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Functional Expression and Site-Directed Mutagenesis of Photoactive Yellow Protein[1]

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The gene encoding photoactive yellow protein (PYP) was isolated from *Ectothiorhodospira halophila*, and a high-level expression system for PYP was constructed in *Escherichia coli*. The molecular weight and the absorption spectrum of PYP expressed in *E. coli* were identical with those of the native PYP isolated from *E. halophila*. The amino acid residues which might interact with the chromophore (Tyr42, Glu46, Thr50, Arg52, and Cys69) were mutated by site-directed mutagenesis and the absorption spectra of these mutants were examined to study the chromophore/protein interaction in PYP. The former three substitutions (Y42F, E46Q, and T50V) brought about red-shifts of the absorption spectra, but the substitution of Arg52 (R52Q) brought about no change and that of Cys69 (C69S) led to no formation of pigments. These results suggest that Tyr42, Glu46, and Thr50 strongly interact with the chromophore, while Arg52 does not contribute the color tuning of PYP.

Key words: chromophore/protein interaction, expression, *in vitro* mutagenesis, opsin shift, photoactive yellow protein.

Photoactive yellow protein (PYP) is a small soluble protein found in a halophilic photosynthetic bacterium, *Ectothiorhodospira halophila* (*1*). Since the action spectrum of the photophobic response agrees with the absorption spectrum of PYP (absorption maximum, 446 nm), PYP is considered to function as a photoreceptor for the negative phototaxis of this organism (*2*). On absorbing light, PYP undergoes a series of spectral changes owing to the formation of red-shifted and blue-shifted intermediates, and then returns to the dark state (*3-6*). This photocycle involves the isomerization of the chromophore (*7*) and is very similar to those of bacteriorhodopsin (bR) and other retinal proteins in *Halobacterium salinarium*.

Unlike other photoreceptor proteins, such as visual pigments and bacterial rhodopsins, PYP is a soluble protein complex composed of a small protein moiety and a simple chromophore. The primary structure of PYP was first investigated by protein sequencing (*8*), and recently deduced from the nucleotide sequence of a DNA fragment encoding PYP (*9*). X-ray crystallography at 1.4 Å resolution has revealed that PYP has an α/β fold structure (*10*). The chromophore of PYP is *p*-coumaric acid (4-hydroxycinnamic acid) bound to Cys69 by a thioester linkage (*9, 11*). It is in the anionic form in the dark state (*9, 12*), and the deprotonated phenolic oxygen interacts with nearby amino acid residues Tyr42, Glu46, and Thr50. Arg52 is located near this region and is thought to stabilize the interaction (Fig. 1a).

PYP is one of the simplest photoreceptor proteins discovered so far and as such holds great promise for the detailed investigation of its structure and function. However, in contrast to the detailed knowledge of the structure of PYP, little is known about the molecular mechanisms of the absorption of a photon by PYP, or what kinds of structural changes take place during the PYP photocycle. Here, we report the isolation of the PYP gene from *E. halophila* and the construction of a high-level expression system for apoPYP in *E. coli*. PYP was reconstituted from expressed apoPYP and *p*-coumaric acid derivatives (*13*) and purified for spectral measurements. Using these techniques, five kinds of site-directed mutants of PYP were prepared, and the effects of the amino acid substitutions on the absorption spectrum was examined to investigate the color regulation mechanism in PYP. The interactions between the chromophore and these amino acid residues are discussed.

MATERIALS AND METHODS

Preparation of PYP from E. halophila—*E. halophila* (BN 9626) was kindly provided by Prof. Keizo Shimada (Tokyo Metropolitan University) and cultured as reported previously (*1*). Native PYP (hPYP) was isolated from *E. halophila* as reported previously (*1, 13*).

