pH-dependent Equilibrium between Long Lived Near-UV Intermediates of Photoactive Yellow Protein*

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The long lived intermediate (signaling state) of photoactive yellow protein (PYP)¹, which is formed in the photocycle, was characterized at various pHs. PYP₅ at neutral pH was in equilibrium between two spectrscopically distinct states. Absorption maxima of the acidic form (PYP₅acid) and alkaline form (PYP₅alkali) were located at 367 and 356 nm, respectively. Equilibrium was represented by the Henderson-Hasselbalch equation, in which apparent pK₅ acid was 6.4. Content of α- and/or β-structure of PYP₅acid was significantly greater than PYP₅alkali as demonstrated by the molar ellipticity at 222 nm. In addition, changes in amide I and II modes of β-structure in the difference Fourier transform infrared spectra for formation of PYP₅acid was smaller than that of PYP₅alkali. The vibrational mode at 1747 cm⁻¹ of protonated Glu-46 was found as a small band for PYP₅acid but not for PYP₅alkali, suggesting that Glu-46 remains partially protonated in PYP₅acid whereas it is fully deprotonated in PYP₅alkali. Small angle x-ray scattering measurements demonstrated that the radius of gyration of PYP₅acid was 15.7 Å, whereas for PYP₅alkali it was 16.2 Å. These results indicate that PYP₅acid assumes a more ordered and compact structure than PYP₅alkali. Binding of citrate shifts this equilibrium toward PYP₅alkali. UV-visible absorption spectra and difference infrared spectra of the long lived intermediate formed from E46Q mutant was consistent with those of PYP₅acid, indicating that the mutation shifts this equilibrium toward PYP₅acid. Alterations in the nature of PYP₅ by pH, citrate, and mutation of Glu-46 are consistently explained by the shift of the equilibrium between PYP₅acid and PYP₅alkali.

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The abbreviations used are: PYP, photoactive yellow protein from Halorhodospira halophilica (2). PYP is an attractive model protein for a common signaling mechanism because it is a structural prototype of the PAS domain superfamily, most of which are involved in the biological sensing (3). PYP is composed of 125 amino acid residues and the p-coumaric acid chromophore linking to the cysteine residue by a thiester bond (4–6). In the dark state, the phenolic oxygen of the chromophore is deprotonated (5) and hydrogen bonded with protonated Tyr-42 and Glu-46 (see Fig. 1), resulting in the yellow color. Photon absorption by the chromophore initiates the photocycle of PYP (7–11). The primary photochemical event of PYP is the trans-cis isomerization of the chromophore (12, 13), like bacterial retinal proteins. Several intermediates are then formed, followed by a return to the dark state in 100 ms under physiological conditions. During the photocycle, photon energy stored in the twisted chromophore is released to the protein moiety to form the putative signaling state (called PYP₅, I₂, or pB). Because of a largely blue-shifted absorption spectrum with respect to the dark state, as well as the long lifetime (~100 ms), PYP₅ is easily detectable by UV-visible spectroscopy. This spectral blue shift is considered to be caused by protonation of the phenolic oxygen of the chromophore (5, 14).

Infrared spectroscopy demonstrated that PYP₅ is formed from its precursor (PYP₅, I₁, or pB) in two steps (15, 16). First, a proton at Glu-46 is transferred to the chromophore, but the global conformational change of the protein moiety does not take place at this stage, as shown by a small absorbance change of the amide mode. This intermediate (pB'') has a protonated chromophore, but the chromophore binding site is considered to be preserved. Second, a global conformational change takes place to form PYP₅, as the lifetime of pB'' (~1 ms) is not markedly different from pR (~100 μs) and the absorption spectra of PYP₅ and pB'' are similar, isolation of pB'' has been difficult in steady-state measurement. Recently, the presence of pB'' between PYP₅ and PYP₅, has been established by time-resolved UV-visible spectroscopy and Raman spectroscopy (17–19).

Although the signaling pathway of the PYP system has not yet been identified, detailed characterization of long lived intermediates (signaling state) is essential for understanding the signal transduction mechanism of PYP. We have demonstrated extensive conformational change upon formation of PYP₅. The dimension of PYP₅ is significantly larger than the dark state as shown by ~1 Å increase of Rg (20, 21) and PYP₅ is in a partially unfolded state (22). The N-terminal cap and the central part undergo a substantial structural change (21, 23); however, the cause of this large conformational change has not yet been determined.

