Evidence for Proton Transfer from Glu-46 to the Chromophore during the Photocycle of Photoactive Yellow Protein

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Photoactive yellow protein (PYP) belongs to the novel group of eubacterial photoreceptor proteins. To fully understand its light signal transduction mechanisms, elucidation of the intramolecular pathway of the internal proton is indispensable because it closely correlates with the changes in the hydrogen-bonding network, which is likely to induce the conformational changes. For this purpose, the vibrational modes of PYP and its photoprotein were studied by Fourier transform infrared spectroscopy and identified several intermediates (10). Irradiation of PYP at −190 °C yields PYPH (λ_{max} = 489 nm) and PYPH_{L} (λ_{max} = 442 nm), which are thermally converted to PYPH_{L} (λ_{max} = 456 nm) through PYPH_{BL} (λ_{max} = 400 nm) and PYPH_{NH}, (λ_{max} = 447 nm), respectively. The two pathways beginning with PYPH_{L} and PYPH join at PYPH_{L} and revert to PYP. Flash photolysis at ambient temperature identified two intermediates, pR (λ_{max} = 465 nm) and pB (λ_{max} = 355 nm) (11, 12). pR is formed within 10 ns after flash excitation. It decays to pB over a submillisecond time scale and reverts to PYP within 1 s. It has been demonstrated that pR is the same species as PYPH (10) (In this paper, pR and pB are called PYPH and PYPH_{L}, respectively, to avoid confusion) and that PYPH_{L} is accumulated by irradiation of PYP at −80 °C (10, 13). However, the precursors of PYPH_{L} have not been discovered by flash photolysis at room temperature.

Recent studies have clarified some details of the events that take place during the PYP photocycle. Crystallography at 1.4-Å resolution (7) and resonance Raman spectroscopy (14) have shown that the phenolic oxygen of the chromophore of PYP is deprotonated in the dark state. The photocycle is initiated by photon absorption, which involves isomerization of the ethylenic bond of the chromophore (15). During the photocycle, observed proton uptake and release correspond with the formation and decay of PYPH_{L} (16).

The largely blue-shifted absorption spectrum of PYPH_{M} suggests that the chromophore/protein interaction of PYPH_{M} is quite different from that of PYP. Therefore, elucidation of the chromophore structure of PYPH_{M} is essential to understand the photocycle of PYP. For the spectral blue-shift of PYPH_{M}, the following explanations would be possible: (i) the phenolic oxygen of the chromophore is protonated; (ii) the conjugated double bond system is broken by extreme distortion of the chromophore (25); and (iii) the π electrons of the conjugated double bond system are localized by a nearby positive charge (26). The first explanation is the most simple and plausible, but there has been no previous experimental evidence to support it, and the others could not be excluded.

In the dark state, the phenolic oxygen of the chromophore interacts with the OH groups of Tyr-42 and Glu-46 by hydrogen bonds (5). As shown in retinal protein systems, the changes in the hydrogen-bonding network centering on the chromophore closely correlates with the protein conformational changes (17). Therefore, it is of importance to study the internal proton movement around the chromophore and nearby amino acid residues to understand the light signal transduction mechanism of PYP. Recently it has been reported that the C=O stretching mode of the COOH group disappears on the formation of PYPH_{M} (18). They reasoned that Glu-46 donates a proton to the chromophore, based on the fact that Glu-46 has a unique COOH group embedded in the protein. This idea arose on elucidation of the tertiary structure of PYP (5) and seems reasonable. However, two vital pieces of experimental evidence required to support this conclusion have never been reported:

far. Namely, the protein moiety of PYP has an α/β fold structure (5) composed of 125 amino acids (6, 7). The chromophore is a p-coumaric acid (7–9) bound to a cysteine residue via a thioester bond.

