Phototaxis is a phenomenon where cyanobacteria move toward a light source. Previous studies have shown that the blue-light-using-flavin (BLUF)-type photoreceptor PixD and the response regulator-like protein PixE control the phototaxis in the cyanobacterium *Synechocystis* sp. PCC 6803. The *pixD*-null mutant moves away from light, whereas WT, *pixE* mutant, and *pixD pixE* double mutant move toward the light. This indicates that PixE functions downstream of PixD and influences the direction of movement. However, it is still unclear how the light signal received by PixD is transmitted to PixE, and then subsequently transmitted to the type IV pil motor mechanism. Here, we investigated intracellular localization and oligomerization of PixD and PixE to elucidate mechanisms of phototaxis regulation. Blue-native PAGE analysis, coupled with western blotting, indicated that most PixD exist as a dimer in soluble fractions, whereas PixE localized in ~250 kDa and ~450 kDa protein complexes in membrane fractions. When blue-native PAGE was performed after illuminating the membrane fractions with blue light, PixE levels in the ~250 kDa and ~450 kDa complexes were reduced and increased, respectively. These results suggest that PixE, localized in the ~450 kDa complex, controls activity of the motor ATPase PilB1 to regulate pilus motility.

**Key Words:** BLUF; cyanobacteria; photoreceptor; phototaxis; PixD; PixE

**Introduction**

Cyanobacteria have various responses to external light stimulation that include balancing chromatic adaptation, cell aggregation, and phototaxis (Bhaya, 2016; Hirose et al., 2010; Kehoe, 2010; Parnasa et al., 2016; Schuergers et al., 2017). The cyanobacterial phototaxis has been well studied in *Synechocystis* sp. PCC 6803 to elucidate whether it moves towards the light (positive phototaxis) or away from light (negative phototaxis) by the type IV pili-dependent twitching motility (Bhaya, 2004; Schuergers et al., 2017; Yoshihara and Ikeuchi, 2004). *Synechocystis* phototaxis is controlled by many photoreceptors that absorb different wavelengths of light (Choi et al., 1999). Among the photoreceptors, the blue-light receptor PixD was shown to elicit critical roles for controlling phototaxis (Masuda and Ono, 2004; Okajima et al., 2005). PixD is a ~17 kDa protein that has the blue-light-using-flavin (BLUF) domain (Fujisawa and Masuda, 2018; Gomelsky and Klug, 2002; Masuda et al., 2004). The BLUF domain binds a flavin as a chromophore, and it induces structural changes when it is excited by blue light. BLUF domain-containing proteins have been identified in many microorganisms, including AppA found in the purple bacterium *Rhodobacter sphaeroides* (Masuda and Bauer, 2002) and photoactivatable adenylyl cyclase (PAC) found in *Euglena* (Iseki et al., 2002). Both AppA and PAC have signal transduction domain modules, whereas PixD has no additional domain other than BLUF. Thus, it can be postulated that PixD, through protein-protein interactions, transmits a light signal downstream (Fujisawa and Masuda, 2018; Masuda, 2013). Previous pull-down and yeast-two-hybrid analysis identified the PixD-interaction-protein PixE (Masuda et al., 2008, 2013; Okajima et al., 2005; Sato et al., 2007; Yuan and Bauer, 2008). PixE has a similarity in its amino-acid sequence to those of CheY-like response regulators; however, PixE does not conserve the amino acids that are phosphorylated in CheY response (Ren et al., 2013). Biochemical analysis indicated that PixD and PixE form a PixD<sub>10</sub>·PixE<sub>4</sub> (or PixD<sub>10</sub>·PixE<sub>5</sub>) complex *in vitro* in which four (or five) monomeric PixE interact with a PixD decamer (Ren et al., 2013; Yuan and Bauer, 2008).

*Corresponding author: Shinji Masuda, Department of Life Science and Technology, Tokyo Institute of Technology, Yokohama 226-8501, Japan. E-mail: shmasuda@bio.titech.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.
In addition, the microscopic analysis indicated that PixE partially co-localizes with the motor ATPase PilB1 in regions close to the cytoplasmic membranes (Jakob et al., 2020). These results suggest that the formation and dissociation of the PixD-PixE complex are controlled by intercellular blue light, and PixD-originated signals are ultimately transmitted to PilB1 that controls the behavior of the type IV pili and directs it to move towards or away from light. However, the in vivo status of the PixD-PixE complex has not been tested, and the mechanism of co-localization of PixE and PilB1 remains unknown.