Accumulated evidence suggests that properties of PYP₅ are highly pH-sensitive. The extent of conformational change evaluated by the difference FTIR spectra under acidic conditions (24, 25) is significantly smaller than at neutral pH (15, 23). The decay rate constant of PYP₅ is reduced by acidification, and the relationship between rate constant and pH agrees with the Henderson-Hasselbalch equation with a pK₅ of 6.4 (26). Proton uptake per PYP₅ formation is also represented by the Henderson-Hasselbalch equation with a pK₅ of 6.6 (27).

On the other hand, we have reported that PYP₅ substantially alters its nature, depending on the buffer system (20). Absorption spectra as well as the lifetime of PYP₅ at acidic pH are affected by multivalent organic ions, such as citrate. Mutation of amino acids nearby the chromophore also affects properties of PYP₅. The absorption maximum of E46Q₅ is 11-nm red-shifted (28). The protein conformational change for formation of E46Q₅ is significantly smaller than wild type (16, 29), suggesting that the photocycle of E46Q may be different from that of wild type. To
study whether there is a simple and consistent explanation for the variety of signaling states or whether each phenomenon has an individual specific reason, the spectroscopic properties of PYPM were studied in detail under various conditions using UV-visible spectroscopy, CD spectroscopy, FTIR spectroscopy, and small angle x-ray scattering measurements. The results are explained by the equilibrium between two states of PYPM.

MATERIALS AND METHODS

Sample Preparation—Wild type PYP and E46Q were prepared as described previously (30). For UV-visible and CD spectroscopy, PYP or mutant was suspended in 10 mM TAPS buffer (pH 8.5–8.0), 10 mM sodium citrate was added instead of NaCl sample concentration/H11011, E46Q were desalted, lyophilized, and resuspended in 100 mM MES buffer (pH 8.0–6.5), or 10 mM MES buffer (pH 6.5–4.0) containing 200 mM NaCl (Dojindo, Kumamoto, Japan). To investigate the effect of binding of citrate to PYP M, 50 mM (see Fig. 6) or 1 M (see Fig. 7) sodium citrate was added instead of NaCl (sample concentration ~0.15 mg/ml). For SAXS measurements, samples at pH 5.0 and 8.0 were concentrated to 2–10 mg/ml with an ultrafiltration membrane (Centricon YM10, Millipore, Billerica, MA). For FTIR measurement, PYP and E46Q were desalted, lyophilized, and resuspended in 100 mM MES buffer (pH 5.0) or TAPS buffer (pH 8.0) containing 200 mM NaCl at a concentration of 150 mg/ml.

UV-visible Spectroscopy—Absorption spectra in the dark and in the photosteady state were obtained by a multichannel CCD/fiber optic spectroscopy system (S2000 system, Ocean Optics, Dunedin, FL) (23). For steady-state measurements, the sample was irradiated with a yellow light obtained from a 150-W cold light source (HL150, HOYA-Schott, Tokyo, Japan) and a Y43 cutoff filter (>410 nm, Asahi Techno Glass, Chiba, Japan). For time-resolved measurements, the sample was excited with a yellow flash obtained with a short arc xenon flash lamp (SA200, Nissin Electronic, Tokyo, Japan) and a Y43 filter. Absorption spectra of PYPM were calculated by subtracting dark spectra from photosteady state spectra (31). At pH > 6, contribution of the small amount of PYPL was also subtracted, as reported previously (31).

CD Spectroscopy—Far-UV CD spectra of dark state and photosteady state were measured using a J-725 circular dichroism spectropolarimeter (JASCO, Tokyo, Japan). Temperature was maintained at 5°C with a PYC-347W Peltier device (JASCO). The sample cell, with a 1-mm light path length, was irradiated at an angle of 30° using a HOYA-Schott HL150 light source and an Y43 cutoff filter. To protect the detector, the band pass filter (214FS10-25, 210 nm < λ < 233 nm, T max = 14%, Andover Corporation, Salem, NH) was set in front of the detector window. It was confirmed that the artifact on CD signal caused by band-pass filter was negligible in this setup (23).

