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Biochemical and Functional Characterization of BLUF-Type Flavin-Binding Proteins of Two Species of Cyanobacteria

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BLUF (a sensor of Blue-Light Using FAD) is a novel putative photoreceptor domain that is found in many bacteria and some eukaryotic algae. As found on genome analysis, certain cyanobacteria have BLUF proteins with a short C-terminal extension. As typical examples, Tll0078 from thermophilic *Thermosynechococcus elongatus* BP-1 and Slr1694 from mesophilic *Synechocystis* sp. PCC 6803 were comparatively studied. FAD of both proteins was hardly reduced by exogenous reductants or mediators except methylviologen but showed a typical spectral shift to a longer wavelength upon excitation with blue light. In particular, freshly prepared Tll0078 protein showed slow but reversible aggregation, indicative of light-induced conformational changes in the protein structure. Tll0078 is far more stable as to heat treatment than Slr1694, as judged from flavin fluorescence. The *slr1694*-disruptant showed phototactic motility away from the light source (negative phototaxis), while the wild type *Synechocystis* showed positive phototaxis toward the source. Yeast two-hybrid screening with *slr1694* showed self-interaction of Slr1694 (PixD) with itself and interaction with a novel PatA-like response regulator, Slr1693 (PixE). These results were discussed in relation to the signaling mechanism of the "short" BLUF proteins in the regulation of cyanobacterial phototaxis.

Key words: blue light sensor, BLUF, FAD, photoreceptor, phototaxis, thermophilic cyanobacterium.

Sensing of light and redox is important for many organisms, especially phototrophic organisms, since they largely depend on light energy, which is so variable in natural environments. One class of typical sensor proteins is the flavoproteins, whose flavin potentially perceives blue light or redox changes. Whereas more than six distinct types of flavoproteins have been identified as oxidoreductases or related enzymes, only four flavin-binding sensor proteins have been found to date (*1*). First, plant phototropin and related proteins contain an FMN-binding PAS (Per-Ant-Sim) domain or LOV (light-oxygen-voltage) domain that exhibits a photocycle of the flavin upon excitation (*2*, *3*). They transmit a blue light signal to the carboxyl-terminal protein kinase domain. Eventually, they are engaged in regulation of the phototropism of shoots, stomatal opening and chloroplast relocation in higher plants (*4*, *5*). Second, bacterial NifL (*Azotobacter vinelandii*) and Aer (*Escherichia coli*) contain an FAD-binding PAS domain that responds to redox changes. NifL is an oxygen-responsive repressor of the *nif* genes for nitrogen fixation, while Aer is an oxygen sensor involved in aerotaxis (*6*, *7*). Third, cryptochrome is an FAD-binding blue light sensor exhibiting high homology to DNA photolyase. It serves as a circadian photoreceptor that regulates growth and development in higher plants (*8–10*). Fourth, a novel group of FAD-binding proteins (BLUF) has recently been reported to be blue light receptors (*11*). AppA of photosynthetic α-proteobacterium *Rhodobacter sphaeroides* contains a novel FAD-binding domain in the N-terminal region, which perceives blue light and modulates the protein–protein interacting domain in the C-terminal region. Upon illumination, AppA shows a spectral shift of the flavin to a longer wavelength of approximately 10 nm (*12*). As a result, light-activated AppA acts as an anti-repressor against PpsR, which is a repressor for photosynthetic genes (*12–14*). PACα and PACβ of eukaryotic alga *Euglena gracilis*, each containing two FAD-binding and two adenylyl cyclase domains in tandem, form a heterodimer, which causes blue-light–induced activation of adenylyl cyclase. The heterodimer is localized in the paraflagellar body as a fluorescent protein and mediates a photophobic response to blue light (*15*).

Cyanobacteria comprise a unique group of bacteria that perform oxygenic photosynthesis. Plant chloroplasts are believed to have evolved from cyanobacteria, although their precise origin has not yet been elucidated. Based on the recent progress in cyanobacterial genome projects, a

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number of genes have been found to encode putative sensory flavoproteins such as LOV proteins and cryptochrome, which are shared with plants (*16–19*). An FAD-binding BLUF domain has also been detected in ORFs in some cyanobacterial genomes (*Thermosynechococcus elongatus* BP-1, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002) (*20*, *21*). These gene products are highly homologous to each other in full length, and also share the conserved BLUF domain with AppA, PAC and many putative sensor proteins in bacteria. Consistently, for cyanobacteria, there have been many reports about responses to blue light and redox conditions (*22–28*). However, little is known about the sensors and signal transduction pathways involved in these signal responses.

Many lines of research seem to be in progress to elucidate the functional role of the cyanobacterial BLUF proteins. The first involves detection of the primary intermediate of the light reaction: Fukushima *et al.* (*29*) found that Tll0078 is converted to a 5 nm–shifted intermediate form, which accumulates upon illumination at 10 K. The second comprises FTIR analysis to determine structural changes upon light excitation and following relaxation in the dark. The RIKEN group has published several key papers regarding the detection of changes in the chemical bond interaction within the Slr1694 protein under normal and restricted conditions (*30*, *31*). On isotope labeling, they found that the primary event during excitation is weakening of the C(4)=O bond in the isoalloxazine ring of FAD. The third is crystallographic analysis: Tll0078 has been crystallized and its three-dimensional structure has been determined (*32*). The fourth involves comparative and functional analyses of Slr1694 and Tll0078, which are presented in this communication. To understand a novel photoreceptor, it is essential to study the protein by means of a wide range of approaches and techniques. Here, we compare the biochemical properties of thermophilic and mesophilic cyanobacteria. We also report the phenotype of the *slr1694*-disruptant in phototaxis of *Synechocystis* and possible signaling models inferred on yeast two-hybrid screening.