In this study, we attempt to further elucidate the molecular mechanism of phototaxis regulation by PixD and PixE by analyzing the in vivo localization and oligomerization status of two proteins. Henceforth, based on the obtained data, we propose a schematic model of the action of PixD and PixE.

Materials and Methods

Growth conditions. All experiments were performed with the Synechocystis sp. PCC 6803 strain PCC-P (Yoshihara et al., 2000). Synechocystis pixD mutant (∆pixD), pixE mutant (∆pixE), pixD-pixE double mutant (∆pixDE), and the FLAG-tagged-PixE expressing strain (PixE-FLAG) that were constructed previously (Jakob et al., 2020; Masuda and Ono, 2004; Sugimoto et al., 2017). These strains were grown in BG11 liquid medium until the mid-log phase were spotted on a 0.8% agar-containing BG11 plate. Spot diameters were measured using a BioRad (BioRad develops, USA) type II spectrophotometer with a 470 nm wavelength detector.

Blue-native PAGE and SDS-PAGE. Blue-native PAGE was performed by the method of Sugimoto et al. (2017) with slight modifications. Cells that were cultured in the BG11 liquid medium until the mid-log phase were centrifuged at 1,200 × g for 10 min at room temperature under dim-light conditions. Each culture was centrifuged at 1.6 mg ml⁻¹. The pellet was washed twice with 50 mM Tris/HCl (pH 7.5), 2 mM NaCl, 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 4% (w/v) SDS. After boiling for 5 min, samples were placed in 15% acrylamide SDS-PAGE with constant current (40 mA).

Blue-native PAGE followed by SDS-PAGE analysis was performed as follows. Each soluble and membrane sample (prepared as mentioned above) was split into two aliquots. One of the aliquots was kept in the dark, and the other aliquot was illuminated with strong blue-light (~250 μmol m⁻² s⁻¹) provided by light-emitting diodes (λmax = 472 nm, full width at half-maximum 30 nm; MIL-B18, Sanyo) for 10 min. 7.5 μl of each soluble fraction was then mixed with 2.5 μl of NativePAGE 4X Sample Buffer (ThermoFisher) and directly applied to blue-native PAGE. For membrane samples, 0.002% (v/v) (final concentration) Triton X 100 was added with the dark-incubated or blue-light-illuminated membrane preparations (50 μg ml⁻¹ of chlorophyll a), and membrane-bound proteins were solubilized in the dark at 4°C for 10 min. The mixture was centrifuged at 17,700 × g for 5 min at 4°C. 0.005% (v/v) (final concentration) CBB G250 (Wako), and 1/4 volume of Invitrogen NativePAGE 4X Sample Buffer was added. Blue-native PAGE was performed with the Novex NativePAGE 4–16% Bis-Tris Gel (ThermoFisher) with constant voltage (150 V) for 90 min. After electrophoresis, the gel was cut for each lane, immersed in the 1X SDS-PAGE sample buffer, incubated for 10 min at room temperature, and then warmed in a microwave oven for 15 s followed by incubation at room temperature for 15 min. The gel was set onto a 15% acrylamide SDS-PAGE gel, and a warmed 1% (w/v) agarose solution was poured onto the gel to seal the spaces. Electrophoresis was performed using a constant current (30 mA).

