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Two cAMP receptor proteins with different biochemical properties in the filamentous cyanobacterium *Anabaena* sp. PCC 7120

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Abstract Two open reading frames (ORFs), *alr0295* and *alr2325*, are found to encode putative cAMP receptor proteins (CRPs) in the genome of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. These ORFs were named cAMP receptor protein-like gene A in *Anabaena* sp. PCC 7120 (*ancrpA*) and cAMP receptor protein-like gene B in *Anabaena* sp. PCC 7120 (*ancrpB*), respectively, and those translated products were investigated. The equilibrium dialysis measurements revealed that AnCrpA bound with cAMP specifically, while AnCrpB bound with both cAMP and cGMP, though the affinity for cGMP was weak. The binding affinity for cAMP of AnCrpA showed the lowest dissociation constant, approximately 0.8 μM, among bacterial CRPs. A gel mobility shift assay elucidated that AnCrpA and AnCrpB formed a complex with the consensus DNA sequence in the presence of cAMP, although AnCrpB did not have ordinary DNA-binding motifs.
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Keywords: cAMP receptor protein; Filamentous cyanobacterium; cAMP-binding affinity; Protein–DNA interaction

1. Introduction

cAMP is an important signaling molecule in prokaryotes and eukaryotes. It is synthesized from ATP by adenylate cyclases in response to extracellular stimuli. In enteric bacteria, cAMP regulates the expression of the alternative catabolic enzymes in response to environmental changes [1]. cAMP binds with a cAMP receptor protein (CRP) in bacteria and *Escherichia coli* CRP is known to regulating the activity of over 100 genes. The crystal structure of CRP has been determined, and the mode of binding to cAMP, consensus DNA sequence and α subunit COOH-terminal domain of RNA polymerase has been revealed [2–6].

Cyanobacteria are Gram-negative and are able to carry out higher plant-type oxygen-evolving photosynthesis. It has been shown that cellular cAMP levels change in response to environmental stimuli such as light–dark, low pH–high pH, oxic–anoxic [7,8], and nitrogen replete–deplete [9]. The filamentous cyanobacterium, *Anabaena* sp. PCC 7120, has the simultaneous abilities of oxygen-evolving photosynthesis and nitrogen fixation. This cyanobacterium has six genes coding adenylate cyclases [10,11], one of which, *cyaB1*, is self-activated by cAMP [12]. However, the physiological function of cAMP in *Anabaena* sp. PCC 7120 remains unknown. In a unicellular cyanobacterium *Synechocystis* sp. PCC 6803, an adenylate cyclase gene *cya1* was identified and the *cya1* disruption mutant displayed immotility [13]. In addition, the gene for cAMP receptor protein, *sycrp1*, was identified from a *Synechocystis* sp. PCC 6803 [14]. The cAMP receptor protein in *Synechocystis* sp. PCC 6803 (SYCRP1) acts as a transcriptional activator in vivo [15] and the phenotype of the gene disruption of *sycrp1* displayed reduction in the number of thick pili and immotility [16]. These results indicated that cAMP and SYCRP1 are involved in the regulatory mechanism of cell motility.

The analysis of entire DNA sequence of the *Anabaena* sp. PCC 7120 genome has been completed [17,18]. Putative CRP coding genes were searched in the whole genome and seven open reading frame (ORF) products of the CRP family have been predicted [18]. Among the seven ORFs, *ntcA* (*alr4392*) [19–22] and *devH* (*alr3952*) [23] annotated to be related to nitrogen metabolism, and the others two ORFs (*alr0295* and *alr2325*) showed a high similarity to *sycrp1*. Consequently, Alr0295 and Alr2325 are named AnCrpA and AnCrpB, respectively. In this study, we investigated biochemical characteristics of these two CRPs of *Anabaena* sp. PCC 7120.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

The *E. coli* strains used as hosts were JM109 [*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(rK$^-$, mK$^+$)], *supE*44, *relA*1, and Δ(*lac-proAB*)/*F′* [*traD*36,

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Abbreviations: CRP, cAMP receptor protein; *ancrpA*, cAMP receptor protein-like gene A in *Anabaena* sp. PCC 7120; *ancrpB*, cAMP receptor protein-like gene B in *Anabaena* sp. PCC 7120; SYCRP1, cAMP receptor protein in *Synechocystis* sp. PCC 6803; ORF, open reading frame; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography

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proAB, *lacI*^{q}, Δ(*lacZ*)M15] for cloning and BL21(DE3) [F^{-}, *ompT*, *hsdS* (rB^{-}, mB^{-}), *dcm*, *gal*, λ(DE3)] for the expression of recombinant proteins. Bacteria were grown in Luria–Bertani medium [24]. When required, kanamycin, chloramphenicol, or ampicillin was added at 25, 30, or 100 μg ml^{-1}, respectively.

