**Structures of Anabaena Calcium-binding Protein CcbP**

**INSIGHTS INTO CA\(^{2+}\) SIGNALING DURING HETEROCYST DIFFERENTIATION**

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Ca\(^{2+}\)-binding proteins play pivotal roles in both eukaryotic and prokaryotic cells. CcbP from cyanobacterium *Anabaena* sp. strain PCC 7120 is a major Ca\(^{2+}\)-binding protein involved in heterocyst differentiation, a process that forms specialized nitrogen-fixing cells. The three-dimensional structures of both Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of CcbP are essential for elucidating the Ca\(^{2+}\)-signaling mechanism. However, CcbP shares low sequence identity with proteins of known structures, and its Ca\(^{2+}\)-binding sites remain unknown. Here, we report the solution structures of CcbP in both Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms determined by nuclear magnetic resonance spectroscopy. CcbP adopts an overall new fold and contains two Ca\(^{2+}\)-binding sites with distinct Ca\(^{2+}\)-binding abilities. Mutation of Asp38 at the stronger Ca\(^{2+}\)-binding site of CcbP abolished its ability to regulate heterocyst formation in *vivo*. Surprisingly, the β-barrel subdomain of CcbP, which does not participate in Ca\(^{2+}\)-binding, topologically resembles the Src homology 3 (SH3) domain and might act as a protein-protein interaction module. Our results provide the structural basis of the unique Ca\(^{2+}\) signaling mechanism during heterocyst differentiation.

Whereas the significance of Ca\(^{2+}\) ions in eukaryotic cells has been well recognized for a long time (1), its importance in prokaryotic cells has only gained increasing interests recently (2–4). There is growing evidence that the intracellular Ca\(^{2+}\) concentration is tightly regulated in prokaryotes, and Ca\(^{2+}\) signaling is involved in cell structure maintenance, gene expression, cell cycle and cell differentiation processes, including the regulation of heterocyst formation in cyanobacteria (2–4).

Cyanobacteria are a group of ancient prokaryotes that appeared on earth at least 2~3.5 billion years ago. Some cyanobacteria can simultaneously carry out oxygenic photosynthesis and nitrogen fixation, which are two biochemically incompatabile processes. When combined nitrogen is scarce, some photosynthetic vegetative cells differentiate into specialized nitrogen-fixing cells called heterocysts (5–9). The signaling network during heterocyst differentiation is highly complex and recalls that of eukaryotic cells. One of the essential triggering signals for heterocyst formation is the increase of intracellular free Ca\(^{2+}\) concentration, and it could represent an earliest example of calcium required cellular differentiation in evolution (9–11).

Protein CcbP (cyanobacterial calcium binding protein) from cyanobacterium *Anabaena* sp. strain PCC 7120 (*Anabaena* sp.) was identified as a major Ca\(^{2+}\)-binding protein involved in Ca\(^{2+}\) sequestration and the regulation of heterocyst differentiation (10, 11). At the early stages of heterocyst differentiation, CcbP is degraded by a serine-type protease HetR, leading to a Ca\(^{2+}\) release and subsequent differentiation processes (11). Nevertheless, CcbP shares low sequence identity with proteins of known structure, and its Ca\(^{2+}\)-binding sites remain unknown. Although CcbP shows certain biochemical and biophysical similarity to the sarcoplasmic Ca\(^{2+}\)-binding protein calsequestrin in vertebrates, its Ca\(^{2+}\)-binding capacity (~2 Ca\(^{2+}\) per molecule) differs from calsequestrin (~40–50 Ca\(^{2+}\) per molecule) (10–12). These results strongly suggest that CcbP may represent a novel class of Ca\(^{2+}\)-binding proteins. Therefore, the structural information of CcbP is essential for elucidating the molecular mechanism of its role in Ca\(^{2+}\) signaling during heterocyst differentiation.

Here, we report the solution structures of CcbP in both Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms determined by nuclear magnetic resonance (NMR) spectroscopy. The structures of CcbP in both forms reveal an overall new fold with an α-subdomain and a β-barrel subdomain. Ca\(^{2+}\) titration experiments by NMR and mutagenesis analysis identified two Ca\(^{2+}\)-binding sites. The stronger Ca\(^{2+}\)-binding site I locates at an α-turn-β region, whereas the weaker Ca\(^{2+}\)-binding site II resembles a single EF-hand motif with defects. Furthermore, the β-barrel subdomain of CcbP unexpectedly reveals an SH3-like topology that might act as a protein-protein interaction module during the degra-

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\(^{b}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental "Results and Discussion," Figs. 1–11, and additional references.

