Roles of Amino Acid Residues near the Chromophore of Photoactive Yellow Protein†

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ABSTRACT: To investigate the roles of amino acid residues around the chromophore in photoactive yellow protein (PYP), new mutants, Y42A, E46A, and T50A were prepared. Their spectroscopic properties were compared with those of wild-type, Y42F, E46Q, T50V, R52Q, and E46Q/T50V, which were previously prepared and specified. The absorption maxima of Y42A, E46A, and T50A were observed at 438, 469, and 454 nm, respectively. The results of pH titration for the chromophore demonstrated that the chromophore of PYP mutant, like the wild-type, was protonated and bleached under acidic conditions. The red-shifts of the absorption maxima in mutants tended toward a pK_a increase. Mutation at Glu46 induced remarkable shifts in the absorption maxima and pK_a. The extinction coefficients were increased in proportion to the absorption maxima, whereas the oscillator strengths were constant. PYP mutants that conserved Tyr42 were in the pH-dependent equilibrium between two states (yellow and colorless forms). However, Y42A and Y42F were in the pH-independent equilibrium between additional intermediate state(s) at around neutral pH, in which yellow form was dominant in Y42F whereas the other was dominant in Y42A. These findings suggest that Tyr42 acts as the hinge of the protein, and the bulk as well as the hydroxyl group of Tyr42 controls the protein conformation. In all mutants, absorbance at 450 nm was decreased upon flash irradiation and afterwards recovered on a millisecond time scale. However, absorbance at 340−370 nm was increased vice versa, indicating that the long-lived near-UV intermediates are formed from mutants, as in the case of wild-type. The lifetime changes with mutation suggest the regulation of proton movement through a hydrogen-bonding network.

Many organisms have a photosensing system. It is initiated by a photoreceptor protein, which has a chromophore that interacts with the protein moiety and has the ability to absorb visible light. Several kinds of chromophores are known, the simplest of which is the *p*-coumaric acid involved in the photoactive yellow protein (PYP)[1] found in the purple phototrophic bacterium, *Ectothiorhodospira halophila* (*1*), and in other bacteria (*2*−*6*).

PYP is a yellow (λ_{max} = 446 nm) and small (MW = 14 000) water-soluble protein. It has a *p*-coumaric acid as its chromophore, binding to the cysteine residue at the position 69 by a thioester bond (*7*−*10*). The protein moiety is composed of 125 amino acids, and has a α/β fold structure. The molar extinction coefficient and quantum yield for formation of the photoproduct are 45 500 M^{-1} cm^{-1} and 0.64 (*11*), respectively, indicating that PYP is an excellent photosensor, comparable to rhodopsin. The photoisomerization of the chromophore from trans form to cis form initiates the photoreaction cycle, which resembles those of the bacterial retinal proteins (*12*−*15*). The phenolic oxygen of the chromophore is deprotonated at neutral pH in the dark (*8*, *16*) due to interactions with the nearby amino acid residues, Tyr42 and Glu46, in the hydrogen-bonding network. PYP from *E. halophila* and *Rhodothalassium salexigens* have Thr at position 50, and it is also involved in the hydrogen-bonding network. PYP is bleached in the acid condition because of the protonation of the chromophore (*1*, *17*). Therefore, this deprotonation is thought to be the most essential factor for the *p*-coumaric acid chromophore to absorb the visible light. In the late stage of the photocycle, a long-lived near-UV intermediate is formed. On formation of this intermediate, the proton at Glu46 is transferred to the chromophore (*18*, *19*). These observations indicate that the hydrogen-bonding network is the core of the active site in the PYP molecule.

The apparent pK_a of the PYP chromophore is 3.0, whereas that of *p*-coumaric acid thioester is 8.8, indicating that pK_a

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[1] Abbreviations: PYP, photoactive yellow protein; λ_{max}, absorption maximum; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.

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is lowered by the protein moiety so that the chromophore can be deprotonated even in the physiological condition. In summary, the roles of the protein moiety of PYP are (i) lowering the pK_a of the chromophore, (ii) moving the absorption spectrum to the visible region, and (iii) transforming the light signal by protein conformational change.

Site-directed mutagenesis is the most powerful tool with which to understand the roles of each amino acid residue (*20*−*23*). The substitution of an amino acid affects the absorption maximum and the lifetimes of photocycle intermediates. In a previous study, we prepared Y42F (λ_{max} = 458 nm), E46Q (λ_{max} = 460 nm), T50V (λ_{max} = 457 nm), and R52Q (λ_{max} = 447 nm) (*20*). The results demonstrated that the extent of the shift of λ_{max} depends on the distance between the phenolic oxygen of the chromophore and the hydroxyl oxygen of the original amino acid residue (Tyr, Glu, or Thr); the positive charge of Arg has little effect. In addition, Y42F is in the mixture of yellow form and $Y42F_{380}$ at neutral pH, suggesting that the stability of Y42F is quite different from that of wild-type and other mutants. The phenolic oxygen of the chromophore of wild-type forms hydrogen bonds with Tyr42 and Glu46. E46Q and T50V retain these two hydrogen bonds, whereas Y42F lacks one of them.

