *hetR* encodes a serine-type protease with DNA-binding activity (20, 21). Even though it has been shown that HetR is autodegrading, no other substrates of HetR have been found so far. One important feature of *hetR* is that its expression is positively autoregulatory (22). Although the binding of the *hetR* promoter by HetR dimer could be important to the autoregulatory process (21), the mechanism of the autoregulation is not well understood. NtcA is a transcription factor that belongs to the cAMP receptor protein superfamily and positively regulates the expression of many genes involved in cell differentiation (23). Its DNA-binding activity is regulated by 2-OG (24). NtcA also has been shown to negatively regulate *rbcL* encoding the large subunit of Rubisco (25). Recent evidence has shown that the expression of *ntcA* and *hetR* is mutually dependent (26).

In this report, we show that CcbP from *Anabaena* sp. strain PCC 7120 (hereafter referred to as *Anabaena* 7120) is specifically degraded by HetR and that the degradation depends on Ca^{2+}. We also demonstrate that NtcA is involved in the down-regulation of *ccbP* in a 2-OG-dependent fashion.

Results

Degradation of CcbP by HetR and the Release of Bound Calcium Ions. It has been demonstrated that mature heterocysts and proheterocysts have an increased $[Ca^{2+}]_i$ (10). To understand the mechanism for regulation of $[Ca^{2+}]_i$ during heterocyst differentiation, we studied the kinetics of $[Ca^{2+}]_i$ increase during heterocyst differentiation. Fig. 1*A* shows that a small increase of $[Ca^{2+}]_i$ could be observed within 1 h after nitrogen step-down. However, the major increase of $[Ca^{2+}]_i$ occurred 4 h after nitrogen deprivation. Fig. 1*A* also shows that, whereas $[Ca^{2+}]_i$ increased 2-fold in *Anabaena* 7120 during the process of heterocyst differentiation as shown by the intensity of obelin-catalyzed coelenteramide fluorescence, which depends on Ca^{2+} (27), the overall cellular Ca^{2+}content remained unchanged

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; 2-OG, α-ketoglutarate.

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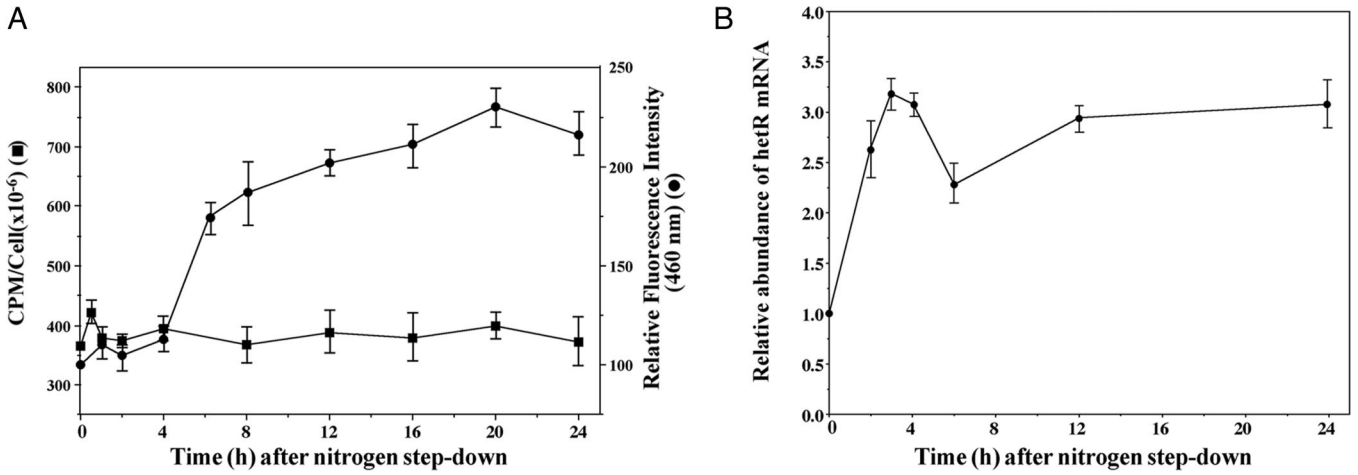