\(^{c}\) The atomic coordinates and structure factors (codes 2P0P and 2K2V) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

\(^{d}\) The chemical shift assignments have been deposited in the Biological Magnetic Resonance Data Bank under accession numbers 15401 and 15402.

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\(^{g}\) The abbreviations used are: NMR, nuclear magnetic resonance; RDC, residual dipolar coupling; HSQC, heteronuclear single-quantum coherence; ITC, isothermal titration calorimetric.
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dation of CcbP by HetR. Our study provides the structural basis for understanding the mechanism of Ca\(^{2+}\) signaling during heterocyst differentiation and further extends our knowledge of Ca\(^{2+}\)-binding proteins.

EXPERIMENTAL PROCEDURES

Sample preparations—The ccbP gene was cloned into a pET15b vector (Novagen) with an N-terminal cleavable His-tag and expressed in an Escherichia coli BL21(DE3) strain. The culture was grown in LB medium, centrifuged, and resuspended in M9 minimal medium with antibiotics and \(^{15}\)NH\(_4\)Cl in the presence or absence of \(^{13}\)C\(_6\)-glucose for preparations of \(^{13}\)C/\(^{15}\)N-labeled or \(^{15}\)N-labeled samples, respectively (13). The CcbP protein was purified by nickel-nitritolactiaceic acid column (Qiagen). The sample was digested using thrombin to remove the N-terminal His-tag and further purified by passing through the nickel-nitritolactiaceic acid column and subsequently the gel filtration column (Superdex-75) using an AKTA FPLC system (Amersham Biosciences). The purity was determined to be >95% as judged by SDS-PAGE. NMR samples were prepared with 1 mM CcbP dissolved in 90% H\(_2\)O/10% D\(_2\)O buffer containing 20 mM Tris-HCl (pH 7.4) and 220 mM NaCl. The sample for Ca\(^{2+}\)-free CcbP was pretreated with excess EGTA and subsequently buffer-exchanged to remove EGTA. Excess Ca\(^{2+}\) ions (40 mM CaCl\(_2\)) were added in the Ca\(^{2+}\)-bound form. In addition, 2,2-dimethyl-2-silapentanesulfonic acid was added as the internal chemical shift reference. CcbP mutants E17A, D21A, E23A, D24A, E26A, E27A, D37A, D38A, T39A, and E41A were expressed in E. coli and purified following the same method as wild type CcbP.

NMR Spectroscopy—All NMR experiments for structural determination were performed on Bruker Avance 500-MHz (equipped with a cryprobe) and 800-MHz spectrometers equipped with a triple-resonance probe with pulsed field gradients at 30 °C. The spectra were processed with the software package NMRPipe (14) and analyzed using the program NMR-View (15). The resonance assignments of backbone and side chain atoms were obtained following the common procedures (16). The three-dimensional \(^{15}\)N- and \(^{13}\)C-edited NOESY-HSQC (mixing time of 100 ms) spectra were recorded to confirm the assignments and generate distance restraints for structure calculations. Hydrogen-deuterium exchange experiments were performed to obtain hydrogen bond information. The \(^{1}\)H-\(^{15}\)N residual dipolar coupling (RDC) constants of CcbP were measured. The measurements were performed by dissolving the CcbP protein in a dilute liquid crystal buffer containing a mixture of alkyl-poly (ethylene glycol) C12E5 and n-hexanol (17). The C12E5/water ratio was 5.5% (w/w), and the molar ratio of C12E5 to n-hexanol was 0.92. The RDC values were extracted from the difference in \(^{1}\)H-\(^{15}\)N splitting measured by \(^{1}\)H-\(^{15}\)N IPAP-HSQC spectra between the weakly aligned and the isotropic samples (18).