Cloning of DNA Fragments Encoding PYP—Genomic

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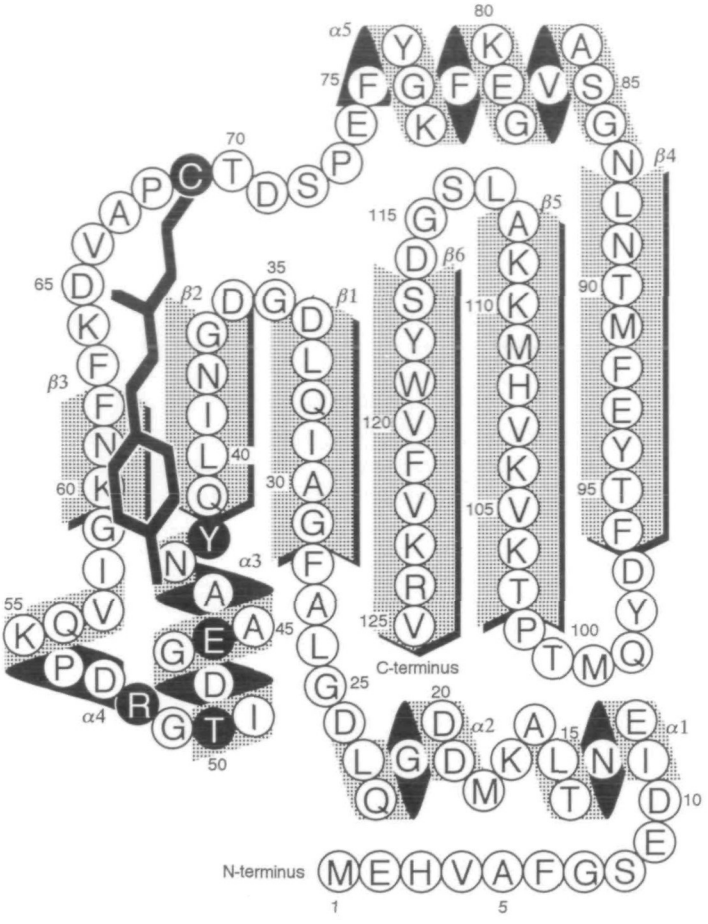
Abbreviations: apo-ePYP, apoprotein of ePYP; apo-hPYP, apoprotein of hPYP; bR, bacteriorhodopsin; ePYP, PYP expressed in *E. coli*; hPYP, native PYP isolated from *E. halophila*; NT-F, CT-R, primers used for cloning of PYP; pET-PYP, the expression vector for ePYP; PYP, photoactive yellow protein.

(a)



(b)

Oligonucleotides used in this study				
Isolation				
NT-F(F)	-7	5' GAATTCCATGGARCAYGTNGCNTTYGG 3'		22
CT-R(R)	389	5' AAGCTTGGATCCTANACNCKYTTNACRAANAC 3'		358
Addition of restriction sites				
PYP-PstI(F)	108	5' CGGCAACATCCTGCAGTACAACGC 3'		131
PYP-EcoRI(R)	234	5' CTTGCCGTAGAATTCCGGGCTG 3'		213
Mutation				
Y42F(F)	117	5' CCTGCAGTTCAACGCCGCGGAG 3'		138
E46Q(F)	117	5' CCTGCAGTACAACGCTGCGCAGGGCGACATCAC 3'		149
T50V(R)	162	5' CGGGTCGCGGCCGACGATGTCGCCCT 3'		137
R52Q(F)	144	5' CATCACCGGTCTGGACCCGAAGCAGG 3'		169
C69S(F)	195	5' CGTGGCCCCGTCCACTGACAGCCCGGAA 3'		222

Fig. 1. **(a) Schematic drawing of the structure of PYP** (modified from Ref. *10*). The amino acid residues replaced in the present experiment are shown as black circles. **(b) The sequence of primers used for cloning of DNA fragments encoding PYP or *in vitro* mutagenesis, containing the additional restriction sites (underlined).** Reversed letters indicate the nucleotide sequences corresponding to the mutated amino acids. Bracketed F and R indicate sense and anti-sense primers, respectively. The numbers correspond to the first and last nucleotide numbers from the translational initiation codon of the insertion site in PYP.

DNA of *E. halophila* was isolated using standard procedures (*14*). About 1 μg of the genomic DNA was used as a template for polymerase chain reactions (PCRs) in amplification mixtures containing 1 μM oligonucleotide mixtures, NT-F and CT-R (Fig. 1b) (*15*). NT-F has the nucleotide sequence corresponding to the first seven amino acids of PYP and the recognition sequences of *Eco*RI and *Nco*I endonucleases. The CT-R has a complementary nucleotide sequence corresponding to the C-terminal six amino acid sequence and restriction sites of *Bam*HI and *Hin*dIII. The amplified DNA fragments were cloned between the *Eco*RI and *Hin*dIII restriction sites of a pUC18 plasmid vector. A clone was digested with *Nco*I and *Bam*HI endonucleases, and the PYP coding region was re-cloned between the *Nco*I and *Bam*HI sites of a pET-16b plasmid vector (Novagen). *E. coli* cells (BL21DE3) were transformed by these plasmids, and the clone (pET-PYP) which showed the highest PYP expression was used for expression of PYP in *E. coli* (ePYP).