In the present study, PYP mutants in which Ala was substituted for Tyr42, Glu46, or Thr50 were prepared to eliminate the hydrogen bond and weaken interaction with the chromophore. The absorption maxima, pK_a of the chromophore, extinction coefficients, and oscillator strengths were analyzed in detail to clarify the roles of amino acid residues near the chromophore. In addition, the light-induced absorbance changes on the millisecond time scale were studied to examine the behavior of the long-lived intermediates of the mutants.

MATERIALS AND METHODS

Wild-type, Y42F, E46Q, T50V, and R52Q were prepared as reported previously (*20*). Oligonucleotide primers, 5′-TCCTGCAGGCTAACGCCGCGGAGGGC-3′, 5′-TCCTGCAGTACAACGCCGCGGCTGGCGACATCACCGGC-3′, and 5′-GCGGAGGGCGACATCGCTGGCCGCGACCCGAAG-3′, were synthesized for Y42A, E46A, and T50A, respectively. Genes of Y42A, E46A, and T50A were amplified using these primers and DNA fragments encoding PYP isolated from *E. halophila* (*20*). Gene of E46Q/T50V was amplified using the primer for T50V (*20*) and gene of E46Q (*20*). They were inserted into the multi-cloning site of expression vector pET-16B (Novagen). *Escherichia coli* BL21(DE3) was transformed by these plasmids. Expressed apo-PYP was solubilized by 8 M urea in Tris buffer (10 mM Tris-HCl, pH 7.4) and reconstituted by adding *p*-coumaric anhydride after 2-fold dilution. Purification was performed by ammonium sulfate precipitation method and DEAE-Sepharose (Pharmacia) column chromatography.

Absorption spectra were recorded by a Hitachi 3210 recording spectrophotometer. The temperature of the sample was kept at 20 °C by circulating the temperature-controlled water in the cell holder. pH of the sample was changed by adding a small volume of HCl or NaOH solution (0.1−1 N), and measured by putting a small portion of the sample on the electrode of a TWIN-pH (Horiba). The sample was manipulated under a red light because the lifetime of the intermediate of PYP is extremely long in the acidic condition.

Flash photolysis experiments at fixed wavelengths were carried out using the Unisoku TPS-501 System. The sample was excited by the yellow flash (>440 nm) obtained by the combination of a strobe lamp and an optical filter (Y46, Toshiba). PYP and mutants were dissolved in CAPS buffer (10 mM CAPS, 150 mM NaCl, pH 9.5) and the absorbance of each sample was adjusted to 0.9−1.0 at its maximum. The transient difference absorption spectra after flash excitation of Y42F were measured by multi-channel CCD spectroscopy system (Ocean Optics S2000 system).

Spectral and kinetic data were analyzed by IGOR Pro ver. 3.14 for Macintosh (Wave Metrics Inc.).

RESULTS

pH Titration. The mutant PYP was expressed in *E. coli* and purified by the ammonium sulfate precipitation method and DEAE-Sepharose column chromatography in the buffer at pH 7.4. Recently the structures of Y42F and T50V were analyzed by crystallography (*21*). They were almost identical to that of wild-type, suggesting that the mutation around the chromophore does not bring the large conformational change. It is known that the chromophore of PYP is protonated and bleached at acidic pH (*1*, *17*). The pK_a of the equilibrium between the yellow and colorless forms is 3.0 for wild-type. However, pK_a of the mutant is possibly shifted because the environment of the chromophore is different (*22*). Therefore, pK_a of the mutant was estimated first, and it is essential to obtain the precise spectrum of the yellow form without the contribution of colorless form.

Figure 1 shows the typical pH-titration experiments of wild-type, Y42F, and Y42A. Because the pH of the wild-type sample was lowered by HCl, the yellow form (λ_{max} = 446 nm) was decreased and the colorless form (λ_{max} = 350 nm) was increased (Figure 1a). In this conversion process, the clear isosbestic point was observed at 383 nm. The absorbance change at λ_{max} (446 nm) was plotted against pH (Figure 2). The pK_a was estimated to be 3.0. Qualitatively similar conversions from the yellow to the colorless form were observed for E46A (469 → 352 nm, pK_a = 7.8), E46Q (460 → 353 nm, pK_a = 5.3), T50A (454 → 352 nm, pK_a = 3.3), T50V (457 → 350 nm, pK_a = 3.6), and R52Q (446 → 350 nm, pK_a = 4.0) (Figure 2). However, those of Y42F and Y42A were strikingly different.