Structure Calculations—The structures of CcbP in both forms were calculated using interproton NOE-derived distance restraints in combination with dihedral angles and hydrogen bonds information. The program TALOS (19) was used to predict dihedral angles \(\psi\) and \(\varphi\) restraints. Hydrogen bond restraints were determined based on hydrogen-deuterium exchange experiments in conjunction with NOEs and secondary structural information. The initial structures were generated using the CANDID module of the CYANA program (20). The 20 conformers with the lowest target function were selected as the models for SANE (21) to extend the NOE assignments. Two hundred structures were iteratively calculated using CYANA (22), and the 100 structures with the lowest target function were selected for further refinement in the AMBER force field using the parm99 parameters (23). The RDC restraints were added during the AMBER refinement procedure. For the Ca\(^{2+}\)-bound form, two Ca\(^{2+}\) ions and distance restraints between Ca\(^{2+}\) and CcbP also were added during the AMBER calculation steps. For each binding site, one Ca\(^{2+}\) ion was restrained by adding Ca\(^{2+}\)-O restraints of 1.8–2.8 Å based on the experimental results from chemical shift perturbations and mutagenesis. Initially, ambiguous Ca\(^{2+}\)-O restraints for the two oxygen atoms of side chain carboxyl groups were used. Based on the calculated structures with Ca\(^{2+}\) ions, the oxygen atoms with Ca\(^{2+}\)-O distances <3 Å were identified to resolve the ambiguous Ca\(^{2+}\)-O restraints and to remove those that were violated. Subsequent calculations were performed using unambiguous Ca\(^{2+}\)-O restraints. Finally, 20 of 100 structures with the lowest AMBER energy were selected as the representative structures of CcbP in the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms, respectively. Programs PROCHECK-NMR (24) and MOLMOL (25) were used to analyze the quality of the structures.

Ca\(^{2+}\) Titration by NMR—For the Ca\(^{2+}\) titrations by NMR, \(^{15}\)N-labeled CcbP protein (final concentration of \(\sim 0.5 \text{ or } 0.05 \text{ mM}\)) was dissolved in 20 mM Tris-HCl buffer (pH 7.4) in the absence of NaCl. CaCl\(_2\) was dissolved in the same buffer and gradually added to the protein sample. A series of two-dimen-sional \(^{15}\)N-edited heteronuclear single-quantum coherence (HSQC) spectra were recorded, and the chemical shift changes of backbone \(^{15}\)N atoms of all residues were analyzed to identify the Ca\(^{2+}\)-binding sites. Ca\(^{2+}\) titration by NMR was also performed in the presence of 220 mM NaCl, and the results were compared with those obtained in the absence of NaCl. In addition, Ca\(^{2+}\) titrations by NMR in the absence of NaCl were performed similarly for CcbP mutant proteins.

Backbone \(^{1}\)H-\(^{15}\)N Heteronuclear NOE Measurements—The backbone steady-state heteronuclear \(^{1}\)H-\(^{15}\)N NOE values of CcbP in the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms were measured on a Bruker Avance 600-MHz NMR spectrometer at 30 °C (26). The experiments were performed in the presence and absence of a 3-s proton presaturation period prior to the \(^{15}\)N excitation pulse.

Ca\(^{2+}\) Titration into CcbP by ITC—Binding of Ca\(^{2+}\) to CcbP was measured by isothermal titration calorimetric (ITC) using a MicroCal VP-ITC MicroCalorimeter (Northampton, MA). The protein samples used in the titration was extensively dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.4), which is the same as used in NMR titration experiments. Stock solutions of CaCl\(_2\) (2.9 and 5.8 mM), used as the titrant, were prepared in the same buffer. Typical ITC experiments were performed at 25 °C according to the manufacturer’s instructions. A total of 272 µl concentrated CaCl\(_2\) (2.9 mM or 5.8 mM) was added into the protein solution (1.43 ml, 0.10 mM, or 0.22 mM)
in 34 aliquots (8-μl each). The additions were 3-min apart to allow heat accompanying each increment to return to baseline prior to the next addition. The reference experiments by titrating CaCl2 ligand into the buffer were subtracted before data analysis. All data were analyzed by fitting with different binding models using the program Origin (version 7.0; MicroCal, Northampton, MA), and best fits were obtained using two-site binding model.

RESULTS

NMR Structure of Ca2+-free CcbP—The solution structure of CcbP in the absence of Ca2+ was determined based on a total of 4,370 restraints derived from multidimensional NMR spectroscopy, including proton-proton distance restraints generated from NOE, hydrogen-bond restraints based on the hydrogen-deuterium exchange experiments, dihedral angle restraints based on chemical shifts, and 1H-15N RDC restraints measured by weakly aligning the protein sample in a dilute liquid crystal buffer (Table 1). The ensemble of the 20 representative structures and a ribbon diagram are depicted in Fig. 1, A and C. The stereo images of the structures are shown in supplemental Fig. 1.

