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Cross-linking analysis reveals the putative dimer structure of the cyanobacterial BLUF photoreceptor PixD

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ABSTRACT

PixD is a blue light using flavin (BLUF)-type blue-light photoreceptor controlling phototaxis in the cyanobacterium *Synechocystis* sp. PCC6803. The crystal structure of PixD shows a decamer, although in solution an equilibrium is maintained between the dimer and decamer. Because the ratio of these two conformers is altered by illumination, the equilibrium state determines photosensitivity. However, no structural information is available for the PixD dimer. Here, we report a predicted structure for the dimer based on docking simulation, mutagenesis, and mass spectrometry-based cross-linking analyses. The results indicate the importance of the PixD C-terminus for dimer preference and photosensitivity.

1. Introduction

BLUF (sensor of blue light using flavin) is a small (~15kDa) flavin-binding domain that functions as a blue light-sensing module conserved in many microorganisms [1,2]. BLUF domain-containing photoreceptors control a wide variety of physiological activities, including gene expression, phototaxis response, motility, and biofilm formation [2]. BLUF-containing proteins and their downstream factors have been studied as models for understanding the molecular basis for light-induced signal transduction in cells. PixD is one of the BLUF-type photoreceptors found in the cyanobacterium *Synechocystis* sp. PCC6803 [3,4]. Although the wild-type *Synechocystis* moves toward a light source (positive phototaxis), a *pixD* mutant lacks such a phototactic response, indicating that PixD is necessary for positive phototaxis of this bacterium [3,4]. Genetic screening and biochemical analysis has indicated that PixD interacts with another protein, PixE, in a light-dependent manner [5,6]. Fig. S1 (Supplementary Material) shows the crystal structure of the PixD decamer and of the putative structure of the PixD–PixE complex. In the PixD crystal, two PixD pentameric rings stack face to face, forming a decamer [7]. Two monomeric PixE may bind to the surface of each ring, and so four PixE can bind the PixD decamer [8]. A complex comprising $PixD_{10}$–$PixE_5$ has also been suggested, although this stoichiometry has not been confirmed experimentally [6,9]. Upon excitation by light, the $PixD_{10}$–$PixE_4$ (or $PixD_{10}$–$PixE_5$) complex dissociates, leaving PixD dimers and PixE monomers [5,6,10]. PixE monomers inhibit positive phototaxis by an unknown mechanism. PixD itself is in equilibrium between dimer and decamer in solution [8,10], and PixE accelerates PixD decamer formation in the dark [6]. Indeed, the equilibrium state of the PixD dimer and decamer determines photosensitivity of PixD-dependent light-induced signal transduction. However, structural information is lacking for the PixD dimer structure.

To gain more insight on the PixD dimer, we performed docking simulation, mutagenesis, mass spectrometry (MS)-based cross-linking analysis of PixD. The results suggest the importance of the PixD C-terminus for the dimer formation.

2. Materials and methods

2.1. Cross-linking protocol

PixD was expressed in *Escherichia coli* and purified as described [11]. PixD (25 or 50 μM final concentration) was incubated for 2 h

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at room temperature with or without 10 mM 1-ethyl-3-[3-dimethy laminopropyl]-carbodiimide hydrochloride (EDC) (Thermo Scientific) in a buffer containing 25 mM MOPS/KOH (pH 7.2) and 1 mM NaCl. The proteins were then separated by SDS–PAGE with a 5–20% gradient polyacrylamide gel. Separated protein bands were excised form the gel and subjected to MS analysis as described below.

2.2. MS-based mapping

Trypsin-based in-gel digestion of SDS–PAGE gel slices was performed with a DigestPro96 instrument (M&S Instruments Inc.). Eluted samples (~15 μL each) were desalted with a ZipTip u-C18 pipette (Millipore, Bedford, MA, U.S.A.). Bound peptides were washed with 0.1% trifluoroacetic acid and then eluted with 0.1% trifluoroacetic acid containing 50% acetonitrile. MALDI mass spectra were obtained using a MALDI-time-of-flight mass spectrometer with Autoflex™-speed (Bruker). Spectra were acquired using the instrument in reflectron mode. A possible cross-linked peptide having MW 2842 (Fig. S2) was further subjected to MALDI-time -of-flight post-source-decay analysis. Each cross-linked position(s) was searched using the on-line tool Mascot (http://www.matrixscience.com/search_form_select.html).

2.3. Blue-native PAGE

The C-terminal-truncated version of PixD (seven amino acid residues deleted from the C-terminus) was expressed in *E. coli*. For constructing the mutant expression plasmid, PCR was carried out using pTYslr1694 (expresses wild-type PixD) [11] as template and the primer pair: 5′-AGTTGTCCTAAGAATTCCTCGAGCCCGGGTG A-3′ and 5′-ATTCTTAGGACAACTCGTAAATCTTGGCAATG-3′. The amplified fragment was circularized with the In-Fusion HD Cloning kit (Clontech), and the resulting plasmid was named pTYslr1694Cdel. *E. coli* strain BL21(DE3) was transformed with pTYslr1694Cdel and used to express the C-terminal-truncated PixD. The truncated PixD was expressed and purified as described for wild-type PixD [11]. His-tagged PixE was expressed and purified as described [5] and used for blue-native PAGE.

