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Structure and Photoreaction of Photoactive Yellow Protein

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ABSTRACT: The chromophore/protein interactions in the photocycle intermediates of photoactive yellow protein (PYP) were probed by site-directed mutagenesis. The absorption spectra of L-intermediates produced from E46Q, T50V, and R52Q mutants were calculated using the absorption spectra of dark states and difference absorption spectra between L-intermediates and dark states, and compared with that of PYP_L. The absorption spectrum of $R52Q_L$ agreed with that of PYP_L, but those of $E46Q_L$ and $T50V_L$ were red-shifted. The effect of these mutations on the absorption spectrum for L-intermediate was comparable to that for the dark state, suggesting that the interaction around the phenolic oxygen of the chromophore is conserved in PYP_L unlike the crystal structure. On the other hand, we have reported that the absorption spectra of $Y42F_M$, $T50V_M$, and $R52Q_M$ agreed with that of PYP_M, but that of $E46Q_M$ was red-shifted, suggesting that the hydrogen bond of the chromophore with Glu46 is conserved but that with Tyr42 is broken in PYP_M. These results suggest that the chromophore interacts with Glu46 throughout the photocycle, but never directly interacts with Arg52. This model conflicts with some of the structural model of PYP intermediates proposed based on the high-resolution X-ray crystallography.

Key words: X-ray crystallography / spectroscopy / photocycle / protein structure / photoreceptor

INTRODUCTION

Photoactive yellow protein (PYP) found in a purple phototropic bacterium, *Ectothiorhodospira halophila*, is proposed to be a photoreceptor protein for the negative phototaxis of the bacterium (for recent review see [1]). It is composed of 125 amino acid residues and a chromophore, *p*-coumaric acid (4-hydroxycinnamic acid) binding to Cys69 by a thioester bond. PYP is comprised of a six-stranded anti-parallel β-sheet, chromophore binding loop, and N-terminal loop [2]. The phenolic oxygen of the chromophore is deprotonated and hydrogen-bonded with Tyr42 and Glu46. Thr50 is hydrogen-bonded with Tyr42, and indirectly interacts with the chromophore. Because these residues are involved in the color regulation of PYP, the mutation for these residues alters the absorption spectrum of PYP [3]. Arg52 is located near this region and thought to stabilize this hydrogen-bonding network. However, because Arg52 forms no direct hydrogen bond with the chromophore, the mutation for Arg52 has little effect on the absorption spectrum. On photon absorption, the chromophore is isomerized from trans form to cis form [4]. As a result, two photocycle intermediates, PYP_B and PYP_H [5] are formed. They are thermally converted to PYP_L, and then revert to PYP via PYP_M. These intermediates can be distinguished by their characteristic absorption spectrum.

Because PYP is a water-soluble protein of 14 kDa, the X-ray crystallographies for photocycle intermediates as well as dark states have been attempted [6-8]. Based on the structural models of the photocycle intermediates revealed by crystallography, the reaction mechanism of PYP has

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been proposed. On the other hand, the photoreaction of PYP in the physiological condition has recently studied by various spectroscopic techniques. However, reaction models based on the crystallography do not explain the recent spectroscopic data [9-12]. In the present study, the chromophore/protein interactions in the photocycle intermediates of PYP were probed by site-directed mutagenesis on the chromophore-binding pocket. On the basis of the results, the behavior of the chromophore during the photocycle is discussed.

MATERIALS AND METHODS

Wild-type PYP, E46Q, T50V, and R52Q mutants were produced using a heterologous overexpression system by *Escherichia coli* and purified by ammonium sulfate precipitation and DEAE-Sepharose column chromatography. Finally they were suspended in 10 mM Tris buffer containing 150 mM NaCl and 66 % glycerol at pH 8.1. Low-temperature UV-visible spectroscopy was carried out using a Shimadzu UV-2400PC spectrophotometer equipped with an optical cryostat (Optistat DN, Oxford) [10]. The absorption spectra of L-intermediates were calculated using the absorption spectra of the dark states and difference absorption spectra between the dark states and L-intermediates assuming that the extinction coefficients of L-intermediates of the mutants are comparable to that of wild type.