FTIR Spectroscopy—Difference FTIR spectra between photosteady state and dark state were measured using an FT6500 Fourier transform infrared spectrometer (23). The sample was put into a CaF2 cell with a 7.2 μm light path length (23). Irradiation light at 436 nm (FWHM = 10 nm) was obtained by an optical interference filter (43161, Edmund Scientific, Barrington, NJ) and a HOYA-Schott HL150 light source.

SAXS Measurements—Small angle x-ray scattering measurements were carried out at BL-10C (Photon Factory, Tsukuba, Japan (20, 21)). The exposure time was 5 min. Three to five sets of independent measurements were averaged to improve the signal to noise ratio. Temperature of the cell was maintained at 5°C. R g and I0(I) were obtained by Guinier approximation in the small angle region (33, 34),

\[ I(Q) \approx I(0) \exp \left( -\frac{1}{3} Q^2 R_g^2 \right) \] (Eq. 1)

where Q = 4πsinθ/λ is the amplitude of the scattering vector, I(Q) is the scattering intensity at Q, 2θ is the scattering angle, λ is the wavelength of the x-ray (1.488 Å), I(0) is the scattering intensity at Q = 0, and R g is the radius of gyration. The sample was irradiated with yellow light obtained using a 1000-W tungsten-halogen lamp (HILUX-HR, Tokyo Master, Japan) and Y43 cutoff filter. The scattering profiles of photoproducts were calculated using scattering patterns of the photosteady state and
**RESULTS**

**pH-dependent Spectral Change of PYP**

- **pH Titration**—Absorbance at 395 nm (see Fig. 2c) or the ellipticity at 222 nm (see Fig. 3b) of PYP was plotted against pH. Data were fitted according to the Henderson-Hasselbalch equation,

\[
\Delta \text{Abs}_{395 \, \text{nm}}, \Delta [\theta]_{222 \, \text{nm}} = k_0 + \frac{k_1}{1 + 10^{pK_a - \text{pH}}} \quad (\text{Eq. 2})
\]

where \(k_0\) and \(k_1\) are a base value and amplitude of the titration curve, respectively.

**Comparison of Secondary Structure Using CD Spectroscopy**—Differences in the secondary structure between PYP\(_{\text{alkali}}\) and PYP\(_{\text{acid}}\) were evaluated using CD spectra in the far-UV region. The CD spectra of PYP at pH 8.0 and 5.0 agreed with each other in the dark state (Fig. 3a, dashed lines), indicating that the secondary structure of the dark state PYP is pH-independent in this pH region. The CD spectra of the photosteady state were then measured, and pure CD spectra of PYP were calculated using the fraction of the dark state PYP estimated by absorption spectroscopy under the same irradiation conditions. Typical CD spectra for PYP\(_{\text{alkali}}\) at pH values 8.0, 6.5, and 5.0 are shown in Fig. 3a. The significant difference indicates that the secondary structure of PYP\(_{\text{alkali}}\) is different from that of PYP\(_{\text{acid}}\). The spectrum at pH 6.5 is in between those at pH 8.0 and 5.0, indicating that it is a 1:1 mixture of PYP\(_{\text{acid}}\) and PYP\(_{\text{alkali}}\). As the photo steady state is in equilibrium between PYP, PYP\(_{\text{L}}\), and PYP\(_{\text{M}}\), the absorption spectrum of PYP\(_{\text{M}}\) at each pH was calculated by subtracting the absorption spectra of dark state and PYP\(_{\text{L}}\) from that of the photosteady state, so that the contributions of PYP and PYP\(_{\text{L}}\) were cancelled (31). Calculated absorption spectra of PYP\(_{\text{M}}\) at pH 8.5 – 4.0 are shown in Fig. 2b. The PYP\(_{\text{M}}\) spectrum at pH 8.5 had a broad shape with the shoulder at around 330 nm (\(\lambda_{\text{max}} = 356 \, \text{nm}\)). Upon acidification, the spectrum was red-shifted (\(\lambda_{\text{max}} = 367 \, \text{nm}\)), and the shoulder disappeared. The pH-dependent spectral change of PYP\(_{\text{M}}\) at pH 8.5 – 4.0 is virtually a two-state transition with a clear isosbestic point at 362 nm. The absorbance change at 395 nm was plotted against pH (Fig. 2c). Absorbance at 395 nm was increased from pH 8.5 to 5.0, but decreased at pH < 5, suggesting that a part of PYP\(_{\text{M}}\) is denatured at low pH. The absorbance change was fitted by the Henderson-Hasselbalch equation (Equation 2). The \(pK_a\) was estimated to be 6.4, which is close to that for the rate constant (6.4) (26) or proton uptake (6.6) (27). The two states of PYP\(_{\text{M}}\) observed at pH 8.5 and at pH 5.0 are hereafter called PYP\(_{\text{alkali}}\) and PYP\(_{\text{acid}}\), respectively.
and PYPM$_{\text{alkali}}$. The loss of ellipticity at 222 nm using light is 36% for PYPM$_{\text{alkali}}$ (pH 8.0) and 9% for PYPM$_{\text{acid}}$ (pH 5.0). In Fig. 3b, the light-induced ellipticity change at 222 nm ($\Delta [\theta]_{222 \text{ nm}}$) is plotted against pH. From pH 8.5 to 5.0, $\Delta [\theta]_{222 \text{ nm}}$ gradually decreased, indicating that the loss of secondary structure is reduced. However, it increased at pH < 5, as demonstrated by the absorption spectroscopy (Fig. 2c), also indicating partial denaturation of PYPM. The apparent $\Delta [\theta]_{222 \text{ nm}}$ for the ellipticity change was 6.4, which agrees with the change in the absorption spectrum. Thus changes in the absorption spectrum of PYPM$_{\text{alkali}}$ correspond well with the secondary structural change. Figs. 2c and 3b show that the fraction of PYPM$_{\text{acid}}$ is maximal at pH 5.0, and the equilibration shift is almost saturated at pH 8.0. PYPM$_{\text{acid}}$ and PYPM$_{\text{alkali}}$ were further characterized using PYP solutions at pH 5.0 and pH 8.0, respectively.