Western blotting. An acrylamide gel was cut into a suitable size. An appropriate size of the PVDF membrane was exposed to methanol followed by incubation with the Blotting buffer containing 1.2% (w/v) Tris, 1.44% (w/v) glycine, and 20% (v/v) methanol. The PVDF membrane and acrylamide gel were sandwiched with a pair of five pieces of filter paper (thickness: 3 mm), and protein blotting was performed at a constant current with the acrylamide gel area (cm²) × 2 mA for 1 h. Then, the PVDF membrane was immersed in the Blocking buffer containing 5% skim milk, 20 mM Tris/HCl (pH 8.0), and 8.8% (w/v) NaCl, and gently shaken at 4°C overnight. The blocked PVDF membrane was immersed in the Blocking buffer containing 1/1,000 volume of the primary antibodies, anti-PixD (Jakob et al., 2020), or anti-FLAG M2 (Sigma), and further incubated for 1 h at room temperature. After the primary antibody treatment, the PVDF membrane was washed five times with TBST buffer containing 20 mM Tris/HCl buffer.
SUGIMOTO and MASUDA

SUGIMOTO and MASUDA

(pH 8.0), 8.8% (w/v) NaCl and 0.05% (v/v) Tween 20 and then immersed in the TBST buffer containing 1/10,000 volume of the anti-Rabbit HRP (Funakoshi) for PixD detection. Alternatively, it was immersed in the TBST buffer containing 1/100 volume of the anti-Mouse HRP (Funakoshi) for PixE-FLAG detection for 1 h at room temperature. The membrane was washed three times with TBST, and luminescence was detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Results

We employed western blotting to characterize the intracellular localization and oligomerization of PixD and PixE in Synechocystis. We prepared the PixD-specific antibody (Jakob et al., 2020); however, the PixE-specific antibody was not available. To detect PixE in vivo, we constructed the Synechocystis strain expressing FLAG-tagged PixE by its native promoter (designated here PixE-FLAG). Given the recombinant gene encoding PixE-FLAG was integrated into the chromosome (Fig. 1A), native PixE did not exist in the PixE-FLAG strain. We first checked the phototaxis response of the PixE-FLAG strain. As shown in Fig. 1B, the PixE-FLAG showed positive phototaxis akin to the WT, indicating that FLAG-tagged PixE could function as a native PixE. Notably, the pixE mutant (ΔpixE) and pixD-pixE double mutant (ΔpixDE) showed strong positive phototaxis; whereas, the pixD mutant (ΔpixD) showed negative phototaxis (Fig. 1B). These results confirmed the previous indication that PixE inhibits positive phototaxis, and dark-adapted PixD negates the PixE function (Sugimoto et al., 2017).

Next, we performed western blotting with isolated soluble and membrane fractions of WT, ΔpixD, and PixE-FLAG strains after SDS-PAGE. When western blotting was performed with the PixD-specific antibody, a ~15 kDa band was detected in WT and PixE-FLAG; however, this was not seen in ΔpixD (Fig. 2A). Given the molecular mass of PixD is 17 kDa, the detected band could be assigned to PixD. PixD was found mostly in the soluble fraction. A ~16 kDa non-specific band was detected in membrane fractions of WT, ΔpixD and PixE-FLAG, which was overlapped with the PixD band in WT and PixE-FLAG, indicating that small amount of PixD was localized in the membrane fraction. The level of PixD in PixE-FLAG was smaller than that in WT, although the phototaxis behavior of WT and PixE-FLAG were similar (Fig. 1).

When we performed the western blotting with the FLAG-specific antibody, a band (~40 kDa) was detected in the PixE-FLAG; however, this was not seen in WT or ΔpixD (Fig. 2A). Given the molecular mass of FLAG-tagged PixE, deduced from its amino acid sequence, is 43 kDa, the detected band could be assigned to FLAG-tagged PixE. The FLAG-tagged PixE was found mostly in the membrane fraction. To confirm whether PixE-FLAG is associated with or integrated into the membranes, we treated the isolated membranes with 0.1 M Na2CO3 and then used for western blotting.

(a) Schematic representation of the construction of the pixD-pixE double mutant (ΔpixDE) and the strain expressing FLAG-tagged PixE (PixE-FLAG). Ω Sp/Sm', spectinomycin/streptomycin-resistance gene cassette with the transcription/translation-termination Ω-motif. B. Phototaxis behavior of the WT, PixE-FLAG, pixD mutant (ΔpixD), pixE mutant (ΔpixE), and ΔpixDE. Cells were spotted onto a plate containing solidified 0.8% agar infused with BG11 medium. The plate was illuminated laterally with light provided by a fluorescent lamp, as illustrated.

