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The PHY domain is required for conformational stability and spectral integrity of the bacteriophytochrome from *Deinococcus radiodurans*

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ABSTRACT

Bacteriophytochrome from *Deinococcus radiodurans* (DrBphP) is a plant phytochrome homolog. To investigate the interaction of chromophore and protein structure, we purified recombinant DrBphP and performed biochemical analyses. Differences of apo- and holo-protein in electrophoretic properties in native gels and their susceptibility to trypsin indicate changes in both the conformation and surface topography of this protein as a result of chromophore assembly. Furthermore, proteolysis to Pr and Pfr conformers displayed distinctive cleavage patterns with a noticeable Pr-specific tryptic fragment. Of interest, a prolonged tryptic digestion showed a more severe impact upon the Pfr form. Most importantly, when we assessed the extent of dark reversion to evaluate the role of the cleaved part, a rapidly accelerated reversion was observed upon cleavage at residues 329–505 corresponding to the PHY domain. Our data thus show that the PHY domain is necessary for the Pfr stabilization and spectral integrity of DrBphP.

Plant phytochrome is the most extensively studied photosensing chromoprotein. A number of developmental and photomorphogenic processes in plants are regulated via phytochrome-mediated light signal transduction. Phytochrome exists as two photoisomers, red light absorbing, Pr and far-red light absorbing, Pfr forms. These isoforms have distinct absorption spectra. Pr has an absorption maximum (λ_{max}) at 660 nm and Pfr has a λ_{max} at 730 nm. Phytochrome is synthesized as a Pr form and transformed to a biologically active Pfr form upon irradiation of red light which is a major portion of white day light and the Pfr is converted back to Pr form by far-red light. This reversible molecular interconversion is accompanied by photoisomerization of the phytochromobilin (PΦB) chromophore which is covalently attached to a N-terminal chromophore binding domain (CBD) [1,2].

Since late 1990s, the presence of phytochrome-like photoreceptors in photoautotrophic prokaryotes has been reported. In a filamentous cyanobacterium, *Fremyella diplosiphon*, a limited resemblance has been identified between the N-terminal half of the bacterial RcaE protein (a regulator of chromatic adaptation) and the CBD of *Arabidopsis* PhyE [3]. Following the completion of genome sequencing of *Synechocystis* sp. PCC6803, further evidence for the existence of phytochrome-like proteins such as Cph1 (cyanobacterial phytochrome) [4], PlpA (phytochrome-like protein) [5], Ppr (PYP-phytochrome-related) [6], CikA (circadian input kinase) [7], and Agp (*Agrobacterium* phytochrome) [8] has been reported. Unexpectedly, nonphotosynthetic eubacteria such as *Deinococcus radiodurans, Pseudomonas aeruginosa* and *Pseudomonas putida* have been reported to possess bacterial phytochromes (BphPs) [9,10]. Because the BphPs and plant phytochromes share major structural basis [11], the BphPs have been used as relatively simple materials to understand the precise molecular structure and mechanism of the plant phytochrome action. Recently, the three-dimensional structure of the N-terminal CBD of bacteriophytochrome from *D. radiodurans* (DrBphP) was determined [12,13]. However, the structural observation was restricted to the ground state Pr form of the N-terminal portion of the DrBphP. Hence, the conformational changes and the differences in the spectral integrities of the full-length photoconverting Pr/Pfr DrBphP have yet to be elucidated.

In the current study, we have utilized a partial tryptic digestion and MALDI-TOF strategy to analyze the topological differences between the Pr and Pfr forms of the DrBphP. Our MS analyses combined with spectroscopic data were then used to examine the structural role of this bacterial phytochrome in its spectral integrities.

Materials and methods

Expression and purification of recombinant DrBphP. BphP (full-length DrBphP) and BphN (N-terminal 1–321) genes were individually cloned into the pET-21(a)+ vector and expressed in *Escherichia coli*, BL-21 cells. The cells were grown at 25 °C and expression was induced with 1 mM IPTG at an OD_{600} of 0.6. After 6 h growth, the cells were collected by centrifugation, resuspended in binding buffer (100 mM Tris–Cl, 150 mM NaCl, and 10 mM immidazole, pH 8.0) supplemented with authentic biliverdin (BV, 2 μg/ml, Frontier Scientific, Carnforth, UK). The mixture was then sonicated and the lysate was subjected to Ni^{+}–NTA affinity column (Qiagen, USA). The purified recombinant DrBphPs were stored at −70 °C. The

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apo-DrBphP was prepared by the same method in the absence of BV. If needed, prepared apo-protein was later reconstituted with BV *in vitro* to obtain holo-protein by spontaneous chromophore assembly.