2.2. Construction of expression plasmids for recombinant proteins

To obtain DNA fragments corresponding to *alr0295* and *alr2325* ORFs (cAMP receptor protein-like gene A in *Anabaena* sp. PCC 7120 (*ancrpA*) and cAMP receptor protein-like gene B in *Anabaena* sp. PCC 7120 (*ancrpB*), respectively), polymerase chain reactions (PCRs) were performed with several sets of synthetic primers and genomic DNA from *Anabaena* sp. PCC 7120. The primers for *ancrpA* were RPA1 (5′-CGGGATCCATGGAAGACCGATATA-3′) and RPA2 (5′-CATCTGGCAGAGAATCT-3′). Primers for *ancrpB* were RPB1 (5′-CGGGATCCATGCAAACTGAGGTTT-3′) and RPB2 (5′-CCTCAAATATCAGGTGT-3′). The sequences of the RPA1 and RPB1 primers were designed to allow the introduction of a *Bam*HI restriction site immediately upstream of the initiator ATG codon. Each PCR product was cloned into pGEM-T Easy vector (Promega, Madison). After verification of the nucleotide sequence, *ancrpA* and *ancrpB* were digested from each plasmid with *Bam*HI and *Eco*RI and cloned between the *Bam*HI and *Eco*RI sites of the pET-28a expression vector (Novagen). The resulting plasmids were named pRPA and pRPB, respectively.

2.3. Expression and purification of recombinant proteins

The transformants, BL21(DE3) cells harboring each of the pRPA and pRPB plasmids, were grown at 37 °C in 1.5 liter Luria–Bertani medium supplemented with kanamycin (25 μg ml^{-1}). The recombinant genes were expressed in an optical density at 600 nm of 0.4 by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation was continued for 3 h at 37 °C for BL21(DE3) harboring pRPA and overnight at 20 °C for BL21(DE3) harboring pRPB.

Following Yoshimura et al. [14], *E.coli* cells were broken using a sonicator (model 200 M, Kubota Co., Tokyo, Japan), and His-tagged proteins were purified through a HiTrap Chelating column (Amersham Pharmacia Biotech; 1.6 × 2.5 cm) and a HiTrap Q column (Amersham Pharmacia Biotech; 1.6 × 2.5 cm) connected to a fast protein liquid chromatography system (FPLC system, Amersham Pharmacia Biotech) by the same methods of His-SYCRP1. His-tagged AnCrpA (His-AnCrpA) or His-tagged AnCrpB (His-AnCrpB) was eluted from the HiTrap Q column with 50 mM Tris–HCl (pH 8.0), 200 mM NaCl and 10% (w/v) glycerol, respectively.

2.4. Equilibrium dialysis and assay for bound nucleotides by reversed-phase HPLC

The procedure of equilibrium dialysis and the measurement of the amounts of nucleotides using a high-performance liquid chromatography (HPLC) system (Shimadzu Co., Kyoto, Japan) have been described previously [14]. To equilibria, samples were incubated for 6 h at 20 or 30 °C in 50 mM Tris–HCl (pH 8.0), 200 mM NaCl and 10% (w/v) glycerol. Dialysis membrane was purchased from Viskase Companies, Inc. (pore size 50 Å). The concentrations of the recombinant proteins and nucleotides are indicated in the legends of Figs. 3 and 4.

A TSK ODS-80Ts column (4.6 mm × 15 cm, TOSOH Co., Tokyo, Japan) was used in HPLC analysis. Samples were assayed with 30 mM sodium phosphate buffer (pH 6.5) containing 5% (v/v) acetonitrile and the effluent was monitored at 259 nm. The flow rate was 1.0 ml min^{-1} and the injection volume was 10 μl.

2.5. Gel mobility shift assay

Gel mobility shift assay was carried out as described previously [14]. A sequence of the oligonucleotide includes a consensus DNA sequence for *E. coli* CRP binding and a *Bam*HI sequence attached to the 5′-end of the consensus sequence (5′-CGGGA TCCGCGAAAAGTGTGACATATGTCACACTTTTCGC-3′) was synthesized. Preparation of double strand DNA was attained by incubating the oligonucloetide at 95 °C for 1 min, 60 °C for 10 min and 25 °C for 1 h in 50 mM Tris–HCl (pH 7.5) containing 100 mM NaCl and 1 mM dithiothreitol. The ^{32}P-labeled DNA (10,000 cpm, approximately 1 ng) was incubated with His-AnCrpA or His-AnCrpB in a total volume of 20 μl of the binding buffer (50 mM Tris–HCl (pH 8.0), 60 mM NaCl, 1 mM EDTA, 8% (w/v) glycerol, and various volumes of poly(dI–dC)) with or without a final concentration of 20 μM cAMP for His-AnCrpA, and 200 μM cAMP for His-AnCrpB for 30 min at room temperature. After the incubation, samples were loaded onto 5% polyacrylamide gels (acrylamide:*N*,*N*′-methylenebisacrylamide, 50:1). The electrophoresis buffer was 0.25× TBE with or without 20 μM cAMP for His-AnCrpA and 200 μM cAMP for His-AnCrpB. The resulting gels were dried and visualized in a Fujix BAS1000 system (Fuji Film).

