Biochemical characterization of the major adenylyl cyclase, Cya1, in the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract **We report herein the biochemical properties of an adenylyl cyclase, Cya1, from the cyanobacterium *Synechocystis* sp. PCC 6803. Heterologously expressed Cya1 catalyzed cyclic AMP formation with a K_m for ATP of approximately 2.2 μM at pH 7.5. Although cellular Cya1 activity is increased by blue light illumination [Terauchi and Ohmori, Mol. Microbiol. 52 (2004) 303], purified Cya1 did not contain any chromophores, and the activity was light-insensitive. This suggests that an unknown blue light-responsive factor interacts with the N-terminal regulatory domain of Cya1 to control its adenylyl cyclase activity. Finally, our results show that the sensor of blue light using FAD (BLUF) protein, Slr1694, does not appear to be involved in the regulation of Cya1-mediated cAMP signal transduction in this bacterium.**

Keywords: Adenylyl cyclase; Blue light receptor; BLUF; cAMP; Cyanobacteria; Motility

1. Introduction

Photosynthetic organisms must respond to light quality and quantity to efficiently capture light energy. cyclic AMP (cAMP) acts as a second messenger in the transduction of a light signal in cyanobacteria [1]. The cellular cAMP concentration in several cyanobacteria changes rapidly in response to environmental light conditions [2–6]. In particular, transfer of the cells from the dark to blue light (380 or 450 nm) conditions results in an increase in cellular cAMP in the cyanobacterium *Synechocystis* sp. PCC 6803 [6]. Biochemical and genetic analyses of the regulatory function of this small molecule have indicated that it is bound to the cAMP receptor protein SYCRP1, which is involved in the biogenesis of pili, cellular structures required for the motility of this bacterium [7,8]. Thus, changes in the environmental light conditions control the activity of specific transcriptional factors through rapid changes in the amount of cAMP. This allows cells to adjust their motility according to the light conditions.

In *Synechocystis* sp. PCC 6803, the cellular cAMP content is maintained by an adenylyl cyclase, Cya1, which catalyzes the formation of cAMP from ATP [9]. A null mutation of the *cya1* gene results in a decrease in cellular cAMP levels (to ~4% of the wild-type level) under photoautotrophic growth conditions. As a result, the mutant strain loses cell motility [9]. Notably, the mutant strain also failed to increase the levels of the cellular cAMP upon dark to blue light transition [6], indicating that Cya1 activity is regulated in response to blue light. There are two possible mechanisms for this regulation of Cya1 activity by blue light. One possibility is that Cya1 contains chromophore(s) that absorb blue light and thereby control the enzymatic activity. The other possibility is that a separate uncharacterized blue light receptor controls the adenylyl cyclase activity of Cya1. Specifically, several recent data have suggested the involvement of a sensor of blue light using FAD (BLUF) protein, Slr1694 [10,11], in controlling Cya1 activity [6].

Slr1694 is a 17-kDa protein composed of 151 amino acids and it possesses one FAD-binding BLUF domain [10,11]. BLUF proteins make up one of the major photoreceptor families. These proteins use FAD as a chromophore and are involved in the perception of blue light by several microorganisms [12–15]. Although the biological function of Slr1694 is still unclear, we recently showed that this protein is photoactive; it shows a typical BLUF-domain photocycle upon blue light irradiation [11]. Interestingly, PAC, a BLUF protein found in the green alga *Euglena gracilis*, harbors two BLUF and two adenylyl cyclase domains [13]. Biochemical and genetic analyses indicated that PAC is involved in the blue light-induced photoavoidance response of *E. gracilis*, and that it requires blue light irradiation for its adenylyl cyclase activity. There is also experimental evidence that flavoprotein is involved in the regulation of blue light-dependent Cya1 activity [6]. Specifically, the flavin antagonist phenylacetic acid inhibits adenylyl cyclase activity of Cya1 in vivo. Therefore, it has been suggested that Slr1694 is involved in the regulation of Cya1 activity in response to blue light [6].

To elucidate the mechanisms of photoreception and signal transduction regulating cAMP in *Synechocystis* cells, we biochemically characterized Cya1 of *Synechocystis* sp. PCC 6803. Our results indicated that purified Cya1 does not contain any chromophores and that its enzymatic activity is light-insensitive. We also examined the interaction of Cya1 with Slr1694.