Comparison of Secondary Structure Using FTIR Spectroscopy—The difference between PYPM$_{\text{acid}}$ and PYPM$_{\text{alkali}}$ was studied by FTIR spectroscopy using PYP solution at pH 5.0 or pH 8.0. Spectra were measured in the dark and under continuous illumination, then difference FTIR spectra between photosteady state and dark state were calculated. Vibrational bands between 1500 and 900 cm$^{-1}$, where chromophore bands appear, were insensitive to pH for the dark state (negative bands); however, the positive bands at 1004 and 1193 cm$^{-1}$ for PYPM$_{\text{alkali}}$ were slightly down-shifted in PYPM$_{\text{acid}}$. This demonstrates a structural difference in the chromophore between PYPM$_{\text{acid}}$ and PYPM$_{\text{alkali}}$ that would cause an 11-nm difference in the absorption maximum, whereas changes in the protein environment may also contribute.

Global conformational change induced by light is evaluated by the absorbance change in the amide I ($\sim 1650$ cm$^{-1}$) and amide II ($\sim 1550$ cm$^{-1}$) mode regions. The negative band at 1646 cm$^{-1}$ at pH 8.0 was reduced and split into two bands at 1644 and 1658 cm$^{-1}$ at pH 5.0. Second derivative spectra (Fig. 4b) show that a 1646 cm$^{-1}$ band at pH 8.0 is composed of 1658, 1645, and 1635 cm$^{-1}$ bands, with the 1645 cm$^{-1}$ band being the largest of them. At pH 5.0, part of the 1645 cm$^{-1}$ mode was shifted to 1658 cm$^{-1}$, resulting in a split peak. A similar shift was observed in N terminus-truncated PYP (23). FTIR spectra and their second derivative spectra clearly show that the 1529 cm$^{-1}$ band, a typical amide II mode of a $\beta$-structure, was reduced by acidification. Together with the CD spectra results, structural change of both $\alpha$-helices and $\beta$-sheets at pH 5.0 is smaller than at pH 8.0. It should be noted that the positive 1747 cm$^{-1}$ band, which indicates protonation of Glu-46, is observed for PYPM$_{\text{acid}}$. The shoulder of this band suggests that Glu-46 is flexible and in two states in PYPM$_{\text{acid}}$. As the peak area of the negative band at 1736 cm$^{-1}$ is 5.3 times larger than that of the positive band at 1747 cm$^{-1}$, it is likely that Glu-46 is partially protonated in PYPM$_{\text{acid}}$.