Y42F has an absorption maximum at 458 nm and a shoulder at 380 nm (Figure 1b). As the pH was lowered, it was converted to the colorless form (λ_{max} = 350 nm). It should be noted that the isosbestic point was slightly shifted and the decrease of the visible band was not uniform (Figure 1b, inset). The shoulder is prominent in the latter stage, indicating the presence of species other than the yellow form ($Y42F_{380}$). $Y42F_{380}$ is more stable against acidic pH than is the yellow form.

Spectral changes of Y42A were more complicated (Figure 1c). At neutral pH, the absorption band at 375 nm with the shoulder at 450 nm was observed (curve 16 in Figure 1c). This band shape is retained over a wide pH range (pH 6.5−9.0), and main band and the shoulder simultaneously decayed to the colorless form at acidic pH (Figure 1c, inset). These features were similar to those of Y42F and $Y42F_{380}$ although

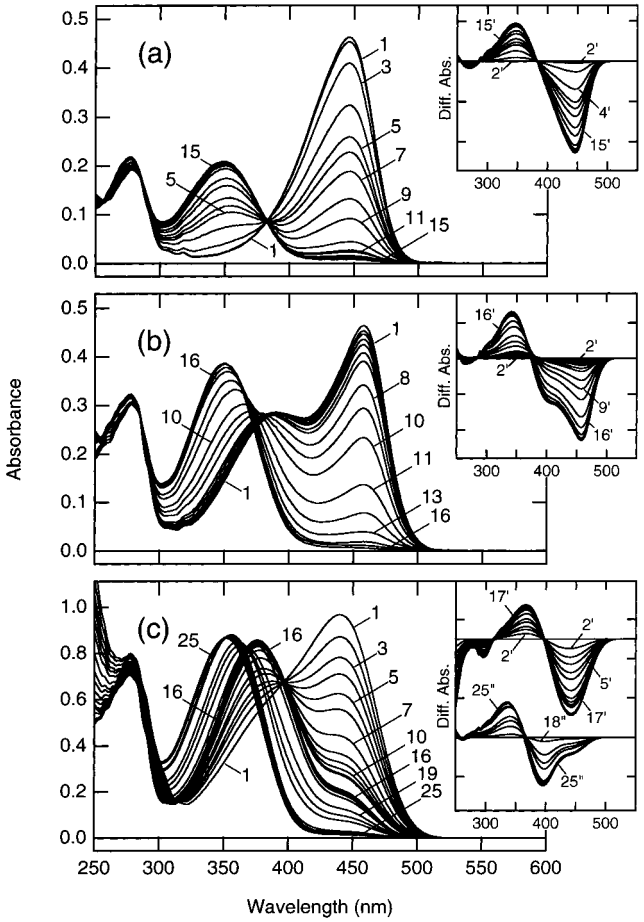

FIGURE 1: pH titration of wild-type, Y42F, and Y42A. (a) pH titration of wild-type PYP. The absorption spectra were recorded at pH 7.3, 4.1, 3.5, 3.2, 3.0, 2.9, 2.8, 2.7, 2.6, 2.3, 2.2, 2.1, 2.0, 1.9, and 1.8 (curves 1-15, respectively). (b) pH titration of Y42F. The absorption spectra were recorded at pH 6.3, 6.1, 5.9, 5.7, 5.5, 5.3, 5.2, 4.9, 4.7, 4.4, 4.0, 3.7, 3.5, 3.2, 3.0, and 2.7 (curves 1−16, respectively). (c) pH titration of Y42A. The absorption spectra were recorded at pH 12.0, 10.9, 10.8, 10.7, 10.5, 10.3, 10.1, 10.0, 9.9, 9.8, 9.7, 9.4, 9.1, 8.4, 7.9, 7.5, 6.9, 6.2, 5.6, 5.3, 5.0, 4.3, 4.1, 3.8, and 3.5 (curves 1-25, respectively). (Insets in a and b) The spectral changes were calculated by subtracting curve 1 in each data set. (Inset in c) Top: curve 1 was subtracted from curves 2−17. Bottom: curve 17 was subtracted from curves 18−25.