The Ca2+-free CcbP shows a mixed α-β fold, which contains an α-helical and a β-barrel subdomains. The three α-helices (residues 9–17 (α1), 25–39 (α2), and 107–122 (α3)) are formed by the N- and C-terminal parts of CcbP, with the short helix α1 packed almost perpendicularly onto the two long helices α2 and α3, forming a triangular shaped conformation. The five anti-parallel β-strands (residues 44–50 (β1), 59–68 (β2), 79–83 (β3), 92–96 (β4), and 100–103 (β5)) form a β-barrel. Two short 3_10 helices H1 and H2 link the β-strands β2–β3 and β4–β5, respectively. The α-helical and β-barrel subdomains are packed tightly with exclusive orientation and form a single globular architecture. The contact between the two subdomains mainly involves hydrophobic interactions. The N terminus of the protein, the extended loops connecting β2–β3 and β3–β4, and the loop linking helix α1-α2 are relatively flexible (supplemental Fig. 2).

A search using DALI (27) or CATH (28) did not find structural homologues with significant overall similarity, suggesting a new protein fold. The best hit by DALI is a partial region of a human cell cycle protein splindlin-1 (Protein Data Bank code

| TABLE 1 | NMR statistics for Anabaena CcbP |
|---------|---------------------------------|
|         | Ca2+-free | Ca2+-bound |
| NMR restraints | Distance restraints | | |
| Total NOE | 4131 | 4430 |
| Total unambiguous NOE | 3246 | 3538 |
| Intra-residue | 1397 | 1506 |
| Inter-residue | | |
| Sequential (| 849 | 875 |
| Medium range (| 352 | 404 |
| Long range (| 648 | 753 |
| Total ambiguous NOE | 885 | 892 |
| Hydrogen bonds | 37 | 42 |
| Total dihedral angle restraints | | |
| Phi (| 67 | 73 |
| Psi (| 67 | 73 |
| Total RDCs | 68 | 64 |
| Total Ca2+-O restraints | 12 | |
| Energy (kcal/mol) | AMBER energy | -7398.06 ± 10.50 - 8022.30 ± 12.24 |
| Distance restraints violation energy | 7.84 ± 1.41 | 20.24 ± 1.42 |
| Torsion angle restraints violation energy | 0.66 ± 0.10 | 0.97 ± 0.15 |
| Structure statistics | Violations \(^{\alpha}\) | 0.33 | 0.35 |
| Maximum distance restraint violation (Å) | 1.45 ± 0.15 | 1.44 ± 0.17 |
| Maximum dihedral angle violation r.m.s.d. \(^{\alpha}\) from mean structure (Å) | 0.90 ± 0.20 | 0.96 ± 0.13 |
| Heavy (for all residues) | 0.99 ± 0.10 | 0.96 ± 0.13 |
| Backbone (for all residues) | 0.49 ± 0.14 | 0.48 ± 0.15 |
| Secondary structure heavy | 0.92 (0.1) | 0.92 (0.2) |
| Secondary structure backbone | 0.61 (0.0) | 0.61 (0.0) |
| Ramachandran statistics (%) | Most favored regions | 82.1 (86.2) | 81.9 (88.3) |
| Also allowed regions | 16.5 (13.7) | 16.6 (11.5) |
| Generously allowed regions | 1.2 (0.1) | 0.9 (0.2) |
| Disallowed regions | 0.2 (0.0) | 0.6 (0.0) |

\(^{\alpha}\) None of the structures have distance violation >0.4 Å or dihedral angle violations >5°. For Ca2+-O restraints, the largest violations were <0.2 Å.

\(^{\alpha}\) The root mean square deviation (r.m.s.d.) values were calculated for 20 refined structures.

\(^{\alpha}\) The values of Ramachandran statistics given outside of the parentheses were calculated for residues 9–50, 58–85, and 92–122 in the 20 refined structures; the excluded residues were the N and C termini and loop regions with high flexibility and few NOE restraints.

