

## Full Paper

# *In vivo* localization and oligomerization of PixD and PixE for controlling phototaxis in the cyanobacterium *Synechocystis* sp. PCC 6803

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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 pili 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 adenyl 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).

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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 substrain PCC-P (Yoshihara et al., 2000). *Synechocystis pixD* mutant ( $\Delta$ PixD), *pixE* mutant ( $\Delta$ PixE), *pixD-pixE* double mutant ( $\Delta$ 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). The cells were grown in a BG11 medium under continuous irradiation with white fluorescent light ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at  $30^\circ\text{C}$ . Kanamycin (final concentration  $10 \mu\text{g ml}^{-1}$ ) or spectinomycin (final concentration  $8 \mu\text{g ml}^{-1}$ ) was used when appropriate. *Escherichia coli* DH5 $\alpha$  was used for the cloning procedure. *E. coli* was grown at  $37^\circ\text{C}$  in Luria Bertani medium. Kanamycin (final concentration  $50 \mu\text{g ml}^{-1}$ ) or spectinomycin (final concentration  $40 \mu\text{g ml}^{-1}$ ) when appropriate.

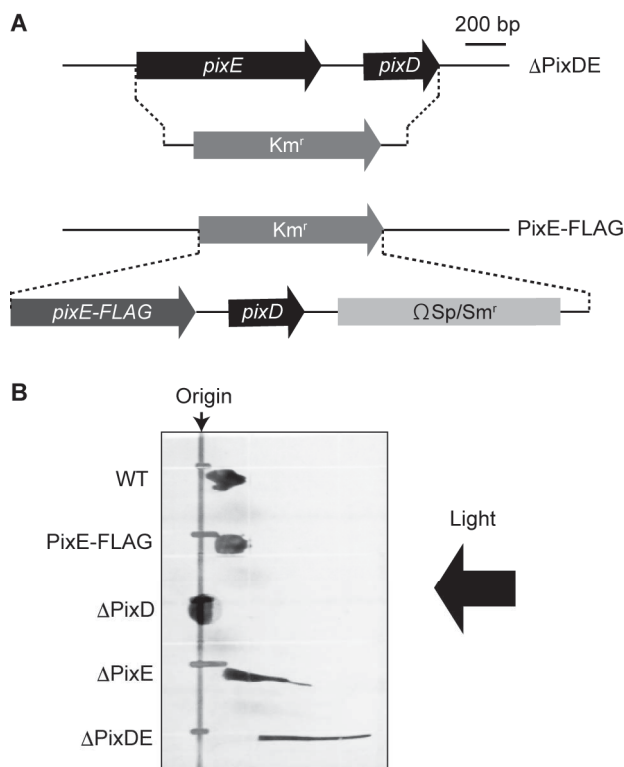
**Phototaxis assay.** Cells that were cultured in the BG11 liquid medium until the mid-log phase were spotted on a 0.8% agar-containing BG11 plate. Spot diameters were uniform at 5–6 mm. The plates were surrounded by a piece of black paper on the top, bottom, and side surfaces; only one of the side surfaces was cut off. The plates were cultured at  $30^\circ\text{C}$  by irradiating  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  of white fluorescent light from the side of the plate not covered with the black paper.

**Blue-native PAGE and SDS-PAGE.** *Synechocystis* strains were grown in the BG11 liquid medium until the mid-log phase, and 6 ml of each culture was centrifuged at  $1,200 \times g$  for 10 min at room temperature under dim-light conditions. The precipitate was suspended in 1 ml of the HEPES buffer containing 50 mM HEPES-KOH (pH7.5) and 10 mM  $\text{MgCl}_2$ . The suspension was centrifuged at room temperature at  $10,000 \times g$  for 30 s. The pellet was suspended in  $300 \mu\text{l}$  of the HEPES buffer, and 0.1 g of glass beads ( $150\text{--}252 \mu\text{m}$  in diameter) was added to the suspensions. Cells were crushed at  $4^\circ\text{C}$  for 1 h by shaking with a BugCrasher GM-01 (TAITEC). PMSF (final concentration 2 mM) was added, and the mixture was centrifuged at  $2,000 \times g$  for 5 min at  $4^\circ\text{C}$  to remove unbroken cells and beads. The supernatant was then centrifuged at  $17,700 \times g$  for 20 min at  $4^\circ\text{C}$ . The supernatant was used as the