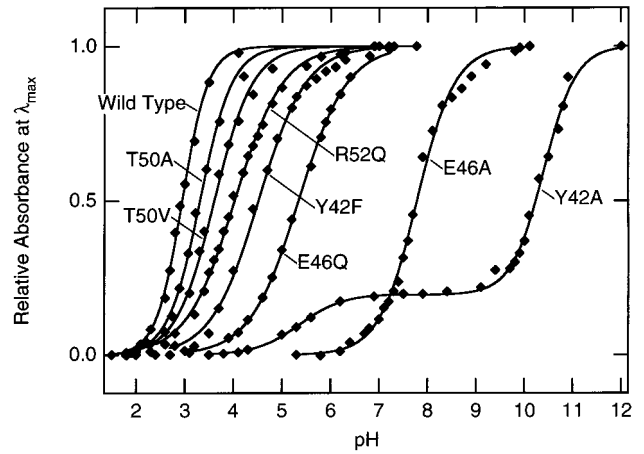

FIGURE 2: pH titration of wild-type and mutant PYP. The absorbances at λ_{max} were plotted against pH.

$Y42A_{375}$ was dominant in Y42A system. At alkaline pH, the yellow form was increased, but it was not saturated even at pH 12. At acidic pH, $Y42A_{375}$ was converted to the 356-nm species and then to the colorless form (λ_{max} = 350 nm). Therefore, Y42A has at least four states at pH 3-12.

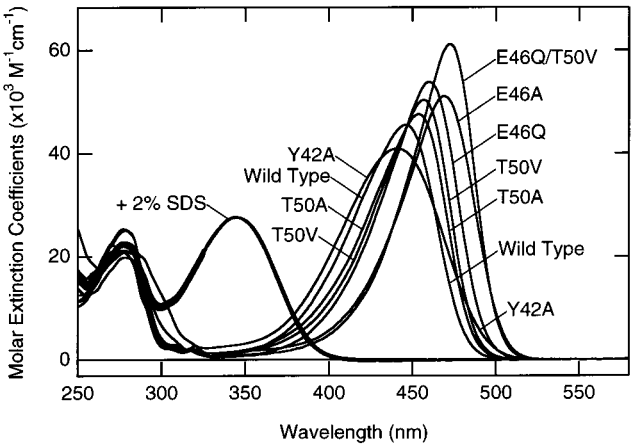

FIGURE 3: Estimation of extinction coefficients. The pH of the sample was adjusted to 8.0 (wild-type, E46Q, T50V, and T50A) or 11.0 (E46A, and E46Q/T50V), and the absorption spectra were recorded. Then PYP or a mutant was denatured by adding SDS to the final concentration of 2%, and pH was immediately adjusted to 3.5 by HCl. The concentration of PYP or a mutant was normalized based on the absorbance of the denatured states. The dilution effects by addition of SDS and HCl were corrected. The absorption spectrum of Y42A is estimated by calculation.

Extinction Coefficients of PYP Mutants. Extinction coefficients of PYP mutants were estimated based on the absorption spectra of the denatured states in the presence of 2% SDS, in which the chromophore/protein interaction is lost and the absorption spectra should be identical (Figure 3). The pH of the sample was adjusted to the pH at which the yellow form is dominant (see Figure 2). Then PYP or a mutant was denatured by adding SDS to the final concentration of 2%, and pH was adjusted to 3.5 by HCl so that no thioester bond was hydrolyzed. Afterward, the absorption spectrum was recorded. As expected, the absorption spectra of the denatured states were identical (Figure 3). On the basis of the absorbance, the protein concentration of each sample was normalized. Because the pure spectra of the yellow forms of Y42F and Y42A could not be recorded, their extinction coefficients could not be estimated by this experiment. However, the absorption spectrum of Y42A could be derived by calculation because the conversion from $Y42A_{375}$ to the yellow form was observed clearly (see below). Using the molar extinction coefficient of wild-type [45500 (*11*)], those of the mutants were calculated (Table 1). These values were plotted against the absorption maxima in frequency (cm^{-1}) (Figure 4a).

Oscillator Strength and Bandwidth. Oscillator strength is calculated from the area of the absorption band. It correlates with the square of the transition dipole moment or transition probability. Therefore, oscillator strength suggests the efficiency of photon absorption by the chromophore.

The abscissa of the normalized absorption spectra (wavelength in nm) (Figure 3) was inverted to the wavenumber (cm^{-1}), and the band area was calculated by integrating the molar extinction coefficients from 16 700 cm^{-1} (600 nm) to 30 300 cm^{-1} (330 nm). Using this value, the oscillator strength (*f*) was calculated as follows:

Table 1: Spectral Properties of PYP Mutants

	λ_{max} (nm)	pK_a	ϵ_{max} (M^{-1} cm^{-1})	H (cm^{-1})	Q (cm^{-1})	f
wild-type	446	3.0	45500	3030	1845	0.637
Y42A	438	5.3	42100	3746	2392	0.795
		10.4				
Y42F	~458	4.5	ND	ND	1710	ND
E46A	469	7.8	51100	2401	1413	0.610
E46Q	460	5.3	53800	2552	1497	0.653
T50A	454	3.3	47400	2763	1633	0.638
T50V	457	3.6	50400	2594	1518	0.628
E46Q/T50V	472	ND	61100	2133	1234	0.632
R52Q	446	4.0	ND	3097	1901	ND