(b) Soluble (S) and membrane (M) fractions, isolated from WT, ΔpixD, and PixE-FLAG, were applied to SDS-PAGE followed by western blotting with specific antibodies for PixD and FLAG-tag. An asterisk indicates a non-specific band. B. Membrane fractions were washed with 0.1 M Na2CO3 and then used for western blotting.

Fig. 2. Localization of PixD and PixE.

A. Soluble (S) and membrane (M) fractions, isolated from WT, ΔpixD, and PixE-FLAG, were applied to SDS-PAGE followed by western blotting with specific antibodies for PixD and FLAG-tag. An asterisk indicates a non-specific band. B. Membrane fractions were washed with 0.1 M Na2CO3 and then used for western blotting.

Fig. 1. Construction and phenotype of the strain PixE-FLAG.

A. Schematic representation of the construction of the pixD-pixE double mutant (ΔpixDE) and the strain expressing FLAG-tagged PixE (PixE-FLAG). Ω Sp/Sm’, spectinomycin/streptomycin-resistance gene cassette with the transcription/translation-termination Ω-motif. B. Phototaxis behavior of the WT, PixE-FLAG, pixD mutant (ΔpixD), pixE mutant (ΔpixE), and ΔpixDE. Cells were spotted onto a plate containing solidified 0.8% agar infused with BG11 medium. The plate was illuminated laterally with light provided by a fluorescent lamp, as illustrated.
In vivo localization of PixD and PixE (Shimada et al., 2007). After washing the membrane fraction with Na₂CO₃, the FLAG-tagged PixE was then seen in the soluble fraction (Fig. 2B), indicating that FLAG-tagged PixE was associated with, however not integrated, into the membranes.

Next, we analyzed the oligomerization status of PixD and PixE in vivo. To elucidate the blue-light effects on oligomerization, isolated soluble and membrane fractions were illuminated by blue-light, and then PixD and/or PixE complexes were separated by blue-native PAGE. Each lane of the blue-native PAGE gel was cut out and used for SDS-PAGE, followed by western blotting to analyze the protein composition of each complex. As shown in Fig. 3A, western blotting with PixD-antibody showed several spots at ~40 kDa on the SDS-PAGE gel (Fig. 3B), which could be assigned to FLAG-tagged PixE. The three spots (I, II, and III) corresponded to ~40, ~250, and ~450 kDa, respectively, in the blue-native PAGE gel. The signals of the ~40 and ~450 kDa bands were increased, and the signal of the ~250 kDa band was decreased when the membrane fractions were illuminated with blue light before the blue-native PAGE (Fig. 3B). The PixD signal was not detected regardless of the light/dark conditions (data not shown), although small levels of PixE-FLAG were observed in the soluble fraction before blue-native PAGE (Fig. 2). Perhaps, PixE-FLAG forms multiple conformations in the soluble region, which were diffused on the blue-native PAGE gel and could not be detected by western blotting.

Next, we checked the oligomerization status of PixD and PixE in membrane fractions. Proteins in the membrane fractions were solubilized by Triton X100 and used for blue-native PAGE, followed by SDS-PAGE, as previously. We chose Triton X100 to solubilize protein complexes, since it has been used for isolation of membrane-associated protein-complexes such as phycobilisomes in cyanobacteria (Gantt, 1980). Western blotting with FLAG-antibody showed several spots at ~40 kDa on the SDS-PAGE gel (Fig. 3B), which could be assigned to FLAG-tagged PixE. The three spots (I, II, and III) corresponded to ~40, ~250, and ~450 kDa, respectively, in the blue-native PAGE gel. The signals of the ~40 and ~450 kDa bands were increased, and the signal of the ~250 kDa band was decreased when the membrane fractions were illuminated with blue light before the blue-native PAGE (Fig. 3B). The PixD signal was not detected regardless of the light/dark conditions (data not shown), although low levels of PixD were still observed in the membrane fractions (Fig. 2). Perhaps, PixD forms multiple conformations in the membrane fractions, which were diffused on the blue-native PAGE gel and could not be detected by western blotting. Alternatively, membrane-associated PixD could not be isolated by Triton X100. The ~16 kDa non-specific protein (Fig. 2) was also not detected (data not shown) perhaps due to the low abundance.