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Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; cAMP, cyclic AMP; BLUF, sensor of blue-light using FAD; FAD, flavin adenine dinucleotide; FHA, forkhead-associated; IPTG, isopropyl β-D-thiogalactopyranoside; K_m, Michaelis constant; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate

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2. Materials and methods

2.1. Expression and purification of Cya1 and Slr1694

Slr1694 protein was expressed in *Escherichia coli* and purified as described previously [11]. The recombinant histidine-tagged Cya1 protein was heterologously expressed using an *E. coli* overexpression system. The *cya1* coding region of *Synechocystis* sp. PCC 6803 was amplified by PCR using isolated genomic DNA as a template and primers Cya1-F (5′-GGGGGGCATATGGATAAGCCTGCCC-TACCC-3′; *Nde*I restriction site underlined) and Cya1-R (GGGGAATTCTTAGGGCCCTTCCGAGGCG-3′; *Eco*RI restriction site underlined). The amplified fragment was digested with *Nde*I and *Eco*RI and then cloned into the *Nde*I and *Eco*RI sites of the pET28(a) vector (Novagen). The resulting plasmid, named pETCya1, was used to transform *E. coli* strain BL21(DE3) (Novagen). The histidine-tagged Cya1 protein was overexpressed by induction for over 16 h at 16 °C with 1 mM IPTG (Takara). Harvested cells were ruptured with a French press and the expressed Cya1 protein was isolated with His-Bind resin (Novagen) according to the manufacturer's instructions. Proteins in 3 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA were further purified by ion exchange chromatography using DEAE-TOYOPEARL 650S resin (TOSOH) and elution with a stepwise gradient of NaCl. Cya1 protein eluted at 300 mM NaCl showing the highest adenylyl cyclase activity was pooled and used as the purified Cya1 for further experiments.

2.2. Construction of the slr1694 deficient mutant

The *slr1694* deficient mutant of *Synechocystis* sp. PCC 6803 was constructed by insertion of a spectinomycin-resistance cassette [16] into the *slr1694* gene. First, the *slr1694* cording region was amplified by PCR using primers Slr1694-F-UP (5′-CGGCTTTTCTGCGT-GATG-3′) and Slr1694-R (5′-GGGAATTCTTAGAGGTCGAG-GAAAAAG-3′). The amplified fragment was then cloned into the pUC18 vector (Takara) at its *Hin*cII site, and the resulting plasmid was digested with *Eco*RV, which cuts the *slr1694* gene 69 bp downstream from the start codon. The digested DNA fragment was then ligated with a *Sma*I-cut spectinomycin resistance cassette [16] and the resulting plasmid was named pUC1694upSp. The pUC1694upSp plasmid was transferred into wild-type *Synechocystis* sp. PCC 6803 as described previously [17]. The recombinants, which were candidate *slr1694* mutants, were isolated on a BG11 plate [18] containing 10 mg/L spectinomycin. The complete segregation of the mutation of *slr1694* in one of the recombinants was confirmed by PCR (data not shown) and the obtained mutant strain was used for further analyses.

2.3. Adenylyl cyclase activity assay

The in vitro assay of adenylyl cyclase activity was performed at 27 °C in a buffer containing 50 mM Tris–HCl (pH 7.5) and 1 mM dithiothreitol in the presence of various concentrations of ATP, $MnCl_2$, and $MgCl_2$ and in the dark or with illumination at 1500 μmol/m^2/s with a halogen lamp. The reaction was stopped by adding an equal volume of ice-cold 10 mM EDTA. Next, the assay mixture was rapidly filtered through an ultrafiltration membrane unit (Millipore) to remove proteins. The cAMP contents were measured by an enzyme immunoassay kit (Amersham).

2.4. Determination of cellular cAMP contents

Cellular cAMP contents were measured essentially as described by Terauchi and Ohmori [6]. Dark-incubated *Synechocystis* cells were incubated in the dark or exposed to blue light (470 nm, 80 μmol/m^2/s) provided by a light emitting diode (MIL-B18; Sanyo).

2.5. Phototactic movement assay

Phototactic movement was examined on 0.4% (w/v) agar-solidified BG11 medium [18] supplemented with 0.3% sodium thiosulfate and under lateral illumination with a white fluorescent lamp at 100 μmol/m^2/s.

3. Results and discussion

A previous study suggested that Slr1694 is involved in the modulation of the Cya1-dependent cAMP formation in *Synechocystis* sp. PCC 6803 [6]. To elucidate this hypothesis, we first tested the effects of the *slr1694* mutation and exogenous addition of cAMP on phototaxis of *Synechocystis* sp. PCC 6803. As shown in Fig. 1, the wild-type strain showed positive phototaxis, but the *slr1694* mutant moved away from the white light on a normal BG11 plate. This observation is consistent with a previous report that showed that *slr1694* is necessary for positive phototaxis [10]. Interestingly, on a plate containing 0.1 mM cAMP, white light did not induce phototactic movement by the wild-type strain, and the *slr1694* mutant showed markedly enhanced negative phototaxis. These results indicate that proper control of the cellular cAMP concentration is essential for normal phototactic behavior of the wild-type and *slr1694* mutant strains.