2.6. Other analytical procedures

The protein concentration was determined using Protein Assay Dye Reagent (Bio-Rad) and bovine serum albumin was used as the standard. SDS–polyacrylamide gel electrophoresis (PAGE) was carried out in polyacrylamide gels containing 0.1% SDS by the method of Laemmli [25].

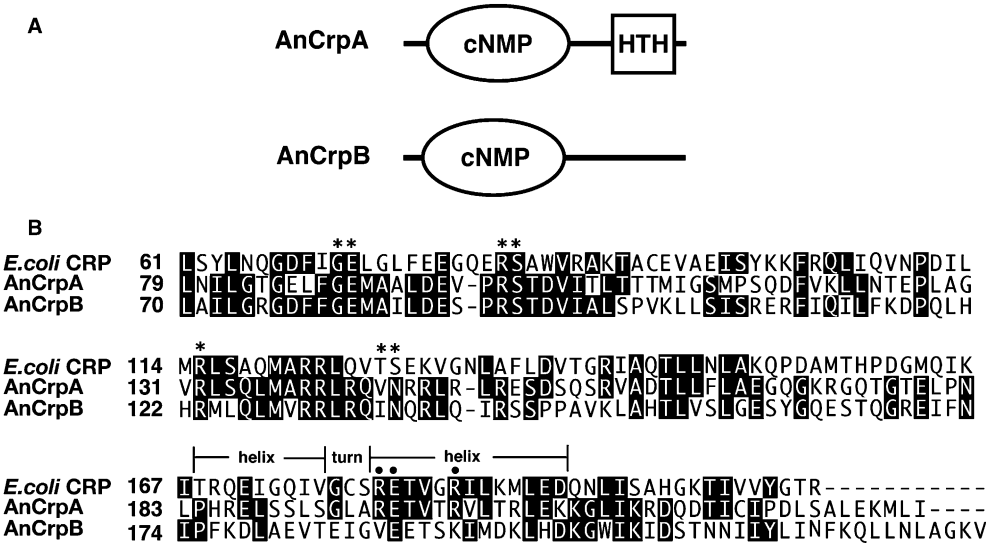

Fig. 1. Modular architecture scheme and amino-acid sequence alignment. (A) The domain abbreviations of AnCrpA and AnCrpB are indicated. cNMP and HTH indicate cyclic nucleotide-monophosphate binding domain and helix–turn–helix domain, respectively. (B) Sequence alignment of *E. coli* CRP, *Anabaena* sp. PCC 7120 AnCrpA and AnCrpB. The asterisks mark amino acid residues that function in binding the cAMP molecule to the *E. coli* CRP structure [4]. The closed circle represents residues that form hydrogen bonds with DNA [4]. Residues identical if more than two of the sequences are shown by black boxes. Numbers at the left of the sequence indicate the corresponding residue positions.

3. Results

The cAMP-binding motif in the prokaryotic cAMP receptor protein and in the eukaryotic regulatory subunit of cAMP-dependent protein kinase is well conserved [26–28]. Using the list of the CRP family in *Anabaena* sp. PCC 7120 [18], we narrowed down the candidates for CRP based on its conserved amino acid residues that interact with the cAMP molecule in *E. coli* CRP. Consequently, two ORFs, *alr0295* and *alr2325*, satisfied the requirements and were named *ancrpA* and *ancrpB*, respectively. Predicted AnCrpA consists of a cNMP-binding domain and a helix–turn–helix domain (Fig. 1A) similar to other bacterial CRPs. Another predicted AnCrpB has a cNMP-binding domain, but lacks the ordinary DNA-binding domain (Fig. 1A). A sequence alignment of *E. coli* CRP, AnCrpA and AnCrpB (Fig. 1B) shows that the respective AnCrpA and AnCrpB share with *E. coli* CRP in five of the seven residues that are involved in binding with the cAMP molecule. Unpreserved residues for AnCrpA and AnCrpB, Thr-127 and Ser-128, have been proved to interact with the adenine ring, and did not appreciably affect the cAMP-binding affinity of *E. coli* CRP [29–32]. Moreover, SYCRP1, which does not conserve these amino acid residues, possessed the cAMP-binding affinity [14]. Thus, though AnCrpA and AnCrpB do not have these residues, they could exhibit cAMP binding. *E. coli* CRP contains a helix–turn–helix domain as a DNA-binding domain in the carboxyl-terminal region [4,33]. AnCrpA shows high homology to the latter helix in that of *E. coli* CRP, a so-called "recognition helix." Also, Arg-180, Glu-181 and Arg-185 of *E. coli* CRP are known to interact directly with the DNA major groove [4], and are conserved in AnCrpA (Fig. 1B). *Anabaena* sp. PCC 7120 genome data were derived from the CyanoBase [34] and the prediction of a secondary structure was established by referring to SMART [35]. Protein identities were calculated from a CLUSTAL W [36].

Fig. 2 shows the each purified recombinant protein, His-AnCrpA and His-AnCrpB, respectively. The respective molecular mass of both His-AnCrpA and His-AnCrpB were estimated to be 29 and 28 kDa by the SDS–PAGE analysis. These values agreed well with the theoretical values.