Fig. 1. Measurement of Ca^{2+} content, cellular free Ca^{2+} concentration, and expression of *hetR* during heterocyst differentiation of *Anabaena* 7120. (*A*) Measurement of cellular Ca^{2+} content. Total cellular Ca^{2+} of *Anabaena* 7120 (■) was determined with $^{45}Ca^{2+}$, and $[Ca^{2+}]_i$ was determined with Ca^{2+}-dependent fluorescence emission at 460 nm in *Anabaena* 7120 expressing the *obelin* gene (●). The values of fluorescence emission were normalized to the initial value at time 0, i.e., when combined nitrogen was removed. (*B*) Relative amount of the *hetR* mRNA during heterocyst differentiation as determined by quantitative PCR. Each point represents an average of six individual measurements, and all values were normalized to the value at time 0 of nitrogen step-down.

during this period based on assays with radioactive $^{45}Ca^{2+}$. These results indicated that the increase of $[Ca^{2+}]_i$ during the heterocyst differentiation of *Anabaena* 7120 was not a result of an up-regulated Ca^{2+} uptake but likely a consequence of the release of bound Ca^{2+}. Because CcbP is a major Ca^{2+}-binding protein in *Anabaena* 7120, we determined the cellular concentration of CcbP by ELISA, and the CcbP concentration was 0.036 ($\pm$0.008) fg per cell, or 2.5 μM. Because CcbP is absent in heterocysts and heterocysts have a relatively high $[Ca^{2+}]_i$, the results shown in Fig. 1 suggest that the increase of $[Ca^{2+}]_i$ in differentiating cells and mature heterocyst could be a result of CcbP degradation. The time course of the increase of $[Ca^{2+}]_i$ is $\approx$1–2 h behind that of *hetR* induction, which reached its maximum level 3 h after nitrogen deprivation (Fig. 1*B*). Because HetR is a protease, we tested whether CcbP could be a substrate of HetR. The results are shown in Fig. 2. Incubation of HetR at 37°C led to an autodigestion as previously demonstrated (20). Incubation of CcbP with HetR at 37°C resulted in the complete digestion of CcbP and HetR. Because CcbP was not digested if HetR was absent (Fig. 2*A*, lane 3), the degradation of CcbP (Fig. 2*A*, lane 4) was not due to digestion by contaminating proteases. The degradation of both proteins could be prevented by 5 mM EGTA (Fig. 2*A*, lane 5) and by 0.2 mM PMSF (Fig. 2*A*, lane 6), a serine-type protease inhibitor. The degradation of CcbP by HetR was probably specific because NtcA of *Anabaena* 7120 and BSA were not degraded by HetR (Fig. 2*A*, lanes 7–10). $HetR_{S152A}$ and $HetR_{S179N}$ are two mutant HetR proteins that show no autodegradation, and the strains carrying these mutant genes could not initiate heterocyst differentiation (17, 28). Incubation of CcbP with $HetR_{S152A}$ or $HetR_{S179N}$ did not result in any digestion