Solution Structure of PYPM$_{\text{acid}}$ and PYPM$_{\text{alkali}}$—The tertiary structures of PYPM$_{\text{alkali}}$ and PYPM$_{\text{acid}}$ were characterized by small angle x-ray scattering measurements. The scattering profile was measured in the dark and under illumination at pH 8.0 and 5.0. The scattering profiles for PYPM$_{\text{alkali}}$ in each preparation (2–10 mg/ml) at pH 8.0 and 5.0 were calculated by subtracting scattering profiles of the dark state from that of the photosteady state, in which the amount of dark state was estimated by UV-visible spectroscopy. Fig. 5 shows Guinier plots ($\ln (I(Q)$ versus $Q^2$) for dark state PYP at pH 5.0 (a) and 8.0 (b), plus PYPM$_{\text{acid}}$ (a) and PYPM$_{\text{alkali}}$ (b). All Guinier plots were fitted in the linear region ($Q^2 < 0.022$), and the square of radius of gyration ($R_g^2$) was obtained from the slope. In all samples, illumination of PYP made the slope of Guinier plots steeper, indicating that $R_g$ is increased upon PYPM$_{\text{alkali}}$ formation. However, the increase in the slope for PYPM$_{\text{alkali}}$ (Fig. 5b) was clearly larger than for PYPM$_{\text{acid}}$ (Fig. 5a). $R_g^2$ values estimated at various concentrations were plotted against concentration (Fig. 5c), and the intrinsic values of $R_g^2$ were obtained by extrapolation to a concentration of zero. $R_g$ values thus obtained are shown in Table 1.

At both pH values, $y$ intersections of Guinier plots were not altered by illumination (Fig. 5, a and b), indicating that neither aggregation nor binding of solute ions to PYPM$_{\text{alkali}}$ (20) take place. Therefore, the difference in $R_g$ between the samples at pH 8.0 and pH 5.0 simply reflects the difference in the structure between PYPM$_{\text{alkali}}$ and PYPM$_{\text{acid}}$. $R_g$ for PYPM$_{\text{alkali}}$ (16.2 ± 0.1 Å) was significantly larger than that for PYPM$_{\text{acid}}$ (15.7 ± 0.2 Å), indicating that PYPM$_{\text{alkali}}$ is swollen compared with the PYPM$_{\text{acid}}$.

Shift in Equilibrium by Citrate—Multivalent organic anions such as citrate bind to PYP$_{\text{M}}$ and stabilize it (20). Here the effect of citrate on the equilibrium of PYPM$_{\text{alkali}}$ was investigated. PYP$_{\text{M}}$ spectra at pH 8.0 and 5.0 in the presence of 50 mM citrate were similarly calculated from the photosteady state spectra (Fig. 6a). Both agreed with the absorption spectrum of PYPM$_{\text{M}}$ at pH 8.0 in the absence of citrate. Under the same conditions, CD spectra of PYPM$_{\text{M}}$ in the presence of citrate were measured (Fig. 6b). Although CD spectra of PYP in the dark were not affected by citrate,
spectra of PYP$\text{M}_{\text{alkali}}$ at pH 5.0 in the presence of citrate agreed with that for PYP$\text{M}_{\text{acid}}$. In addition, $R_g$ values also agreed with each other (20), indicating there is no difference in protein structure. These results indicate that binding of citrate shifts the equilibrium between PYP$\text{M}_{\text{acid}}$ and PYP$\text{M}_{\text{alkali}}$ toward PYP$\text{M}_{\text{alkali}}$. In other words, $pK_a$ of the equilibrium shifts to less than 5 because of the binding of citrate to PYP$\text{M}_{\text{alkali}}$.