In Vitro Mutagenesis—The PCR-mediated mutagenesis of the PYP gene was carried out using the oligonucleotide primers (Fig. 1b). To make mutations easier, additional

restriction sites for *Pst*I and *Eco*RI were made at the positions corresponding to Leu40 and Glu74 by using PYP-*Pst*I and PYP-*Eco*RI, respectively (Fig. 1b). Mutation primers were designed to induce the amino acid substitutions at the specific residues (Fig. 1b).

Expression of apo-ePYP—E. coli cells (BL21DE3) containing pET-PYP or pET-PYP mutants were cultured at 37°C in 2×YT medium containing 50 μg/ml ampicilin. When the absorbance at 600 nm of the culture solutions reached about 1.0, expression of ePYP and of the mutants was induced by the addition of isopropyl-thio-β-galactoside (IPTG; final concentration 1 mM). After about 6 h, cells were harvested by centrifugation at 5,000×*g* for 15 min. Expressions of apoproteins of ePYP and the mutants were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (*16*).

Reconstitution and Purification of ePYP—Harvested cells were suspended in the extraction buffer (8 M urea, 25 mM Tris-HCl, pH 8.1) and stirred for 15 min at 0°C. The supernatant was diluted by the addition of an equivalent volume of buffer A (25 mM Tris-HCl, pH 8.1), followed by addition of a 2-3-fold molar excess of *p*-coumaric anhydride, which was synthesized as described (*13*). The reaction mixture was stirred at room temperature overnight and centrifuged (100,000×*g*, 30 min). The supernatant was dialyzed against buffer B (10 mM Tris-HCl, pH 7.4) and applied to a DEAE-Sepharose CL6B (Pharmacia) column pre-equilibrated with buffer B. After washing the column with buffer B, ePYP was eluted with a linear gradient of NaCl (100-200 mM) in buffer B, and the yellow fractions eluted around 150 mM NaCl were collected. Ammonium sulfate powder was added to the fraction to 60% saturation, and the mixture was stirred at 4°C for 30 min and centrifuged (4,800×*g*, 20 min). The supernatant was dialyzed against buffer B at 4°C (overnight), and DEAE-Sepharose CL6B column chromatography was repeated until the optical purity index (Abs_{275}/Abs_{446}) of ePYP reached ~0.5. Fractions containing ePYP were collected and concentrated by using an ultrafiltration membrane (Amicon, Centriprep-10).

Spectroscopy—Absorption spectra were recorded with a Hitachi 3210 recording spectrophotometer interfaced with a personal computer (NEC 9801 NS/R) to store and analyze the spectral data.

RESULTS

The amount of apo-ePYP in *E. coli* was estimated by SDS-PAGE to be 20-30% of the total proteins (data not shown). The apparent molecular weight of apo-ePYP was the same as that of the native apoPYP (apo-hPYP) from *E. halophila*. Apo-ePYP appeared to be in the insoluble fraction, suggesting that it was contained in inclusion bodies.

Because apo-ePYP was insoluble, it had to be solubilized with a denaturant before reconstitution. In the present study, apo-ePYP was solubilized in 8 M urea, and the extract was 2-fold diluted. ePYP was then reconstituted by adding *p*-coumaryl thiophenyl ester in 4 M urea. These manipulations were carried out gently and all the buffers used were extensively deaerated to avoid oxidization of the thiol group of Cys69, because such oxidation inhibits the formation of a thioester bond. After addition of *p*-coumaric anhydride, the reaction mixture turned yellow and the formation of the pigment was saturated after stirring for 12 h. The absorption spectrum of purified ePYP was identical with that of hPYP in the visible region (Fig. 2). Furthermore, the optical purity index (Abs_{275}/Abs_{446}) of ePYP was almost same as that of hPYP. These findings indicate that ePYP folded correctly and the chromophore was accommodated in the same manner as hPYP. We obtained 50 mg (150 OD·ml) ePYP from a 1-liter culture of *E. coli*.