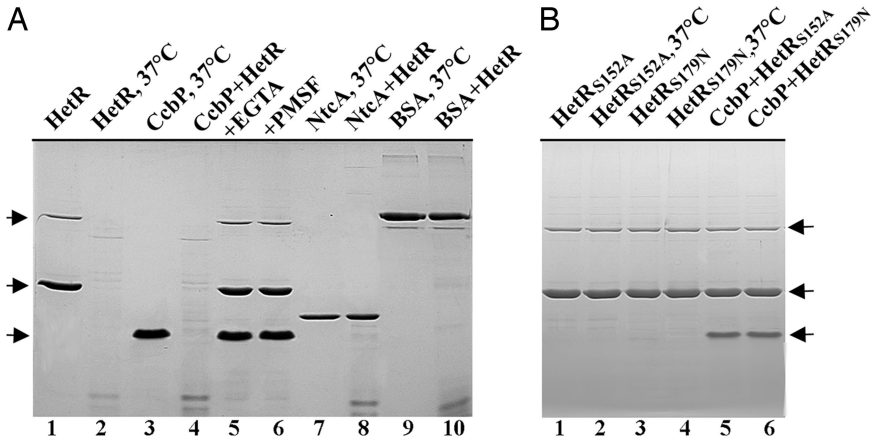

Fig. 2. SDS/PAGE analysis of CcbP degradation by HetR. (*A*) Degradation of CcbP by wild-type HetR under the conditions indicated above the gel. The duration of incubation at 37°C was 2 h. The initial concentrations of HetR, CcbP, NtcA, and BSA were 1, 2, 1, and 2 mg·ml^{-1}, respectively. The concentrations of EGTA and PMSF were 5 and 0.2 mM, respectively. Lanes: 1 and 2, HetR before and after incubation at 37°C, respectively; 3 and 4, CcbP after incubation without or with HetR at 37°C, respectively; 5 and 6, CcbP after incubation with HetR in the presence of 5 mM EGTA or 0.2 mM PMSF, respectively; 7 and 8, NtcA after incubation without or with HetR at 37°C, respectively; 9 and 10, BSA after incubation without or with HetR at 37°C, respectively. The thin bands below the major BSA bands in lanes 9 and 10 were from BSA stock and were present before treatment. (*B*) No digestion of CcbP by two mutant HetR proteins that lack autodegradation activity. The concentrations of CcbP, $HetR_{S152A}$, and $HetR_{S179N}$ and the digestion conditions were the same as for CcbP and HetR in *A*. Lanes: 1 and 2, $HetR_{S152A}$ before and after incubation at 37°C, respectively; 3 and 4, $HetR_{S179N}$ before and after incubation at 37°C, respectively; 5 and 6, incubation of CcbP with $HetR_{S152A}$ and $HetR_{S179N}$ at 37°C, respectively. The upper arrows in *A* and *B* indicate the position of the HetR dimer, the middle arrows indicate the position of HetR monomer, and the lower arrows indicate the position of CcbP.

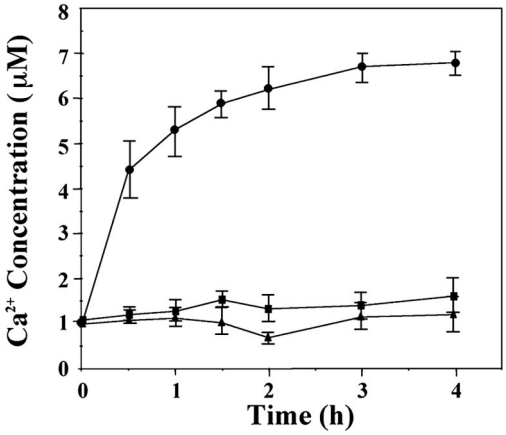

Fig. 3. Release of bound Ca^{2+} from CcbP during its digestion by HetR. Solutions (50 mM Tris·HCl, pH 7.4/100 mM KCl) containing both HetR at 0.1 mg·ml^{-1} and CcbP at 0.43 mg·ml^{-1} (3 μM) (●), HetR only (■), or CcbP only (▲) were incubated at 37°C, and the concentrations of free Ca^{2+} were measured with a Ca^{2+} electrode. The initial concentration of free Ca^{2+} was adjusted to 1.0 μM.

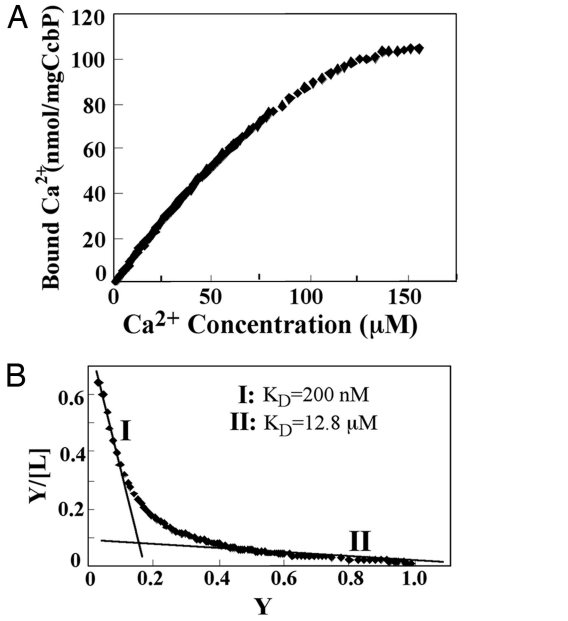

Fig. 4. Measurement of the number of bound Ca^{2+} per molecule of CcbP. (*A*) Determination of the stoichiometry of CcbP and bound Ca^{2+}. $CaCl_2$ from a stock solution of 1 M was added incrementally to a 10-ml 0.83 mg·ml^{-1} CcbP solution in 10 mM Tris·HCl buffer (pH 7.5) containing 100 mM KCl, and free Ca^{2+} in solution was measured with a Ca^{2+} electrode. An average of 1.7 Ca^{2+} bound per CcbP was determined. (*B*) Scatchard plot for the determination of Ca^{2+} dissociation constants (K_d) of CcbP. Y represents the percentage of CcbP with bound Ca^{2+} and [L] represents the free Ca^{2+} concentration in micromolar. Curves I and II were obtained by curve fitting. Curve I has a slope of −4.99, corresponding to a K_d of 200 nM; curve II has a slope of −0.078, corresponding to a K_d of 12.8 μM.