MATERIALS AND METHODS

Culture and Growth Conditions—A motile strain of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 was obtained from the Pasteur Culture Collection and a clone showing vigorous motility of positive phototaxis (PCC-P) was selected as the parent strain for gene disruption (*33*). The thermophilic potentially motile cyanobacterium *Thermosynechococcus elongatus* BP-1, isolated from a hot spring in Beppu in Japan, was used as the genetic source of *tll0078*. *Synechocystis* cells were grown in liquid BG11 medium containing 1% (v/v) CO_2, with air-bubbling at 31°C and a light intensity of 50 μE m^{-2} s^{-1}.

For cloning and subcloning of DNA in plasmids, strains XL10 (Epicurian Coli XL10-Gold Kan, Stratagene, La Jolla, CA, USA) and JM109 of *Escherichia coli* were used, while BL21 (DE3) pLysS was used for expression in pET28a (Novagen, Madison, WI, USA).

Construction of a slr1694-Disruptant and Phototaxis Assaying—A fragment of 1.0 kbp carrying *slr1694* was amplified by PCR with primers (5′-CCTTTCGGGCTTTAACGG-3′ and 5′-AGTCTCGCCTTTAGTTTGG-3′) using the genome sequence of *Synechocystis* and *Taq* polymerase (Ampli-Taq, Applied Biosystems, Foster City, USA), and then cloned into the pT7Blue-T vector (Novagen) with *E. coli* XL10-Gold cells according to the manufacturer's instructions. The coding region of *slr1694* was interrupted at the *Stu*I site by insertion of a Tn-5 derived kanamycin resistance cassette without a transcriptional terminator. Mutants were generated by transformation of wild-type cells with this DNA and selected on BG11 plates containing 20 μg ml^{-1} kanamycin (*34*). Complete segregation was confirmed by PCR with the same primers as those above (data not shown). For the phototaxis assay, 1 μl aliquots of a concentrated cell suspension (*ca.* 2×10^9 cells ml^{-1}) were spotted on to agar-BG11-glucose-diuron plates, dried, and then incubated for 44 h under lateral illumination with monochromatic light at 460 and 660 nm of 0.7 μE m^{-2} s^{-1} at 31°C (*35*). Monochromatic light was supplied by Okazaki Large Spectrograph (*36*).

Cloning of tll0078 and slr1694 for Expression—The coding regions of *tll0078* of *T. elongatus* and *slr1694* of *Synechocystis* were amplified from the genomic DNA by PCR with primers (*tll0078*: 5′-ACATATGGGACTACATCGCCTG-3′ and 5′-CAGATCTAGGATCCTTGACTCA-3′; *slr1694*: 5′-ACATATGAGTTTGTACCGTTT-3′ and 5′-AGTCTCGCCTTTAGTTTGG-3′). PCR was performed with *Pfu* DNA polymerase (Stratagene), and the PCR products were cloned into *Srf*I-digested pPCR-Script (Stratagene). The DNA sequence of the resultant was confirmed by nucleotide sequencing by means of the BigDye terminator fluorescence detection method (Applied Biosystems) and a capillary sequencer (PRISM 310 Genetic Analyzer, Applied Biosystems). The sequenced DNAs were excised with *Nde*I and *Bgl*II (*tll0078*) or *Nde*I and *Bam*HI (*slr1694*), and then inserted into pET28a (Novagen) to allow expression with an N-terminal $(His)_6$-tag.

Expression and Purification—$(His)_6$-tagged Tll0078 and Slr1694 proteins (designated as His-Tll0078 and His-Slr1694) were expressed in *E. coli* BL21 (DE3) pLysS transformed with the recombinant plasmids. Expression was induced by adding isopropyl β-D-galactopyranoside (1 mM) to cultures at OD_{600} between 0.5 and 0.6 at 25°C in LB medium containing 20 μg ml^{-1} kanamycin for 6 h. Cells were harvested, frozen at –80°C, thawed at 4°C, and then resuspended in 20 mM HEPES-NaOH (pH 7.5) buffer containing 1 M NaCl. After sonication for disruption, samples were centrifuged at 48,000 × *g* for 1 h at 4°C. His-tagged fusion proteins were purified from the supernatants by nickel affinity column chromatography (HiTrap Chelating HP; Amersham Biosciences, Piscataway, NJ, USA). A sample was loaded on a column, washed, and then eluted with 20 mM HEPES-NaOH (pH 7.5) buffer containing 1 M NaCl with a linear gradient of imidazole, from 0 to 500 mM. Proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie Brilliant Blue R-250. Protein concentrations were estimated by the Bradford method as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Flavin was extracted from His-Slr1694 by heat treatment at 65°C and extracted from His-Tll0078 by 2% SDS-heat treatment at 95°C. Flavin was analyzed by TLC (silica gel 60 plates; Merck, Darmstadt, Germany) using *n*-butanol/acetic acid/water (4:1:5) as the developing solvent. The

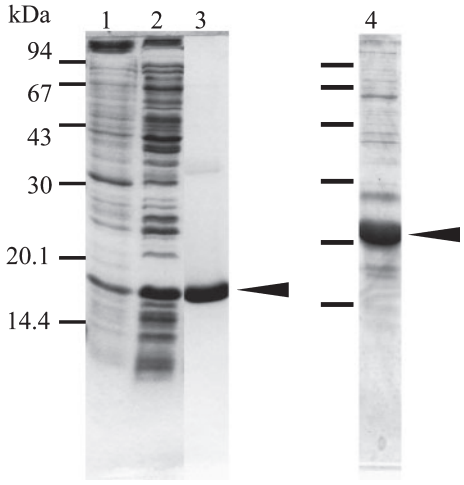

Fig. 1. **Overexpression and purification of the His-Tll0078 and His-Slr1694 proteins.** Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. Lane 1, insoluble fraction; lane 2, soluble fraction; lane 3, His-Tll0078 protein purified by Ni affinity chromatography; lane 4, purified His-Slr1694 protein.

