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Biochemical Properties of a cAMP Phosphodiesterase in the Cyanobacterium *Anabaena* sp. strain PCC 7120

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A gene for cAMP phosphodiesterase, designated *cpdA*, was identified in the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. The predicted amino acid sequence of the gene was similar to the sequences of cAMP phosphodiesterases from *Thermosynechococcus elongatus*, *Escherichia coli* and *Haemophilus influenzae*. The recombinant protein was purified by sequential column chromatography and its biochemical properties were determined. The *Anabaena* cAMP phosphodiesterase hydrolyzed cAMP and cGMP with similar levels of activity. The K_m value for cAMP was 45 μM and the Vmax was 4.9 μmol min^{-1} mg $protein^{-1}$. These values are similar to those of *Escherichia coli* cAMP phosphodiesterase. The enzyme was activated by divalent cations such as Fe^{2+} and Mn^{2+}. The tertiary structure of this enzyme was predicted by homology modeling. The deduced structure has two metal-binding sites in the catalytic domain.

Key words: cyclic nucleotide phosphodiesterase, cAMP signal transduction, cyanobacteria

cAMP is an intracellular second messenger that plays a key role in many physiological processes in both prokaryotes and eukaryotes[3]. cAMP is formed by an adenylate cyclase and hydrolyzed by a cAMP phosphodiesterase. In cyanobacteria, the cellular cAMP level changes in response to several environmental factors such as light-dark, low-high pH, oxic-anoxic and nitrogen repletion-depletion[17,18]. Cells regulate the intracellular concentration of cAMP by balancing its synthesis and degradation. Disruption of an adenylate cyclase gene, *cya1*, of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 caused a decrease in the cellular cAMP level and at the same time immovability of the cells[20]. Overexpression of another adenylate cyclase gene in the filamentous cyanobacterium *Anabaena* sp. PCC 7120 caused an increase in the cellular cAMP level and fragmentation of the filaments[10]. Thus, not only adenylate cyclase but also phosphodiesterase is important to maintain the cellular concentration of cAMP at an appropriate level.

The 3':5'-cyclic nucleotide phosphodiesterase (PDE) [EC 3.1.4.17] catalyzes the hydrolysis of 3':5'-cyclic nucleotides to the corresponding nucleoside 5'-monophosphates. In eukaryotes, PDEs represent a large divergent group of enzymes, and two classes, Class I and Class II, have been recognized[2]. Few reports however have been published about the biochemical nature of PDE in prokaryotes. CpdA in *Escherichia coli*[7] and Icc in *Haemophilus influenzae*[13] were classified as Class III enzymes, while the periplasmic enzyme CpdP in *Vibrio fisheri*[5] was assigned to the eukaryotic Class II group.

We tried to identify the cyanobacterial PDE gene in the *Synechocystis* sp. PCC 6803 genome but could not because no sequence characteristic of a hitherto known PDE was detected in this species. In the present study, we searched the

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Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis.

genome of another cyanobacterium, *Anabaena* sp. PCC 7120[8], and found the PDE gene. Then, some biochemical characteristics of the recombinant protein were determined for the first time in cyanobacteria.

Materials and Methods

Bacterial strains, plasmids, culture media and growth conditions

The cyanobacterial strain *Anabaena* sp. PCC 7120, which was supplied by H. Böhme at the Institute of Botany, University of Bonn, Bonn, Germany, was grown in BG11 medium[19] in the light at 30°C. The *Escherichia coli* strain JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*(r_K^-, m_K^+), *supE44*, *relA1*, Δ(*lac-proAB*)/F' [*traD36*, *proAB*, *lacIq*, *lacZ*ΔM15]) (Takara Bio Inc. Otsu, Japan) was used for cloning, and BL21(DE3) (F^-, *ompT*, *hsdS*(r_B^-, m_B^-), *dcm*, *gal*, λ(DE3)) (Novagen, Madison, WI) for expression of the recombinant protein. The *E. coli* strain was grown in Luria Bertani medium[14]. When required, kanamycin at 25 μg ml^{-1} or chloramphenicol at 30 μg ml^{-1} was added.