Discussion

Based on the results obtained here and from previous studies, a schematic model of the action of PixD and PixE for controlling phototaxis has been constructed (Fig. 4). We here showed that most FLAG-tagged PixE associates with membrane fractions (Fig. 2), which is consistent with a previous study that showed that the fluorescent protein (mVenus)-fused PixE localizes in a specific site near the plasma membrane, as examined by fluorescent microscopy (Jakob et al., 2020). In the dark condition, PixE is in a
large ~250 kDa complex (Fig. 3B) and inactive to control positive phototaxis (Fig. 4). Upon blue-light illumination, the amount of the PixE in the ~450 kDa complex is increased (Fig. 3B, spot III). We showed that mVenus-fused PixE colocalized with the main motor ATPase PilB1 under light conditions (Jakob et al., 2020), which suggests that PixE in the ~450 kDa complex switch the direction of phototaxis from positive to negative through interaction with PilB1. Given the molecular mass of PilB1 monomer is 75 kDa, which may function as a hexamer (Mancl et al., 2016; McCallum et al., 2017), the largest ~450 kDa band detected on the blue-native PAGE (Fig. 3B, spot III) seems to contain PilB1 and PixE (Fig. 4). Although PixD is in equilibrium between a dimer and decamer in vitro (Ren et al., 2013), our in vivo localization analysis showed that PixD mostly exists in a dimer in soluble fractions, even in the dark (Fig. 3A). Given monomeric PixE promotes decamer formation of PixD in vitro (Yuan and Bauer, 2008), intracellular levels of monomeric PixE in a soluble region may not be large enough to form stable PixD-PixE complex. In other words, monomeric PixE, dissociated from the membrane complexes, could be rapidly captured by PixD to negate PixE function. Such high abundance of dimeric PixD in soluble regions may be required for fine-tuning the blue-light-dependent regulation of positive phototaxis. This model needs to be clarified in future to further understand the molecular mechanism of how a single cell of cyanobacteria can sense light direction.

Acknowledgments

This work was supported, in part, by MEXT/JSPS KAKENHI Grant Number 19K22148 and 19H04719 (to SM).

References

Bhaya, D. (2004) Light matters: Phototaxis and signal transduction in unicellular cyanobacteria. Mol. Microbiol., 53, 745–754.

Bhaya, D. (2016) In the limelight: Photoreceptors in cyanobacteria. Microbiol. Mol. Biol. Rev., 7, e00741-16.

Choi, J. S., Chung, Y. H., Moon, Y. J., Kim, C., Watanabe, M. et al. (1999) Photomovement of the gliding cyanobacterium Synechocystis sp. SCC 6803. Photochem. Photobiol., 70, 95–102.

Fujiwasa, T. and Masuda, S. (2018) Light-induced chromophore and protein responses and mechanical signal transduction of BLUF proteins. Biophys. Rev., 10, 327–337.

Gantt, E. (1980) Structure and function of phycobilisomes: Light harvesting pigment complexes in red and blue-green algae. Int. Rev. Cytol., 66, 45–80.

Gomelsky, M. and Klug, G. (2002) BLUF: a novel FAD-binding domain involved in sensory transduction in microorganisms. Trends Biochem. Sci., 27, 497–500.

Hirose, Y., Narikawa, R., Katayama, M., and Ikeuchi, M. (2010) Cyanobacteriochrome CcaS regulates phycocyanin accumulation in Nostoc punctiforme, a group II chromatic adapted. Proc. Natl. Acad. Sci. USA, 107, 8854–8859.

Iseki, M., Matsunaga, S., Murakami, A., Ohno, K., Shiga, K. et al. (2002) A blue-light-activated adenyl cyclase catalyzes photooxidation in Euglena gracilis. Nature, 415, 1047–1051.

Jakob, A., Nakamura, H., Kobayashi, A., Sugimoto, Y., Wilde, A. et al. (2020) The (PATAN)-CheY-like response regulator PixE interacts with the motor ATPase PilB1 to control negative phototaxis in the cyanobacterium Synechocystis sp. SCC 6803. Plant Cell Physiol., 61, 296–307.