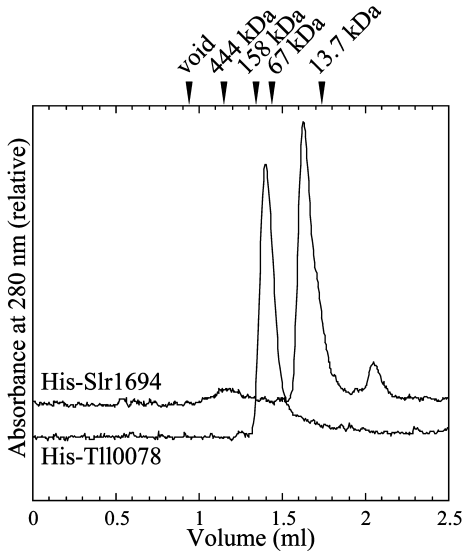

Fig. 2. **Elution profile of the His-Tll0078 (lower line) and His-Slr1694 (upper line) proteins on a Superdex 200 PC 3.2/30 column.** Molecular markers: 444 kDa, ferritin; 158 kDa, aldolase; 67 kDa, BSA; 13.7 kDa, ribonuclease.

molecular masses of the purified proteins were determined by size exclusion column chromatography (Superdex 200 PC 3.2/30, SMART system, Amersham Biosciences) with 20 mM HEPES-NaOH (pH 7.5), 1 M NaCl and 400 mM imidazole.

Chemical Reduction of Flavin—Sodium dithionite powder (final ~7 mg ml^{-1}) was added to a solution of each purified His-tagged protein, which was covered with mineral oil to avoid autooxidation by air. Reduction of flavin was monitored as the absorbance of FAD and the data were collected after the addition of mineral oil.

Spectroscopy—Ultraviolet and visible absorption spectra of the proteins were recorded with a spectrophotometer (model UV-3100 PC; Shimadzu, Kyoto, Japan). Fluorescence spectra were obtained with a fluorometer (model RF-5300 PC, Shimadzu). Flavin was excited by irradiation with a 150 W xenon arc lamp (2.6 μmol cm^{-2} s^{-1}, L2195; Hamamatsu Photonics, Shizuoka, Japan) with a 390 nm long cut filter (B-390; HOYA, Tokyo, Japan) or a blue laser (19 μmol cm^{-2} s^{-1} at 405 nm, MLXF-A16-405-30; Kikoh Giken, Hyougo, Japan). These experiments were performed at room temperature.

Yeast Two-Hybrid Screening—The screening was performed according to (*37*). The coding region of *slr1694* was excised from the pET28a expression construct with *Nde*I and *Bam*HI, and the subcloned into a bait vector, pAS2–1 (Clontech, Palo Alto, CA, USA). The bait clone was then transformed into the AH109 (mat α) yeast strain. A cyanobacterial genomic library was generated by subcloning of randomly fragmented DNA into a prey vector, pACT2-AS, a derivative of pACT2 (Clontech). The library clones were then introduced into the Y187 (mat a) yeast strain according to standard lithium acetate–mediated procedures. The diploid yeast cells generated on mating of the two yeast strains containing the bait clone and genomic library were transferred to histidine-lacking synthetic medium supplemented with 5 or 10 mM 3-aminotriazole, and then grown at 30°C for 4 days for analysis of hybrid formation. Reverse hybrid screening was performed with *slr1694* in the pACT2-AS vector, and the six *patA*-like genes (*sll0038*, *slr1041*, *sll1291*, *slr1693*, *slr1594* and *slr1214*) and *slr1694* in the pAS2-1 vector. The construction of the latter plasmids will be reported elsewhere.

RESULTS

Purification of the His-Tagged Protein—When expression of His-Tll0078 and His-Slr1694 was induced with isopropyl β-D-galactopyranoside, *E. coli* cells were colored yellow. After fractionation of the crude extract by centrifugation, about half of the proteins was recovered in the soluble fraction. Notably, the particulate fraction comprising inclusion bodies was also colored yellow, suggestive of partial assembly of the proteins. The soluble yellow proteins were purified by nickel affinity column chromatography. On SDS-PAGE, the molecular masses of the purified His-Tll0078 and His-Slr1694 proteins were estimated to be ~18 kDa and ~21 kDa, respectively, which are consistent with the molecular masses calculated from the amino acid sequences (Fig. 1). The purity of the His-Tll0078 and His-Slr1694 proteins was ~95% and ~85%, as judged on dye staining.

Molecular Masses—The molecular mass of the native His-Tll0078 protein was estimated to be ~100 kDa by size exclusion chromatography (Fig. 2). Taking into account the deduced molecular mass (~18 kDa), we assumed that the native His-Tll0078 protein formed a 5–6 mer. On the other hand, the molecular mass of the native His-Slr1694 protein was estimated to be ~40 kDa, while its deduced mass was ~21 kDa. This indicates that the native His-Slr1694 protein formed a homo dimer. These findings suggest that both proteins are present in an oligomeric form in cyanobacterial cells.

Flavin Content and Heat Stability—The absorption spectra of these proteins indicated a typical oxidized flavin.

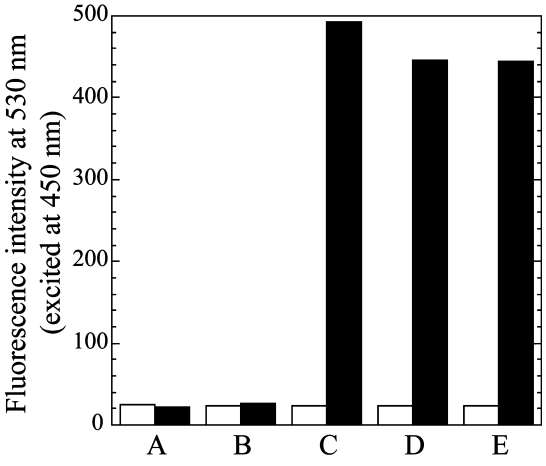

Fig. 3. **Fluorescence intensity of the His-Tll0078 (white box) and His-Slr1694 (black box) proteins after heat treatment.** A, non-treated: B, 30°C, 5 min: C, 60°C, 5 min: D, 90°C, 5 min: E, 90°C, 10 min.