Construction of the expression plasmid for the recombinant protein

The complete structural *cpdA* gene in *Anabaena* sp. PCC 7120 was amplified by PCR. The primers (*cpdA*-P1; 5'-ACGGATCCATGAACGAAAAGTTACCCA-3', *cpdA*-P2; 5'-GCAAGCTTCTACGCTTACTCTATCT-3') were designed to anneal sequences for *Bam*H I and *Hin*dIII restriction sites at each end. The resulting PCR products were ligated into pGEM-T3 Easy Vector (Promega Corporation, Madison, WI) and introduced into competent *E. coli* JM109 cells, and the plasmids were isolated. After verification of the nucleotide sequence, the coding region was digested with *Bam*H I and *Hin*dIII and inserted between the *Bam*HI and *Hin*dIII sites of the pET28a expression vector (Novagen). The resulting construct, pCPDA, contains the entire *cpdA* gene fused to the His-tag sequence from pET28a, which is placed under the control of the phage T7 promoter.

Expression and purification of recombinant His-CpdA in E. coli

Competent *E. coli* BL21 (DE3) cells were transformed with the plasmid pCPDA and grown at 37°C in 300 ml of Luria Bertani medium until they reached at an OD_{600} of about 0.5. Then expression of His-CpdA was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.15 mM. Cells were harvested after 3 hours by centrifugation, then resuspended in 20 ml of buffer A (50 mM Hepes-NaOH, pH 7.5, 500 mM NaCl, and 20% (v/v) glycerol) containing 1 mM phenylmethylsulfonyl fluoride and 0.2 mg ml^{-1} of lysozyme, and placed on ice for 60 min. The cells were then disrupted by sonication using an Insonator model 201M (Kubota Co., Tokyo, Japan), the resulting crude extract was centrifuged at 20,000×g for 15 min, and the supernatant was further centrifuged at 245,000×g for 60 min. According to methods reported previously[21], the supernatant was loaded onto a HiTrap Chelating column (Amersham Biosciences, NJ) connected to a FPLC system that had been equilibrated with buffer A, and then eluted using a step gradient of 50 and 200 mM imidazole in buffer A. The 200 mM imidazole fractions were collected and concentrated by Ultrafree-15 (Millipore, MA). The concentrated eluate was loaded onto a Superdex 75 HR 10/30 column (Amersham Biosciences) connected to the FPLC system, which was equilibrated with buffer A. Fractions were assayed for enzyme activity as described below. Fractions having high enzyme activity and purity were collected and stored at –80°C.

Assay of cyclic nucleotide phosphodiesterase activity

The activity of cyclic nucleotide phosphodiesterase was determined by measuring the amount of 5'-AMP formed by the enzyme reaction. The reaction was performed in a total volume of 0.2 ml containing 40 mM Tris-HCl, pH 8.0, 0.05 mM $MnCl_2$, 5 mM DTT, 0.05 mM cAMP, and 50 ng of enzyme as the standard condition. The reaction mixture was incubated at 30°C for 10 min and then boiled for 5 min to terminate the reaction. After that, the reaction mixture was centrifuged at 18,000×g for 10 min at 4°C to remove protein. The 5'-AMP in the supernatant was measured using a HPLC system (Shimadzu Co., Kyoto, Japan). An aliquot (10 μl) of the supernatant was applied to the TSK ODS-80Ts HPLC column (TOSOH Co., Tokyo, Japan), which was equilibrated with 30 mM sodium phosphate buffer, pH 5.0, containing 5% (v/v) acetonitrile. The flow rate was 1.0 ml min^{-1} and the effluent was monitored at 259 nm. The protein concentration was determined by the Bradford method[4] as described in the instructions of the Bio-Rad protein assay kit. Bovine serum albumin was used as the standard.