Blue-native PAGE was performed using the NativePAGE Novex Bis-Tris Gel system (Invitrogen). Purified wild-type or C-terminal-truncated PixD (150 μM final concentration) was mixed with purified PixE (20 μM final concentration) in a buffer containing 10 mM Tris–HCl (pH 8.0) and 135 mM NaCl for 15 min at room temperature. The mixtures were then subjected to blue-native PAGE following the manufacturer's instructions.

2.4. Docking simulation

Coordinate data for the PixD monomer structure were taken from the 1.8 Å resolution X-ray structure of the PixD decamer (PDB entry 2HFN). Water molecules or ions included in the PixD decamer structure were removed before docking. A PixD monomer was the docked to another PixD monomer using ZDOCK [12].

3. Results and discussion

For cross-linking analysis, purified PixD was treated with 1-et hyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC), which activates carboxyl groups for spontaneous reaction with primary amines, generating amide bonds between Lys and acidic residues (Asp or Glu) that come into close proximity [13]. Fig. 1A shows the SDS–PAGE profiles of EDC-treated and untreated PixD. Three bands were detected for EDC-treated PixD; band 1 (~30kDa) band 2 (~17kDa), and band 3 (~14kDa). Band 2 was

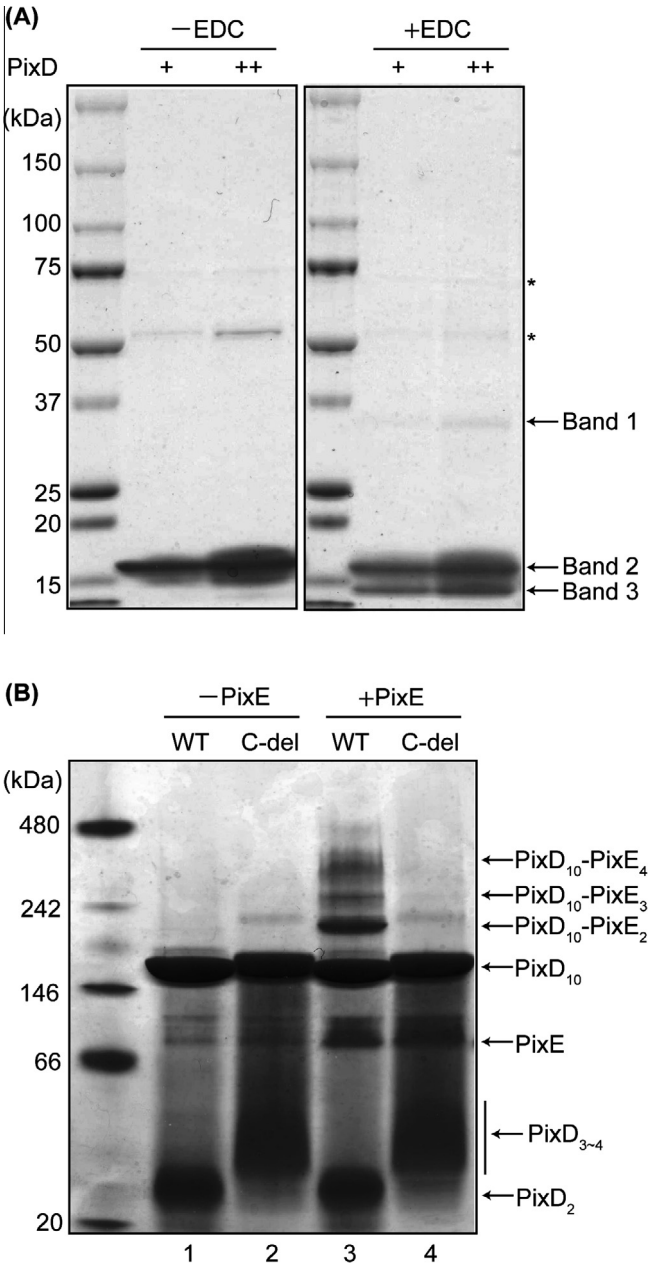

Fig. 1. (A) Cross-link analysis of PixD. Purified PixD, either 25 μM (+) or 50 μM (++), was left untreated or treated with 5 mM EDC, and the reactions were subjected to SDS–PAGE. Band 1: putative cross-linked PixD dimer; Band 2: PixD monomer; Band 3: putative intramolecularly cross-linked PixD monomer. (B) Blue-native PAGE profiles of wild-type and C-terminal-truncated PixD (C-del) in the presence or absence of PixE.

observed for untreated PixD (left), indicating that this band represents monomeric/non-cross-linked PixD. PixD has a predicted molecular mass of 17.5kDa, indicating that bands 1 and 3 represent the intermolecular cross-linked PixD dimer and intramolecular cross-linked PixD monomer, respectively. Indeed, PixD in band 1 may contain an intramolecular as well as intermolecular cross-link(s), although these two putative forms were not electrophoretically separated under our experimental conditions.

Bands 1 and 3 were excised from the gel, partially digested with trypsin, and subjected to MS to identify intermolecular cross-linked peptides. As shown in Fig. S2 (Supplementary Material), a trypsin-digested peptide of molecular weight (MW)