RESULTS AND DISCUSSION

The absorption maximum of wild-type PYP is located at 446 nm, whereas those of E46Q, T50V and R52Q are at 460, 456, and 446 nm, respectively (Figure 1) [3]. It is consistent with the crystal structure of dark-state PYP in which phenolic oxygen of the chromophore is hydrogen-bonded with Tyr42, Glu46, and Thr50, but not with Arg52. Therefore, the site-directed mutagenesis is the most simple and straightforward method to probe the chromophore/protein interaction. Similarly, the absorption spectra of the photocycle intermediates of PYP mutants were examined. We have previously recorded the difference absorption spectra between L-intermediates and dark states [10]. However, because L-intermediate is a red-shifted intermediate, the wavelength at which only dark state has absorbance was not present. That makes it difficult to estimate the amount of the L-intermediate, which is essential to calculate the absorption spectra of L-intermediates. Here, they were calculated assuming that the extinction coefficients of L-intermediates of the mutants are comparable to that of wild type L-intermediate (PYP_L).

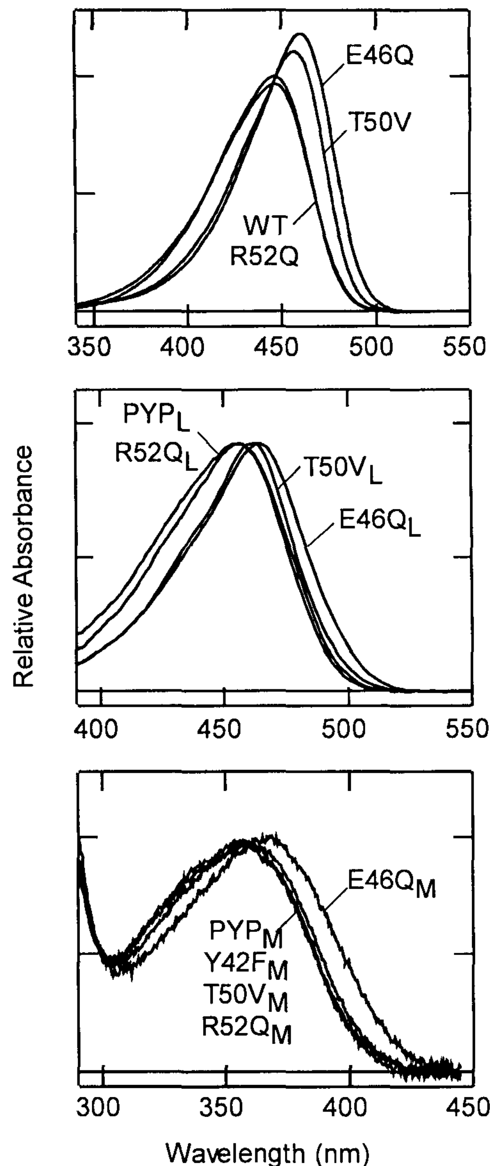

Figure 1: The absorption spectra of PYP and mutants. *Top*: The absorption spectra of PYP, E46Q, T50V, and R52Q in the dark. *Middle*: The absorption spectra of L-intermediates measured at 193 K. *Bottom*: Absorption spectra of M-intermediates measured at 293 K (reproduced from [10]).

The absorption spectra of dark states were measured at 193 K. Then they were irradiated with blue-light, and the difference absorption spectra between the dark states and L-intermediates were recorded. The absorption spectra of L-intermediates were calculated by adding difference spectra to the dark-state spectra. The factors were determined so

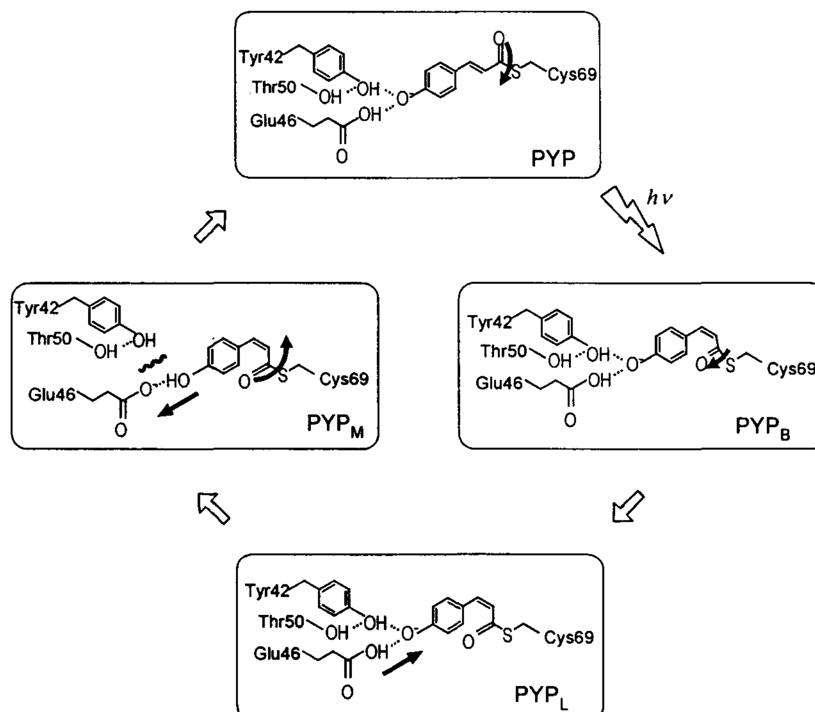

Figure 2: Model for the structural change of the chromophore based on the spectroscopic evidence.