Results and Discussion

Identification of a gene for Anabaena *sp. PCC 7120 cAMP phosphodiesterase*

To identify the cAMP phosphodiesterase gene in *Ana-*

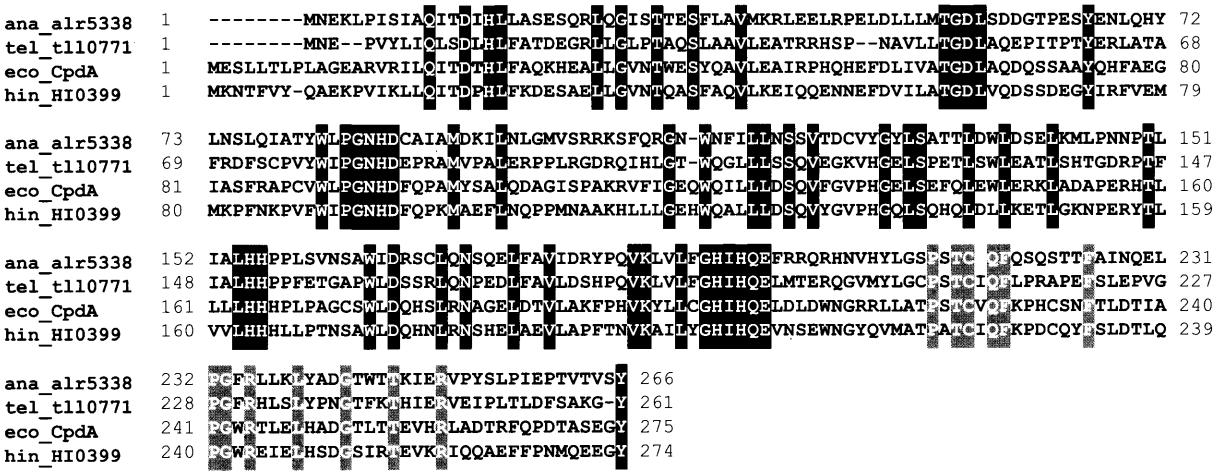

Fig. 1. Alignment of the amino acid sequences among cAMP phosphodiesterases in bacteria. Black boxes indicate conserved residues in all sequences. Red boxes indicate putative metal-binding sites. Yellow boxes indicate putative substrate-binding sites. ana, *Anabaena* sp. PCC 7120; tel, *Thermosynechococcus elongatus* BP-1; eco, *Escherichia coli*; hin, *Haemophilus influenzae*. The accession numbers for *E. coli* and *H. influenzae* are F65090 and E64065, respectively.

baena 7120 cells, we first performed a search for a putative phosphodiesterase in the genome sequence data in CyanoBase (http://www.kazusa.or.jp/cyanobase/index.html). The nucleotide sequence of 3',5'-cyclic nucleotide phosphodiesterase of *E. coli* was used as a query[7]. No homologous gene was found in the *Synechocystis* genome[9], but one homologous gene (*alr5338*) existed in the *Anabaena* genome[8]. The predicted *Anabaena* PDE consists of 266 amino acids and is similar overall to the sequences of PDEs in *Thermosynechococcus elongatus* BP-1 (T110771)[15], *E. coli* (CpdA) and *H. influenzae* (HI0399) (Fig. 1). The identity is 33.5–35.3% among these four proteins.

Expression and purification of Anabaena *phosphodiesterase*

To isolate the PDE protein, we constructed an expression vector (pCPDA) that encodes a hybrid PDE protein with the His-tag sequence as described in Materials and Methods. Figure 2 shows the results of SDS-PAGE obtained by a sequential column chromatographic analysis. The PDE protein was expressed by adding 1 mM IPTG (lane 3) and recovered in the soluble fraction (lane 4). The PDE protein was purified with Ni-affinity chromatography (lane 5) followed by gel filtration chromatography (lane 6). The molecular mass of the purified protein was estimated to be 35 kDa by SDS-PAGE analysis, which closely corresponds to the theoretical value.

Phosphodiesterase activity of the recombinant protein

Figure 3 shows the correlation between the concentration of cAMP and the enzyme activity of the purified protein. Maximum activity (4.9 μmol min^{-1} mg $protein^{-1}$) was exhibited at a cAMP concentration of 1 mM. The K_m value for cAMP was 45 μM, which is near the value of *E. coli* PDE[7]. It is suggested that the PDE functions in cells in which

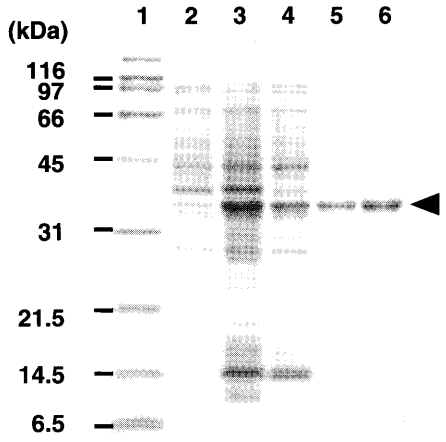

Fig. 2. Purification of His-CpdA protein by sequential column chromatography. SDS-PAGE was carried out using a 15% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. Lane 2, cell extract (without isopropyl-β-D-thiogalactopyranoside); lane 3, extract of cells treated with 0.1 mM isopropyl-β-D-thiogalactopyranoside; lane 4, 245,000×*g* supernatant; lane 5, HiTrap Chelating column chromatography; lane 6, Superdex 75 column chromatography. The arrowhead indicates the position of His-CpdA. Lane 1, molecular size markers.