Based on the results of protein determination and the general molecular extinction coefficient of flavin, the flavin content of His-Tll0078 was roughly estimated to be 0.9–1.0. The flavin content of His-Slr1694 was approximately 10% lower than that of His-Tll0078. Judging from the purity of the preparations, we can conclude that His-Slr1694 also includes a flavin in a stoichiometric amount. Flavin was released from His-Slr1694 by heat or 2% SDS treatment, and from His-Tll0078 by SDS-heat treatment. The fluorescence intensity of these extracts at pH 7 was ~50% of that at pH 3 (data not shown). Generally, free FAD shows a similar decrease in fluorescence yield at higher pH due to the formation of an intramolecular complex between the isoalloxazine ring and the adenine ring, whereas riboflavin or FMN does not (*38*). TLC of the extracted flavin showed that the R_f value of the flavin from both proteins corresponded to that of authentic FAD, although the extract from His-Tll0078 contained a degradation product, probably due to the harsh treatment (data not shown). These findings suggest that the His-Tll0078 and His-Slr1694 proteins bind FAD non-covalently.

To evaluate heat stability, we monitored the fluorescence yield of FAD in the proteins at 530 nm after heat treatment. Since the fluorescence of FAD is quenched by the surrounding protein matrix, the fluorescence intensity of FAD bound to a native protein is much lower than that of a free molecule of FAD. Notably, the intensity of the His-Tll0078 protein hardly changed even on treatment of 95°C for 10 min (Fig. 3). In contrast, the fluorescence intensity of the His-Slr1694 protein increased more than 20-fold upon heat treatment for 5 min at 60°C. Further treatment at higher temperature did not increase the intensity any more. These findings indicate that the His-Tll0078 protein of thermophilic *T. elongatus* is far more stable than the His-Slr1694 protein of mesophilic *Synechocystis* sp. PCC 6803.

Chemical Reduction of Flavin—To gain insights into the accessibility of FAD to exogenous reductants, we measured the reduction of FAD with dithionite with or without a redox mediator. Generally, oxidized FAD shows an absorption peak around 450 nm, while reduced FAD shows little absorption in this region. As shown in Fig. 4, FAD of the His-Tll0078 protein was hardly reduced with dithionite alone or together with a mediator such as NADPH (1 mM), NADH (1 mM), ferredoxin from spinach (100 μg ml^{-1}), or dimethylbenzoquinone (50 μM). The hydrophobic mediator methylviologen (1 μM) accelerated the reduction of His-Tll0078 by dithionite (Fig. 4, left). On the other hand, FAD of the His-Slr1694 protein was appreciably reduced by dithionite with or without NADPH, NADH, ferredoxin or dimethylbenzoquinone (Fig. 4, right). Methylviologen accelerated the reduction by dithionite at a rate much faster than that for His-Tll0078. When dithionite was added to free FAD in solution, the reduction of FAD was too fast to be followed (data not shown). This suggests that FAD is embedded in a hydrophobic

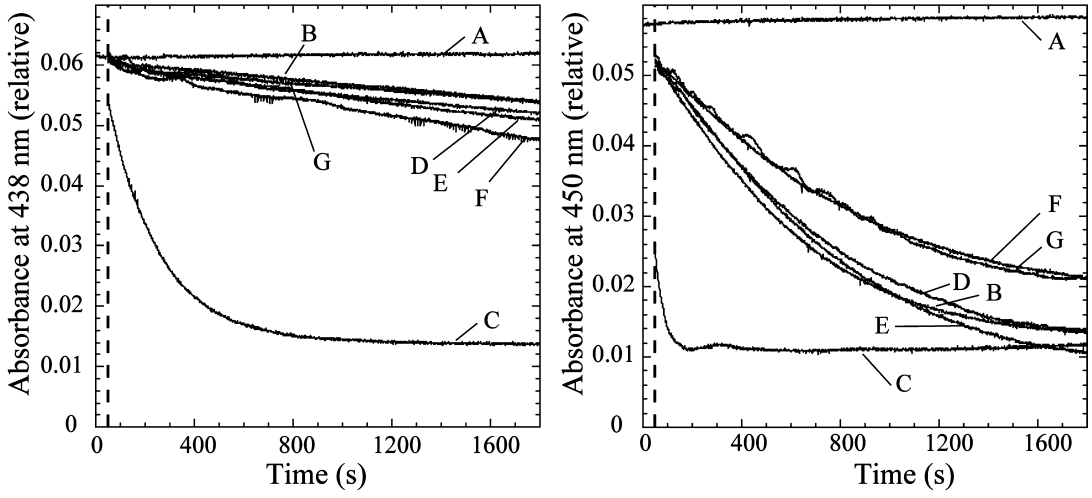

Fig. 4. **Reduction of the His-Tll0078 (left) and His-Slr1694 (right) proteins with dithionite in the presence of electron mediators.** A, no addition; B, dithionite; C, dithionite plus methylviologen (1 μM); D, dithionite plus NADPH (1 mM); E, dithionite plus NADH (1 mM); F, dithionite plus ferredoxin (100 μg ml^{-1}); G, dithionite plus dimethylbenzoquinone (50 μM). Data were collected after mixing with the addition of mineral oil (dashed line).