Treatment of protein with denaturing conditions. Pretreatment in different temperatures (4–60 °C) or with various concentrations (0–8 M) of urea at 25 °C for 10 min was administered to the apo-DrBphP. After another 10 min incubation with BV (2 μg/ml) at room temperature, samples were resolved by SDS–PAGE. The extent of the chromophore association was then measured using a zinc blot as described below.

Detection of chromophore bearing peptide. Zinc blot was performed to visualize the chromophore bearing peptides. Briefly, the polyacrylamide gel was soaked in zinc acetate solution (20 mM zinc acetate, 150 mM Tris–Cl, pH 7.0) for 20 min at room temperature and the fluorescence signal was captured under UV light.

Trypsin digestion and analysis of the enzymatic fragments. To investigate the effect of chromophore binding upon the surface topology of DrBphP, partial enzymatic digestion pattern was compared between apo- and holo-protein. BV assembly was achieved by adding BV (2 μg/ml) to apo-DrBphP for 10 min at room temperature. The reconstituted holo-protein (0.2 mg/ml) was then digested for 1 h at room temperature with 10 μg/ml trypsin under white light. In the case of apo-protein sample, trypsin treatment was conducted prior to the addition of BV. The reaction products were then subjected to SDS–PAGE along with full-length holo-protein as a control followed by Coomassie staining and zinc blot. For the analysis of Pr/Pfr-specific tryptic cleavage, the holo-DrBphP (0.2 mg/ml) was irradiated either with far-red or red light for 10 min. Thereafter, trypsin (10 μg/ml) was added to each Pr and Pfr protein sample for various reaction times (0–90 min) under continuous far-red and red irradiation, respectively. The reaction was terminated by addition of a trypsin inhibitor (50 μg/ml, Sigma, MO, USA).

Identification of digested tryptic fragments. For N-terminal sequencing, tryptic fragments were separated by SDS–PAGE and transferred to a PVDF membrane. Alternatively, each Coomassie stained peptide species was excised from the gel and extracted by electro-elution then concentrated in acetone at −20 °C to determine the molecular weight. Further procedures for N-terminal sequencing and MALDI-TOF MS were performed at the KBSI (Korea Basic Science Institute, Daejeon, Korea). Based on the obtained data and the full-length amino acid sequence of DrBphP as queries, the residues of tryptic fragments were estimated from the PROWL database (http://prowl.rockefeller.edu/prowl/peptidemap.html).

Spectroscopic analysis of trypsin cleavage and dark reversion. Pr or Pfr holo-DrBphP (0.2 mg/ml) was subjected to a prolonged trypsin digestion under continuous far-red and red light irradiation, respectively. The absorbance change of each conformer was then recorded by spectrophotometer (V-550, Jasco, Japan). The rate of dark reversion was assessed by monitoring the absorbance change of each photochromic molecule under darkness. To obtain the predominantly produced 36 kDa and 55 kDa peptides, both the Pr- and Pfr-proteins (0.4 mg/ml, each) were cleaved by trypsin (20 μg/ml) for 30 min under far-red and red irradiation, respectively. After addition of 100 μg/ml trypsin inhibitor, the expected stoichiometry was re-confirmed by an additional zinc blot. Red light irradiation was then performed to induce photoconversion of each peptide to the Pfr form prior to a spectroscopic measurement with a 10 min interval under dark condition. Additionally, the BphP (full-length) and BphN (residues 1–321) were examined by the same method.