To further investigate if Cya1 controls the cellular cAMP levels in the light dependent manner, we next achieved the biochemical characterization of Cya1. For this application, we purified heterologously expressed histidine-tagged Cya1 protein by affinity and ion exchange chromatography. As shown in Fig. 2, the purified Cya1 protein appeared as a single band on SDS–PAGE. The predicted molecular mass according to SDS–PAGE was approximately 40 kDa, which closely corresponds to the value predicted by its amino acid sequence (39.7 kDa).

We then examined the dependence of the adenylyl cyclase activity of Cya1 on the concentration of ATP in the presence of Mn^{2+} or Mg^{2+}. As shown in Fig. 3, at pH 7.5 and 27 °C and in the presence of Mn^{2+} (closed circles), purified Cya1 showed adenylyl cyclase activity. Under these conditions, the K_m value for ATP was 2.2 ± 0.3 μM and the maximum velocity (V_{max}) was 66.3 ± 2.2 pmol cAMP/min/nmol. The enzyme activity in the presence of Mg^{2+} (open circles) was about 50-fold lower than in the presence of Mn^{2+}. Previous phylogenetic analyses have indicated that Cya1 of *Synechocystis* sp. PCC 6803 belongs to class III adenylyl cyclase [19,20]. The observed preference for Mn^{2+} is similar to other class III adenylyl cyclases [21,22]. Furthermore, the K_m value for ATP (2.2 ± 0.3 μM) for

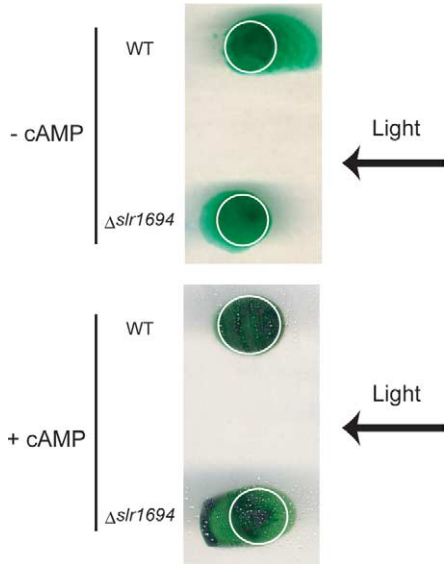

Fig. 1. Phototactic movement of wild-type and *slr1694* mutant *Synechocystis* sp. PCC 6803 in the presence or absence of 0.1 mM cAMP. Cells were grown in lateral white-light (100 μmol/m^2/s).

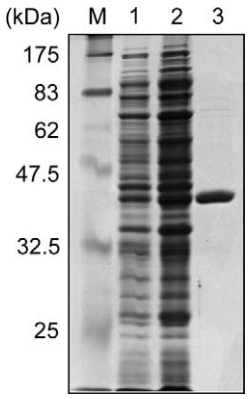

Fig. 2. Purification of Cya1 protein. SDS–PAGE was carried out on a 12% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue. Lane 1, extract from uninduced cells; lane 2, extract from cells induced with 1 mM IPTG; lane 3, purified histidine-tagged Cya1. The positions of molecular weight standards are indicated on the left.

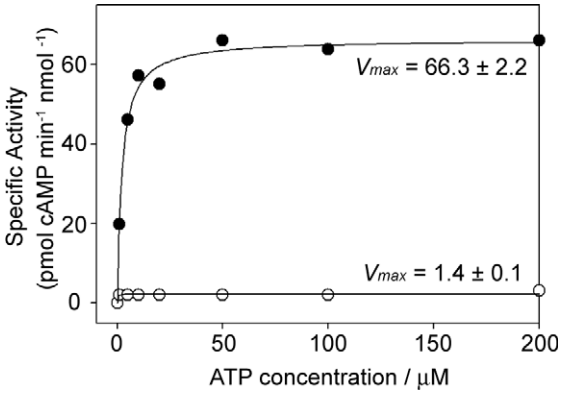

Fig. 3. ATP concentration dependence of Cya1 adenylyl cyclase activity. Reactions were carried out at 27 °C in 50 mM Tris–HCl (pH 7.5) and 1 mM dithiothreitol and in the presence of 5 mM $MnCl_2$ (closed circles) or 5 mM $MgCl_2$ (open circles).