[a] λ_{max}, absorption maximum; ϵ_{max}, molar extinction coefficient at λ_{max}; H, half-bandwidth; Q, quarter-bandwidth; f, oscillator strength; ND, not determined.

$$f = 4.319 \times 10^{-9} \times \int \epsilon(\nu)\, d\nu$$

where $\epsilon(\nu)$ denotes the molar extinction coefficient at ν (cm^{-1}). The calculated oscillator strengths are shown in Table 1.

Oscillator strength and half-bandwidth were plotted against the absorption maxima in frequency (cm^{-1}), resulting in the linear correlation (Figure 4, panels b and c). Because the contribution of $Y42F_{380}$ to the half-bandwidth could not be neglected, the quarter-bandwidth (the bandwidth at 75 % of maximal absorbance) was also calculated and plotted in Figure 4c.

Absorption Spectrum of Y42A. Y42A was in the mixture of the yellow form and $Y42A_{375}$ even at pH 12. Because the protein moiety was denatured above pH 12, the absorption spectrum of Y42A was estimated by calculation.

The half- or quarter-bandwidth was correlated to the absorption maximum (Figure 4). First, we extrapolate the spectral change from curve 13 to curve 1 in Figure 1c so that the band area and bandwidth are close to the value expected from Figure 4. However, this calculation produced a negative absorbance region. Next, we noted the ratio of the quarter-bandwidth to the half-bandwidth. Because the ratio was 1.68 ± 0.05 for all mutants, the extrapolation was carried out to obtain a ratio close to this value. The results are shown in Figure 3, and the calculated values of absorption maximum (438 nm), extinction coefficient (42 100), oscillator strength (0.795), and bandwidth are shown in Table 1 and Figure 4. The spectral analysis for Y42F was not carried out because the yellow form could not be separated from $Y42F_{380}$.

Flash Photolysis. The chromophore of PYP is protonated in the long-lived intermediate (called I2, PYP_M, or pB), resulting in a large conformational change. To investigate the effect of changes in the hydrogen-bonding network on proton movement, the behaviors of M intermediates of mutants were studied by flash photolysis experiments. Because E46A is mainly in the colorless form at neutral pH, the pH was adjusted to 9.5. Optical density at λ_{max} of the PYP mutant samples was adjusted to 0.9−1.0, and the absorbance changes after excitation by yellow flash (>440 nm) were monitored at 450 and 370 nm (340 nm for Y42F). Because yellow form of Y42A was a minor component even at pH 9.5, it was not subjected to the flash photolysis.

In all mutants, absorbance decrease at 450 nm took place upon flash excitation, then absorbance was recovered on a millisecond time scale. The absorbance change at 370 nm was complimentary, and the ratio of absorbance decrease at 450 nm and increase at 370 nm was similar among the mutants. These observations indicate the formation of intermediates having spectral properties similar to those of PYP_M. To estimate the lifetimes, the traces of absorbance changes were fitted by single-exponential curves, which were superimposed to the traces. As is obvious in the figure, the recoveries from M intermediates were expressed by single-exponential curves. The decay rate constants of M intermediates of wild-type (PYP_M), E46Q ($E46Q_M$), Y42F ($Y42F_M$), and T50V ($T50V_M$) are qualitatively in agreement with findings in a previous report (*21*, *23*). However, rate constants we estimated are several times smaller than those in the previous report, because our sample contained 150 mM NaCl. The decay of $R52Q_M$ was several times slower than that of wild-type, which is consistent with findings on R52A (*23*). The decay time constants of $E46A_M$ and $Y42F_M$ were also slower than that of PYP_M, probably due to the loss of hydrogen bonding. Unexpectedly, the mutation at Thr50

