Identification and Characterization of a Novel cAMP Receptor Protein in the Cyanobacterium Synechocystis sp. PCC 6803*

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Three open reading frames of Synechocystis sp. PCC 6803 encoding a domain homologous with the cAMP binding domain of bacterial cAMP receptor protein were analyzed. These three open reading frames, sll1371, sll1924, and slr0593, which were named sycrp1, sycrp2, and sypk, respectively, were expressed in Escherichia coli as His-tagged or glutathione S-transferase fusion proteins and purified, and their biochemical properties were investigated. The results obtained for equilibrium dialysis measurements using these recombinant proteins suggest that SYCRP1 and SYPK show a binding affinity for cAMP while SYCRP2 does not. The dissociation constant of His-tagged SYCRP1 for cAMP is approximately 3 µM. A cross-linking experiment using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide revealed that His-tagged SYCRP1 forms a homodimer, and the presence or absence of cAMP does not affect the formation of the homodimer. The amino acid sequence reveals that SYCRP1 has a domain similar to the DNA binding domain of bacterial cAMP receptor protein in the COOH-terminal region. Consistent with this, His-tagged SYCRP1 forms a complex with DNA that contains the consensus sequence for E. coli cAMP receptor protein in the presence of cAMP. These results strongly suggest that SYCRP1 is a novel cAMP receptor protein.

Cyclic AMP (cAMP) is a global signaling molecule that exists in both prokaryotes and eukaryotes. It mediates a signal from outside the cell to a target protein that regulates gene expression or the enzyme activity of various enzymes. cAMP plays a central role in the regulation of the response to different nutritional states in enteric bacteria (1). Glucose is known to lower intracellular cAMP levels under certain conditions in Escherichia coli (2, 3). cAMP functions to regulate gene transcription in conjunction with the cAMP receptor protein (CRP) in Gram-negative bacteria. E. coli CRP has been well characterized in vitro by a variety of physical and biochemical techniques, including x-ray analysis of CRP crystals (4, 5). The CRP forms a homodimer; each subunit of this dimer is composed of two domains. The larger NH2-terminal domain contains the cAMP binding site and shows homology to a class of cAMP-dependent protein kinases (6–8). The dimer form of CRP is able to bind to specific DNA sequences when it is liganded with cAMP. The COOH-terminal domain can bind to DNA at helix-turn-helix motifs. The cAMP-CRP complex functions as a transcriptional regulator, activating transcription at several promoters and repressing transcription from others (1, 9–12).

In the green algae Chlamydomonas, cAMP is reported as a key second messenger in the mating reaction, but the adenylate cyclase and the mediator of cAMP have not yet been identified. In this case, the cAMP signal cascade is poorly understood (13). In Euglena, Carrie and Edmunds (14) have reported that cAMP mediates the phasing of the cell division cycle through the circadian clock. Recently, it was reported that the catalytic subunit of PKA had been identified in Euglena gracilis (15), although the adenylate cyclase and regulatory subunit of PKA have not been reported. In higher plants, many attempts have been made to clone the adenylate cyclase and PKA genes. Nevertheless, these genes have not been identified in higher plants.

Cyanobacteria, Gram-negative bacteria, are able to perform higher plant-type oxygen evolving photosynthesis. The intracellular cAMP levels of cyanobacteria change in response to changes in environmental conditions such as light-dark, low pH-high pH, oxic-anoxic (16, 17), and nitrogen replete-deplete (18). Exogenous cAMP stimulates the gliding movement of Spirulina platensis (19) and also enhances the cell motility of Synechocystis sp. PCC 6803 (20). These results suggest that cAMP functions as a signaling molecule in cyanobacteria as well. However, no intracellular molecule that mediates the cAMP signal has yet been identified in cyanobacterium.

