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Purification and Reconstitution of PYP-Phytochrome with Biliverdin and 4-Hydroxycinnamic Acid

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Abstract

PYP-phytochrome (Ppr) is a unique photoreceptor that contains a blue light-absorbing photoactive yellow protein (PYP) domain, a red light-absorbing phytochrome domain, and a histidine kinase domain. This chapter describes overexpression of Ppr in a strain of *Escherichia coli* that allows covalent attachment of substoichiometric amounts of biliverdin *in vivo*. Ppr is then fully reconstituted with biliverdin, followed by attachment of 4-hydroxycinnamic acid (*p*-coumaric acid), *in vitro*. Holo-Ppr with both chromophores is then isolated via an affinity tag and quantified for chromophore attachment by analysis of the absorption spectrum for biliverdin and 4-hydroxycinnamic acid. We also provide conditions for measuring autophosphorylation of Ppr.

Introduction

The purple photosynthetic bacterium *Rhodospirillum centenum* has a novel photoreceptor called PYP-phytochrome (Ppr) (Jiang *et al.*, 1999). Ppr has been shown to control the regulation of chalcone synthase expression in response to changes in light intensity (Jiang *et al.*, 1999). The amino terminus of Ppr is composed of a photoactive yellow protein (PYP) domain (Cusanovich and Meyer, 2003) that covalently attaches the blue light-absorbing chromophore, 4-hydroxycinnamic acid (*p*-coumaric acid). The PYP domain is followed by a bilin-binding domain similar to that observed in many prokaryotic phytochromes known to covalently attach the red light-absorbing chromophore, biliverdin (Montgomery and Lagarias, 2002). These blue light-and red light-absorbing chromophore attachment domains are followed by a carboxyl-terminal histidine kinase domain (Montgomery and Lagarias, 2002).

Because Ppr is ancestral to cyanobacterial and plant phytochromes (Montgomery and Lagarias, 2002), photochemical analysis of Ppr provides an ancestral view to the function of cyanobacterial and plant phytochromes. This chapter describes the overexpression, isolation, and *in vitro* reconstitution of apo-Ppr with biliverdin and with 4-hydroxycinnamic acid. We also provide spectral features of reconstituted Ppr and assays for autophosphorylation.

Vector Construct

The *R. centenum ppr* gene is amplified by polymerase chain reaction (PCR) and cloned into the *NdeI*–*EcoRI* site of pET28a(+) (Novagen, Madison, WI) to form the Ppr expression plasmid pET28ppr. To make biliverdin in the host cell as a chromophore, the *R. palustris hmuO* gene is PCR amplified with forward (5′-GGCATATGGTGGTGGGAAGCAGCGAAAC-3′) and reverse primers (5′-GGGATCCGTAGCGCTCTAGGCGTCGAG-3′) that contain *NdeI* and *BamHI* sites, respectively, and then cloned into *NdeI*–*BamHI* sites of the expression plasmid pET11a (Novagen), resulting in the recombinant heme oxygenase expressing plasmid pETHmuO. Coexpression of both proteins is accomplished by cotransforming pET28ppr that is kanamycin resistant and pETHmuO that is ampicillin resistant into BL21(DE3) (Novagen). Cotransformed strains are selected on LB agar growth medium containing 50 µg/ml kanamycin and 100 µg/ml ampicillin and stored in 20% glycerol at –80°.

Preparation of 4-Hydroxycinnamic Acid Anhydride and Biliverdin

We use 0.25 M 4-hydroxycinnamic acid anhydride to reconstitute apo-Ppr with its blue light-absorbing chromophore. 4-Hydroxycinnamic acid anhydride is made according to Imamoto *et al.* (1995) and Kroon *et al.* (1996). To make 4-hydroxycinnamic acid anhydride, 0.5 ml of a 3 M dicyclohexylcarbodiimide (DCC) solution (6.25 g DCC dissolved in 2.5 ml of *N,N'*-dimethylformamide [DMF]) is added to 4-hydroxycinnamic acid solution, which is made by dissolving 0.164 g 4-hydroxycinnamic acid (Sigma) in 3.5 ml of DMF on ice and shaken at 4° overnight. Dicyclohexyl urea precipitate is removed by centrifugation for 2 min at 15,000g in an Eppendorf centrifuge, and the clarified supernatant containing 4-hydroxycinnamic acid anhydride is then used for *in vitro* reconstitution with Ppr (see later).

Biliverdin hydrochloride (Frontier Scientific, UT) is dissolved in dimethyl sulfoxide to 20 mM for use in reconstitution of apo-Ppr with biliverdin *in vitro*.