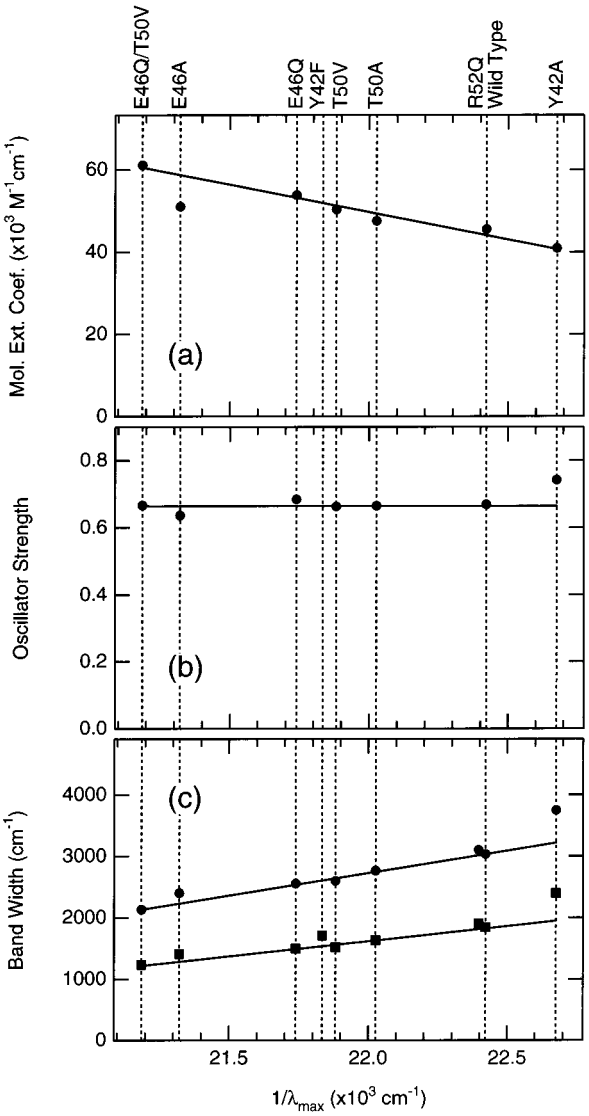

FIGURE 4: Molar extinction coefficients (circles in a), oscillator strength (circles in b), half-bandwidth (circles in c), and quarter-bandwidth (squares in c) were plotted against the inverse of the absorption maxima. The values for Y42A were estimated by calculation.

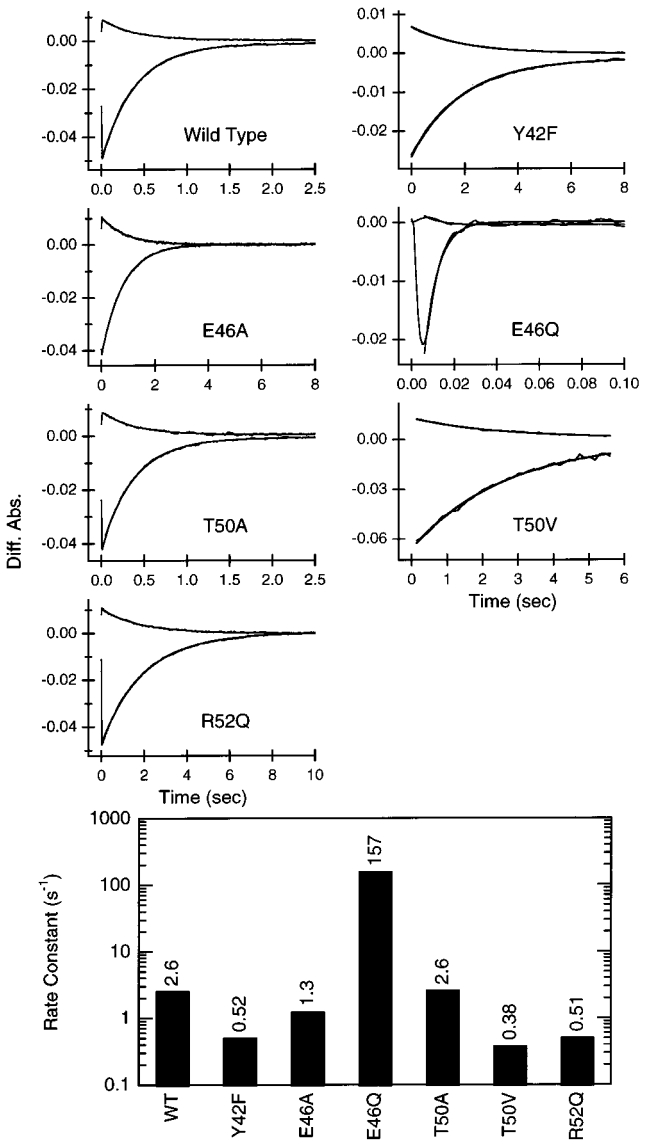

FIGURE 5: The decay of M intermediates of PYP and its mutants. The absorbance changes were monitored at 450 nm (lower traces) and 340 (Y42F) or 370 nm (upper traces). Bottom: the lifetimes of M intermediates of PYP mutants.

largely affected the decay of the M intermediate. While the decay rate constant of $T50A_M$ was 2.6, which was comparable to that of PYP_M, that of $T50V_M$ was 0.38 at pH 9.5. The decay rate constant was 0.45 for $T50A_M$ and 0.49 for $T50V_M$ at pH 7.4 (data not shown).