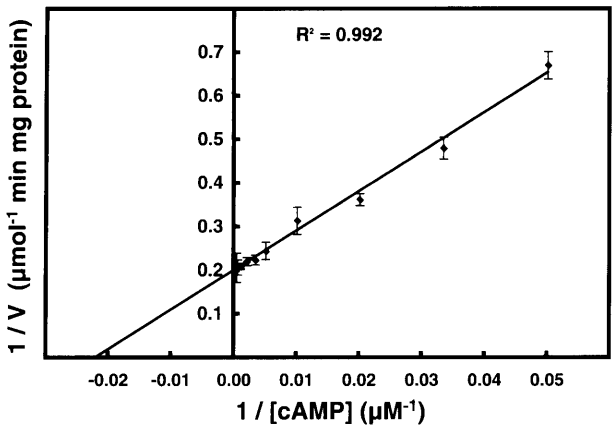

Fig. 3. Activity of His-CpdA in response to variations in the cAMP concentration. The data are plotted in a Lineweaver-Burk relationship. The enzyme activity was determined as described in Materials and Methods.

Table 1. Hydrolysis of cAMP and cGMP by His-CpdA protein

Substrate	PDE activity (μmol min^{-1} mg $protein^{-1}$)
cAMP	4.73±0.27
cGMP	4.36±0.04

The activity was measured as described in Materials and Methods.

cAMP levels are fairly high, about 40 to 50 μM. The substrate specificity was determined using cAMP or cGMP as a substrate. Table 1 shows that *Anabaena* PDE hydrolyzed cAMP and cGMP similarly, which is a general characteristic of PDEs[2]. The effect of divalent metal ions on the PDE activity was also determined. Figure 4 shows that the PDE activity of the recombinant protein depended upon the presence of 50 μM Fe^{2+} and Mn^{2+}. The activation of PDE by Fe^{2+} has been reported in *E. coli*[7]. The other six divalent cations tested (Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Ni^{2+} and Co^{2+}) little activated the enzyme.

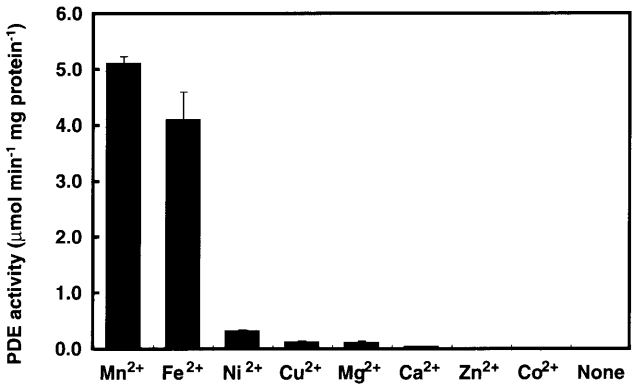

Fig. 4. Effects of divalent metal ions on the PDE activity. Metal ions were added at a concentration of 50 μM. The reaction conditions are shown in Materials and Methods. Means±S.D. of three experiments are shown.

Tertiary structure of CpdA predicted using homology modeling

From the results obtained in this experiment, CpdA is categorized as a bacterial PDE and is different from the PDEs of eukaryotes. To predict the tertiary structure of CpdA, we

Fig. 5. Predicted tertiary structure of CpdA in *Anabaena* sp. PCC 7120. The schematic drawing was made with FAMS software based on an analysis of the crystal structure of the protein phosphatase[16]. The figure was prepared with the Pymol program (http://pymol.sourceforge.net/). (a) The entire structure. The protein backbone is shown in a ribbon representation. (b) Putative reaction site in the CpdA model.

first carried out a PSI-blast search[1] against a non-redundant database of proteins with similar amino acid sequences. Twenty families of proteins were extracted from the database and three were analyzed bioinformatically. These proteins were Ser/Thr-type protein phosphatase[6], 5'-nucleotidase[12] and purple acid phosphatase[11]. All of them contain a binding site for divalent metal ions in their catalytic domain. It is assumed that CpdA contains amino acids enabling it to bind with two metal ions. To construct a three dimensional model of CpdA, the FAMS homology modeling program was used[16]. After examining 15 different structural models, we chose one based on Ser/Thr-protein phosphatase (Fig. 5a). We also predicted the tertiary structure of the reaction site based on other bacterial PDE proteins. In this model, two metal ions are surrounded by four histidines, three aspartic acids and one asparagine (Fig. 5b). This core structure would be essential to hydrolyze the cyclic nucleotides.