PYP absorbs a photon and enters the photocycle. We have analyzed the photocycle of PYP in detail by low temperature spectroscopy and identified several intermediates (10). Irradiation of PYP at −190 °C yields PYPH (λ_{max} = 489 nm) and PYPH_{L} (λ_{max} = 442 nm), which are thermally converted to PYPH_{L} (λ_{max} = 456 nm) through PYPH_{BL} (λ_{max} = 400 nm) and PYPH_{NH}, (λ_{max} = 447 nm), respectively. The two pathways beginning with PYPH_{L} and PYPH join at PYPH_{L} and revert to PYP. Flash photolysis at ambient temperature identified two intermediates, pR (λ_{max} = 465 nm) and pB (λ_{max} = 355 nm) (11, 12). pR is formed within 10 ns after flash excitation. It decays to pB over a submillisecond time scale and reverts to PYP within 1 s. It has been demonstrated that pR is the same species as PYPH (10) (In this paper, pR and pB are called PYPH and PYPH_{L}, respectively, to avoid confusion) and that PYPH_{L} is accumulated by irradiation of PYP at −80 °C (10, 13). However, the precursors of PYPH_{L} have not been discovered by flash photolysis at room temperature.

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1 The abbreviations used are: PYP, photoactive yellow protein from E. halophilus; FTIR, Fourier transform infrared.
one is that the chromophore of PYPM is protonated, and the other is assignment of the C=O stretching mode.

Recent progress in experimental techniques made it possible to prepare PYP with an isotope-labeled chromophore (9) and a site-directed mutant of PYP (19, 27). These techniques have enabled assignment of the vibrational modes. In the present study, to study the movement of the proton around the chromophore and nearby amino acid residues, the vibrational modes of the chromophore and COOH group of PYP and its intermediates were analyzed by low temperature FTIR spectroscopy. The first experimental findings indicating proton transfer from Glu-46 to the chromophore during the photocycle are presented.

**MATERIALS AND METHODS**

PYP was isolated from *E. halophila* BN 9626 according to previously reported methods (1, 10). The Glu-46 → Gln mutant of PYP (E46Q) was expressed by *Escherichia coli* and reconstituted with p-coumaric anhydride (19). p-Coumaric-8-13C-acid was prepared by p-hydroxybenzaldehyde and triethylphosphonoacetate-2-13C followed by alkaline hydrolysis of the ester. 13C-Labeled PYP was prepared by reconstitution of PYP with 13C-labeled p-coumaric anhydride and apoPYP (9). They were then desalted by dialysis and applied to a small DEAE-Sepharose column (Pharmacia Biotech Inc.). After washing the column with 10 mM phosphate buffer (pH 7.2), PYP was eluted with a linear gradient of NaCl (100–200 mM) in the same buffer. PYP was then concentrated with an ultrafiltration membrane (Centriprep 10, Amicon) and diluted with 10 mM phosphate buffer. Dilution and concentration steps were repeated several times to remove NaCl. Finally, PYP was concentrated to 3–4 mg/ml.

A 20 μl sample was placed on a BaF2 window (10 mm in diameter) and dried under a gentle stream of N2 gas. The dried sample was sealed using silicon rubber spacer and another BaF2 window and set in the sample cell holder. Before sealing, 0.2 μl of H2O or D2O was put inside the spacer for hydration or deuteration of PYP. The sample cell holder was mounted in an optical cryostat (DN1704, Oxford) connected to a temperature controller (ITC502, Oxford).

Absorption spectra in the UV-visible region were recorded with a Hitachi U-3210 recording spectrophotometer. Infrared spectra were recorded with a Horiba FT-210 Fourier transform infrared spectrophotometer equipped with an MCT detector. The sample was irradiated with a 1 kW slide projector (HILLUX-HR, Tokyo Master) using glass optical filters (Y47, Toshiba). The difference FTIR spectra shown in this paper are the means of four to eight independent recordings, each of which was the mean of 64 scans (resolution = 2 cm⁻¹).