In this report, we investigated the biochemical properties, especially the cAMP binding affinity, of putative cyanobacterial CRPs. Among them, a novel cAMP receptor protein was identified as a cyanobacterial cAMP receptor protein and named SYCRP1.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media—The E. coli strains used as hosts were JM109 (recA1, endA1, gyrA96, thi-1, hsdR17(rK-, mK-)), supE44, relA1, Δlac-proABF<sup>−</sup> (traD36, proAB, lac<sup>+</sup>, ΔlacZΔM15) for cloning and BL21(DE3)pLysS (F<sup>−</sup>, ompT, hsdS<sup>−</sup>(rB<sup>−</sup>, mB<sup>−</sup>), dcm, gal, λ<sup>−</sup>(DE3), pLysS) for the expression of recombinant proteins. Bacteria were grown in Luria-Bertani medium (21). When required, kanamycin or chloramphenicol was added at 25 or 30 µg ml<sup>−1</sup>, respectively.
Construction of Expression Plasmids for Recombinant Proteins—To obtain DNA fragments corresponding to sll1371, sll1924, and slr0593 ORFs (syrp1, syrp2 and sypk, respectively), polymerase chain reactions were performed with several sets of synthetic primers and genomic DNA from Synechocystis sp. PCC 6803. The primers for syrp1 were CA1 (5′-CGGGATCCGCTGATCCC-3′) and CA2 (5′-CCCACTAAGTGTGACATATG-3′). Primers for syrp2 were HY1 (5′-CGGGATCCGCTGATCCC-3′) and HY2 (5′-GGCAACGTCTGACCGTATT-3′). Primers for sypk were PKA1 (5′-CGGGATCCGCTGATCCC-3′) and PKA2 (5′-CGGGATCCGCTGATCCC-3′). The sequences of the CA1, HY1, and PKA1 primers were designed to allow the introduction of BamHI restriction site immediately upstream of the initiator ATG codon. Each polymerase chain reaction product was cloned into pCRII vector (Invitrogen). After verification of the nucleotide sequence, syrp1 and syrp2 were digested from each plasmid with BamHI and EcoRI and cloned between the BamHI and EcoRI sites of the pET-28a expression vector (Novagen). The resulting plasmids were named pCGA and pHYP. The sypk was partially digested with Alul, and a DNA fragment corresponding to a putative cAMP binding site (from 291 to 872 relative to the start site of the open reading frame) was cloned into the Smal site of pGEX-2T (Amersham Pharmacia Biotech). The direction of the open reading frame was confirmed by restriction mapping. The resulting plasmid was named pPKA.

Expression and Purification of Recombinant Proteins—The transformants, BL21(DE3)plysS cells harboring each of the pCGA, pHYP, and pPKA plasmids, were grown at 37 °C in 1.5 liter of Luria-Bertani medium supplemented with kanamycin or ampicillin (25 or 100 μg ml−1, respectively) and chloramphenicol (50 μg ml−1). The recombinant genes were expressed in exponentially growing cells by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of incubation, the cells were harvested by centrifugation, washed with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10% (v/v) glycerol. The cell suspension was frozen and thawed, and then sonicated at 4 °C for 9 min (3 min three times) using a sonicator (model 200M, Kubota Co., Tokyo, Japan). The cell extract was centrifuged at 16,000 × g for 10 min, and the supernatant was further centrifuged at 150,000 × g for 45 min.

Recombinant proteins in the 150,000 g supernatants were purified with several affinity columns connected to a fast protein liquid chromatography system (FPLC system, Amersham Pharmacia Biotech). The 150,000 × g supernatant containing His-tagged SYCRP1 (His-VSYCRP1) was loaded onto a Hitrap chelating column (Amersham Pharmacia Biotech; 1.6 × 2.5 cm) connected to an FPLC system, and the proteins were eluted using a step gradient of 30, 60, and 100, and 200 mM imidazole in Buffer A. The 200 mM imidazole fraction was loaded onto a Hitrap Q column connected to an FPLC system, and eluted using a step gradient of 100, 200, and 400 mM NaCl in Buffer B. The 400 mM NaCl fraction was loaded onto a Hitrap chelating column connected to an FPLC system, and eluted using a step gradient of 100, 200, and 400 mM imidazole in Buffer A. The 400 mM imidazole fraction contained purified His-SYCRP2. The predicted molecular mass of His-SYCRP2 is 30.4 kDa.