The question of why *Synechocystis* sp. PCC 6803 does not have a PDE as that of the *Anabaena* is difficult to answer. This *Synechocystis* species may possess a completely different type of PDE from other cyanobacteria. A protein with a domain for phosphate ester hydrolysis, which appears in the *Synechocystis* genome[9], may exhibit activity as a nucleotide phosphodiesterase. Since PDE is very important to all organisms, systematic analyses of this enzyme in biochemistry and molecular biology should be carried out.

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References

1) Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389–3402.
2) Beavo, J.A. 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol. Rev. **75**: 725–748.
3) Botsford, J.L. and J.G. Harman. 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56**: 100–122.
4) Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.
5) Dunlap, P.V. and S.M. Callahan. 1993. Characterization of a periplasmic 3':5'-cyclic nucleotide phosphodiesterase gene, *cpdP*, from the marine symbiotic bacterium *Vibrio fischeri*. J. Bacteriol. **175**: 4615–4624.
6) Goldberg, J., H.B. Huang, Y.G. Kwon, P. Greengard, A.C. Nairn and J. Kuriyan. 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature **376**: 745–753.
7) Imamura, R., K. Yamanaka, T. Ogura, S. Hiraga, N. Fujita, A. Ishihama and H. Niki. 1996. Identification of the *cpdA* gene encoding cyclic 3',5'-adenosine monophosphate phosphodiesterase in *Escherichia coli*. J. Biol. Chem. **271**: 25423–25429.
8) Kaneko, T., Y. Nakamura, C.P. Wolk, T. Kuritz, S. Sasamoto, A. Watanabe, M. Iriguchi, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, N. Nakazaki, S. Shimpo, M. Sugimoto, M. Takazawa, M. Yamada, M. Yasuda and S. Tabata. 2001. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res. **8**: 205–213.
9) Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda and S. Tabata. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. **3**: 109–136.
10) Katayama, M. and M. Ohmori. 1998. Expression of an adenylate cyclase gene of *Anabaena cylindrica* in the cyanobacterium *Anabaena* sp. strain PCC 7120. Plant Cell Physiol. **39**: 786–789.
11) Klabunde, T., N. Strater, R. Frohlich, H. Witzel and B. Krebs. 1996. Mechanism of Fe(III)-Zn(II) purple acid phosphatase based on crystal structures. J. Mol. Biol. **259**: 737–748.
12) Knofel, T. and N. Strater. 1999. X-ray structure of the *Escherichia coli* periplasmic 5'-nucleotidase containing a dimetal catalytic site. Nat. Struct. Biol. **6**: 448–453.
13) Macfadyen, L.P., C. Ma and R.J. Redfield. 1998. A 3',5' cyclic AMP (cAMP) phosphodiesterase modulates cAMP levels and optimizes competence in *Haemophilus influenzae* Rd. J. Bacteriol. **180**: 4401–4405.
14) Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15) Nakamura, Y., T. Kaneko, S. Sato, M. Ikeuchi, H. Katoh, S. Sasamoto, A. Watanabe, M. Iriguchi, K. Kawashima, T. Kimura, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, N. Nakazaki, S. Shimpo, M. Sugimoto, C. Takeuchi, M. Yamada and S. Tabata. 2002. Complete genome structure of the Thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1. DNA Res. **9**: 123–130.
16) Ogata, K. and H. Umeyama. 2000. An automatic homology modeling method consisting of database searches and simulated annealing. J. Mol. Graph. Model. **18**: 258–272, 305–256.
17) Ohmori, M. and S. Okamoto. 2004. Photoresponsive cAMP signal transduction in cyanobacteria. Photochem. Photobiol. Sci. **3**: 503–511.
18) Ohmori, M., K. Terauchi, S. Okamoto and M. Watanabe. 2002. Regulation of cAMP-mediated photosignaling by a phytochrome in the cyanobacterium *Anabaena cylindrica*. Photochem. Photobiol. **75**: 675–679.
19) Rippka, R., J. Deruelles, J.B. Waterbury, M. Herdman and R.Y. Stanier. 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111**: 1–61.
20) Terauchi, K. and M. Ohmori. 2004. Blue light stimulates cyanobacterial motility via a cAMP signal transduction system. Mol. Microbiol. **52**: 303–309.
21) Yoshimura, H., T. Hisabori, S. Yanagisawa and M. Ohmori. 2000. Identification and characterization of a novel cAMP receptor protein in the cyanobacterium *Synechocystis* sp. PCC 6803. J. Biol. Chem. **275**: 6241–6245.