of CcbP, demonstrating that the active serine of HetR is required for the degradation of CcbP (Fig. 2*B*). $HetR_{C48A}$, a mutant protein that cannot form dimer but retains protease activity, can digest CcbP (data not shown).

The release of CcbP-bound Ca^{2+} during the process of CcbP degradation by HetR was investigated (Fig. 3). The initial concentration of CcbP was adjusted to 3 μM, similar to the CcbP concentration *in vivo*. Once HetR was added, free Ca^{2+} concentration increased rapidly from 1 μM to 7 μM, although no such increase was observed when HetR was omitted (Fig. 3). If the initial free Ca^{2+} concentration was adjusted to 1 μM in the absence of CcbP, addition of HetR to the solution did not lead to an increase of free Ca^{2+} concentration. This result indicated that complete degradation of 1 μmol of CcbP could release ≈2 μmol of Ca^{2+}. Measurement of the Ca^{2+}/CcbP ratio showed that one CcbP binds approximately two (1.73 ± 0.41) Ca^{2+} (Fig. 4*A*). The K_d values for CcbP's two Ca^{2+}-binding sites were 12.8 μM and 200 nM (Fig. 4*B*). It is estimated that the intracellular concentration of the CcbP-bound Ca^{2+} is ≈1.5 μM based on the fact that the cellular concentration of CcbP was 2.5 μM and the fact that the physiological concentrations of free Ca^{2+} are between 100 nM and 200 nM (15). These results demonstrate that the Ca^{2+} released from CcbP by HetR digestion could contribute significantly to the increase of $[Ca^{2+}]_i$ during heterocyst differentiation.

Down-Regulation of the Expression of *ccbP* During Heterocyst Differentiation. It has been shown that the expression of *ccbP* in heterocysts is down-regulated, which could be critical to the increase of $[Ca^{2+}]_i$ in heterocysts and proheterocysts (10). Analysis of the *ccbP* promoter region of *Anabaena* 7120 showed that there was a potential NtcA-binding site, **GT**TCTGAGTGGTC**ACA** (23), 154 bp upstream of the start codon of *ccbP* (nucleotides in bold indicate the conserved binding sequence). To investigate whether NtcA was directly involved in the regulation of *ccbP* expression, we studied the effect of coexpression of *ntcA* and *gfp* (encoding GFP) controlled by the *ccbP* promoter from *Anabaena* 7120 in *Escherichia coli* (Fig. 5). When *E. coli* cells containing the plasmid pPccbP-gfp, which bears a *gfp* gene under control of the *ccbP* promoter (10), were excited with a blue light (460 nm), a GFP-specific emission spectrum was obtained, suggesting that the *ccbP* promoter is functional in *E. coli* and that functional GFP was produced. When the *E. coli* cells containing both pPccbP-gfp and pET-ntcA were grown in the presence of 0.1 mM isopropyl β-D-thiogalactoside (IPTG) to induce the expression of *ntcA*, emission of GFP was reduced by ≈70%. If the medium contained 0.5 mM 2-OG as well as 0.1 mM IPTG, emission from GFP was not detected. When *E. coli* cells containing pPccbP-gfp and pET-psaE, which was used for production of PsaE of photosystem I (29), were induced with IPTG and 2-OG, emission of GFP was same as that from *E. coli* containing only pPccbP-gfp. These results indicate that NtcA negatively regulated the activity of the *ccbP* expression in *E. coli* and that this regulation was influenced by 2-OG. The role of NtcA on the *ccbP* expression of *Anabaena* 7120 was investigated by measuring the amount of *ccbP* mRNA after nitrogen step-down in both the wild-type and $ntcA^-$ strains (Fig. 5*B*). The *ccbP* mRNA in the wild type remained unchanged within the first 3 h after nitrogen step-down. In contrast, the *ccbP* transcript in $ntcA^-$ increased by 50% in the same period, indicating that NtcA negatively regulates *ccbP* expression in the early stage of heterocyst differentiation. The amount of *ccbP* mRNA in both strains reached to the same level 12 h after nitrogen step-down followed by a gradual decrease. Whether NtcA affected the activity of the *ccbP* promoter was further tested by EMSA using DNA fragments in the *ccbP* promoter region that contain the possible NtcA-binding sequence noted before. As shown in Fig. 5*C*, the presence of 3 nM recombinant NtcA in the binding buffer resulted in a retardation of migration of the DNA fragment in gel electrophoresis (Fig. 5*C*, lanes 1–5), whereas BSA alone did not have such an effect (Fig. 5*C*, lane 6). The presence of increasing concentrations of 2-OG (Fig. 5*C*, lanes 1–5) in the binding buffer led to an increasing amount of DNA retarded in electrophoresis, indicating that 2-OG enhanced interaction of NtcA with the DNA fragment. Fig. 5*D* shows that the NtcA-binding sequence ($GTN_{11}ACA$) in the *ccbP* promoter region was required for NtcA-induced gel mobility shift of a synthetic DNA fragment (Fig. 5*D*, lane 2). When the

