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

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A B S T R A C T
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.

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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 PixD10–PixE5 has also been suggested, although this stoichiometry has not been confirmed experimentally [6,9]. Upon excitation by light, the PixD10–PixE4 (or PixD10–PixE5) 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-dimethylaminopropyl]-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'-AGTTGTCCTAAGAATTCCTCGAGCCCGGGTGAA-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 docked to another PixD monomer using ZDOCK[12].

3. Results and discussion

For cross-linking analysis, purified PixD was treated with 1-ethyl-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 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)
2842 was observed for band 1 but not for band 3. Given that two other peptides of MW of 2595 and 2634 were similarly observed in both bands 1 and 3, the 2842 peptide was assigned as an intermolecular cross-linked peptide. To determine the composition of this peptide, it was further subjected to MS/MS analysis (see Section 2 for details). This analysis revealed that peptide 2842 contained an intermolecular cross-link between Lys22 and Glu26.

The position of the potential cross-link was mapped onto the crystal structure of the PixD decamer. There are two possible dimer pairs in the PixD decamer (α–β and α–γ pairs) (Fig. 2A and B). In both conformations, the relevant amino acid residues (Lys22 and Glu26) are not proximal between each PixD molecule and thus could not form an intermolecular cross-link (Fig. 2C and D). Thus, this cross-link may form in the PixD dimer but not the decamer.

We next modeled the PixD dimer by docking simulation. The data for the PixD monomer were obtained from the crystal structure of the decamer, and two PixD monomers were subjected to docking simulation with ZDOCK [12]. Fig. S3 (Supplemental Material) shows the top five most-stable structures (Models 1–5). Models 1–4 show very similar conformations each other in which the dimer forms via interaction between the C-terminal regions of the monomers. Among the five structures, Model 1 and 4 showed the close distance between Lys22 and Glu26 (2.5 and 1.3 Å, respectively), which are accessible for forming an EDC-induced cross-link (Figs. 2E and S3). Previously, Kondo et al. performed pulsed electron paramagnetic resonance analysis of light-induced radical pair formation in PixD and calculated an interprotein distance of flavin radicals in the dimer [14]. The result indicated a distance of ~40 Å between flavin radicals, which is close to the distance predicted in the PixD dimer (35–40 Å; Fig. 2B).

We next characterized the C-terminal-truncated version of PixD to check the importance of the C-terminus for dimer formation and complexation with PixE. The oligomerization states of the proteins were studied by blue-native PAGE. In the absence of PixE, wild-type PixD was in equilibrium between the dimer and decamer (Fig. 1B, lane 1) as reported [6,8,10]. On the other hand, the C-terminal-truncated PixD mutant did not form a dimer, although smeared bands indicated formation of a trimer and/or tetramer (Fig. 1B, lane 2). Mixing PixE with the truncated mutant of PixD yielded no PixD–PixE complex, although wild-type PixD clearly could form the complex with PixE (Fig. 1B, lane 3, 4). The mutant PixD still could form the decamer (Fig. 1B, lane 2), however, and thus these results indicated that the C-terminus of PixD is necessary for stable dimer formation as well as complex formation with PixE but is not necessary for decamer formation.

What is the physiological relevance of PixD dimer formation? One possibility is that dimerization is important for downstream interaction with PixE. Although the C-terminal-truncated PixD clearly could form a decamer (Fig. 1B, lane 2), it could not form the higher-order PixD–PixE complex (Fig. 1B, lane 4). This result

Fig. 2. (A) and (B) Top and side views of the X-ray crystal structure of the Synechocystis PixD decamer (PDB entry 2HFN). Three adjacent monomers are labeled by α, β and γ. Flavins are indicated as ball-and-stick structures. (C) and (D) Two dimeric conformations (α–β and α–γ pairs) in the crystal structure of the PixD decamer. (E) Predicted structure of the PixD dimer, as revealed by docking simulation (Model 1 shown in Fig. 3S). The Lys22 in one subunit, and Glu26 in another subunit, are indicated as ball-and-stick structures. Each flavin is indicated as a stick model. The C-terminal seven residues are colored magenta.
indicated that PixE can associate with the PixD dimer, but not decamer, for forming the stable PixD–PixE complex. Fig. 3 shows an alignment of partial amino-acid sequences of PixD from Synechocystis and Thermosynechococcus elongatus. The C-terminal region differs between these two species. Notably, the T. elongatus PixD in solution is in equilibrium between a pentamer and decamer, but it does not form a dimer even when exposed to light [15]. Furthermore, no pixE ortholog has been found in the fully sequenced genome of T. elongatus [16]. These observations suggest that PixD dimer formation is necessary for physical and functional interaction with PixE. Perhaps the C-terminus of Synechocystis PixD has evolved to facilitate dimerization and to control PixE activities for fine-tuning photosensitivity of the phototaxis response. Future characterization of PixD–PixE signaling should provide crucial information concerning not only the molecular mechanism but also the evolution of light-induced signal transduction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.05.019.

References