Overexpression and Reconstitution of apo-Ppr with Chromophores

Cells are streaked onto an LB agar plate from a -80° frozen stock and incubated overnight at 37°. Five to 10 colonies are inoculated in 50 ml of LB liquid media containing 50 µg/ml kanamycin and 100 µg/ml ampicillin and incubated overnight at 37°. A volume of 20 ml of overnight culture is added to 1 liter of Terrific Broth media containing 50 µg/ml kanamycin and 100 µg/ml ampicillin. Cells are shaken at 37° until the OD₆₀₀ reaches 0.4 to 0.6 at which point 0.5 ml of filter-sterilized 1 M isopropyl-β-D-thiogalactopyranoside (Sigma) and 0.5 ml of 1 mM of δ-aminolevulinic acid hydrochloride (Sigma) are added to the culture. The culture is then shaken at 18° for 20 to 24 h. δ-Aminolevulinic acid is used to make hemes in the host cells as a precursor (Beale, 1990). Also, it has been reported that introduction of exogenous δ-aminolevulinic acid results in a rapid increase of spectrophotometrically detectable phytochrome in seedlings where the phytochrome level was inhibited by gabaculine (Elich and Lagarias, 1987), as well as effective chromophore attachment in *E. coli* expressed phytochrome (Gambetta and Lagarias, 2001).

Cells are harvested by centrifugation at 3000g at 4° for 10 min and are resuspended in 30 ml of 4° 1 × His-binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). Resuspended cells are disrupted three times with a cell cracker (Schmidt and Huttner, 1998) and then clarified by centrifugation at 27,000g at 4° for 30 min. About 40 ml of the supernatant is placed in a 200-ml beaker wrapped with aluminum foil to guard against light exposure and slowly mixed with a stirring bar at 4°. Ten-microliter aliquots of activated 0.25 M 4-hydroxycinnamic anhydride acid are slowly added at 5-min intervals until a total of 60 µl has been added. Next, 7 µl of biliverdin is added every 5 min for a total of 35 µl of biliverdin. Fully reconstituted Ppr is then affinity purified from other cellular proteins as described later.

Purification of Ppr Reconstituted with Chromophores

An FPLC system (Amersham Biosciences) at 4° is used for purification of reconstituted Ppr. The supernatant reconstituted with chromophores is applied to a 5-ml HiTrap chelating column (Amersham Biosciences). The column is washed with 20 column volume of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and 70 column volume of 5.5% of 1 × elute buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). Reconstituted Ppr is then eluted with a gradient of 5.5 to 100% of elute buffer at a flow rate of 2 ml/min. Fractions with the green color of Ppr are pooled and checked by SDS-PAGE. Salt in the pooled fractions is removed by overnight dialysis against 20 mM Tris-HCl, pH 8.0, at 4°. Reconstituted Ppr is eluted at approximately 150 mM imidazole.

An Amicon Centriprep YM10 filter (10,000 MWCO, Millipore Co., Bedford, MA) is then used to concentrate purified Ppr typically to a volume of about 2 ml. The protein concentration is adjusted to 1 mg/ml with Tris-HCl, pH 8.0, to measure absorption spectra. Dialysis against storage buffer (50% glycerol, 20 mM Tris-HCl, pH 7.6, 50 mM KCl) is also performed for long-term storage at -20° . From this method, more than a 10-mg amount of homogeneous, spectrally active Ppr with both chromophores is readily produced from 1 liter of starting cell culture. Purified reconstituted Ppr is also typically more than 95% pure as measured by SDS-PAGE analysis.

Spectroscopic Measurements of holo-Ppr, Ppr-BV, and Ppr-pCA

Absorption spectra are recorded using an Agilent 8453E ultraviolet-visible spectroscopy system (Agilent Technologies, Germany). The absorption spectrum of Ppr fully reconstituted with only 4-hydroxycinnamic acid has a single peak at 431 nm (Fig. 1). This is contrasted by Ppr reconstituted with only biliverdin, which has absorbance bands at 700 and 394 nm, as is typical of other bacteriophytochromes that are in the Pr form in the ground state. The spectrum of Ppr fully reconstituted with both chromophores (solid line in Fig. 1) displays absorbance maximum at 700 and 400 nm, plus a shoulder at 430 and at 640 nm.

In Vitro Autophosphorylation of Ppr

The *in vitro* phosphorylation experiment of reconstituted Ppr is performed under green safety light (Kodak 7B, Cat. No. 8070112) at room temperature. Protein in storage buffer is diluted with $10 \times$ kinase buffer (200 mM Tris-HCl, pH 7.8, 60 mM $MgCl_2$, 1 M NaCl) to yield a final concentration of 10 μ M of Ppr in individual reaction mixtures. Half of the reaction mixtures is incubated in the dark and the other half is irradiated with specific light condition, such as red or blue light with intensity of 1 or 10 μ mol/m²/s. Phosphorylation of Ppr is initiated by adding 1/10 volume of $10 \times$ ATP mix composed of 36 μ l of 10 mM ATP and 3 μ l (0.024 m μ) of [γ -³²P]ATP (>7000 Ci/mmol, MP Biochemicals). Immediately upon illuminating, 10- μ l aliquots (2 μ g of protein) are removed at time intervals, mixed with SDS-loading buffer, and placed on ice until analyzed by SDS-PAGE. The samples are subjected to SDS-PAGE, and the ³²P-labeled Ppr band is quantified by the Typhoon 9200 variable mode imager (Amersham Biosciences).

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Fig. 1. Absorbance spectra of Ppr reconstituted with different chromophores in the dark. Ppr with both chromophores, including activated 4-hydroxycinnamic acid and biliverdin (solid line). Ppr with biliverdin (dashed line). Ppr with the activated 4-hydroxycinnamic acid (dotted line).