**RESULTS**

Our recent low temperature spectroscopy results with PYP in 66% glycerol buffer showed that PYPM is formed by the irradiation of PYP at −80 °C, but PYPM was not observed in the thermal reaction (10). This could be due to the effect of the presence of glycerol at high concentration, because it suppresses the decay of PYPM but accelerates the decay of PYP M (20). In the method used here, the film sample can be frozen with no increase in turbidity, and the addition of glycerol was not necessary for the low temperature experiments, thus removing impediments to the formation of PYPM. We tested several irradiation conditions above −80 °C to accumulate PYPM without PYPl for FTIR spectroscopy. Upon irradiation of PYP film with >450-nm light at −40 °C, the absorbance at 350 nm was increased (Fig. 1). Because this product had largely blue-shifted absorption spectrum similar to PYP, and directly reverted to PYP (data not shown), it would correspond to pB (called PYPM hereafter). In this difference spectrum, the absorbance at 480 nm was not increased, indicating that only PYPM was formed. Under this irradiation condition, the difference FTIR spectra were recorded with hydrated and deuterated PYP films. The difference spectra before and after irradiation with >450-nm light at −40 °C are the difference between PYPM and PYP (PYPM/PYP spectrum), in which positive and negative peaks are attributed to PYPM and PYP, respectively (Fig. 2). The prominent bands of PYP were observed at 1736, 1558, 1498, 1439, 1301, 1163, 1058, 1041, 983, and 831 cm⁻¹. On the other hand, the intensities of the bands of PYPM were relatively small, but 1175, 1081, and 994 cm⁻¹ bands were characteristic for PYPM. Several bands of them were affected by D2O substitution.

Recent resonance Raman spectroscopy covering the range 1750–1000 cm⁻¹ (14) was helpful for the implication of our FTIR data. It was reported that the prominent vibrational modes of PYP were observed at 1633, 1558, 1498, 1389, 1165, 1059, and 1043 cm⁻¹ in H2O buffer (14) and were shifted 0–4 cm⁻¹ by D2O substitution. It was concluded that the chromophore of PYP is deprotonated; otherwise the chromophore bands would be D2O-sensitive. It was further pointed out that the deprotonated chromophore shows bands at 1498, 1437, and 1163 cm⁻¹ but when protonated there is only one band at 1176 cm⁻¹ (14). The PYPM/PYP spectrum reported here shows corresponding bands: negative at 1482, 1437, and 1163 cm⁻¹ and positive at 1175 cm⁻¹. The bands at 1482, 1437, and 1163 cm⁻¹ were observed only on the negative side, indicating that PYPM does not have vibrational modes corresponding to these. Moreover, the fact that the 1175 cm⁻¹ band was strongly affected by D2O substitution suggests that the chromophore of PYPM is protonated. To confirm that the 1175 cm⁻¹ band of PYPM is the vibrational mode of the chromophore, PYPM/PYP spectrum was recorded using PYP containing 13C-labeled chromophore at 8 position under the same irradiation condition (>450-nm light, −40 °C) (Fig. 3a). The shift of 1175 cm⁻¹ band with 13C-label (Fig. 3a) was similar to that with D2O substitution (Fig. 2). Therefore, this band is attributed to the chromophore, which proves that the chromophore of PYPM is protonated.

The negative band at 1736 cm⁻¹ should be noted because it could be the C=O stretching mode of the COOH group of either aspartic acid or glutamic acid. A similar band was found recently by FTIR spectroscopy (18), although the irradiation condition was considerably different from that of the present experiment. The appearance of the negative band without the complementary positive band indicates deprotonation of the COOH group. Among 19 acidic residues of PYP (7), the most plausible candidate for the origin of this band would be Glu-46 because its OH group directly interacts with the phenolic oxygen of the chromophore and it is buried inside the protein. To test this, a Glu-46 → Gln mutant (E46Q) of PYP was prepared.
and its vibrational modes were studied by FTIR spectroscopy. Whereas the absorption maximum of E46Q in the visible region is slightly red-shifted from wild type PYP (19), the intermediate corresponding to PYPM accumulated under the same irradiation conditions (450-nm light, 240 °C) (data not shown). FTIR spectroscopy was then carried out using E46Q, and a PYPM/PYP spectrum was obtained (Fig. 3b). As expected, the PYPM/PYP spectrum of E46Q did not show a 1736-cm\(^{-1}\) band, indicating that the C=O stretching mode of the COOH group of Glu-46 is indeed the origin of this band. Namely, Glu-46 is protonated in PYP and releases its proton in the PYPM state.

