Amplification of Light-induced Molecular-Shape Change by Supramolecular Machines

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1. Introduction

Along with the drastic advances in structural biology and protein science, the main interests in biological research is being shifted from characterization of individual proteins to comprehensive understanding of their roles in the highly sophisticated cellular networks and how they regulate biological events. Thereby the development of methodologies to control protein functions would provide essential tools for intracellular imaging, synthetic biology, and molecular diagnostics. In that sense, light-controllable proteins are particularly attractive because light offers high spatiotemporal resolution. Actually, chemical modification of the target proteins with photoresponsive molecules has been used to reversibly control their functions by UV or visible light irradiation.¹ Although such chemical approach is versatile and targets various biomolecules including protein, nucleic acids, and lipids, the structural change of photochromic molecules is sometimes so small as not to affect the property of an target molecule. In contrast, in cases of photoreceptor proteins, a small molecular-shape change of the photoresponsive molecule is amplified to the whole molecule. Thereby it is expected that these photoreceptors possibly allow for the effective manipulation of large molecules by photoirradiation. In that sense, photoreceptors could be useful candidates as light-responsive units to control activity of biomolecules.²

PYP known as a photosensor protein has been isolated from Ectothiorhodospira halopila.³ Exposure of PYP to visible light (λ = 446 nm) causes partial unfolding of α-helices at N-terminal region.³ PYP contains p-coumaric acid derivative as a chromophore, which binds to Cys69 via a thioester bond. This chromophore undergoes trans-to-cis photoisomerization by irradiation with visible light (λ = 446 nm), followed by thermal cis-to-trans isomerization to recover the initial trans-configuration at room temperature (Scheme 1). It is known that the overall photo-induced configurational and conformational changes of the chromophore and surrounding peptide chains occur within 1 s.⁴ Compared with conventional photochromic molecules, an optical signal could be more efficiently converted into a mechanical motion of molecules in the case of PYP.

Scheme 1 Trans/cis isomerization of PYP chromophore.
Therefore, PYP is expected to act as an effective photoresponsive module for controlling the function of large molecules such as proteins.

(a) (b)

Fig. 1. (a) Crystal structure of a heptameric assembly of α-Hemolysin (Hla). Left and Right: top and side views, respectively. (b) The model structure of C-PYP-Hla based on crystal structures of Hla and PYP. Green: Cap and Rim domains; Blue: Stem domain.

α-Hemolysin (Hla), isolated from Staphylococcus aureus, is a pore-forming toxin that causes lysis of red blood cells. While Hla is secreted as a soluble monomeric protein, it assembles into a cyclic heptamer (Fig. 1) on the surface of the cell membrane. After the formation of the heptameric ring, the stem region forms a β-barrel channel, which penetrates into the membrane to cause lysis of the cell. The pore is inherently stable and acts as a nonspecific ion channel for application to some engineered tools. We have already prepared a chimeric protein N-PYP-Hla, where PYP is connected to the N-termini of Hla, and found that the hemolytic activity of this chimeric protein could be controlled by visible-light irradiation. Here we report construction of chimeric protein C-PYP-Hla, where PYP is connected to the C-termini of Hla, and its hemolytic activity in the dark and under irradiation.

2. Methods

1H NMR spectra was recorded on 400 MHz FT NMR JEOL JNM-LA400 spectrometer, where the chemical shifts were determined with respect to tetramethylsilane (TMS, δ 0.00) as an internal standard. Matrix-assisted laser desorption/ ionization time-of-flight mass (MALDI-TOF MS) spectrometry was performed in reflector mode with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix on Bruker Daltonics REFLEX III spectrophotometer. UV-Vis spectrum was recorded on JASCO V-650 UV-Vis spectrophotometer. Turbidity was recorded on JASCO V-530 UV-Vis spectrophotometer. Irradiation was carried out by a xenon lamp (ASAHI SPECTRA LAX-102).

3. Results and Discussion

We first prepared an apo-protein without a chromophore at the PYP domain, which was then treated with p-coumaric anhydride to afford C-PYP-Hla. The hemolytic activity of C-PYP-Hla was investigated by monitoring the turbidity of the sheep red blood cell suspension at 700 nm after addition of C-PYP-Hla in the dark and under irradiation with visible light (λ = 450 ± 10 nm) at 25°C.

3.1. Synthesis of p-coumaric anhydride

To a dry dichloromethane (20 mL) solution of p-coumaric acid (328 mg, 2.0 mmol) was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (192 mg, 1.0 mmol) at 0°C, and then the resulting mixture was stirred for 5 h at 20°C.

Then, to the reaction mixture was added tetrahydrofuran (20 mL) and the resulting mixture was stirred overnight. The reaction mixture was washed with water (2 × 10 mL) and brine (10 mL) to allow isolation of p-coumaric anhydride in 47% yield (289 mg, 0.93 mmol). 1H NMR (400 MHz, CDCl3 containing 0.03% TMS, 22°C): δ 7.79 (d, J = 15.6 Hz, 2H), 7.48 (d, J = 8.8 Hz, 4H), 6.87 (d, J = 8.8 Hz, 4H), 6.38 (d, J = 15.6 Hz, 2H) ppm; MALDI-TOF MS (CHCA, positive mode): m/z: calculated for C30H13O5: 310.32; found: 333.59 [M+Na]+, 349.44 [M+K]+.