To determine the cAMP-binding ability of each expressed protein, equilibrium dialysis measurements were performed using these recombinant proteins and nucleotides at 20 °C. Fig. 3A shows that approximately 3.5 μM cAMP was bound with 10 μM His-AnCrpA, whereas neither cGMP nor 5′-AMP were thus bound. To investigate the binding specificity of His-AnCrpA to cAMP, equilibrium dialysis measurements were performed using 10 μM His-AnCrpA and high concentrations of 25 μM and 50 μM cGMP and 5′-AMP, respectively. His-AnCrpA did not bind with either cGMP or 5′-AMP even at high concentrations (data not shown). As for His-AnCrpB, approximately 0.75 μM cAMP and 0.2 μM cGMP bound with

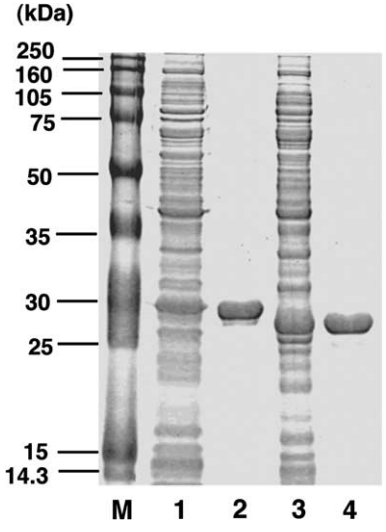

Fig. 2. Overexpression and purification of His-AnCrpA and His-AnCrpB. SDS–PAGE was preformed using a 12% polyacrylamide gel, stained with Coomassie Brilliant Blue R-250. Lane 1, cell extract of BL21(DE3) harboring pRPA after the addition of 1 mM IPTG (15 μg); Lane 2, His-AnCrpA (4 μg); Lane 3, cell extract of BL21(DE3) harboring pRPB after the addition of 1 mM IPTG (15 μg); Lane 4, His-AnCrpB (4 μg). M represents molecular mass markers.

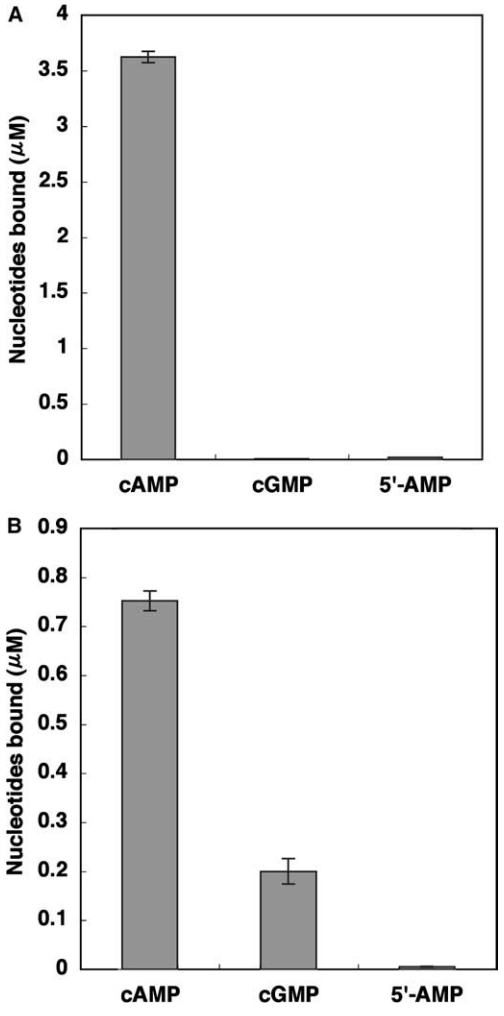

Fig. 3. Binding activity of nucleotides to recombinant proteins measured by equilibrium dialysis. (A) 10 μM His-AnCrpA was incubated for 6 h in the presence of 10 μM cAMP, 10 μM cGMP, or 10 μM 5′-AMP at 20 °C. (B) 25 μM His-AnCrpB was incubated for 6 h in the presence of 10 μM cAMP, 10 μM cGMP, or 10 μM 5′-AMP at 20 °C. The amount of each bound nucleotide was measured by HPLC.

25 μM His-AnCrpB, respectively, but 5′-AMP did not (Fig. 3B). These data indicate that His-AnCrpA has a specific and strong affinity to cAMP, while His-AnCrpB has lower specificity and weaker affinity to the cyclic nucleotide than His-AnCrpA.