soluble fraction, and the pellet was used as the membrane fraction. The protein concentration of each soluble fraction was determined by the Bradford assay kit (BioRad), and the concentration was adjusted to  $1.6 \text{ mg ml}^{-1}$ . The pellet was suspended in the HEPES buffer with an equal volume of respective supernatant. For SDS-PAGE, soluble and membrane fractions were mixed with an equal volume of the 2X SDS-PAGE sample buffer containing 0.25 M Tris/HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, 10% (v/v)  $\beta$ -mercaptoethanol and 0.01% (w/v) bromophenol blue. 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 (two-dimensional PAGE) was performed as follows. Each soluble and membrane sample (prepared as mentioned earlier) was split into two aliquots. One of the aliquots was kept in the dark, and the other aliquot was illuminated by strong blue-light ( $\sim 250 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) provided by light-emitting diodes ( $\lambda_{\text{max}} = 472 \text{ nm}$ , full width at half-maximum 30 nm; MIL-B18, Sanyo) for 1 min.  $7.5 \mu\text{l}$  of each soluble fraction was then mixed with  $2.5 \mu\text{l}$  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 mixed with the dark-incubated or blue-light-illuminated membrane preparations ( $50 \mu\text{g ml}^{-1}$  of chlorophyll *a*), and membrane-bound proteins were solubilized in the dark at  $4^\circ\text{C}$  for 10 min. The mixture was centrifuged at  $17,700 \times g$  for 5 min at  $4^\circ\text{C}$ . 0.0005% (w/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 ( $\text{cm}^2$ )  $\times$  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 shaking at  $4^\circ\text{C}$  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



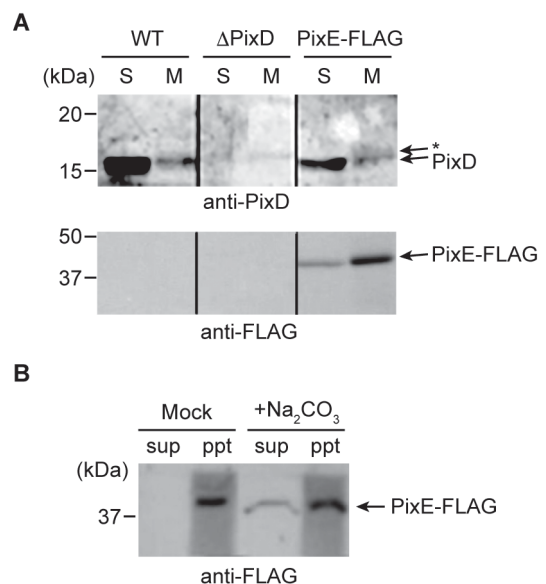
**Fig. 1.** Construction and phenotype of the strain PixE-FLAG.

A. Schematic representation of the construction of the *pixD-pixE* double mutant ( $\Delta$ PixDE) and the strain expressing FLAG-tagged PixE (PixE-FLAG).  $\Omega$ -Sp/Sm<sup>r</sup>, spectinomycin/streptomycin-resistance gene cassette with the transcription/translation-termination  $\Omega$ -motif. B. Phototaxis behavior of the WT, PixE-FLAG, *pixD* mutant ( $\Delta$ PixD), *pixE* mutant ( $\Delta$ PixE), and  $\Delta$ PixDE. 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.

(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



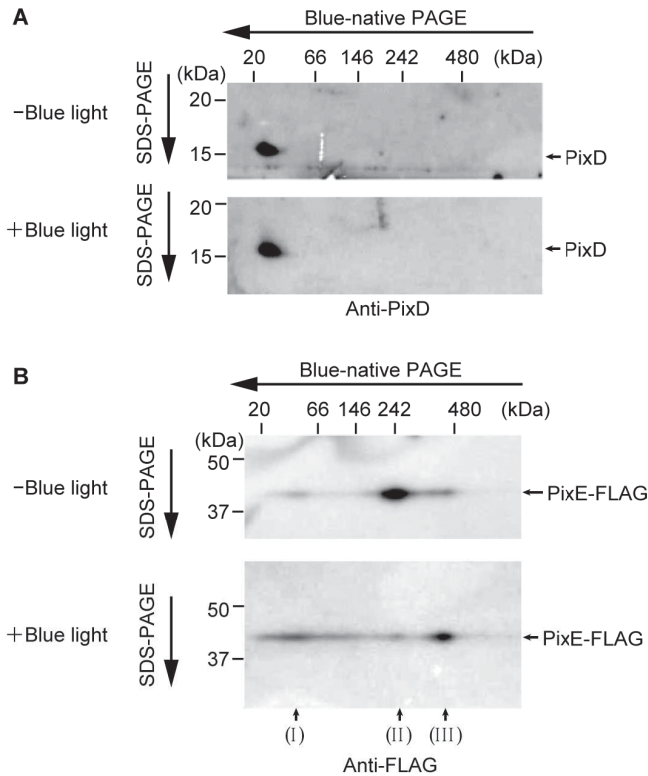
**Fig. 2.** Localization of PixD and PixE.

