# Photoreactions of Tyr8- and Gln50-Mutated BLUF Domains of the PixD Protein of Thermosynechococcus elongatus BP-1: Photoconversion at Low Temperature without Tyr8 †

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# Photoreactions of Tyr8- and Gln50-Mutated BLUF Domains of the PixD Protein of *Thermosynechococcus elongatus* BP-1: Photoconversion at Low Temperature without Tyr8[†]

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ABSTRACT: We studied the photoreaction of a blue-light sensor PixD protein of *Thermosynechococcus elongatus* that has the blue-light-using flavin (BLUF) domain. The Tyr8 and Gln50 residues of the protein were modified to phenylalanine, alanine, or asparagine (Y8F, Y8A, Q50N, and Q50A) by site-directed mutagenesis. The following results were obtained. (1) At room temperature, blue-light illumination induced the red shift of the absorption bands of flavin in the wild-type (WT) protein but not in the Y8F, Y8A, Q50A, and Q50N mutant proteins, as reported [Okajima, K., et al. (2006) *J. Mol. Biol. 363*, 10−18]. (2) At 80 K, neither the Q50N nor the Q50A mutant protein accumulated the red-shifted form. (3) At 80 K, the Y8F protein photoaccumulated the red-shifted forms to an extent that was half that in the WT protein at a 43-fold slower rate, and the Y8A protein to the one-fourth the extent at a 137-fold slower rate. (4) The red-shifted form in the Y8F protein was stable below 240 K and became unstable above 240 K in the dark. (5) The illumination of the Y8F protein at 150 K accumulated the red-shifted form at the beginning, and the prolonged illumination accumulated the flavin anions by the secondary photoreaction. (6) The results indicate that Tyr8 is not indispensable for the accumulation of the red-shifted form at least at 80 K. (7) Photoconversion mechanisms in the WT and Tyr8-mutated proteins are discussed in relation to the schemes with and without the electron transfer between Tyr8 and flavin in the first step of the photoconversion.

Recent genomic analysis revealed a family of blue-light receptors containing flavin in various organisms. A BLUF[1] (sensor of blue light-using flavin) domain is a recently discovered flavin adenine dinucleotide (FAD)-binding blue-light sensory domain that is widely distributed among the genome of purple photosynthetic bacteria, cyanobacteria, and the unicellular eukaryote *Euglena* (*2*). PixD is a BLUF domain protein of cyanobacteria *Thermosynechococcus elongatus* BP-1 and *Synechocystis* sp. PCC6803 (*3*; orf, Tll0078 and Slr1694). Tll0078 and Slr1694 were named TePixD and SyPixD, respectively. The deletion of the SyPixD protein gene was shown to modify the positive phototaxis of *Synechocystis* cells (*3*). A BLUF domain protein AppA of a purple photosynthetic bacterium *Rhodobacter sphaeroides* was shown to regulate the expression of photosynthesis genes in response to the blue light (*4*, *5*). Another BLUF domain protein of *R. sphaeroides* BlrB awaits clarification of its physiological role. A BLUF protein of *Euglena gracilis* PAC is known to regulate the photoavoidance reaction (*6*, *7*).

All the BLUF proteins show reversible 10−20 nm light-induced red shifts of the absorption bands of flavin moieties (*1*, *3*, *5*, *7*−*14*). The red-shifted form is considered to be the signaling state that transmits the photic information to the receiver domain. In cryptochrome and LOV domain proteins, the photoinduced electron transfer from a nearby amino acid residue and the photoinduced triplet state of flavin are known to initiate their photoconversion reactions (see refs *15*−*18* for reviews). The photoconversion mechanism of the BLUF protein that forms the unique red-shifted form, thus, makes a clear contrast to those of the other flavoproteins that never exhibit red shifts.

The photoreaction mechanisms of BLUF domain proteins have been studied by spectroscopy at low temperatures. In TePixD or SyPixD proteins, the intermediate state with a red shift of 5 or 9 nm ($I_5$ or $I_9$ form), respectively, was formed after illumination at 5 K (*19*, *20*). The shift widths were narrower than the 11 or 14 nm red shift seen in the signaling state formed at room temperature. Upon warming to 230 K in the dark, the $I_5$ form turns into further red-shifted forms ($J_{11}$ form); then, warming to 280 K causes the transition from

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[1] Abbreviations: BLUF, sensor of blue-light-using FAD; FAD, flavin adenine dinucleotide.