FIGURE 1. Solution structures of Anabaena CcbP in the Ca2+-free and Ca2+-bound forms. A and B, superimpositions of the 20 representative structures of Anabaena CcbP in the Ca2+-free (A) and Ca2+-bound forms (B). C and D, ribbon diagram representations of CcbP in Ca2+-free (C) and Ca2+-bound (D) forms, with the secondary structures labeled. The α-helical subdomain is shown in red, the β-barrel subdomain is shown in green, and the two short 3_10 helices located in the β-barrel subdomain are shown in yellow. The Ca2+ ions in the Ca2+-bound form are not shown. The figures were prepared by MOLMOL (25).
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2NS2, chain A) with a Z score of 4.8, and a root mean square deviation of 4.1 Å over 73 aligned Cα atoms. However, these aligned residues are strictly limited to the β-barrel subdomain of CcbP. Interestingly, a closer inspection reveals that this β-barrel subdomain is topologically reminiscent of the eukaryotic SH3 domain (Fig. 2A). A structural alignment by DaliLite (29) between the SH3-like subdomain of CcbP and the structure of c-Src SH3 domain (Protein Data Bank code 1QWE) showed that the overall structures are similar, with a 2.0 Å root mean square deviation for 44 aligned backbone Cα atoms. The loops consisting of residues 51–58, 69–78, and 84–91 in CcbP correspond to the RT loop, the n-Src loop, and the distal loop in the c-Src structure, respectively (Fig. 2B). The structure-based sequence alignment (Fig. 2C) shows no identity (0%) between the two protein sequences. However, several residues located on the β-strands are relatively similar in amino acid types and biochemical properties. Most of these residues have hydrophobic side chains and form the hydrophobic core of the β-barrel, indicating their importance in maintaining the overall structure.

NMR Structure of Ca2+-bound CcbP—Similar to the Ca2+-free CcbP, the structure of Ca2+-bound CcbP was determined with a total of 4,694 restraints, including 12 Ca2+-O restraints (Table 1). The addition of excess CaCl2 appears to slightly stabilize the whole structure of CcbP, as evidenced by the increased number of interproton NOE restraints. This was also supported by the fact that under a Ca2+-free condition, residues Ser53–Ser56 were missing in the two-dimensional 15N-edited HSQC spectrum, which appeared in the presence of excess CaCl2. Nevertheless, comparison of the overall architecture of CcbP between the Ca2+-bound and Ca2+-free forms reveals considerable similarity (Fig. 1, B and D). All secondary structural elements as well as the relative orientations between the two subdomains are retained essentially upon Ca2+-binding. The backbone root mean square deviation between the mean structures of the two forms is 1.0 Å for all residues and is only 0.5 Å for residues in regular secondary structures. The results indicate that Ca2+ binding does not induce notable conformational changes in CcbP. In addition, we compared the motional flexibility on picosecond-to-nanosecond time scales of both forms of CcbP by measuring the backbone (1H)-15N heteronuclear NOE values (for more details see supplemental “Results and Discussion”). The results demonstrated that Ca2+ binding does not induce significant changes in the motional flexibility of CcbP as well (supplemental Fig. 2).

Because CcbP exists as oligomer under low ionic strength, which forbids its structure determination by NMR, the structures of CcbP in both forms were determined in the presence of 220 mM NaCl. Taking into consideration that the intracellular environments generally contain high K+ concentration (~100–200 mM) (30), we compared the two-dimensional 15N-edited HSQC spectra of both forms of CcbP in excess K+ with those in excess Na+. The HSQC spectra with Na+ or K+ are similar, demonstrating essentially identical conformations of CcbP (supplemental Fig. 3). Therefore, under conditions near physiological environment, the binding of Ca2+ ions does not induce considerable conformational changes of CcbP.

Ca2+ Titration by NMR—Ca2+ titration experiments by NMR were performed to identify the Ca2+-binding sites of CcbP, which were monitored by HSQC spectroscopy (additional discussions are available in supplemental “Results and Discussion” and Figs. 3–10). During the gradual increase of Ca2+ concentrations, two regions in CcbP showed responses (supplemental Fig. 10). When the molar ratio of Ca2+-CcbP changed from 0:1 to 1:1, the backbone nitrogen chemical shifts of several residues located at the C terminus of helix α2 were largely perturbed, suggesting that these residues constitutes the stronger Ca2+-binding site (site I). When the molar ratio Ca2+:
CcbP continued to increase, another region (residues Ile^{19}–Glu^{27}) located at the loop linking helix α1 and α2 showed moderate sensitivity to Ca^{2+} ions, suggesting a weaker Ca^{2+}-binding site (site II).