The shift in equilibrium caused by the binding of citrate to PYP$\text{M}_{\text{alkali}}$ was observed by transient spectroscopy at a millisecond time scale (Fig. 7). PYP suspended in 1 M citrate buffer (pH 5.0) was excited by a yellow flash, then transient difference spectra were measured. Just after the flash, an absorbance increase at 360 nm was observed, indicating formation of PYP$\text{M}_{\text{acid}}$. It was blue-shifted over time, and finally the positive band of the difference spectra had a maximum at 355 nm and shoulder at 330 nm. This spectral shape agreed with that for PYP$\text{M}_{\text{alkali}}$. Absorbance change at 383 nm was plotted against time after flash (inset), fitted to an exponential curve, and the time constant of this shift was estimated to be 300 ms. In 1 M acetate buffer (pH 5.0), PYP$\text{M}_{\text{acid}}$ was

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**TABLE 1**

| pH | PYP$\text{M}_{\text{acid}}$ | PYP$\text{M}_{\text{alkali}}$ |
|----|-----------------|-----------------|
| 2.0 | $15.9 \pm 0.2^a$ | $15.8 \pm 0.2$ |
| 5.0 | $15.2 \pm 0.2$ | $15.2 \pm 0.1$ |
| 8.0 | $15.2 \pm 0.1$ | $15.2 \pm 0.1$ |

$a$ Bleached.
**pH-dependent Equilibrium between PYP Intermediates**

The present results clearly demonstrate that the characteristics of PYP\textsubscript{M} are altered by pH. The pH-dependent changes in absorption spectra and CD spectra were represented with the Henderson-Hasselbalch equation, for both of which the apparent \( pK_a \) was 6.4. Together with the results of FTIR and SAXS, the pH-dependent change in the nature of PYP\textsubscript{M} is explained by a pH-dependent equilibrium between two types of PYP\textsubscript{M} (PYP\textsubscript{M\textsubscript{alkali}} and PYP\textsubscript{M\textsubscript{acid}}). This is consistent with the previous suggestion by NMR that PYP\textsubscript{M} (pB) is in an equilibrium between a well ordered state and a partially unfolded state (39). They are different in secondary and tertiary structures as well as the chromophore-protein interaction. The pH-dependent changes in decay rate constant of PYP\textsubscript{M} (26) and proton uptake (27) are explained by this equilibrium.

The characteristics of E46QM are comparable with that of PYP\textsubscript{M\textsubscript{acid}}, suggesting that replacement of Glu-46 by Gln shifts this equilibrium toward PYP\textsubscript{M\textsubscript{alkali}}. This seems to suggest the protonation of Glu-46 converts PYP\textsubscript{M\textsubscript{alkali}} into PYP\textsubscript{M\textsubscript{acid}} and vice versa. In fact, a small positive band at 1747 cm\(^{-1}\) was observed in difference FTIR spectrum between PYP\textsubscript{M\textsubscript{alkali}} and PYP\textsubscript{M\textsubscript{acid}}. This band is assigned to Glu-46 in protonated form, although its negative charge is required for a global conformational change. Therefore it is unlikely that the protonation state of Glu-46 solely switches between PYP\textsubscript{M\textsubscript{alkali}} and PYP\textsubscript{M\textsubscript{acid}}, although its negative charge is required for a global conformational change (16, 29).

The apparent \( pK_a \) value of 6.4 in this equilibrium is comparable with that of the imidazole group of the histidine side chain, suggesting the involvement of histidine. The equilibrium shifts toward PYP\textsubscript{M\textsubscript{acid}} at low pH agreed with spectra of PYP\textsubscript{M} at pH 2.0 (acid denatured state of PYP\textsubscript{M}) and pH 8.0 (PYP\textsubscript{M\textsubscript{alkali}}) (Fig. 8a). As it is unlikely that E46QM is converted into its alkaline form by acidification, E46QM is readily denatured at pH 4.0. \( pK_a \) of phenolic oxygen of the chromophore of E46Q is higher than wild type, suggesting that E46Q is less resistant to acidification than wild type. The apparent \( pK_a \) of E46QM was estimated to be 5.2 (data not shown).