Cya1 is the lowest reported value of class III adenylyl cyclases, suggesting that the purified Cya1 is in an active form. The UV–vis absorption spectrum indicated that purified Cya1 did not contain any chromophores (data not shown). Furthermore, when assayed under sub-optimal conditions (relatively low concentrations of Mn^{2+}, 0.5 mM, and ATP, 5 μM) to allow the detection of either enzyme stimulation or inhibition, exposure to light had no effect on the adenylyl cyclase activity of Cya1 (Table 1). These results strongly indicate that it is not Cya1

Table 1
Effects of light and Slr1694 protein on the adenylyl cyclase activity of Cya1 under sub-optimal conditions

	Specific activity (pmol cAMP min^{-1} $nmol^{-1}$)	
	−Slr1694	+Slr1694
Dark	16.8 ± 1.1	18.5 ± 1.7
Light	17.7 ± 1.4	18.0 ± 1.5

Reactions were carried out at 27 °C in the dark or under white light illumination (1500 μmol/m^2/s) in 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 5 μM ATP, and 0.5 mM $MnCl_2$ and in the presence or absence of a 50-fold molar excess of purified Slr1694 protein.

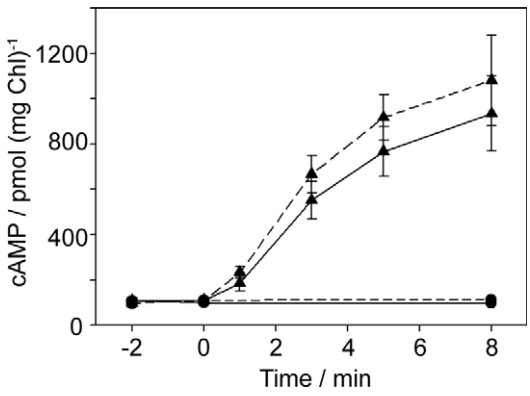

Fig. 4. Changes in cellular cAMP content in wild-type (solid lines) and *slr1694* mutant (dashed lines) *Synechocystis* sp. PCC 6803. Dark-incubated cells were exposed to blue light (470 nm, 80 μmol/m^2/s) at time 0 (triangles) or incubated continuously in the dark (circles).

itself but rather another unknown photoreceptor that is responsible for the blue light-dependent adenylyl cyclase activity of Cya1 in vivo.

To address weather Slr1694 is directly involved in blue light-dependent cAMP formation by Cya1, we measured blue light-induced changes in cellular cAMP levels in wild-type and the *slr1694* deficient strain of *Synechocystis* sp. PCC 6803. As shown in Fig. 4, the cellular cAMP contents significantly increased within a few minutes when dark-adapted wild-type cells were exposed to blue light (470 nm; triangles, solid line). This observation is consistent with a previous report that studied the effects of various wavelengths of blue light (380 and 450 nm) [6]. This report also showed that a mutant strain lacking the *cya1* gene does not show a blue light-dependent increase in cellular cAMP levels [6]. Similar treatments also caused an increase in cAMP in the *slr1694* mutant (triangles, dashed line). Finally, when incubated continuously in the dark, both wild-type and *slr1694* mutant cells showed no increase in cAMP (circles). These results indicated that Slr1694 is not involved in the control of Cya1 activity in response to blue-light.

Because we could not exclude the possibility that there are redundant mechanisms in vivo, we next tested the effect of the Slr1694 protein on Cya1 activity in vitro. To allow detection of positive or negative effects of Slr1694, we performed the assays under sub-optimal conditions, including relatively low metal ion (0.5 mM Mn^{2+}) and substrate (5 μM ATP) concentrations. As shown in Table 1, addition of a 50-fold molar excess of Slr1694 protein did not affect the adenylyl cyclase activity of Cya1 under either dark or light conditions. The light intensity used in this assay (1500 μmol/m^2/s) was strong enough to induce the photocycle reaction of the Slr1694 protein [11]. Therefore, this result further supports the conclusion that Slr1694 is not involved in the regulation of Cya1 activity in vivo.

The mechanism of blue light-dependent control of Cya1 activity remains open to speculation. The class III adenylyl cyclases, including Cya1, show considerable variability in topology and physiological roles [20], and a variety of regulatory domains associate with the catalytic regions of these enzymes. These could stimulate or inhibit the cyclase activity in response to different environmental signals. In the case of Cya1, a

N-terminal Forkhead-associated (FHA) domain [23] is linked to the C-terminal cyclase domain [9]. This suggests that the FHA domain works by unknown mechanism to regulate the C-terminal catalytic activity. It was recently shown that FHA domain recognizes intramolecular or intermolecular phosphothreonine epitopes [24,25]. Genome sequence data have indicated that *Synechocystis* sp. PCC 6803 comprises several genes coding for putative serine/threonine kinases [26], suggesting that FHA domain of Cya1 interacts with one of such protein kinases, which has blue-light sensing capability.

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