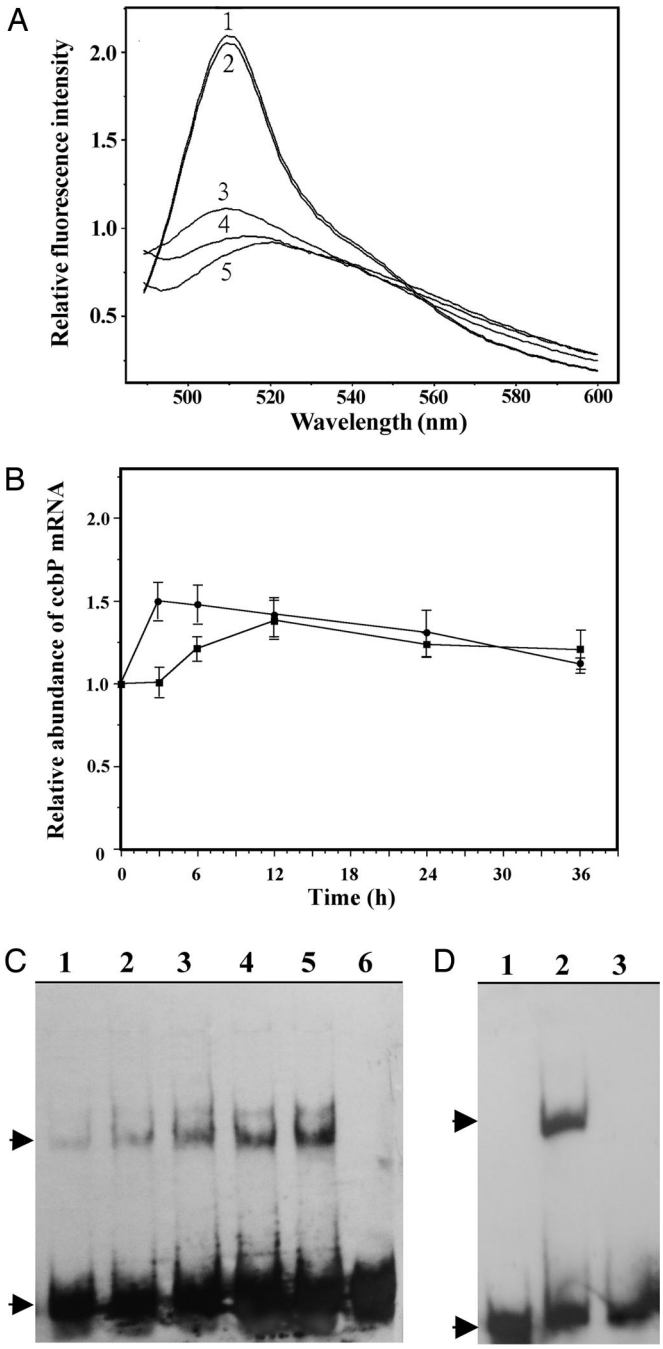

Fig. 5. Down-regulation of the *ccbP* gene of *Anabaena* 7120 by NtcA. (*A*) GFP fluorescence spectra of *E. coli* cells expressing a *gfp* gene under control of the *ccbP* promoter and the *ntcA* gene from *Anabaena* 7120 inducible by IPTG. Curves: 1, fluorescence spectrum obtained from the *E. coli* cells containing pPccbP-gfp; 2, emission spectrum obtained when the *E. coli* cells containing pPccbP-gfp and pET-psaE were in the presence of 0.1 mM IPTG and 0.5 mM 2-OG; 3 and 4, emission spectra obtained when the *E. coli* cells containing both pPccbP-gfp and pET-ntcA were in the presence of 0.1 mM IPTG without or with 0.5 mM 2-OG, respectively; 5, a spectrum obtained from *E. coli* cells containing pRL25C (from which pPccbP-gfp is derived) and pET-ntcA. No GFP fluorescence emission peak was obtained. The optical densities at 600 nm of all cultures were adjusted to 1.0 before the measurement of the fluorescence spectra. (*B*) Quantitative PCR analysis of *ccbP* expression in the wild type (■) and *ntcA*$^-$ (●) after nitrogen step-down. Total RNA was isolated at the times indicated for quantitative PCR. All values were normalized to that at time 0. (*C*) NtcA-induced gel mobility shift of a 100-bp DNA fragment in the *ccbP* promoter region of *Anabaena* 7120. DNA (400 ng) was incubated with 3 nM NtcA in the binding buffer with 2-OG at the concentrations described below for 10 min before analysis with polyacrylamide gel (6%) electrophoresis. Lanes: 1–5, incubation of the DNA fragment with NtcA in the presence of 2-OG at concentrations of 0, 0.05, 0.1, 0.2, and 0.5 mM, respectively; 6, the DNA fragment incubated with BSA alone. (*D*) The NtcA-binding sequence was required for the NtcA-induced gel mobility shift. The conditions for EMSA were the same as in *C*, except that synthetic DNA fragments of the *ccbP* promoter region (from nucleotides −179 to −130 upstream of the start codon) were used. The sequence $GTN_{11}ACA$ was retained in one of the fragments (lane 2), and it was changed to $CCN_{11}CCC$ in the other fragment (lane 3). The conditions for lane 1 were the same as those for lane 2, except that no NtcA was included in the binding buffer. (*C* and *D*) The upper arrows indicate the positions of the shifted DNA bands and the lower arrows indicate the free DNA fragments.

binding sequence was absent, no retardation of the DNA band was observed (Fig. 5*D*, lane 3).