To obtain the K_d values of His-AnCrpA and His-AnCrpB for cAMP, various concentrations of cAMP were incubated with 2 μM His-AnCrpA or 25 μM His-AnCrpB for 6 h at 20 or 30 °C for equilibrium dialysis. The K_d value was determined from the slope of the regression line by least-square analysis on a Scatchard plot (Fig. 4A and B). The obtained K_d value of His-AnCrpA for cAMP was 0.78 μM at 20 °C and 0.84 μM at 30 °C, whereas the value of His-AnCrpB for cAMP was 57.1 μM at 20 °C and 57.5 μM at 30 °C. These K_d values were almost constant in this range of temperature. The predicted maximum amounts of bound cAMP on His-AnCrpA and His-AnCrpB were approximately 0.45 and 0.4 molecule on one molecule, respectively. Therefore, both His-AnCrpA and AnCrpB might form a dimer and bind one molecule of cAMP. Indeed, the cross-linking experiment was performed to demonstrate dimer formation (data not shown). That means each His-AnCrpA and His-AnCrpB form a homodimer binding one molecule of cAMP. The K_d value of cGMP in His-AnCrpB was also measured with the same procedures using 50 μM His-AnCrpB and 1–500 μM cGMP. The K_d value of His-AnCrpB for cGMP was estimated to be approximately 150 μM at both 20 and 30 °C (data not shown).

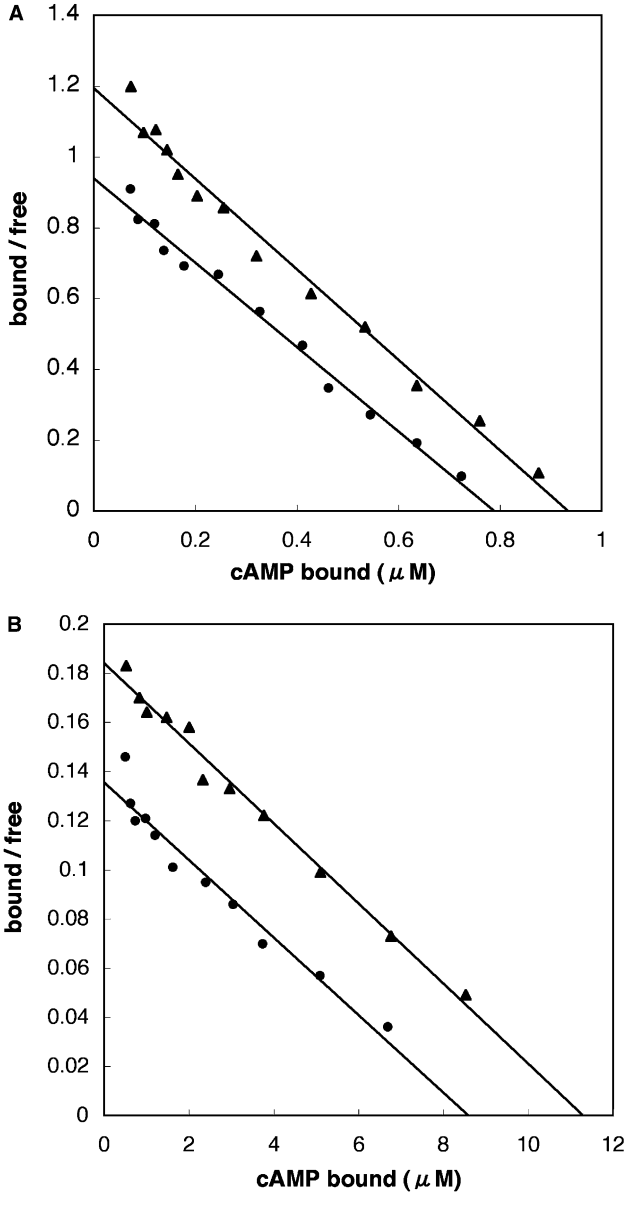

Fig. 4. Scatchard plot from cAMP bound to recombinant proteins by equilibrium dialysis. (A) 2 μM His-AnCrpA was incubated for 6 h in the presence of 0.5–25 μM of cAMP at 20 °C (▲) or 30 °C (●). (B) 25 μM His-AnCrpB was incubated for 6 h in the presence of 0.5–50 μM of cAMP at 20 °C (▲) or 30 °C (●). The amounts of bound cAMP were measured by HPLC.

Various bacterial CRPs are known to bind with the consensus DNA sequences in the presence of cAMP. To confirm whether AnCrpA and AnCrpB bind to the consensus DNA sequences, a gel mobility shift assay was performed. As shown in Fig. 5A, retarded bands appeared in the presence of cAMP (lanes 2, 3 and 5), while the band was not detected without cAMP (Fig. 5A, lanes 6–10) despite the addition of a 30-fold amount of His-AnCrpA (Fig. 5A, lane 10). The addition of excess non-labeled DNA probe resulted in the disappearance of the retarded band (Fig. 5A, lane 4) and the addition of 3-fold amount of poly(dI–dC) had no effect (Fig. 5A, lane 5). These results indicate that His-AnCrpA recognizes the CRP consensus DNA sequence specifically in the presence of cAMP. As well as AnCrpA, gel mobility shift assay was also performed using His-AnCrpB and the consensus DNA sequence. Though AnCrpB lacks ordinary DNA-binding motifs in its amino-acid sequence and the residue identity of the carboxyl-terminal region is low in comparison with other CRPs (Fig. 1A and B), retarded bands were detected at a high concentration of His-AnCrpB in the presence of 200 μM cAMP (Fig. 5B, lanes 4–8). These bands were not detected under 100 ng His-AnCrpB concentration (Fig. 5B, lanes 1–3) or in the absence of cAMP (Fig. 5B, lanes 10–14). Similar to His-AnCrpA, an increase of poly(dI–dC) did not affect the retarded bands (Fig. 5B, lanes 4–8), while the excessive non-labeled DNA probe addition had an effect (Fig. 5B, lane 9). These results indicate that His-AnCrpB, with its protein structure very different from that of common CRP, was able to recognize the consensus DNA sequence.