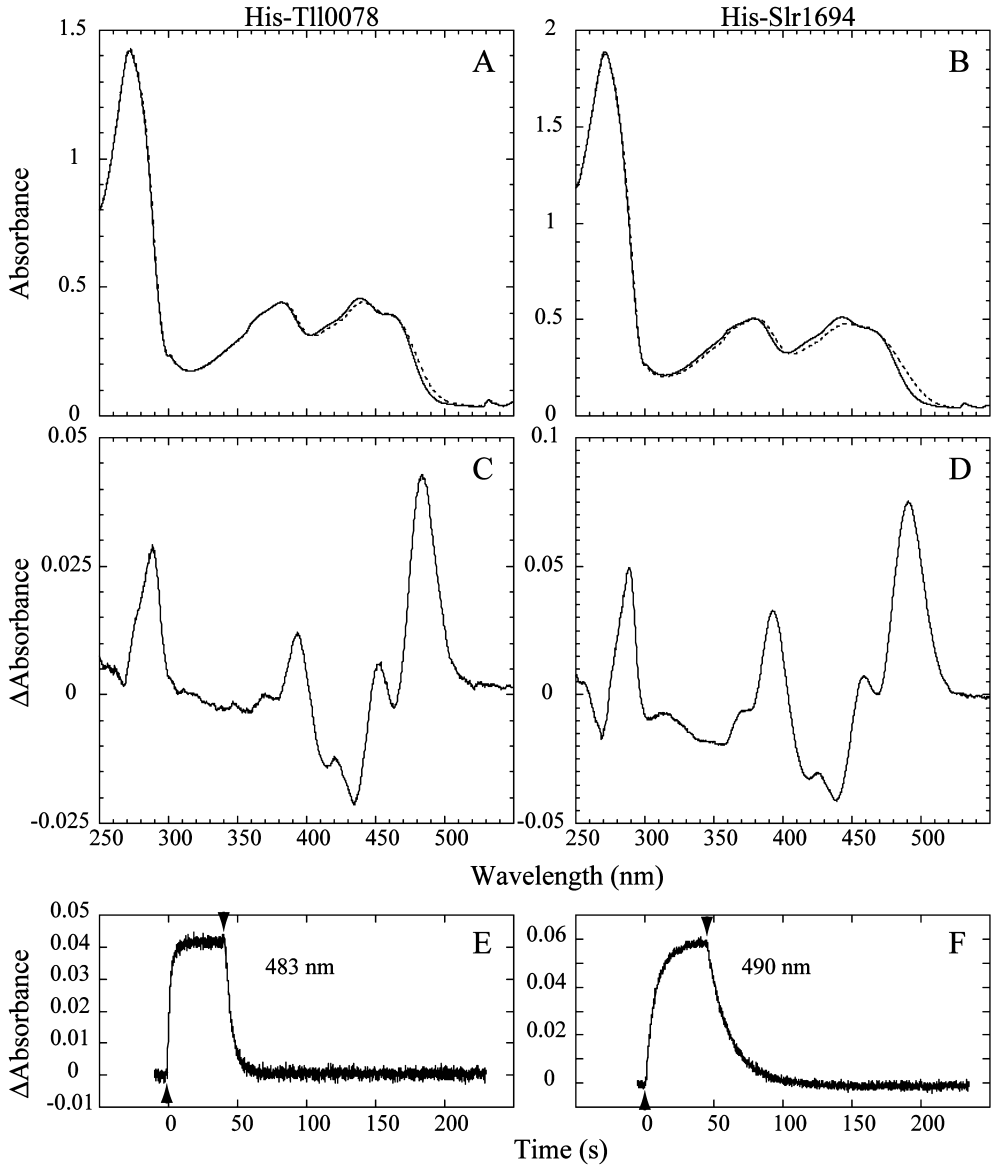

Fig. 5. **Absorption changes of the His-Tll0078 and His-Slr1694 proteins.** A, absorption spectra of His-Tll0078 dark-adapted (solid line) and excited with a xenon lamp (dashed line). B, absorption spectra of His-Slr1694 dark-adapted (solid line) and excited (dashed line). C and D, light minus dark difference spectra of His-Tll0078 and His-Slr1694. E and F, time courses of difference absorbance at 483 nm and 490 nm for His-Tll0078 and His-Slr1694. Upward arrowhead, light on; downward arrowhead, light off. The proteins used in this figure were cured by incubation for more than 3 days.

pocket of His-Tll0078 and even His-Slr1694, to which the hydrophilic reductant can not readily gain access. Further, the proteinous shield surrounding the flavin appears to be more stringent in Tll0078 than in Slr1694.

Spectroscopy—The absorption peaks of the dark-adapted His-Tll0078 protein and His-Slr1694 protein were located at 382, 439 and 460 nm (shoulder), and 379, 443 and 465 nm (shoulder), respectively (Fig. 5, A and B). The prominent shoulder on the lowest energy band suggests that flavin is located in a nonpolar environment (*39*). When excited with a xenon lamp, the spectra were clearly red shifted. The difference spectrum of His-Tll0078 showed positive peaks (394, 454 and 483 nm) and a negative peak (434 nm). Similarly, the difference spectrum of the His-Slr1694 protein showed positive peaks (393, 459 and 491 nm) and a negative peak (439 nm) (Fig. 5, C and D). The outline of the difference spectrum of His-Tll0078 was almost the same as that of His-Slr1694. Interestingly, the positive peak of His-Tll0078 below 400 nm was slightly longer than that of His-Slr1694, while the peaks between 450 nm and 500 nm were shorter than those of His-Slr1694 by 5–8 nm. These difference spectra are practically the same as that of the AppA of photosynthetic bacterium *Rhodobacter sphaeroides* (positive peaks at 387, 461 and 495 nm, and a negative peak at 442 nm) (*40*). This light-induced red shift and dark reversion was observed more than 10 times, this being indicative of fully reversible photocycles.

We measured the time course of the absorption changes of His-Tll0078 and His-Slr1694 at the peak positions in the difference spectra. Samples were incubated in the dark for at least 2 min and then illuminated with a xenon lamp. The absorbance at 483 nm and 490 nm increased rapidly upon illumination and decreased instantaneously when the illumination was cut off. The half times of this dark decay of His-Tll0078 and His-Slr1694 were calculated to be ~3.5 and ~11 s, respectively. The decay rate of Tll0078 was about 3-fold faster than that of Slr1694 at room temperature (Fig. 5, E and F).