M-Intermediate of Y42F. Y42F was in the mixture of yellow form and $Y42F_{380}$. Only little spectral change was observed at pH 6.0-9.5 (Figures 1b and 6a), indicating that the equilibrium between Y42F and $Y42F_{380}$ was pH-independent in this pH range. For further characterization of $Y42F_{380}$ and $Y42F_M$, the transient difference absorption spectra after excitation of Y42F were recorded at pH 7.0 and 9.5 (Figure 6). Y42F was excited with >450-nm flash so that $Y42F_{380}$ was not excited. Just after excitation, the positive band at 280−370 nm and negative band at 370−500 nm were observed, indicating the formation of $Y42F_M$. It decayed to Y42F with the time constant of 0.52 at pH 9.5 and 0.71 at pH 7.0. It should be noted that the negative band had the spectral shoulder although Y42F was excited with >450-nm flash. The rate constants estimated from the

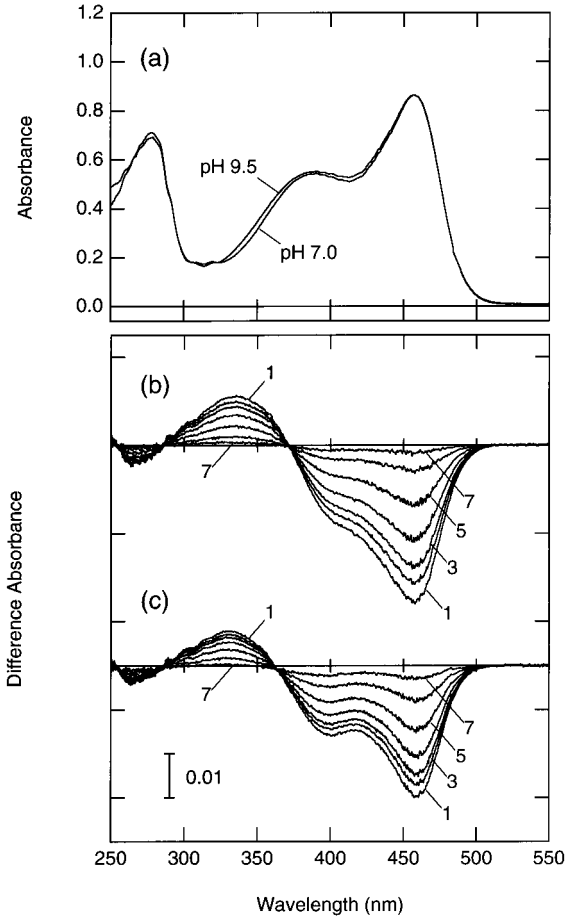

FIGURE 6: Transient difference absorbance spectra after flash excitation of Y42F. (a) Absorption spectrum of Y42F in 10 mM MOPS buffer (150 mM NaCl, pH 7.0) or 10 mM CAPS buffer (150 mM NaCl, pH 9.5). It was excited by >450 nm flash at 20 °C at pH 7.0 (b) or pH 9.5 (c), and the spectra were measured at 0, 0.17, 0.35, 0.70, 1.40, 2.80, and 5.60 s after excitation.

absorbance changes at 455, 400, and 340 nm agreed with each other within the experimental error. Therefore, Y42F was converted to $Y42F_M$ by excitation, and the equilibrium between Y42F and $Y42F_{380}$ would be immediately formed. At pH 9.5, the shoulder was more prominent than that at pH 7.0, indicating that the repopulation of Y42F was faster.

DISCUSSION

In the present study, preparation of Y42A, E46A, and T50A was designed to eliminate the hydrogen bond and weaken interaction. The results showed that the pK_a of the chromophore as well as the absorption maximum is shifted by mutation. The absorption maximum correlated to pK_a except in the cases of R52Q and Y42A. Because Arg52 is a positively charged residue, unlike Tyr42, Glu46, and Thr50, the interaction with the chromophore would be different. The spectral change of the pH titration of Y42A was quite different from those of other mutants, and the protein structure may be largely altered.

Because the pK_a of free *p*-coumaric acid is 8.8 (*17*), the pK_a of the chromophore is lowered by the effect of the hydrogen-bonding network. The present findings on E46A along with those on E46Q demonstrated that the mutation at position 46 has the largest effect on the absorption maximum. The shift in E46A was larger than that in E46Q,

probably due to the loss of hydrogen bonding. These results suggest that the absorption maximum is shifted to red and the pK_a becomes larger as the hydrogen bond is weakened.