4. Discussion

We have identified two cAMP receptor proteins, AnCrpA and AnCrpB, in *Anabaena* sp. PCC 7120. The equilibrium dialysis measurements demonstrated that His-AnCrpA bound with cAMP specifically, while His-AnCrpB shows affinity to both cAMP and cGMP (Fig. 3). It is generally known that bacterial CRPs bind with both cAMP and cGMP [37,38], while AnCrpA and SYCRP1 bind specifically with cAMP (Fig. 3A, [14]). Moreover, the K_d value of His-AnCrpA for cAMP was lower than 1 μM at 20 and 30 °C (Fig. 4A). This value is quite low as compared to the K_d values for other bacterial CRPs, in the order of 10^{-5} M [37,38]. The K_d value of His-AnCrpB for cAMP was approximately 60 μM at 20 and 30 °C (Fig. 4B), which is almost the same as for *Vivrio harveyi* CRP [39]. The distinctions of specificity and affinity for these CRPs are

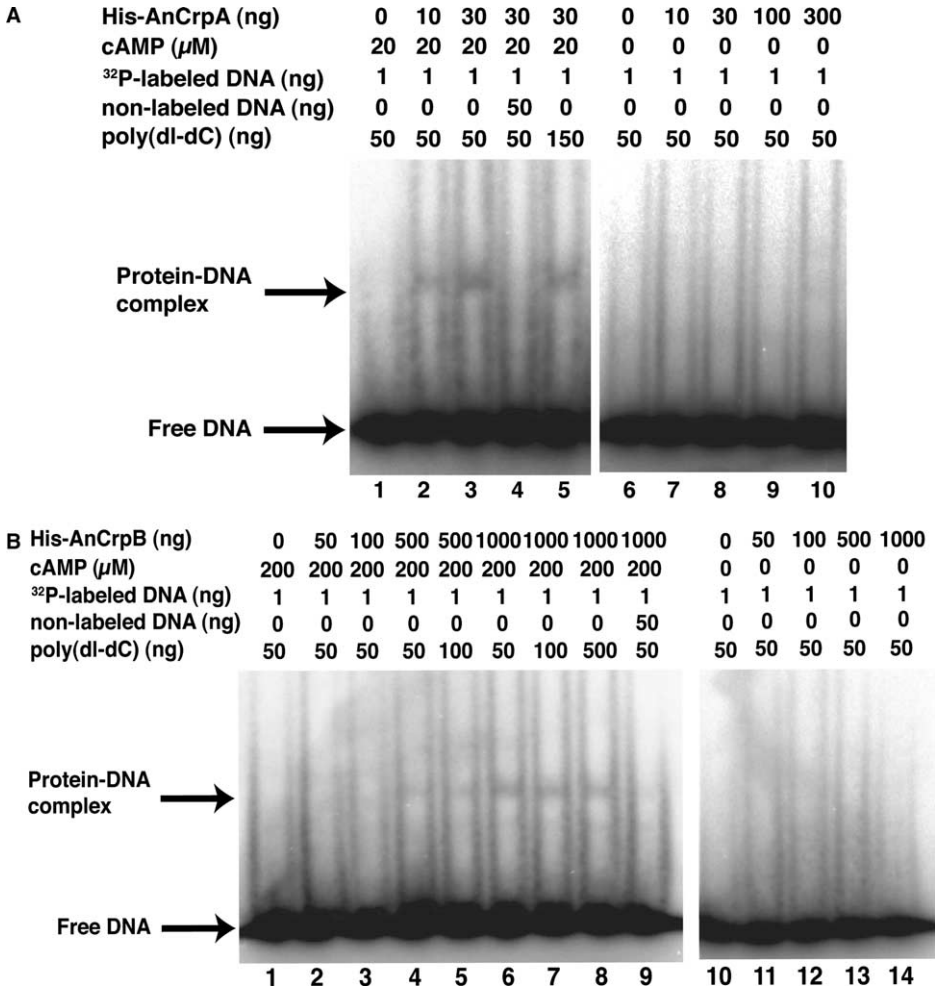

Fig. 5. DNA-binding of AnCrpA and AnCrpB. The binding of His-AnCrpA to the bacterial consensus sequence for CRP in the presence (A, lanes 1–5) or absence (A, lanes 6–10) of cAMP and the binding of His-AnCrpB to the consensus sequence in the presence (B, lanes 1–9) or absence (B, lanes 10–14) of cAMP were examined. ^{32}P-labeled probe DNA (approximately 1 ng) was incubated with the His-AnCrpA or His-AnCrpB, poly(dI–dC) and with or without non-labeled DNA, which was added in the amounts indicated above each lane in the presence or absence of cAMP. The positions of the protein–DNA complex and free DNA are indicated by arrows.