The chromophore of PYP is deprotonated at the phenolic oxygen and negatively charged (*9, 12*). From the tertiary structure of PYP (*10*), we noted five amino acid residues (Tyr42, Glu46, Thr50, Arg52, and Cys69) (Fig. 1a). Tyr42, Glu46, and Thr50 interact with the phenolic oxygen of the chromophore through the hydrogen-bonding network (*10*), Arg52 stabilizes the negative charge of the chromophore and protects the chromophore from the solvent, and Cys69 is the chromophore-binding site; so these residues were expected to be essential for PYP to absorb visible light. Indeed, they are perfectly conserved in all PYPs whose primary structures have been studied so far (*17-19*). To investigate their functions, they were replaced by other amino acid residues which have different physicochemical properties: Phe was substituted for Tyr42 (Y42F), Gln for Glu46 (E46Q), Val for Thr50 (T50V), Gln for Arg52

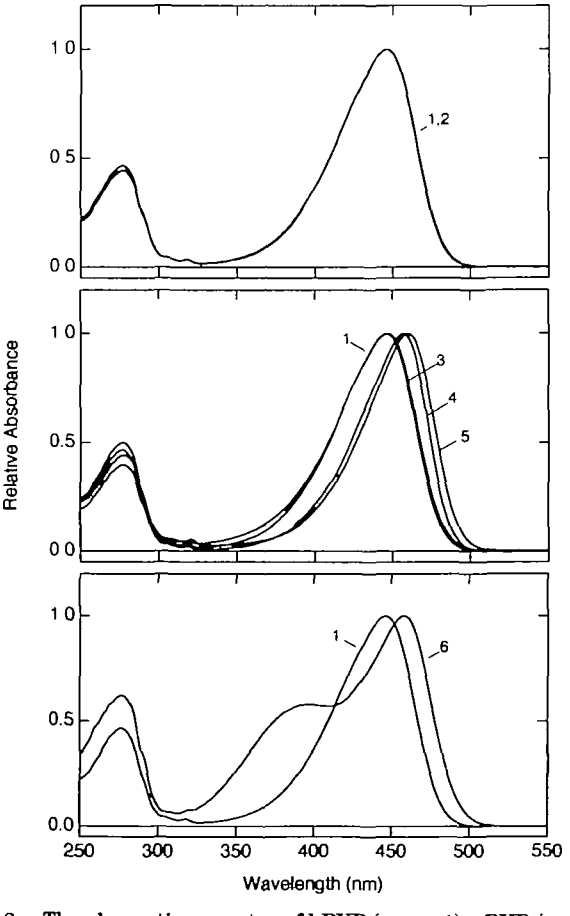


Fig. 2. **The absorption spectra of hPYP (curve 1), ePYP (curve 2), R52Q (curve 3), T50V (curve 4), E46Q (curve 5), and Y42F (curve 6).** The absorption maxima were 446 nm for hPYP and ePYP, 458 nm for Y42F, 460 nm for E46Q, 457 nm for T50V, and 447 nm for R52Q.

(R52Q), and Ser for Cys69 (C69S). The mutated PYPs were expressed in *E. coli* and reconstituted in the same manner as for ePYP.

The absorption maxima of Y42F, E46Q, and T50V were located at 458, 460, 457 nm, respectively, which were red-shifted by 10-14 nm from the wild-type ePYP (446 nm) (Fig. 2). In the absorption spectrum of Y42F, a spectral shoulder was observed at about 370 nm. Because it was considerably separated from the absorption maximum, it was attributed not to the vibrational fine structure of Y42F, but to the presence in the sample of a 370-nm species. Unexpectedly, R52Q had an absorption maximum at 447 nm, and the shape of the absorption spectrum was identical with that of ePYP (Fig. 2), indicating that Arg52 has no electrostatic interaction with the chromophore, and has no role in the color tuning of PYP.

DISCUSSION

PYP mutants were required for the further studies of PYP which specify the functional regions of the protein moiety. In the present study, we established a high-level expression system of PYP in *E. coli*, methods for reconstitution of ePYP, and the site-directed mutagenesis of PYP.