We also found that the His-Tll0078 protein showed aggregation reproducibly, when freshly prepared samples

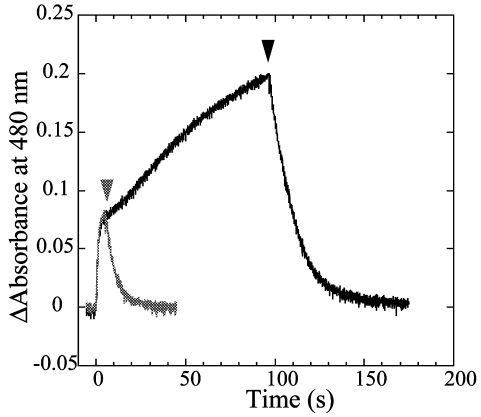

Fig. 6. **Absorption changes of freshly prepared His-Tll0078 protein upon illumination for ~5 s (gray line) and ~90 s (black line).** Downward arrowhead, light off.

were illuminated for a prolonged time (Fig. 6). The difference spectrum after prolonged illumination (90 s) showed a red-shift and an absorbance increase at a broad wavelength, indicative of light scattering (data not shown). This scattering quickly reverted to the original level in the subsequent dark (Fig. 6). Such light-dependent scattering seems to reflect conformational changes of His-Tll0078, which were generated from the excited state. It should be noted that the induction of scattering during prolonged illumination was not observed for aged preparations of His-Tll0078, which were kept on ice in the dark for longer than 3 days. It should be noted that the spectral data shown in Fig. 5 were obtained for cured preparations.

Phototaxis Assay—The phototactic motility of a mutant of *Synechocystis* sp. PCC 6803 was evaluated as the movement of colonies on a soft agar plate with lateral illumination with monochromatic light. The parent strain (PCC-P) used for gene disruption reproducibly

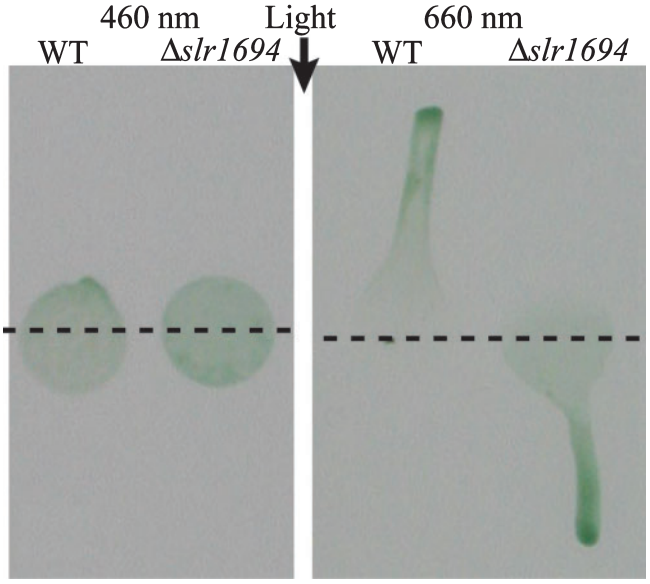

Fig. 7. **Phototactic movements of colonies of the *slr1694* gene–disrupted mutant (Δ*slr1694*) and its parent strain, PCC-P.** 1 μl of each cell suspension was spotted and grown for 44 h under lateral illumination (arrow) at 460 nm (left) and 660 nm (right). The dotted line shows the initial position of inoculation before the illumination.

moved toward the light source from 500 to 700 nm (positive phototaxis). The *slr1694*-disruptant showed negative phototaxis away from the light source, while its motility was almost the same as that of the parent strain (Fig. 7). On the other hand, both the wild type and the disruptant showed very little phototaxis toward the light source of 460 nm. We confirmed that all independent transformants tested showed the same negative phototaxis. This suggests that *slr1694* encodes a factor essential for positive phototaxis.

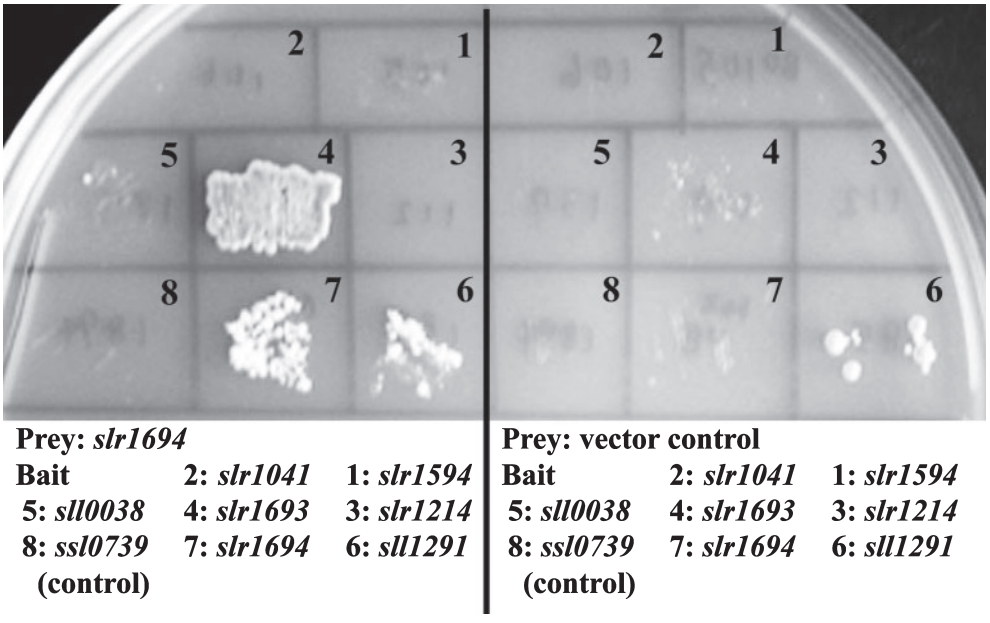

Fig. 8. **Yeast two-hybrid analysis of the prey (*slr1694*) and baits (*pat*A-like response regulator genes and *slr1694*).** A bait clone carrying *ssl0739* was used as a negative control. Cells were grown with 10 mM 3-aminotriazole.