3.2. Construction of an expression vector

A DNA fragment encoding staphylococcal α-toxin without the signal sequence was amplified using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan), with S. aureus strain Mu50 (ATCC 700699) genomic DNA as the template and the following primers: α-Hemolysin-S (5′-CATG CCATGGCAGATTTCTGATATTAAAACC GG-3′), α-Hemolysin-AS (5′-CGCTCGAG AC-3′).
ATTTGTATTTTCTTTTTCCAAAATCG-3\'), (recognition sites for the restriction enzyme are underlined). The PCR products were inserted into the NeoI and XhoI sites of the pET28-b vector (Merck, Whitehouse Station, NJ). A His\(_6\) tag was fused at the C terminus for purification by nickel-chelate affinity chromatography. A DNA fragment encoding photoactive yellow protein was amplified using KOD-Plus DNA polymerase with the following primers: 15_PYP_Back (5’- GAAGA AATGACAATATGGAACACGTCAGCTCAG-3’) and 15_PYP_Forward (5’- GTGCTGGTGCTCG AGGACGCGCTTGACGAAGAC-3’). The amplified fragment was introduced downstream of the staphylococcal α-toxin gene with In-Fusion Advantage PCR Cloning kit (Takara Bio Inc., Shiga, Japan).

3.3. Preparation of C-PYP-Hla

*Escherichia coli* BL21(DE3) transformed with the expression vector was grown at 28 °C in 2× YT medium containing 16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl, and 30 mg/L kanamycin. After growing the culture up to an optical density of 0.8 at 600 nm, IPTG (a final concentration: 500 mM) was added to the culture to induce expression of the desired protein. Then the culture was incubated overnight and centrifuged at 7000 rpm for 15 min at 4 °C. After removal of the supernatant, the resulting pellet was resuspended in 50 mL Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl). The resulting cell suspension was sonicated for 20 min on ice and then centrifuged at 40,000 xg for 30 min at 4 °C. The supernatant was loaded on 1 mL of resin (Ni Sepharose 6 Fast Flow, GE Healthcare), which was then washed with 20 mL of washing buffer (5 mM Imidazole, 200 mM NaCl, and 50 mM Tris-HCl, pH 8.0). The target protein was eluted with 10 mL of elution buffer (500 mM imidazole, 200 mM NaCl, and 50 mM Tris-HCl, pH 8.0) at 4 °C. Fractions containing the desired protein were collected, dialyzed against Tris-HCl buffer (50 mM, pH 8.0 containing 200 mM NaCl) and then further purified on a size-exclusion chromatography (HiLoad\textsuperscript{TM} 26/60 Superdex 200, GE Healthcare). The purity and homogeneity of the protein were evaluated by means of sodium dodecyl sulfate–polyacylamide gel electrophoresis (SDS-PAGE).

The PYP unit attached to C-terminus of Hla was then reconstituted by the following procedure\textsuperscript{11}. To a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of apo-PYP-Hla (3 mg/mL) was added a DMF solution of p-coumaric anhydride (10-fold molar excess), and the resulting mixture was stirred overnight at 4 °C. The reaction mixture was filtered to remove insoluble substances, and then subjected to size-exclusion chromatography (HiLoad\textsuperscript{TM} 26/60 Superdex 200, GE Healthcare) to allow isolation of the reconstituted protein C-PYP-Hla. C-PYP-Hla was obtained as a monomeric form in Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl).

3.4. UV-Vis absorption spectroscopy

Successful introduction of p-coumaric acid in C-PYP-Hla was confirmed by electronic absorption spectroscopy. C-PYP-Hla displayed an absorption band around 446 nm, as with wild-type PYP in the dark (\(\lambda_{\text{max}} = 446\) nm), indicating the presence of the p-coumaric acid moiety attached to PYP via thioester bond.

![UV-Vis absorption spectrum of C-PYP-Hla in Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) at 25 °C](image)

3.5. Hemolytic activity assay

Preserved blood collected from sheep (Nippon Biotest Laboratories inc., Tokyo, Japan) was centrifuged at 1,500 xg for 5 min and washed several times with PBS. The erythrocytes were then resuspended in PBS (pH 7.4) so that the
resulting mixture has a turbidity of 0.6 at 700 nm with a volume of 2.95 mL. Then the resulting suspension was incubated at 25 °C for 1 h. To the erythrocyte suspension thus prepared, was added a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of the protein (15 μL), and the activity was evaluated by measuring the turbidity at 700 nm. Of importance, under irradiation with visible light (\(\lambda = 450 \pm 10\) nm), hemolysis by C-PYP-Hla was retarded significantly comparing to that in the dark at 25 °C (Fig. 3). It has been reported that the partial unfolding of the C-terminal region is required for the channel formation of Hla.\(^{12}\) Hence, it may be possible that the PYP intermediate generated by irradiation would interfere in the flexibility of the C-terminal of Hla.

![Graph](image_url)

Fig. 3. Time course curves of hemolysis of sheep red blood cells by C-PYP-Hla (11.5 μg/mL) in PBS in the dark (blue) and under irradiation with visible light (\(\lambda = 450 \pm 10\) nm) (green) at 25 °C, monitored by optical density at 700 nm (OD\(_{700}\)).

References