**Characterization of Ca^{2+}-binding Site I—**Site I locates at the segment Asp^{37}–Glu^{41}, which is an α-turn-β motif connecting helix α2 and the first β-strand of the β-barrel subdomain (Fig. 3, A and B). Although CcbP has a large negatively charged surface, this Ca^{2+}-binding site is highly specific and shows the highest sensitivity to the presence of Ca^{2+} ions (Fig. 3D, supplemental Figs. 7–10). In NMR spectroscopy, metal coordination to a backbone carbonyl can cause deshielding of the backbone nitrogen atom of the succeeding residue (31). The backbone ^{15}N atoms of residues Asp^{38}, Thr^{39}, and Glu^{41} at this region showed significant downfield chemical shift changes, suggesting the main chain carbonyls of the preceding residues Asp^{37}, Asp^{38}, and Leu^{40} might be potential Ca^{2+} ligands.

To further characterize this binding site, plasmids carrying genes encoding CcbP mutants D37A, D38A, T39A, and E41A were constructed. The Ca^{2+} titrations by NMR were performed similarly using these mutant proteins, and the Ca^{2+}-binding abilities of this site were examined by comparing the backbone nitrogen chemical shift changes with the wild type CcbP. Results showed that the Ca^{2+} sensitivity of this site was impaired largely by D37A, D38A, and E41A mutants, but not T39A mutant (data not shown). Thus, it is highly possible that the side chain oxygen atoms of Asp^{37}, Asp^{38}, and Glu^{41} also are involved in Ca^{2+} coordination. The local structure of site I was further calculated based on the above results using the AMBER force field (see “Experimental Procedures”). The bound Ca^{2+} ion is in proximity (Ca^{2+}–O distances < 3 Å) with six oxygen atoms, three from the carboxyl group of Asp^{37}, Asp^{38}, and Glu^{41} and three backbone carbonyls of Asp^{37}, Asp^{38}, and Leu^{40} (Fig. 3B).

**Characterization of Ca^{2+}-binding Site II—**Site II locates at the loop linking helix α1 and α2 (Fig. 3C and E, and supplemental Fig. 10B). Similarly, we investigated the possible Ca^{2+} ligands of this site. The backbone ^{15}N chemical shifts of residues Val^{20}, Asp^{21}, and Ala^{22} were most perturbed during Ca^{2+} titration experiments by NMR, although the chemical shift changes were small (Fig. 3E and supplemental Figs. 7F, 9F, and 10B). CcbP mutants E17A, D21A, E23A, D24A, E26A, and E27A were purified, and Ca^{2+} titrations by NMR were performed. Results showed that the E17A and E26A mutations did not affect Ca^{2+} binding, D21A mutation only had minor effects, whereas E23A, D24A, and E27A mutations significantly decreased Ca^{2+} binding (data not shown). The local structure of site II (Fig. 3C) shows that the second Ca^{2+} ion is surrounded closely by the side chains of Glu^{23}, Asp^{24}, Glu^{47}, and the backbone carbonyl of Asp^{31}.

**Mutation at Ca^{2+}-binding Site I Abolishes CcbP Function in Vivo—**Because site I of CcbP binds Ca^{2+} significantly stronger than site II, it appears that site I may contribute prominently to Ca^{2+} sequestration in vivo. To assess the functional significance of this site in heterocyst differentiation regulation, in vivo functional assays were performed.

We constructed plasmids carrying ccbP genes encoding the wild type protein or mutants at site II under the control of the petE promoter (32). The plasmids were used to transform Anabaena sp., so that the expression of ccbP was inducible with copper. During nitrogen step down, and in the absence of added copper, the Anabaena strain transformed with plasmid carrying wild type ccbP gene was able to form heterocysts with slightly decreased heterocyst frequency (10). When the expression of wild type CcbP was induced with copper under nitrogen limiting conditions, the free Ca^{2+} was sequestered and the heterocyst formation was suppressed completely as reported previously (Fig. 3F) (10). However, overexpressing a mutant protein CcbP-D38A failed to suppress heterocyst formation (Fig. 3G). The fact that the point mutation D38A abolishes the Ca^{2+}-binding ability of CcbP in vivo demonstrates the biological importance of site I in heterocyst differentiation regulation.
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**DISCUSSION**