Discussion

Free $[Ca^{2+}]_i$ increases in differentiating cells and mature heterocysts, and the increase of free $[Ca^{2+}]_i$ is required for the process of heterocyst differentiation (10). CcbP is a recently identified calcium-binding protein present in *Anabaena* 7120 and other heterocyst-forming cyanobacteria. CcbP regulates heterocyst differentiation by sequestering Ca^{2+}. In this study, we focused our investigation on these two aspects of the regulation of $[Ca^{2+}]_i$ by CcbP during heterocyst formation: A mechanism for the release of the CcbP-bound Ca^{2+} during the process of differentiation and a mechanism for down-regulation of the *ccbP* expression in heterocysts.

Although CcbP lacks apparent Ca^{2+}-binding motifs, such as EF hands, it binds two Ca^{2+} per molecule (Fig. 4). Based on the cellular concentration of CcbP as determined by ELISA, the CcbP-bound Ca^{2+} is a significant pool of Ca^{2+}, and they could increase $[Ca^{2+}]_i$ 6- to 8-fold if completely released. The reduced amount of CcbP in heterocysts (10) suggests that CcbP is degraded during differentiation.

HetR, a serine-type protease (20), has been recognized as the master switch of heterocyst differentiation (17). Although HetR could be specifically labeled by the serine-type protease inhibitors and showed autodegradation (20), no other physiological substrate of its protease activity was known. In this report, we demonstrate that HetR can degrade CcbP (Fig. 2). Although the mechanism of degradation of CcbP by HetR is not entirely clear, the reaction appears specific because HetR digests neither BSA nor NtcA. Among many proteins, such as phycobiliproteins, PsaE, and PsaD, CcbP was the only protein digested by HetR. Both autodigestion of HetR and degradation of CcbP were dependent on Ca^{2+} because EGTA completely prevented both reactions. The active serine of HetR (Ser-152) was required to digest CcbP. This result is consistent with the conclusion that HetR is a serine-type protease and that the active serine is required for heterocyst differentiation (28). Fig. 2 also demonstrates that $HetR_{S179N}$ was unable to digest CcbP, indicating that, although Ser-179 is not the active serine, it is required for the protease activity. This suggestion is in agreement with early reports that no heterocysts were formed in the strain carrying $hetR_{S179N}$ (17) and that $HetR_{S179N}$ showed no autodegradation (20).