A. Soluble (S) and membrane (M) fractions, isolated from WT,  $\Delta$ PixD, 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 Na<sub>2</sub>CO<sub>3</sub> and then used for western blotting.

a native PixE. Notably, the *pixE* mutant ( $\Delta$ PixE) and *pixD-pixE* double mutant ( $\Delta$ PixDE) showed strong positive phototaxis; whereas, the *pixD* mutant ( $\Delta$ PixD) 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,  $\Delta$ PixD, 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  $\Delta$ PixD (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,  $\Delta$ PixD 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).

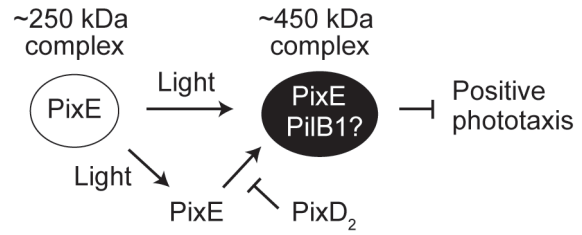
When we performed the western blotting with the FLAG-specific antibody, a band (~40 kDa) was detected in the PixE-FLAG; however, this was not seen in WT or  $\Delta$ PixD (Fig. 2A). Given the molecular mass of FLAG-tagged PixE, deduced from its amino acid sequence, is 43 kDa, the detected band could 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 Na<sub>2</sub>CO<sub>3</sub> by which proteins associated with the thylakoid membranes, such as ferredoxin-NADP<sup>+</sup> reductase, were detached from the



**Fig. 3.** Blue-native PAGE and SDS-PAGE analysis of PixD and PixE. Soluble (A) and membrane (B) fractions isolated from PixE-FLAG were kept in the dark (-Blue light) or illuminated with blue light ( $\lambda_{\max} = 472$  nm,  $\sim 250 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 1 min (+Blue light), and then applied to blue-native PAGE. The blue-native PAGE gel was cut out and applied to SDS-PAGE, followed by western blotting with anti-PixD (A) and anti-FLAG (B) antibodies. NativeMark (Invitrogen) was used as a size marker for blue-native PAGE, which contains apoferritin band 2 (480 kDa), B-phycoerythrin (242 kDa), lactate dehydrogenase (146 kDa), bovine serum albumin (66 kDa) and soybean trypsin inhibitor (20 kDa).

membranes (Shimada et al., 2007). After washing the membrane fraction with  $\text{Na}_2\text{CO}_3$ , 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 a 15 kDa band after SDS-PAGE that could be assigned to PixD. The PixD band corresponded to  $\sim 30$  kDa in the blue-native PAGE gel, suggesting that most PixD in the soluble fraction is in a dimeric form. Although PixD is in equilibrium between a dimer and decamer *in vitro* (Masuda et al., 2004; Ren et al., 2013, 2015; Yuan and Bauer, 2008), decameric PixD was not observed regardless of the light/dark conditions (Fig. 3A). We also performed the western blotting analysis in an attempt to detect FLAG-tagged PixE with anti-FLAG antibody; however, no specific bands for PixE-FLAG were detected after blue-native PAGE (data not



**Fig. 4.** Proposed model for the PixD and PixE action for controlling phototaxis.

Under dark conditions, PixE localizes in the  $\sim 250$  kDa complex in the membrane fraction. Upon blue-light excitation, PixE localizes in a large  $\sim 450$  kDa complex perhaps with PilB1 at a specific site of the plasma membrane to negatively control positive phototaxis. PixD dimer in soluble regions inhibits the  $\sim 450$  kDa complex formation of monomeric PixE.

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  $\sim 40$  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  $\sim 40$ ,  $\sim 250$ , and  $\sim 450$  kDa, respectively, in the blue-native PAGE gel. The signals of the  $\sim 40$  and  $\sim 450$  kDa bands were increased, and the signal of the  $\sim 250$  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  $\sim 16$  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.

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