The 150,000 × g supernatant containing a fusion protein (GST-pSYPK) between GST and a portion of SYPK was loaded onto a glutathione-Sepharose 4B column (10 ml, Amersham Pharmacia Biotech), and eluted with 5 mM glutathione in Buffer A. The eluate was loaded onto a Hitrap Q column connected to an FPLC system, and eluted using a step gradient of 100 and 200 mM NaCl in Buffer B. The 200 mM NaCl fraction contained purified GST-pSYPK. The predicted molecular mass of GST-pSYPK is 45.5 kDa.

Gel Mobility Shift Assay—An HPLC system (Shimadzu Co., Kyoto, Japan) was used to measure the amounts of cAMP bound to recombinant proteins. Aliquots from the sample side were denatured for 5 min with cold trichloroacetic acid, final concentration 4.5% (v/v), or by heating at 95 °C for 2 min, and then centrifuged at 18,000 × g for 5 min at 4 °C to remove proteins. The 10 μl aliquot of the supernatant from the sample side or 10 μl from the reference side was applied to a TSK ODS-80Ts column (4.6 mm × 30 cm, Tosoh Co., Tokyo, Japan) equilibrated with 30 mM sodium phosphate buffer (pH 6.5) containing 5% (v/v) acetonitrile; the effluent was monitored at 259 nm. The flow rate was 1.0 ml/min.

Cross-linking of His-SYCRP1 with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)—Purified His-SYCRP1 (4 μg) was incubated in 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10% (w/v) glycerol. The gel was stained with Coomasie Brilliant Blue R-250. Gel Mobility Shift Assay—An oligonucleotide (5′-CGGGATCCGCGAATAATGAGCTATGACACCTTTTGCC-3′) was synthesized and used in the assay. The sequence of the oligonucleotide includes a consensus DNA sequence for E. coli CRP binding and a BamHI site attached to the 5′ end of the consensus sequence. Since the consensus
sequence is composed of two palindromic copies of a 16-bp sequence, preparation of double strand DNA was achieved by incubating the oligonucleotide 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), 100 mM NaCl, and 1 mM dithiothreitol. The DNA fragment was labeled with Klenow fragment and [γ-32P]dCTP (ICN Biomedicals Inc., Costa Mesa, CA), and used as a probe. The 32P-labeled probe (10,000 cpm, approximately 1 ng) was incubated with His-SYCRP1 in a total volume of 20 µl of the binding buffer (50 mM Tris-HCl (pH 7.5), 60 mM NaCl, 1 mM EDTA, 8% (v/v) glycerol, 50 ng of poly(dI-dC)) with or without a final concentration of 20 µM cAMP for 30 min at room temperature. Samples were loaded onto 5% polyacrylamide gels (acylamide:N,N'-methylenebisacrylamide, 50:1). The electrophoresis buffer was 0.25× TBE with or without 20 µM cAMP. Electrophoresis was conducted at constant current (15 mA). When the binding reactions were carried out in buffer containing cAMP, electrophoresis buffer supplemented with 20 µM cAMP was also used. After electrophoresis, the gels were dried and autoradiographed.

Other Analytical Procedures—Protein concentration was determined by the method of Bradford (23). Bovine serum albumin was used as the standard. SDS-PAGE was carried out in polyacrylamide gels containing 0.1% SDS by the method of Laemmli (24).