Together with PatS (30, 31), HetN (31–35), and PatA (36, 37), HetR controls heterocyst pattern. One of the critical factors for the control of pattern formation is positive autoregulation (38). Although it has been demonstrated that the expression of *hetR* is positively autoregulated (22), how the positive feedback of HetR is achieved was not clear. The evidence that HetR regulates $[Ca^{2+}]_i$ (Fig. 3) provides a mechanism for achieving the positive autoregulation of HetR at posttranslational level because its enzymatic activity depends on Ca^{2+} (Fig. 2). It is likely that digestion of CcbP by HetR under physiological conditions is positively autoregulatory

because the released Ca^{2+} would stimulate HetR activity when $[Ca^{2+}]_i$ is low. The increased $[Ca^{2+}]_i$ could play other important roles in heterocyst differentiation, such as Ca^{2+}-dependent proteolysis (39). Heterocyst-forming cyanobacteria contain many regulatory proteins, such as kinases and enzymes for cyclic nucleotides, and Ca^{2+} may also regulate the activities of some of these enzymes.

Down-regulation of *ccbP* in heterocysts, which also led to an increase of $[Ca^{2+}]_i$, depended on NtcA (Fig. 5). Although only a minimal NtcA-binding sequence was present in the region of the *ccbP* promoter (23), NtcA bound to the fragment containing this sequence (Fig. 5). *rbcL* of *Anabaena* 7120 is negatively regulated by NtcA (25). The NtcA-binding sequence of *ccbP*, like that of *rbcL*, is located downstream of a putative −10 box of a predicted promoter (our unpublished results). Therefore, the down-regulations of *ccbP* and of *rbcL* by NtcA are similar. As in *Synechococcus* sp. PCC 7942 (24), the NtcA-binding activity was enhanced by 2-OG based on assays of the GFP reporter gene *in vivo* and gel mobility shifting *in vitro* (Fig. 5). These results suggest that 2-OG also is involved in the regulation of $[Ca^{2+}]_i$, enforcing its signaling in the initiation of heterocyst differentiation (8). The difference of *ccbP* expression after nitrogen deprivation between the wild type and *ntcA*$^-$ (Fig. 5) suggests that repression of *ccbP* expression in the initiation stage of heterocyst differentiation could be critical to the increase of $[Ca^{2+}]_i$. The expression of *ntcA* and *hetR* in heterocyst differentiation is mutually dependent, and the up-regulation of *hetR* requires NtcA (24, 26). Because the *hetR* promoter contains no NtcA-binding sequence, it is generally believed that the regulation of *hetR* expression by *ntcA* is indirect. The results shown in Fig. 5 suggest that the regulation of *ccbP* expression by NtcA could contribute to the regulation of *hetR* up-regulation by NtcA because it contributes to the increase of $[Ca^{2+}]_i$. The increase of $[Ca^{2+}]_i$ in differentiating cells is likely due to the release of CcbP-bound Ca^{2+}, although it cannot be ruled out that some Ca^{2+} could be imported from vegetative cells. The degradation of CcbP by HetR assures that only those differentiating cells with high HetR content would increase their $[Ca^{2+}]_i$, whereas $[Ca^{2+}]_i$ in vegetative cells remains low. The results in Fig. 2 show that HetR significantly degrades itself *in vitro*. Because HetR is likely to be modified *in vivo* (37, 40, 41), autodegradation of HetR may be prevented in differentiating cells and heterocysts *in vivo*.

In this study, we demonstrate that HetR, CcbP, and NtcA collaborate in the control of $[Ca^{2+}]_i$ in heterocyst differentiation. We predict that the identification of CcbP as a substrate of HetR will help with the understanding of the proteolytic mechanism of HetR. The digestion of CcbP by HetR for increasing $[Ca^{2+}]_i$ may represent a primitive mechanism for the regulation of $[Ca^{2+}]_i$ because it requires complete digestion of a Ca^{2+}-binding protein, whereas more sophisticated mechanisms of Ca^{2+} homeostasis are evolved in eukaryotic cells (42).