explained by the model of Ochoa de Alda et al. [40], which suggested that Asn-80 and Asn-145 of CRP-*Syn* (synonymous with SYCRP1) could create a new hydrogen bond with cAMP. The AnCrpA conserves both amino acid residues, but AnCrpB does not conserve the residue equivalent to Asn-80 of SYCRP1 or AnCrpA (Fig. 1B), which supports the model.

A consensus DNA sequence for an *E. coli* CRP-specific binding site has been established [41–46]. This sequence is palindromic: a six-base-pair spacer separates two core motifs that consist of the five-base-pair sequence 5′-TGTGA-3′. This sequence is also recognized in other bacterial CRPs. Crystallographic and genetic arguments for the involvement of constants between this core sequence and functional amino acids, especially Arg-180, Glu-181 and Arg-185, that participate in DNA-binding have been reviewed [4,5,47,48]. The carboxyl-terminal region of AnCrpA shows a high homology to the latter part of the helix–turn–helix motif of *E. coli* CRP and includes the three main functional amino acids that participate in DNA-binding (Fig. 1B). As a result of the gel mobility shift assay using His-AnCrpA and the bacterial CRP consensus DNA sequence, it was concluded that His-AnCrpA bound to the consensus DNA sequence in a cAMP-dependent manner (Fig. 5A).

The AnCrpB lacks an ordinary DNA-binding motif, and shows a less homologous sequence to the carboxyl-terminal region of *E. coli* CRP, SYCRP1, and AnCrpA. Moreover, it does not have three direct DNA-binding amino acids with the exception of 181-Glu of *E. coli* CRP (Fig. 1B), though, surprisingly, His-AnCrpB formed a complex with the consensus DNA sequence in the presence of 200 μM cAMP (Fig. 5B). It is suggested that AnCrpB functions as the transcriptional regulator. Therefore, a prediction of the binding manner of AnCrpB for the consensus DNA sequence was performed (Fig. 6B). The corresponding residues of AnCrpB for Arg-180, Glu-181, and Arg-185 in *E. coli* CRP are 187-Val, 188-Glu and 192-Lys, respectively (Fig. 1B). The 187-Val of AnCrpB could not form a hydrogen bond with the DNA because the properties between Arg and Val are extremely different. However, Glu-188 in AnCrpB is considered to bind with the C4 nucleotide in the consensus DNA sequence similar to *E. coli* CRP as illustrated in Fig. 6B. Furthermore, 192-Lys in AnCrpB possibly forms a hydrogen bond with the G4 or T5 nucleotide in the consensus DNA sequence because both Arg and Lys are basic amino acid residues, although Arg belongs to a branched amino group. Hence, 192-Lys in AnCrpB could interact with