RESULTS AND DISCUSSION

Selection of ORFs That Predictably Encode cAMP Receptor Proteins—The cAMP binding motif in the prokaryotic cAMP receptor protein and the eukaryotic regulatory subunit of cAMP-dependent protein kinase is substantially conserved (6–8). We performed a search for a putative cAMP receptor protein in the Synechocystis PCC 6803 genome sequencing data (25) and Cyanobase, with reference to the entire amino acid sequence of E. coli CRP, and 18 candidate ORFs were first selected. Based on the presence of functional amino acids that bind cAMP (indicated by the open stars in Fig. 1), the identical residues in this segment are Gly-89, Glu-90, Glu-95, Glu-96, Arg-99, Ser-100, and Val-103, which correspond to Gly-72, Glu-73, Glu-78, Glu-79, Arg-83, Ser-84, and Val-87 in E. coli CRP, respectively. The next highly homologous region, residues 138–141 of SYCRP1, is highly conserved. Arg-124 stabilizes Glu-73 in E. coli CRP (4, 26), but these residues are replaced by Leu-144 and Asn-145 in SYCRP1. A check with the Sequence Motif Search (the GenomeNet web server) revealed the cAMP binding motif 1 to be [LIVMF]-[VIC]-[STACV]-[LIVMF]-[GAC]-[X]-[LIVMFY]-[4 amino acid residues]-[X]-[LIVMFA]-[X]-[STACV]. These motifs are in pairs. SYCRP1 does not participate directly in the binding of cAMP (indicated by the open stars in Fig. 1). The identical residues in this segment are Gly-89, Glu-90, Glu-95, Glu-96, Arg-99, Ser-100, and Val-103, which correspond to Gly-72, Glu-73, Glu-78, Glu-79, Arg-83, Ser-84, and Val-87 in E. coli CRP, respectively. The next highly homologous region, residues 138–141 of SYCRP1, is highly conserved. Arg-124 stabilizes Glu-73 in E. coli CRP through an ionic interaction (4, 26). Thr-128 and Ser-129 participate in the binding of cAMP in E. coli CRP (4, 26), but these residues are replaced by Leu-144 and Asn-145 in SYCRP1. A check with the Sequence Motif Search (the GenomeNet web server) revealed the cAMP binding motif 1 to be [LIVMF]-[VIC]-[X]-[G]-[DENQTA]-[X]-[GAC]-[X]-[LIVMFA]-[G1]-[LIVMFA]-[X]-[STACV]. These motifs are in pairs. SYCRP1 does not participate directly in the binding of cAMP (indicated by the open stars in Fig. 1). The identical residues in this segment are Gly-89, Glu-90, Glu-95, Glu-96, Arg-99, Ser-100, and Val-103, which correspond to Gly-72, Glu-73, Glu-78, Glu-79, Arg-83, Ser-84, and Val-87 in E. coli CRP, respectively. The next highly homologous region, residues 138–141 of SYCRP1, is highly conserved. Arg-124 stabilizes Glu-73 in E. coli CRP through an ionic interaction (4, 26). Thr-128 and Ser-129 participate in the binding of cAMP in E. coli CRP (4, 26), but these residues are replaced by Leu-144 and Asn-145 in SYCRP1. 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A Novel cAMP Receptor Protein in Cyanobacteria

SYCRP1 (4 m) Brilliant Blue R-250.

polyacrylamide gel for electrophoresis. The gel was stained with Coo-

Experimental Procedures. The samples were loaded onto a 10% SDS-

regulatory subunit of PKA in eukaryotes. However, the rest of

that the cAMP binding site of SYPK is similar to that of the

Experimental Procedures. When the entire

constructed expression vectors (pCGA, pHYP, and pPKA) en-

chemical analysis of the putative cAMP receptor proteins, we

prepare recombinant proteins encoded by these ORFs for bio-

Fig. 1; tions. In motif 1 [VIC] and G (underlined) are replaced by L and

not match these motifs because of several amino acid substitu-

tions. In motif 1 [VIC] and G (underlined) are replaced by L and H, respectively. In motif 2, A (underlined) is replaced by T (see

Fig. 1; E. coli CRP motif 1 is the segment from Leu-30 to Gly-46

and motif 2 is the segment from Ile-71 to Ala-89). Together, these data indicate that SYCRP1 must carry a novel cAMP

binding motif. E. coli CRP has a helix-turn-helix motif for

DNA-binding in the carboxyl-terminal region (5, 27). From

residues Arg-196 to Glu-207 in SYCRP1, this region shows high

homology to the latter helix region in E. coli CRP. The specific

amino acids that interact with DNA in E. coli CRP are Arg-181,

Glu-182, Thr-183, Arg-186, and Lys-189 (5).