Results and discussion

Most members of phytochrome superfamily, including those of plants, cyanobacteria and fungi commonly have P2/PAS, P3/GAF, and P4/PHY domains in the N-terminal photosensory core followed by a dimerization motif and protein kinase domain in their C-terminal regulatory regions, whereas the serine-rich P1 domain is exclusively found in plant phytochromes [14,15]. The PAS functions as signal sensor and GAF stabilizing domain [16]. Chromophore binding occurs in the GAF domain [17]. However, the precise role of the PHY domain is not clear. Several reports have indicated that this region of other phytochrome members may be functionally important. Deletion of this PHY domain from oat phyA results in alterations of the spectral properties of this phytochrome [18]. For the Cph1, mutations within the PHY domain showed a complete loss of spectroscopic activity, suggesting the requirement of this region for native bilin–apoprotein interactions [19]. Since the photochromic behavior of DrBphP is derived from its association with a bilin ligand, we examined the protein domains and intra/inter molecular interaction underlying their structural and functional coordination with the associated chromophores.

Proper protein folding is required for the chromophore assembly

To confirm that the purified recombinant DrBphP reliably undergoes photoconversion, its absorption spectra under red and far-red irradiation were measured. DrBphP assembled *in vitro* with BV was reported to show Pr/Pfr-specific absorbance spectra [9]. The holo-DrBphP in our purification and reconstitution system displayed such typical photochemical properties (Pr, λ_{max}=698 nm; Pfr, λ_{max}=750 nm) (Fig. 1A). The absorbance spectra of DrBphP measured after reconstitution with BV indicated that the chromophore autoassembly is completed in less than 10 min. It is consistent with the result obtained from Cph1 study [20]. We further examined the relative extent of BV binding to the apo-DrBphP by zinc blot under denaturing conditions to determine the importance of protein folding in this process. At temperatures of over 37 °C, the efficiency of the BV incorporation was markedly decreased (Fig. 1B, upper). In addition, little fluorescence was detectable for the 2 M urea treatment, indicating that the BV fails to bind to apo-DrBphP (Fig. 1B, lower). The results thus suggest that proper folding of DrBphP is necessary for the chromophore assembly.

Chromophore binding influences the protein conformation

Next we examined whether chromophore binding conversely affects the formation of the unique conformation of holo-DrBphP. Upon binding of the chromophore, Coomassie staining of the native-PAGE gel revealed two major bands representing the dimeric and weaker putative tetrameric forms of the holo-DrBphP whereas the apo-protein itself produced a ladder of high molecular weight protein complexes in addition to the dimer form (Fig. 1C, bracket). It has been reported that PCB chromophore attachment triggers dimerization of the Cph1 from an equilibrium mixture of monomers and dimers [21]. The authors did not observe oligomeric protein complexes for apo- or holo-Cph1 by native-PAGE analysis. In the current study, BV binding seems to promote the conversion from a mixed state into a more homogenous condition in which dimers predominate. Of significance, the chromophore bearing peptide migrated faster than the apo-protein (Fig. 1C, arrows). This mobility change suggests an alteration of globular structures upon chromophore assembly.

The conformational change in DrBphP caused by chromophore binding was further supported by the enzymatic cleavage patterns of SDS–PAGE gel obtained before and after BV association (Fig. 1D). Following incubation with BV, holo-DrBphP was subjected to a trypsin digestion for 1 h under white light, which produced three chromophore bearing peptide bands of 55, 36, and 25 kDa. However, no protein band was observed by Coomassie and zinc blots when a trypsin treatment was performed prior to BV incubation, suggesting an extensive degradation of the apo-DrBphP. We postulate from this result that BV chromophore assembly certainly influences the overall protein conformation of DrBphP so that the cleavage target sites nestle into a hydrophobic cluster.

Pr and Pfr forms have a different surface topology

Tryptic digestions were also performed to analyze the differences in the protein conformations of the Pr and Pfr forms of DrBphP (Fig. 2A–D). The trypsin digestion of either Pr or Pfr form of the full-length (85 kDa) DrBphP generated 55, 36, and 25 kDa enzymatic fragments. It is likely that the 36 and 25 kDa peptides are end products of the 90 min digestion and that the 55 kDa mid-sized peptide is a reaction intermediate. This result is consistence with the tryptic digestion pattern performed under a laboratorial white light condition in which the Pfr form dominates (Fig. 1D).

The appearance of 55 kDa peptide with a concomitant reduction of the full-length 85 kDa origin occurred after about 3 min in the