The shift in pK_a and λ_{max} in T50A and T50V is relatively small, suggesting that Thr50 is not essential for the spectral properties of PYP. This finding is consistent with the facts that Thr50 does not form a direct hydrogen bond with the chromophore and Thr50 is not conserved in PYP of *Rhodobacter sphaeroides* (*6*) and *Rhodobacter capsulatus* (*24*). However, the shifts of T50V were somewhat larger than those of T50A, suggesting that the presence of the large hydrophobic residue alters the environment around the chromophore.

The extinction coefficients were negatively correlated with the inverse of the absorption maxima, whereas bandwidth was positively correlated (Figure 4). As a result, the band area and oscillator strength were almost constant, except in the case of Y42A. Because the oscillator strength correlates with the transition probability, the sensitivity to the photon is not largely changed if Tyr42 is conserved. The oscillator strengths calculated from the band area were 0.61−0.65, which were close to the values of rhodopsin (*25*). Moreover, the quantum yield of the formation of the first photoproduct is comparable to that of rhodopsin (*11*, *26*). Therefore, the sensitivity of PYP to light is as high as that of rhodopsin. The molar extinction coefficient of E46A was somewhat small, but the absorption band was broad. Because the phenolic oxygen of the chromophore of E46A forms only one hydrogen bond, the chromophore would be loosely fixed. The broad absorption band of E46A would be caused by the fluctuation of the chromophore.

If the mutants have Tyr at position 42, they are in the equilibria between the yellow and colorless forms at neutral-acidic pH. That is, only two protein conformations are possible and the protonation of the chromophore switches the protein conformation from one form to the other. However, Y42F and Y42A have three or four states. Because E46A, which lacks one of two hydrogen bonds of phenolic oxygen of the chromophore, has only two states, the disorder of this switch is due to the loss of Tyr42 rather than to hydrogen bonding. Therefore, Tyr42 would act as the hinge of the protein, preventing it from taking any conformation other than the yellow or colorless form.

Because the chromophore of PYP_M is protonated (*18*, *19*) and in a partially unfolded state, the colorless form of PYP in the acid condition would be regarded as the model for PYP_M. PYP is thought to be the photoreceptor for the negative phototaxis of the bacteria (*27*). PYP_M would interact with the putative transducer protein to transmit the light signal. If conformation other than the yellow form or PYP_M is possible for PYP, it may cause the dark noise of the signal. Therefore, a signaling protein like PYP requires the switch in conformational change to avoid the dark noise.

Our transient difference absorption spectra demonstrated that the repopulation of Y42F and $Y42F_{380}$ is much faster than the decay of $Y42F_M$. Therefore, $Y42F_{380}$ is not in an irreversible denatured state, but $Y42F_{380}$ and Y42F are in the equilibrium. It is recently reported that the chromophore of $Y42F_{380}$ is deprotonated based on the Fourier transform Raman experiments (*21*). In addition, $Y42F_{380}$ is converted to Y42F by cooling to liquid nitrogen temperature or by adding ammonium sulfate at high concentration (*21*). Therefore, the equilibrium depends on the protein conformational change. Similar equilibrium was observed in Y42A system at pH 6.5−9.0. It should be noted that $Y42A_{375}$ was dominant in this equilibrium unlike Y42F system. Both of alanine and phenylalanine are hydrophobic residues which forms no hydrogen bond, but the bulk is different. Therefore, the bulk as well as the hydroxyl group of Tyr42 would be necessary to maintain the protein conformation of yellow form.

The decay of $E46A_M$ and $Y42F_M$ were slower, whereas that of $E46Q_M$ was much faster, than that of PYP_M. It is reported that the decay of $E46Q_M$ is highly pH-dependent and it is slower in acidic pH (*23*). The lack of one of the hydrogen bonds of the phenolic oxygen of the chromophore caused the slower decay of PYP_M. Notably, the mutation at Thr50 had a great effect on the behavior of the M intermediate despite the similarity in absorption spectra and the pK_a of the chromophore in the dark. The decay rate constants of $T50A_M$ and $T50V_M$ were 2.6 and 0.38 at pH 9.5, and 0.45 and 0.49 at pH 7.4, respectively. The optimal pH at which the decay rate constant of the M intermediate is the largest would be shifted to the alkaline pH for T50A. Because the Thr residue is similar in size to Val, the change in hydrophilicity would cause electrostatic interaction in the M state. The comparison of the structures of the mutants in the dark states is suggestive to understand the reaction mechanism. However, because chromophore of M intermediate is in a configuration different from that of dark state and the protein conformation is largely altered, the interaction manner of the chromophore of the intermediate should be different. The structural analysis of the intermediates in the mutants in a high-resolution would be necessary for the elucidation.

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