Materials and Methods

Strains and Growth Conditions. *Anabaena* 7120 was grown in BG11 or $BG11_0$ media illuminated with cool fluorescent light (43). *E. coli* was grown in LB medium at 37°C. The strain DH5α was used for all general cloning purposes. The strain BL21(DE3) was used for protein overproduction and for coexpression of *ntcA* of *Anabaena* 7120 and *gfp*.

DNA Manipulation and Protein Overproduction. The sequences of primers used in this study and the procedures for overproduction of NtcA and HetR are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. CcbP was overproduced as described previously (10). The NtcA-induced DNA mobility shift was performed according to Huang *et al.* (21). A100-bp fragment from the *ccbP* promoter for EMSA was amplified by PCR with primers 1 and 2 (see Table 1, which is published as supporting information on the PNAS web site). The DNA bands after polyacrylamide gel (6%) electrophoresis were visualized by x-ray films (Kodak). To confirm whether the sequence $GTN_{11}ACA$ in the *ccbP* promoter region was required for NtcA binding, EMSA was performed with two synthetic DNA fragments based on the DNA sequence from nucleotides −179 to −130 upstream of the start codon of the *ccbP* gene. The $GTN_{11}ACA$ sequence was changed to $CCN_{11}CCC$ in one of the fragments. Coexpression of the *ntcA* of *Anabaena* 7120 and *gfp* was carried out by transformation of *E. coli* strain BL21(DE3) with pET-ntcA or pET-psaE (29), both of which confer resistance of ampicillin, and pPccbP-gfp, which contains the *gfp* gene under control of the *ccbP* promoter (10) and confers resistance to kanamycin, with selection on ampicillin and kanamycin. Quantitative PCR for determination of *hetR* and *ccbP* mRNA in *Anabaena* 7120 was performed according to Huang *et al.* (21). The primers used for determination of *ccbP* transcripts were primers 3 and 4 (Table 1). The values obtained with quantitative PCR were normalized to that obtained by quantitative PCR from 16S rRNA with primers 5 and 6 (Table 1).

Characterization of CcbP. The stoichiometry of CcbP and its bound Ca^{2+} was determined as follows. To a 10-ml solution containing 0.83 mg·ml^{-1} CcbP, 10 mM Tris·HCl (pH 7.5), and 100 mM KCl, portions of a 1 M stock solution of $CaCl_2$ were added incrementally, and free Ca^{2+} in solution was measured with a Ca^{2+} electrode according to Baudet *et al.* (44). The amount of Ca^{2+} per CcbP was determined based on the titration curve. To calculate the Ca^{2+} dissociation constants (K_d) of CcbP, Scatchard plotting of the above-described titration was determined and curves were fit with the software Sigmaplot (Systat). To measure the release of bound Ca^{2+} from CcbP during its digestion by HetR, solutions (50 mM Tris·HCl, pH7.4/100 mM KCl) containing HetR at 0.1 mg·ml^{-1} and CcbP at 0.43 mg·ml^{-1} (3 μM), HetR only, or CcbP only were incubated at 37°C, and the free Ca^{2+} concentrations were determined. The initial free Ca^{2+} concentration was adjusted to 1.0 μM.

Cellular concentration of CcbP was determined by ELISA with a Protein Detector Elisa kit from KPL (Gaithersburg, MD). Total soluble proteins of *Anabaena* 7120 were serially diluted in the coating buffer. The amount of CcbP was determined by using rabbit anti-CcbP antibodies as primary antibodies according to the instruction of the supplier. The cellular concentration of CcbP was estimated according to Laurent *et al.* (8) in their estimation of cellular concentrations of 2-OG.

Detection of $[Ca^{2+}]_i$. Ca^{2+}-dependent fluorescence emission by obelin was detected as described by Zhao *et al.* (10). Changes of fluorescence emission at 460 nm were used to determine changes of $[Ca^{2+}]_i$ in *Anabaena* 7120. Estimation of the total cellular calcium content of *Anabaena* 7120 was performed by $^{45}Ca^{2+}$ labeling according to Smith *et al.* (45).

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