that their extinction coefficients are comparable to that of wild type (Figure 1). The shape of the spectrum of R52Q$_L$ agreed with that of PYP$_L$, while those of E46Q$_L$ and T50V$_L$ were red-shifted. The effect of these mutations on the spectral shift for L-intermediate was comparable to that for the dark state and PYP$_B$, a precursor of PYP$_{BL}$ [10], suggesting that the hydrogen-bonding network around the phenolic oxygen of the chromophore is conserved in PYP$_L$. For comparison, the absorption spectra of PYP$_M$, E46Q$_M$, T50V$_M$, and R52Q$_M$ [10] are shown in Figure 1. The absorption spectrum of E46Q$_M$ was red-shifted from PYP$_M$, while those of Y42F$_M$, T50V$_M$ and R52Q$_M$ agreed with that of PYP$_M$.

The structures of the photocycle intermediates of PYP have been extensively analyzed by a high-resolution X-ray crystallography. The structure of PYP$_M$ (also called I2 or pB) was first proposed by time-resolved crystallography on a millisecond time scale. It has shown that the phenol part of the chromophore rotates on photo-isomerization, and the hydrogen-bonding network among the chromophore, Tyr42 and Glu46, is broken and a new hydrogen bond is formed between the chromophore and Arg52. If this reaction takes place in the solution, the absorption spectrum of PYP$_M$ should agree not with R52Q$_M$ but with E46Q$_M$ unlike the spectroscopic data.

The structure of PYP$_L$ (also called I1 or pR) was later proposed by time-resolved crystallography on a nanosecond time scale. Structural modeling revealed that the phenol part rotates for formation of PYP$_L$. As a result, while the movement of the phenol ring is small, the phenolic oxygen of the chromophore is removed from Glu46, and the hydrogen bond is broken. This model conflicts with the present spectroscopic data which suggests that the hydrogen-bonding network in the dark state is conserved in PYP$_L$.

PYP$_{BL}$, the intermediate that appears between PYP$_B$ and PYP$_L$, has been trapped at 150 K and the structure was analyzed. In this model, the thioester part is flipped upon isomerization, and the hydrogen-bonding network around the phenolic oxygen of the chromophore is maintained in PYP$_{BL}$. This structure is consistent with the previous spectroscopic data [10].

The analysis based on the UV-visible absorption spectra demonstrated that the hydrogen-bonding network around the chromophore is conserved up to PYP$_L$, but it is rearranged upon the formation of PYP$_M$. Our recent low-temperature FTIR spectroscopy of PYP demonstrated that the C=O stretching mode of Glu46 of PYP$_B$, PYP$_H$ and PYP$_L$ was constant (1732-1733 cm^{-1}). It indicates that the environment of the hydrogen bond is not altered in these intermediates. In contrast, remarkable difference in the vibrational mode of ethylene bond is observed among these

intermediates. Therefore, the structural difference of the chromophore in these intermediates lies in the ethylene bond region rather than the phenolic oxygen.

The possible model based on our spectroscopic data is shown in Figure 2. The chromophore of PYP in the dark is in a deprotonated *trans* form, and Glu46 is protonated. On photon absorption, the *trans* chromophore is isomerized to *cis* form. Because it is considered that no large change of the hydrogen-bonding network takes place in PYP_B, the ester part of the chromophore would be flipped. PYP_B has the chromophore in twisted and deprotonated *cis* form and Glu46 is still protonated. On the conversion process to PYP_L, only a small structural alternation around the phenol part and Glu46 takes place. In contrast, the distortion of the chromophore at the ethylene bond would be relaxed by rotating the ester part. PYP_L then converts to PYP_M with proton transfer from Glu46 to the chromophore [13]. The red shift of the absorption spectrum of $E46Q_M$ strongly suggests that the hydrogen bond of chromophore with Glu46 is maintained but that with Tyr42 is broken in PYP_M, resulting in the global conformational change.

Our spectroscopic evidence supports the structural model of PYP_{BL} derived from crystallography. However, there are inconsistencies with the models for PYP_L and PYP_M from crystallography. The crystallization of PYP would alter the reaction pathway of PYP especially in the latter stage.

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