We prepared five mutants of PYP. The absorption spectra of Y42F, E46Q, and T50V were red-shifted, like the D85N and D212N mutants of bacteriorhodopsin. The absorption maximum of R52Q was located at 447 nm, which was almost identical with that of ePYP (Fig. 2). No pigment was formed from C69S (data not shown). E46Q, T50V, and R52Q had broad absorption spectra similar to ePYP and hPYP, but that of Y42F had a spectral shoulder at about 370 nm. Because it was considerably separated (5,200 cm^{-1}) from the absorption maximum, this was attributed not to the vibrational fine structure of Y42F, but to the coexistence of a 370-nm species. The denatured PYP at neutral pH has its absorption maximum at 340 nm. Therefore, it is unlikely that the 370-nm product is in the denatured state. Some effects of the nearby amino acid residues still remained, though the near-UV absorption maximum strongly suggests that the chromophore is not in the anionic form. Tyr42 is located in the center of the hydrogen-bonding network around the chromophore, and its mutation may destabilize the phenolic anion.

The absorption maximum of PYP is 446 nm, while that of free *p*-coumaric acid is 309 nm. The latter shifts to 340 nm on forming the thioester bond with cysteine, and is further shifted to 400 nm by ionization of the phenolic oxygen (*9*). The shift from 400 to 446 nm is attributed to the effects of the surrounding amino acid residues and is thought to correspond to "opsin shift," which is the parameter used in the color tuning mechanism of retinal proteins. "Opsin shift" is denoted as the shift from the protonated Schiff base (440 nm) to the intact pigment. In the case of rhodopsin, whose absorption maximum is at 500 nm, the opsin shift is calculated to be 2,700 cm^{-1}. The opsin shift of PYP (PYP shift) is calculated to be 2,600 cm^{-1} (400→446 nm), which is comparable to that of rhodopsin. Additional PYP shifts were observed in mutants, namely, 600 cm^{-1} for Y42F, 700 cm^{-1} for E46Q, and 550 cm^{-1} for T50V. The PYP shifts of the mutants are considerable, suggesting that the electrostatic and/or the steric interaction between the chromophore and the protein moiety is largely altered.

Crystallography (*10*) had shown that the distances from phenolic oxygen (O4) of the chromophore to Oη of Tyr42 and Oε of Glu46 are 2.71 and 2.69 Å, respectively, and that these form a hydrogen-bonding network. The Oγ of Thr50 is located 3.84 Å from O4 but 2.83 Å from Oη of Tyr42. It appears that the distance from O4 to the hydroxyl group which was truncated by the mutagenesis correlates with the extent of the red-shift.

Lack of hydroxyl groups of Tyr42 and Glu46 induced red-shifts of 600-700 cm^{-1}. Because they are hydrogen-bonded with O4, alternation of the electrostatic interaction would take place. This is similar to the fact that D85N mutant of bacteriorhodopsin, which lacks the counter ion, has an absorption maximum at 605 nm, red-shifted by 1,000 cm^{-1} from the wild-type (570 nm) (*20*). Though the hydroxyl group of Thr50 has been reported not to form a hydrogen-bond with O4, its absence causes a 550-cm^{-1} red-shift. Therefore, changes in the hydrogen-bonding network which overlies Thr50---Tyr42---chromophore---Glu46 are suggested to cause the shift of the absorption spectrum.

Glu46 has recently been suggested to be a proton donor of the chromophore during the photocycle (*21*). This work, however, lacks experimental evidence for the assignment of the residue which functions as a proton donor, because the methods for the preparation of mutant PYP had not been established. The experiments using our mutant will clarify which amino acid residue is the proton donor of the chromophore.

It should be noted that R52Q has an absorption spectrum almost identical with ePYP, although Arg52 is considered to stabilize the hydrogen-bonding network surrounding the O4 of the chromophore. This strongly suggests that Arg52 has no effect on the hydrogen-bonding network. Arg52 thus appears to be involved not in the stabilization of the hydrogen-bonding network around the chromophore, but in the proton transfer and/or the conformational change during the photocycle. The precise mechanism of the PYP-shift and the role of Arg52 will be clarified by analysis of the change in tertiary structure induced by the mutation. This is now under investigation and will be reported elsewhere.

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