Difference FTIR spectrum between E46QM and E46Q was recorded at pH 6.0 and then compared with that between PYP\textsubscript{M\textsubscript{acid}} and PYP recorded at pH 5.0 (Fig. 8b). The intensity of difference spectra in the amide I and amide II regions shows protein structural change. The shape and intensity of 1608–1575 cm\(^{-1}\) band for E46QM were comparable with those of PYP\textsubscript{M\textsubscript{acid}} PYP at pH 5.0 shows two bands at 1658 and 1644 cm\(^{-1}\). The negative band of E46Q at 1646 cm\(^{-1}\) was not clearly separated, but the shoulder at ~1655 cm\(^{-1}\) would correspond to 1658 cm\(^{-1}\) band of PYP. The negative band of E46Q at 1646 cm\(^{-1}\) is larger than PYP, whereas the positive band of E46QM at 1625 cm\(^{-1}\) was smaller than PYP\textsubscript{M\textsubscript{acid}}. Consequently, the difference absorbance between peak (1625 cm\(^{-1}\)) and valley (1646 cm\(^{-1}\)) for E46Q was 89% of PYP. This indicates that the protein structure of E46QM is comparable with that of PYP\textsubscript{M\textsubscript{acid}}. Therefore, absence of negative charge at position 46 would prevent photoactivated PYP from large structural change (16, 29). Absorption spectra of PYPM at pH 2.0, 5.0, and 8.0 are shown for comparison.

**FIGURE 8. Equilibrium of E46QM.** a, absorption spectra of E46QM at pH 6.5 and 4.0 (solid lines). Absorption spectra of PYP\textsubscript{M} at pH 2.0, 5.0, and 8.0 are shown for comparison (broken lines). b, difference FTIR spectra between E46Q (negative signals) and E46QM (positive signals) at pH 6.0. PYP\textsubscript{M\textsubscript{acid}}/PYP spectrum at pH 5.0 was reproduced from Fig. 4 (broken line).

formed just after the flash, but little spectral shift was observed in this time scale (data not shown).

**Role of Glu-46 on Global Conformational Change—Mutation of Glu-46 to Gln results in a significantly reduced conformational change upon the formation of M intermediate (E46QM) (16, 29). Absorption maximum of E46QM at pH 7 is 11-nm red-shifted from PYP\textsubscript{M\textsubscript{alkali}} (28), whereas PYP\textsubscript{M\textsubscript{alkali}} is dominant at pH 7 for wild type. These characteristics of E46QM are similar to those of PYP\textsubscript{M\textsubscript{acid}}. To examine the similarity between PYP\textsubscript{M\textsubscript{acid}} and E46QM, spectroscopic properties of E46QM were compared with PYP\textsubscript{M\textsubscript{acid}} (Fig. 8).

Absorption spectrum of E46QM at pH 6.5 was calculated from the absorption spectrum of the photosteady state (Fig. 8a). At pH > 6.5, difference spectra between dark state and photosteady state became significantly smaller because of the short lifetime of E46QM. Absorption spectrum of E46QM at pH 6.5 agreed with that at pH 7.0 obtained from the transient difference spectra 10 ms after the flash (28). At a higher pH, the blue shift of E46QM was not observed, but a significant amount of E46Q was formed (37).

Absorption spectra of E46QM at pH 6.5 agreed with that of PYP\textsubscript{M\textsubscript{acid}} at pH 5.0. At pH 4.0, the spectrum of E46QM was blue-shifted. This

\[ ^{4} \text{M. Harigai, M. Kataoka, and Y. Imamoto, unpublished result.} \]
salt concentration (pK_a = 6.7, data not shown). As electrostatic interaction is enhanced at low salt concentration, it is likely that PYP_M^{acid} is stabilized by the basic amino acid residue, which is charged at acidic pH. PYP has two histidine residues (His-3 and His-108). Although His-3 is fully exposed to the solvent, His-108 is buried between the β-scaffold and the N-terminal cap. Because the structure of the N-terminal cap changes upon PYP_M formation at neutral pH, the protonation state of His-108 possibly correlates with the structure of PYP_{M} (25, 27).

Citrine blue shifts the absorption spectra of PYP_M and increases R_{G} at pH 5 (20). Here the citrate-bound form of PYP_M was characterized in detail, demonstrating that the absorption spectrum, the amount of secondary structure, and the primary effect of binding of citrate is a shift of the equilibrium toward PYP_M^{alkali}. NMR analysis of the photoprocess of E46Q has shown that the structural change is limited to α3 and the chromophore (29). Assuming that the structure of E46Q is comparable with PYP_M^{acid}, the main structural change for formation of PYP_M^{acid} is the conformational change of α3. It should be noted that citrate binds to PYP_M^{acid} to shift the equilibrium, indicating that the citrate binding site is active in PYP_M^{alkali}. This finding is consistent with our previous speculation that Arg-52 located in between α3 and α4 in PYPM, which contains the citrate binding site (20).