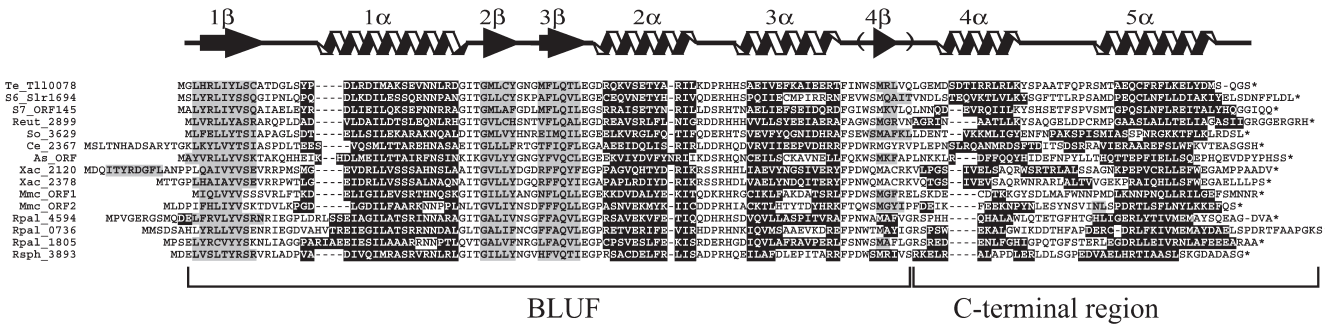

Fig. 9. **Sequence alignment and 2D structure prediction of "short" BLUF proteins with the 3D-PSSM program.** Te, *T. elongatus* BP-1; S6, *S.* sp. PCC 6803; S7, *Synechococcus* sp. PCC 7002; Reut, *Ralstonia metallidurans*; So, *Shewanella oneidensis* MR-1; Ce, *Corynebacterium efficiens* YS-314; As, *Acinetobacter* sp. ADP-1; Xac, *Xanthomonas axonopodis* pv. citri str. 30 ; Mmc, *Magnetococcus* sp. MC-1; Rpal, *Rhodopseudomonas palustris*; Rsph, *Rhodobacter sphaeroides*. The black box is α-helix and the gray box β-sheet.

Yeast Two-Hybrid Screening—To follow the signal transduction pathway from Slr1694 to phototaxis, we performed yeast two-hybrid screening with full-length *slr1694* as a bait. From a genomic prey library, we obtained several clones carrying either *slr1694* itself or *slr1693* (data not shown). Although *slr1693* is located upstream of *slr1694*, clones carrying *slr1693* did not cover the coding region of *slr1694*. The *slr1693* gene codes for a PatA-like response regulator of the two-component regulatory system (*20*). In *Synechocystis*, there are six homologous *patA*-like genes in the genome. To confirm the specificity of the screening, we introduced the coding region of *slr1694* into the prey vector and then assayed it with bait clones carrying the full-length coding regions of the six *patA*-like genes and the *slr1694* gene. Again, *slr1693* as well as *slr1694* gave positive results (Fig. 8). The self-interaction of Slr1694 supports the homo-oligomeric structure, as shown on gel filtration chromatography (Fig. 2). The interaction of the Slr1694 and Slr1693 proteins, which was confirmed in both directions, strongly suggests a possible signal transduction pathway as discussed below.

DISCUSSION

As found on genome analysis, some cyanobacteria have a gene for a BLUF protein. They are categorized as "short" BLUF proteins, as they do not carry any discernible signal-output domain such as the adenylyl cyclase domain in *Euglena* PAC proteins. It is known that free flavin is responsive to light and redox changes. We examined the accessibility of various reductants and mediators to FAD bound to Tll0078 and Slr1694. The poor reactivity of the flavin with various reductants other than methylviologen suggested that FAD is embedded in a hydrophobic pocket of the proteins and is thus well shielded from the aqueous environment. We also demonstrated that the BLUF protein from a thermophilic organism (Tll0078) is far more stable than that from a mesophilic one (Slr1694), as revealed by the heat-induced fluorescence of the flavin. Consistently, Tll0078 showed less leaky reaction to reductants than Slr1694. Based on this, we chose Tll0078 for crystallization and determined its 3D structure (*32*).

Both cyanobacterial BLUF proteins showed a light-induced spectral shift of the flavin absorption to a longer wavelength, which is similar to that of AppA. The light-induced spectral shift of the flavin of AppA upon illumination has been accounted for as the occurrence of a π-π stacking interaction between the isoalloxazine ring of FAD and a Tyr residue, hydrogen bonding interaction between C(4)=O of the ring and unknown residue(s), and/or deprotonation of the ring (*12*, *30*, *40*, *41*). Spectroscopic analysis of Tll0078 at liquid helium temperature revealed light-induced accumulation of a primary intermediate, whose peaks shifted to approximately half of the regular shift to a longer wavelength (*29*). During subsequent warming in the dark above 50 K, the intermediate was converted to the fully shifted form. Nevertheless, nothing is known about the signaling mechanism for the Tll0078 or Slr1694 protein. It should be noted that the His-Tll078 preparation showed slow aggregation in an aqueous solution upon prolonged illumination (Fig. 6). Moreover, the reversion in the subsequent dark was rather fast and fully reversible. These events indicate light-induced reversible changes in the conformation or surface charge of the protein, which may be linked with the signaling process. Detailed monitoring of the changes in the protein structure will be needed to elucidate the signaling mechanism. Tll0078 and Slr1694 have a unique short extension in the C-terminal region. Although this region shows marginal sequence homology with the C-terminal extension of other "short" BLUF proteins from other than cyanobacteria, the length of the extension is 40–50 residues in common. Moreover, prediction of the secondary structure by 3D-PSSM (*42*) revealed that the C-terminal region of "short" BLUF proteins consists of two consecutive α-helices with similar arrangements (Fig. 9).