Kehoe, D. M. (2010) Chromatic adaptation and the evolution of light color sensing in cyanobacteria. Proc. Natl. Acad. Sci. USA, 107, 9029–9030.

Mancl, J. M., Black, W. P., Robinson, H., Yang, Z., and Schubot, F. D. (2016) Crystal structure of a type IV pilus assembly ATPase: Insights into the molecular mechanism of PilB from Thermus thermophilus. Structure, 24, 1886–1897.

Masuda, S. (2013) Light detection and signal transduction in the BLUF photoreceptors. Plant Cell Physiol., 54, 171–179.

Masuda, S. and Bauer, C. E. (2002) AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in Rhodobacter sphaeroides. Cell, 110, 613–623.

Masuda, S. and Ono, T. (2004) Biochemical characterization of the major adenylcyclase, Cya1, in the cyanobacterium Synechocystis sp. SSC 6803. FEBs Lett., 577, 255–258.

Masuda, S., Hasegawa, K., Ishii, A., and Ono, T.-A. (2004) Light-induced structural changes in a putative blue-light receptor with a novel FAD binding fold sensor of blue-light using FAD (BLUF); Strl1694 of Synechocystis sp. SCC 6803. Biochemistry, 43, 5304–5313.

Masuda, S., Hasegawa, K., Ohta, H., and Ono, T. A. (2008) Crucial role in light signal transduction for the conserved Met93 of the BLUF protein PixD/Srl1694. Plant Cell Physiol., 49, 1600–1606.

Masuda, S., Nakatani, Y., Ren, S., and Tanaka, M. (2013) Blue-light-mediated transcriptional regulation of two species of cyanobacteria. J. Biochem., 137, 741–750.

Parnasa, R., Nagar, E., Sendersky, E., Reich, Z., Simkovsky, R. et al. (2016) Small secreted proteins enable biofilm development in the cyanobacterium Synechococcus elongatus. Sci. Rep., 6, 1–10.

Ren, S., Sato, R., Hasegawa, K., Ohta, H., and Masuda, S. (2013) A predicted structure for the PixD-PixE complex determined by homology modeling, docking simulations, and a mutagenesis study. Biochemistry, 52, 1272–1279.

Ren, S., Sugimoto, Y., Kobayashi, T., and Masuda, S. (2015) Cross-linking analysis reveals the putative dimeric structure of the cyanobacterial BLUF photoreceptor PixD. FEBs Lett., 589, 1879–1882.

Sato, S., Shimoda, Y., Muraki, A., Kohara, M., Nakamura, Y. et al. (2007) A large-scale protein protein interaction analysis in Synechocystis sp. SCC 6803. DNA Res., 14, 207–216.

Schugers, N., Mullineaux, C. W., and Wilde, A. (2017) Cyanobacteria in motion. Curr. Opin. Plant Biol., 37, 109–115.

Shimada, H., Mochizuki, M., Ogura, K., Froehlich, J. E., Osteryoung, K. W. et al. (2007) Arabidopsis cotyledon-specific chloroplast biogenesis factor CYO1 is a protein disulfide isomerase. Plant Cell, 19.

Sugimoto, Y., Nakamura, H., Ren, S., Hori, K., and Masuda, S. (2017) Genetics of the blue light-dependent signal cascade that controls phototaxis in the cyanobacterium Synechocystis sp. SCC 6803. Plant Cell Physiol., 58, 445–465.

Yoshisaki, S. and Ikeuchi, M. (2004) Phototactic motility in the unicellular cyanobacterium Synechocystis sp. SCC 6803. Photochem. Photobiol. Sci., 3, 512–518.

Yoshisaki, S., Suzuki, F., Fujita, H., Geng, X. X., and Ikeuchi, M. (2000) Novel putative photoreceptor and regulatory genes required for the positive phototactic movement of the unicellular motile cyanobacterium Synechocystis sp. SCC 6803. Plant Cell Physiol., 41, 1299–1304.

Yuan, H. and Bauer, C. E. (2008) PixE promotes dark oligomerization of the BLUF photoreceptor PixD. Proc. Natl. Acad. Sci. USA, 105, 11715–11719.