SYCRP2 shows high homology (40% similarity) to SYCRP1.

However, SYCRP2 lacks several amino acids corresponding to

Glu-73, Arg-83, and Ser-84 in E. coli CRP, which participate

directly in CAMP binding.

The amino acid sequence of SYPK containing a putative cAMP

binding domain is almost the same as that of SYCRP1.

SYPK has common cAMP binding motifs; however, the entire

predicted amino acid sequence of SYPK comprises 434 amino

acids, and the amino- and carboxyl-terminal regions are differ-

ent from those in E. coli CRP and SYCRP1. As a result of

BLAST search (the GenBankTM BLAST server), it is shown

that the cAMP binding site of SYPK is similar to that of the

regulatory subunit of PKA in eukaryotes. However, the rest of

the SYPK sequence shows less homology to the regulatory

subunit of eukaryotic PKA.

Expression and Purification of Recombinant Proteins—To

prepare recombinant proteins encoded by these ORFs for bio-

chemical analysis of the putative cAMP receptor proteins, we

constructed expression vectors (pCGA, pHYP, and pPKA) en-

coding hybrid proteins with His tag or GST as described under

“Experimental Procedures.” When the entire sypk was cloned

into pET-28a, expression of the desired protein was not ob-

served. Therefore, we cloned the ORF into pGEX-2T expression

vector (Amersham Pharmacia Biotech) and succeeded in ex-

pressing the desired protein as a fusion protein with GST.

Unfortunately, the product was insoluble (data not shown).

Therefore, the deduced amino acid sequence of the sypk con-
taining a putative cAMP-binding site (193 amino acids; 98–290

amino acids in the entire predicted amino acid sequence, see

Fig. 1) was examined. When the partial sypk was cloned into

the pGEX-2T expression vector and expressed, a soluble prod-

uct named GST-pSYPK was obtained. Then, this fusion protein

was purified by glutathione-Sepharose 4B column and ion ex-

change chromatography. Other recombinant proteins (His-

tagged SYCRP1 and His-tagged SYCRP2) were purified by Ni2+

-nitrilotriacetic acid affinity chromatography and ion ex-

change chromatography. The molecular masses of both His-

tagged SYCRP1 (His-SYCRP1) and His-tagged SYCRP2 (His-

SYCRP2) were estimated to be 30 kDa by SDS-PAGE analysis,

corresponding closely to the theoretical values (Fig. 2). The

molecular mass of a major band of GST-pSYPK was estimated

to be 45 kDa, but another minor band was also detected at 45.5

dkda, a value that corresponds to the theoretical molecular

mass of GST-pSYPK. Probably several amino acids in the car-

boxyl-terminal region were cleaved without any effect on the

binding to CAMP (Fig. 2, lane 6).

cAMP Binding Activity of Recombinant Proteins—To deter-
mine the cAMP binding ability of each of the expressed pro-
teins, equilibrium dialysis measurements were performed at

25 °C as described under “Experimental Procedures.” The re-

sults are shown in Fig. 3. Three-fifths of the amount of 5 μM

cAMP was bound to 20 μM His-SYCRP1, suggesting that His-

SYCRP1 can bind cAMP. His-SYCRP2 showed low cAMP bind-

ing activity. We had carried out the equilibrium dialysis mea-
surements using 10 μM CAMP, 10 μM His-SYCRP2, and 10 μM

His-tagged galactosidase (Novagen, control sample) as nega-
tive control under the same conditions as in Fig. 3 several times

(data not shown) and found no CAMP binding activity of His-

SYCRP2. Based on these results, we concluded that His-SY-

CRP2 had no CAMP binding activity. It was noted that very

high amounts of CAMP, more than the amounts initially added,

were released from 20 μM GST-pSYPK, indicating that GST-
pSYPK was purified together with endogenously bound CAMP.