Here we demonstrated that the *slr1694* gene is essential for positive phototaxis in *Synechocystis* sp. PCC 6803. It has been shown that *Synechocystis* cells exhibit twitching motility via thick pili on the cell surface (*35*, *43–45*), and move toward a light source (positive phototaxis) depending on the light intensity or quality (*33*, *46–48*). Previously, we reported that disruption of the phytochrome-like *pixJ1* gene resulted in a phenotype of negative phototaxis (*33*). Thus, the change from a positive to a negative direction of motility appears to be shared with disruption of the flavin sensor gene *slr1694* and phytochrome-like sensor gene *pixJ1*. FAD of Slr1694 perceives blue light. Recently, the PixJ1 product, which was isolated from *Synechocystis* cells, showed novel reversible photoconversion between the 435 nm–absorbing Pb and 535 nm–absorbing Pg forms (*49*). Based on spontaneous

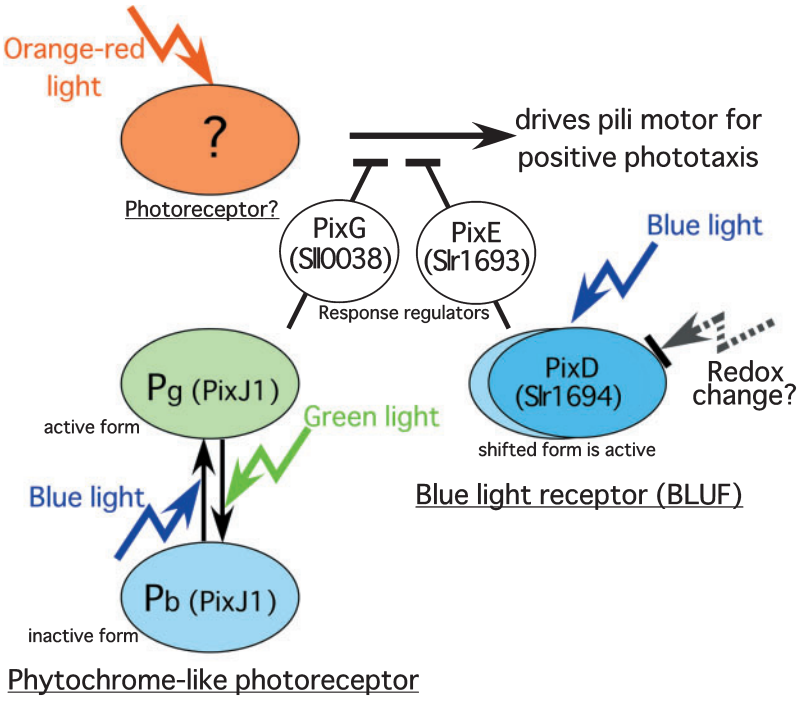

Fig. 10. **Schematic diagram of the light-induced signaling pathways involved in positive phototaxis in *Synechocystis* sp. PCC 6803.** The spectral shifted form of Slr1694 (designated as PixD) is illustrated as a slightly shifted blue circle. Note that a putative orange-red photoreceptor for positive phototaxis has not yet been identified.

dark reversion from Pg to Pb, we suggested that Pb is the blue light sensor and light-activated Pg is the active form that suppresses positive phototaxis like Slr1694. Finally, a *patA*-like gene, *pixG*, which is located upstream in the *pixJ1* gene cluster, is essential for positive phototaxis (*33*). Another PatA-like Slr1693 interacts specifically with Slr1694, as shown on yeast two-hybrid screening (Fig. 8). Based on these parallel lines of evidence, we propose a model for multiple light-signaling pathways in the regulation of positive phototaxis (Fig. 10). Here, we use the designation *slr1694 pixD*, since it is essential for positive phototaxis. We also provisionally use the designation *slr1693 pixE*.

In this model, an unknown photoreceptor is hypothesized to perceive red to orange light, toward which cells move. This is based on the results of spectrograph analysis of phototaxis. Namely, monochromatic light of 500–700 nm was effective, while light below 500 nm was ineffective for driving the positive phototaxis of *Synechocystis*. By contrast, disruptants of *pixD* (*slr1694*) and *pixJ1* showed negative phototaxis toward monochromatic light of 500–700 nm. A part of the results is presented in Fig. 7. These results strongly suggested that the two blue light sensors (PixD and PixJ1) are a kind of master switch between positive and negative phototaxis. Since from blue to UV-A light is harmful to cyanobacteria as well as other organisms (*50*), it is tempting to speculate that the blue light sensing systems are engaged in switching on the negative phototaxis to avoid photoinhibition even though photosynthetic light is available. At present, we do not have a good explanation for why *Synechocystis* has two different blue light sensing systems. A major difference between PixD and PixJ1 is in the absorption peaks: PixD absorbs 380 and 440 nm, while the Pb form of PixJ1 absorbs 435 nm. Further, flavin of PixD may respond to redox changes in addition to blue light, although we could not detect quick responses to hydrophilic reductants in contrast with light excitation.

At present, nothing is known about the physiological role of Tll0078. Regarding the phototactic motility of *T. elongatus*, contradictory facts have been reported to date. Kondou *et al.* (2001) reported that thermophilic *Synechococcus elongatus* BP-1 (equivalent to *T. elongatus* BP-1) shows positive phototaxis toward light at 500 to 750 nm (*51*). However, the complete genome of *T. elongatus* BP-1 revealed that *pilC*, which is essential for the pili-dependent motility of *Synechocystis*, is inactivated by a frameshift mutation (*21*, *52*). We confirmed the non-motile phenotype of *T. elongatus* carrying frameshifted *pilC* and the motile phenotype of a closely related species, *T. vulcanus* RKN, which carries non-interrupted *pilC* (Shingai, T., and Ikeuchi, M., unpublished results). We are now performing mutational analysis of *tll0078* in a motile strain of *T. vulcanus* RKN.

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