*Anabaena CcbP Represents a Novel Family of Ca²⁺-binding Proteins—Ca²⁺ binds to a variety of proteins, including those that mediate cell adhesion, enzymes that need Ca²⁺ to be activated, as well as Ca²⁺ buffers and Ca²⁺ sensors. The Ca²⁺-binding protein CcbP from *Anabaena* displays unique structural characteristics compared with other known families of Ca²⁺-binding proteins. The structure of CcbP shows an overall new fold containing a triangular shaped α-helical region packed tightly onto a β-barrel subdomain. Multiple sequence alignment of CcbP proteins from different cyanobacteria species and proteobacterium *P. naphthalenivorans* CJ2 (Fig. 4A) indicates that the sequence conservation is much higher in the three helices than in the β-barrel region. Because the three α-helices of CcbP together form the highly acidic surface containing the two Ca²⁺-binding sites (Fig. 4, B and C), the amino acid conservation in these regions might be critical for maintaining the structural scaffold in CcbP for Ca²⁺ binding. Although binding of Ca²⁺ ions does not result in significant conformational changes of the protein, it alters the local charge at the two sites and also partially neutralizes the nearby area, which might influence the protein-protein interaction pattern of CcbP (supplemental Fig. 11).

**Ca²⁺-binding Sites of CcbP**—The Ca²⁺-binding site I of CcbP locates at a short α-turn-β region, which is distinct from previously reported Ca²⁺-binding sites. *In vivo* functional assays showed that a single amino acid mutation at this site abolished the activity of CcbP in regulating heterocyst formation, demonstrating its biological importance. Furthermore, the amino acids at this site are conserved highly among the CcbP family (Fig. 4A). In particular, residues Tyr²⁵, Leu²⁶, Leu⁴⁰, Pro⁴³, and Phe⁴⁴ are strictly conserved. In addition, residues 37, 38, and 41, whose side chains may participate in Ca²⁺ binding, are restricted to carbonyl-containing Asp, Glu, Asn, or Gln. The *in vivo* and *in vitro* experimental results, together with bioinformatics analysis, strongly suggest that this novel Ca²⁺-binding site plays essential role in Ca²⁺ sequestration in *Anabaena*.

On the other hand, Ca²⁺-binding site II of CcbP consists of an α-loop-α region, which is similar to a single EF-hand motif. Interestingly, the 12-residue sequence of Glu¹⁵–Glu²⁶ (ETEIIVDAEDKE) fits well to an EF-hand Ca²⁺-binding loop. According to the classical EF-hand, the residue Glu²⁶ in position 12 is the most critical and strictly conserved in the EF-hand motif. The acidic residues Glu¹⁵ and Glu¹⁷ at positions 1 and 3 and the water mediating Glu²³ at position 9 are ideal for Ca²⁺ binding, whereas the amino acids at positions 5 and 6 show deviations from canonical EF-hands (33). However, the NMR titration experiments and mutagenesis results indicate that unlike canonical EF-hands, the acidic side chains of residues Glu²³, Asp²⁴, and Glu²⁷ in CcbP appear important in Ca²⁺ binding.

The affinity of Ca²⁺ binding by CcbP was determined in the previous study with the Ca²⁺ electrode method, which identified a Ca²⁺-binding site with $K_d$ of 200 nm and a second Ca²⁺-binding site with $K_d$ of 12.8 μM (11). In this study, we also determined $K_d$ values of CcbP with NMR titration at both high (~0.5 mM) and low (0.05 mM) protein concentrations. The results show that site I binds Ca²⁺ with a dissociation constant $K_d \sim 40-100 \mu M$, whereas site II shows much weaker Ca²⁺-binding affinity ($K_d$ in the millimolar range). Thus, both methods clearly identified the Ca²⁺-binding site with a $K_d$ at low micromolar range, demonstrating that CcbP is a Ca²⁺-binding...
protein. However, there is an unexpected result from our present study; the high affinity site \( K_d \sim 200 \text{ nM} \) was not observed in NMR titrations. Based on the facts that the Scatchard plot used to determine \( K_d \) values in \( \text{Ca}^{2+} \) electrode method could be affected by certain errors (34), we speculate that the apparent “high affinity binding site” of CcbP observed in the \( \text{Ca}^{2+} \)-electrode method could be introduced artificially, and the much weaker \( \text{Ca}^{2+} \)-binding site II observed in NMR titration was not observable by the Scatchard plot. This was further supported by the results from ITC experiments, which demonstrated that CcbP contains a \( \text{Ca}^{2+} \)-binding site I with a \( K_d \) value of \( 39 \pm 9 \text{ \mu M} \) and a \( \text{Ca}^{2+} \)-binding site II with a \( K_d \) value in the millimolar range (Fig. 5). Therefore, CcbP binds \( \text{Ca}^{2+} \) with micromolar and millimolar range affinities. This calcium-binding ability is observable by the Scatchard plot. This was further supported by the integrated binding isotherm (lower panel). The binding isotherm in the lower panel was fit using a two-site binding model where \( N_1 = 1 \) (fixed), \( K_1 = (2.57 \pm 0.49) \times 10^5 \text{ [M }^{-1}] \), \( \Delta H_1 = -(2.92 \pm 0.12) \times 10^3 \text{ [cal-mol}^{-1}] \); \( N_2 = 1 \) (fixed), \( K_2 = 565 \pm 63 \text{ [M}^{-1}] \), \( \Delta H_2 = -(5.79 \pm 0.36) \times 10^3 \text{ [cal-mol}^{-1}] \); \( N \) is the stoichiometry of binding, \( K \) is the \( \text{Ca}^{2+} \)-binding association constant \((1/K_d)\), and \( \Delta H \) is the heat change per mol.