We previously studied conformational change upon the formation of PYP_M^{alkali} by CD spectroscopy (23). Results indicated that the difference maximum in CD spectrum was 222 nm, indicating that a structural change in α-helices is mainly involved. However, this change cannot be explained by the short α-helices like α1 and α2 located in the N-terminal cap segment. Thus an unfolding of the long helices was strongly suggested. Therefore, the difference between PYP_M^{acid} and PYP_M^{alkali} is considered to be the unfolding of α3 and/or α5. Gln-46 and Thr-50 are located in α3, and Tyr-42 is located between β2 and α3. They are hydrogen-bonded with the phenolic oxygen of the chromophore. It should be noted that Gln-46 is in the central part of α3. As negative charge at position 46 is required for the conformational change, further structural change in α3 would take place in addition to that in α5. FTIR spectra demonstrate that the amide II mode of the β-sheet (1530 cm\(^{-1}\)) is largely changed upon formation of PYP_M^{alkali}, indicating that the conformation of the β-sheet is also changed.

At neutral pH, two types of PYP_M (I_{2}/I_{2}' or pB'/pB) are sequentially formed (17, 18). The latter has a lifetime of ~100 ms and is accumulated by steady-state illumination. The former has a lifetime close to PYP_L (~1 ms) but the absorption spectrum is close to the latter. The characteristics of pB' are a relatively small protein conformational change (15, 16) and slightly red-shifted absorption spectrum (17). In detailed analysis of pH dependence of the PYP photocycle, data were explained without an equilibrium between pB and pB' (17). However, recent double flash experiments detected a species, which has photoreversal kinetics similar to I_{2} over ~100-ms time scale, and the authors suggested an equilibrium between I_{2} and I_{2}' (18). As this experiment was carried out at pH 6, at which 70% of PYP_M^{acid} is in the acidic form, this shows the photoreversal kinetics of PYP_M^{acid} agree with that of I_{2} (pB'). Absorbance change in amide mode of transient difference FTIR spectrum 450 μs after excitation (15) is comparable with that of the PYP_M^{acid}/PYP spectrum. Therefore, it is reasonable to conclude that pB' (I_{2}) and PYP_M^{acid} are the same species and that pB' is trapped at acidic pH.

Recently, the crystal structures of photocycle intermediates formed from PYP and E46Q were analyzed by time-resolved crystallography (40–42). They showed the presence of two blue-shifted intermediates of PYP (pB_1 and pB_2) whose decay time constants are ~10 and ~100 ms (42). These values are consistent with the decay time constants of pB' (PYP_{M}^{acid}, ~1 ms) and pB (PYP_{M}^{alkali}, ~100 ms), respectively. To examine whether or not the structures of pB_1 and pB_2 correspond to PYP_{M}^{acid} and PYP_{M}^{alkali}, respectively, the SAXS profiles of pB_1 and pB_2 were calculated from their crystal structures (PDB codes, 1T50 and 1T56, respectively) (42) using CRYSSOL software (43). Guinier plots of calculated SAXS profile gave the equal values of R_g for pB_1 and pB_2, indicating that pB_1 and pB_2 cannot be distinguished by SAXS experiments. Because experimental R_g values for PYP_M^{acid} and PYP_{M}^{alkali} are significantly different (Table 1), pB_1 would not correspond to PYP_{M}^{alkali}. PYP_{M}^{acid} state in solution would be the mixture of pB_1 and pB_2, and the large structural change for formation of PYP_{M}^{alkali} is restricted by crystal packing. For E46Q, three late intermediates were found (IL1, IL2, and IL3) (41). IL1 and IL2 show the similar change in electron density observed in PYP. However, no difference in R_g values was resulted from their crystal structures. Together with our results that E46Q_M is comparable with PYP_M^{acid}, blue-shifted intermediates formed in the crystal are qualitatively the same as PYP_M^{acid}.

The present data provide detailed characteristics of pB' (I_{2} or PYP_M^{alkali}), which is in the stage just before the global conformational change takes place in the photocycle. These observations are of importance in elucidating the mechanism of light-induced global conformational change of PYP.
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