**DISCUSSION**

Based on our present observations, the model for proton movement during the PYP photocycle is shown in Fig. 4. On absorption of a photon, the chromophore of PYP isomerizes to the cis form (15). There are two possible models for the isomerization of the chromophore: one is that the phenol part rotates, and the other is that the ester part rotates. Xie et al. (18) proposed that the hydrogen bond between the OH group of Glu-46 and the phenolic oxygen of the chromophore remains in PYPL and supported the latter model. However, they irradiated PYP at 80 K to trap PYPL. At 80 K, irradiation yields a mixture of PYPB and PYPH (10, 21), so the changes in C=O stretching mode of PYP observed at 80 K cannot be attributed to PYP L. Moreover, the intermediate formed readily absorbs a photon, and it is uncertain whether or not the photoproduct accumulated at 80 K has the cis chromophore. Therefore, few data are available to discuss the mechanism of the isomerization of the chromophore. However, the latter model is likely because the phenolic oxygen interacts with Glu-46 and Tyr-42, and it is fixed by the hydrogen bond. In the case of retinal proteins, the protein moiety has the binding site for the \(\beta\)-ionone ring of the chromophore, and the Schiff base portion (rather than the \(\beta\)-ionone ring region) rotates on photoisomerization (22). That is, in both PYP and retinal protein systems, the smaller moiety rotates on isomerization. Minimization of the conformational change upon isomerization would contribute to the fast and highly efficient photoreaction of photoreceptor proteins.

The present experiments have demonstrated the first evidence that the phenolic oxygen of the chromophore is protonated in PYPM. The absorption maximum of PYPM is 355 nm (12), which is close to that of denatured PYP at neutral pH (11, 23). Because the phenolic oxygen of PYP is protonated, the largely blue-shifted absorption spectrum of PYPM is due mainly to protonation of the chromophore. However, the possibility that steric interactions and the nearby positive charge affect the absorption spectrum of PYPM is not completely excluded. These effects will be examined in future research.
In retinal proteins, the chromophore of the dark state is protonated at the nitrogen atom of the Schiff base linkage and positively charged. It is deprotonated and neutral in the near-UV intermediates like M and metarhodopsin II. In contrast, the p-coumaryl chromophore is negatively charged in PYPM but is neutral in PYPM. Despite the striking differences in the chromophores, protein moieties, and linkages, the chromophores of both retinal proteins and PYP turn neutral in their near-UV intermediates, which are thought to be formed as the result of large conformational changes (17, 28). In addition, cis/trans isomerization of the chromophore is involved in both systems (15). These are the common events for the protein conformational changes among the photoreceptor proteins and would be the most efficient mechanism. Namely, the charged chromophore is the core of the hydrogen-bonding network that determines its tertiary structure in the dark state. Isomerization of the chromophore on photon absorption results in the loss of its charge. As a result, the hydrogen-bonding network is disordered, and the protein conformational change takes place.

In PYP, the phenolic oxygen of the chromophore interacts with Tyr-42 and Glu-46, and the chromophore binding site is Cys-69. The loss of this interaction would make the structure of the chromophore binding site remarkably flexible.

The present work confirmed that the 1736 cm⁻¹ band of PYP is the C=O stretching mode of the COOH group of Glu-46. Absence of this mode in PYPM indicates that Glu-46 is protonated in PYP but deprotonated in PYPM. Concurrently, the chromophore of PYPM is protonated. This indicates that the pKᵦ values of the chromophore and Glu-46 of PYPM are close to those in solution but that those of PYP are considerably different due to the hydrophobic environment in the protein. Therefore, the chromophore binding pocket would be exposed to the solvent in PYPM as the result of the protein conformational changes.

Because the protonation states of the chromophore and Glu-46 are complementary, the simplest model for proton pathways would be that the proton at Glu-46 is transferred to the chromophore in PYPM state, recovering to Glu-46 in the recovery step (Fig. 4). In E46Q, the chromophore would uptake proton from the solvent or Tyr-42 would act as the alternative proton donor. This model does not explain the proton release and uptake coupled with the formation and decay of PYPM. Therefore, another amino acid residue and/or water might be involved in the proton transfer, resulting in vectorial movement of the proton, as with bacteriorhodopsin (24). Further FTIR studies on the other intermediates using various site-directed mutants will elucidate the pathway of proton transfer.

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