Previous studies demonstrated that CcbP is directly associated with calcium sequestration in cyanobacterial cells and acts as a negative regulator in heterocyst differentiation. The detailed mechanism of the function of CcbP in calcium sequestration \( \textit{in vivo} \), however, is yet unclear. Our structural study of CcbP demonstrated that it is indeed a calcium binding protein with a novel calcium-binding motif \((\text{Ca}^{2+} \)-binding site I\), which has biological significance. A \( \text{Ca}^{2+} \)-buffering function was suggested previously based on functional studies (11). Because the \( \text{Ca}^{2+} \)-binding affinity of site I is in the micro-molar range, whereas the intracellular concentrations of free \( \text{Ca}^{2+} \) in cyanobacteria are between 100 nM and 200 nM (11), the calcium ions bound by CcbP alone might not be a major pool of bound calcium under nitrogen-replete conditions. CcbP could be more important in regulation of free calcium concentration during the process of heterocyst formation when calcium concentration increased significantly in heterocysts and proheterocysts (10). The increase of calcium concentration could come from an increased influx of calcium and/or a release of bound calcium ions, which remains to be further investigated.

**Structure of Anabaena CcbP**—Apart from the three acidic \( \alpha \)-helices that contain the two \( \text{Ca}^{2+} \)-binding sites, the structure of CcbP unexpectedly reveals a \( \beta \)-barrel subdomain topologically and structurally reminiscent of eukaryotic SH3 domain. The SH3 domain is a small module with 55–70 residues commonly found in eukaryotic signaling pathways, and it mediates transient protein-protein interactions with moderate affinity (35, 36). It recognizes specific proline-rich sequences and prefers sequences with a PxxP core motif (where \( x \) is any amino acid, and \( + \) is a basic residue, usually arginine) of the class II motif that interacts with the canonical SH3 domains. Moreover, this sequence in HetR also closely resembles the consensus RRxxPφPφR + SxxP motif recognized by the p53bp2 SH3 domain (39). Therefore, a likely scenario is that the proline-rich sequence of HetR recognizes and interacts with the SH3-like domain of CcbP, which facilitates the degradation of CcbP and the release of \( \text{Ca}^{2+} \) ions during heterocyst differentiation.

Other prokaryotic domains that are sequentially unrelated but topologically reminiscent of eukaryotic SH3 domains have also been discovered in recent years (40–45). Among these, the SH3-like domain in the bacterial histidine kinase CheA mirrors the SH3 domains in mammalian tyrosine kinases and suggests the ubiquitous involvement of this common topology in cell signaling among different life kingdoms (40). Our study reveals the presence of an SH3-like subdomain in \textit{Anabaena} CcbP, which demonstrates a direct association of the SH3-like domain with a \( \text{Ca}^{2+} \)-binding protein. This represents another paradigm for the coupling of SH3 topology to prokaryotic signaling processes, and in particular, is the first example for the involvement of SH3-like domains in prokaryotic \( \text{Ca}^{2+} \) signaling.

**Conclusions**—In summary, the present study reveals that \textit{Anabaena} CcbP adopts an overall new fold with two \( \text{Ca}^{2+} \)-binding sites. Site I consists of an \( \alpha \)-turn-\( \beta \) region unreported previously, whereas site II resembles a single EF-hand motif. Furthermore, CcbP harbors an SH3-like subdomain which might play a role in \( \text{Ca}^{2+} \) release. Our study provides the structural basis for understanding the function of CcbP in the \( \text{Ca}^{2+} \)
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signaling in *Anabaena* and offers novel insights for future investigations into the molecular mechanism of heterocyst differentiation regulation.

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