To investigate the binding specificity of His-SYCRP1 to CAMP,
equilibrium dialysis measurements were performed using 10
**SYCRP1 Is a Novel CRP in Cyanobacteria**—Although a sequence-specific interaction between His-SYCRP1 and a DNA sequence was observed in competition experiments (lanes 4 and 5 in Fig. 6A), the identification of the optimum sequence for the binding of His-SYCRP1 remains to be elucidated. The results obtained in the present experiments suggest that SYCRP1 is a novel cAMP receptor protein, which is involved in transcriptional regulation as a mediator of the cAMP signal.

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μM His-SYCRP1 and 10 μM 5′-AMP or 10 μM cGMP. His-SYCRP1 bound neither 5′-AMP nor cGMP entirely (data not shown); thus, the data indicate that His-SYCRP1 binds cAMP specifically.

Dissection Dissociation Constant (K_d) of His-SYCRP1 from cAMP—To obtain the K_d value of His-SYCRP1 from cAMP, 0.5–20 μM cAMP was incubated with 5 μM His-SYCRP1 for 6 h at 20 °C or 30 °C for equilibrium dialysis. The K_d value was determined from the revolution lines by least-square analysis on a Scatchard plot (Fig. 4). The K_d value was 2.57 ± 0.08 μM at 20 °C and 2.82 ± 0.09 μM at 30 °C. In this range of temperature, the K_d values were almost constant. These values are physiologically relevant in bacteria because the intracellular cAMP concentration is within 0–10 μM (28). The K_d values of bacterial CRPs are known to be in a order of 10^-6 M in general (29–31). The obtained K_d value for His-SYCRP1 is comparatively lower than that of other bacteria. The bacterial CRPs bind both cAMP and cGMP, and the K_d values of both nucleotides are very similar, that is 10^-6 M (29–31). Considering the predicted maximum amounts of bound cAMP on His-SYCRP1, approximately 0.6 molecule of cAMP bound on 1 molecule of His-SYCRP1. This means that a His-SYCRP1 dimer binds 1 molecule of cAMP, as in the case in *E. coli* CRP (32).

Cross-linking Analysis of His-SYCRP1—To determine whether cyanobacterial CRP forms a dimer, a protein cross-linking experiment of His-SYCRP1 with the zero-length cross-linking reagent EDC was performed. Fig. 5 shows the detection of a protein-protein cross-linking dimeric His-SYCRP1 band in the absence of cAMP. In *E. coli* CRP, cAMP has been reported to stabilize the CRP dimer form (33). If cAMP stabilized His-SYCRP1, the Western blotting analysis after incubation of His-SYCRP1 with the DNA probe. His-SYCRP1 alone did not enter the gel in the presence of 20 μM cAMP.

It was concluded that His-SYCRP1 bound to an *E. coli* CRP consensus DNA sequence, and the binding of His-SYCRP1 to this sequence is dependent on the presence of cAMP *in vitro* (Fig. 6A). It is unlikely that the His-SYCRP1 protein preparation is contaminated with *E. coli* CRP, because no band was observed except for His-SYCRP1 on denaturing polyacrylamide gel (Fig. 2), and a band migrating at a position corresponding to the shifted band was observed in Western blotting analysis (Fig. 6B).

SYCRP1 Is a Novel CRP in Cyanobacteria—Although a sequence-specific interaction between His-SYCRP1 and a DNA sequence was observed in competition experiments (lanes 4 and 5 in Fig. 6A), the identification of the optimum sequence for the binding of His-SYCRP1 remains to be elucidated. The results obtained in the present experiments suggest that SYCRP1 is a novel cAMP receptor protein, which is involved in transcriptional regulation as a mediator of the cAMP signal.