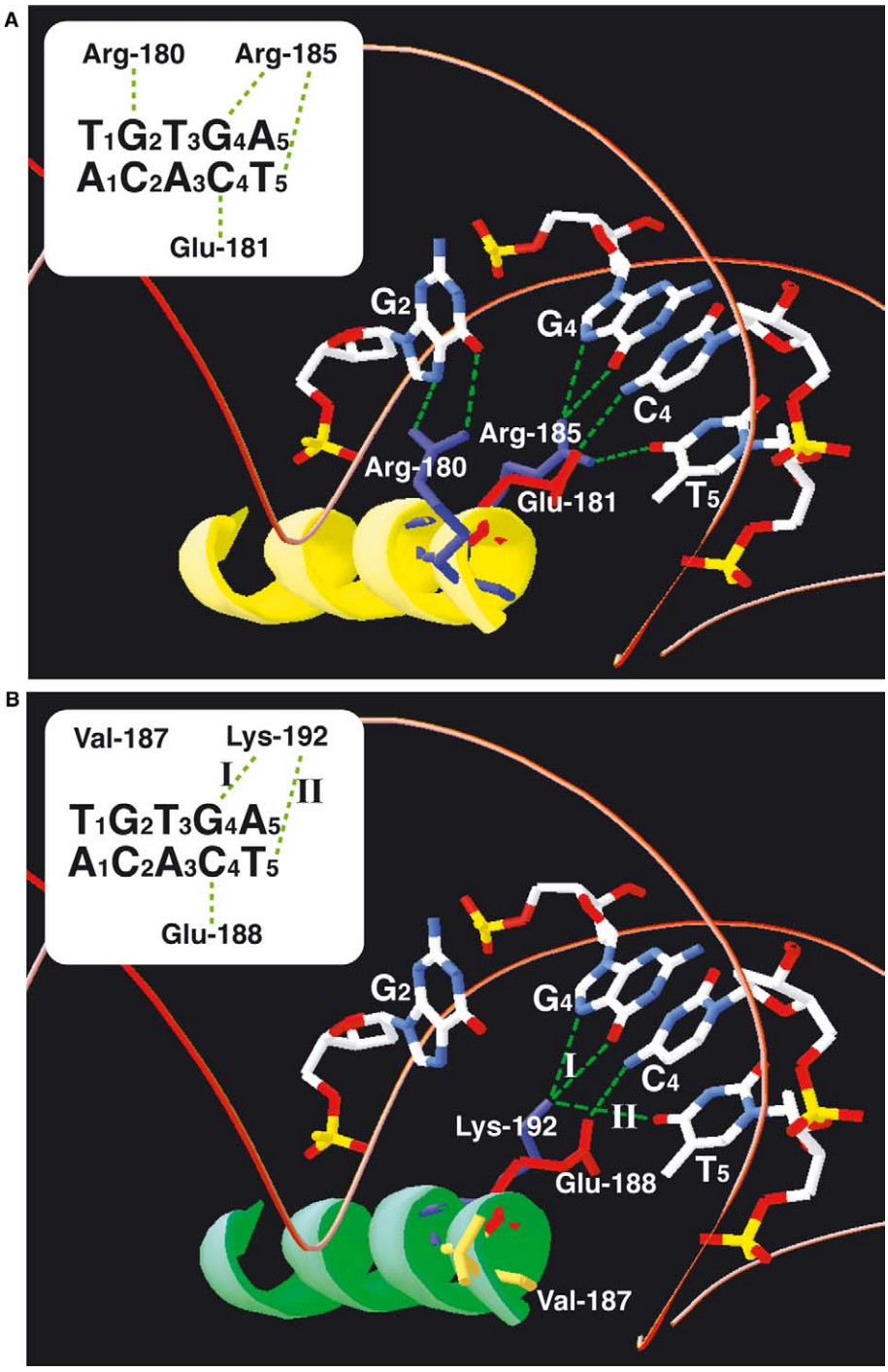

Fig. 6. Prediction of hydrogen bonds of AnCrpB for consensus DNA sequence. The schematic drawing was made by FAMS software [53] based on the crystal structure analysis of the *E. coli* CRP–cAMP–DNA complex [4]. The plain scheme is indicated at the upper left position of respective panels. Only nucleotides that interact with functional amino acid residues directly in *E. coli* CRP (G_2, G_4, C_4 and T_5 in the consensus sequence, 5′-$T_1G_2T_3G_4A_5$-3′ and 5′-$T_5C_4A_3C_2A_1$-3′) are illustrated on each stereo scheme. Ribbon-shaped helixes indicate recognition helix of *E. coli* CRP (A), or putative recognition helix of AnCrpB (B). The amino acid residues are colored according to type: basic, blue; polar, red; and non-polar, yellow. (A) Mode of binding between the recognition helix of *E. coli* CRP and the half-side consensus DNA sequence. Hydrogen bonds between the protein side chains and the consensus DNA sequence (dotted green lines) were quoted from Schultz et al. [4]. (B) Putative schematic drawing between the predicted recognition helix of AnCrpB and the half-side consensus DNA sequence. Putative hydrogen bonds were denoted by dotted green lines as in (A). Lys-192 in AnCrpB is considered to interact with either the G4 nucleotide (represented by I) or the T5 nucleotide (represented by II).

either the G4 nucleotide or T5 nucleotide as described in Fig. 6B.

The second structural prediction of AnCrpB from 3D-PSSM ver. 2.6.0 [49] suggested that the corresponding region for the *E. coli* CRP helix–turn–helix region of AnCrpB possibly forms two helices that straddle the residues of 184-Glu, 185-Ile and 186-Gly of AnCrpB (data not shown). It is unclear whether the residues of 184-Glu, 185-Ile and 186-Gly of AnCrpB form a

turn structure, although most of the α-helical DNA-binding proteins in bacteria have a helix–turn–helix motif [50]. The OmpR is an extreme case of the helix–turn–helix variant that is defined as a "winged-helix–turn–helix" DNA-binding protein [51,52]. Based on these predictions, it is anticipated that AnCrpB perhaps belongs to a new subfamily of "helix–turn–helix" DNA-binding protein.

A specific sequence interaction in the presence of cAMP between His-AnCrpA or His-AnCrpB and the consensus DNA sequence was observed by competition experiments (Fig. 5A and B), although the respective optimal sequences and the roles in vivo remain to be identified. In *Synechocystis* sp. PCC 6803, SYCRP1 regulated expression of the genes of slr1667 and slr1668 that could be involved in cell motility [15,16]. However, *Anabaena* sp. PCC 7120 shows no cell motility, suggesting that AnCrpA and AnCrpB regulate genes for not cell motility but other signal transduction pathways. Particularly, AnCrpB may function in an extreme cellular condition where the cAMP level becomes very high. It is very interesting that *Anabaena* sp. PCC 7120 possesses two cAMP receptor proteins that show different biochemical properties, and their physiological roles should be elucidated.

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