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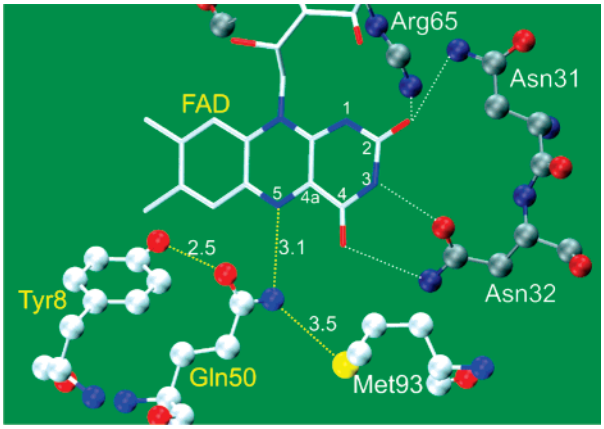



FIGURE 1: Spatial arrangement of FAD and estimated networks of surrounding hydrogen bonds in the TePixD protein (PDB entry 1X0P, chain A).

$J_{11}$ to the final signaling state ($F_{490}$ form) above 280 K (*20*). This result suggested that the limited structural changes of the amino acid residues around flavin occur at low temperatures, and it propagates into different domains of protein upon further warming, as revealed by the FTIR study that detected smaller changes in the FTIR difference spectrum in the protein moiety at 238 K (or under the dehydrated conditions at 288 K) compared to the hydrated condition at 288 K (*21*). The smaller shift widths detected in the intermediate states at low temperatures might also be interpreted as a light-induced redistribution between the cold-trapped inhomogeneous conformations.

Ultrafast spectroscopy of BLUF proteins detected the formation of the red-shifted form within 100 ps after the laser excitation in parallel with the decay of the singlet excited state at room temperature (*22*−*25*). The result is consistent with the fast decay of the flavin fluorescence with a time constant of 120 ps (*19*). A recent study in SyPixD (Slr1694) suggested the charge separation between flavin and amino acid residues with time constants of 7, 40, and 180 ps and proposed that the charge separation and the following charge recombination reactions between flavin and nearby Tyr8 trigger the alteration of the H-bond network around flavin (*23*). A similar charge separation−recombination mechanism was also proposed in the analysis of the Y21 and W104 mutant proteins of AppA (*26*).

The photochemical properties of the BLUF domain seem to come from the unique protein structure around flavin. All the structures of TePixD, SyPixD, AppA, and BlrB determined by X-ray crystallography and NMR spectroscopy (*27*−*32*) show that the flavin molecules are ligated by H-bonding with well-conserved Gln and Asn residues and surrounded by hydrophobic residues. In the structure of TePixD in Figure 1, we can see Gln50, Asn32, and Asn31 residues that bind flavin by H-bonding (*31*).

FTIR and Raman studies have reported that the H-bonding of the C4═O group of the flavin isoalloxazine ring to the apoprotein is strengthened with the red shift of flavin (*8*, *21*, *33*−*39*). It was also proposed that Gln50 forms a H-bond with N5 of flavin in the dark and alters it in the photoreaction because a mutant protein, which has Ala in place of Gln50 (Q50A), did not form the red-shifted form (*31*). The NMR study of AppA also proposed the configuration change of H-bonding of the conserved Gln63 (corresponding to Gln50 of TePixD) (*40*). The importance of the conserved Gln residue of AppA was also shown by biochemical analysis (*41*). On the other hand, N32A and N31A mutant proteins that have Ala in the place of Asn32 and Asn31 gave normal red shifts (*31*). These results indicated the direct involvement of the Gln50 residue, but not the Asn32 and Asn31 residues, in the photoconversion.

Tyr8, which is not in contact with flavin, is also predicted to be important for the photoconversion of BLUF proteins in a model that assumes the rearrangement of H-bonds between Gln50 and Try8. It is assumed that a hydroxyl group of Tyr8 is hydrogen-bonded to Gln50 and Gln50 to N5 atoms of flavin. It was also proposed that Tyr8 acts as a H-bond donor on the basis of FTIR studies of the [4-$^{13}$C]Tyr-labeled mutant protein of TePixD (*42*). The mutations of the corresponding tyrosine residues (Y8F in TePixD and SyPixD, Y21F in AppA, and Y472F in PACα-F2) abolished the photoconversion activities at physiological temperatures (*1*, *7*, *22*, *26*, *34*, *43*). It was proposed in SyPixD that the electron transfer to the photoexcited flavin from Tyr8 takes place with time constants of 7, 40, and 180 ps, the formation of flavin anion/semiquinone modifies the H-bonds between Tyr8 and Gln50, and then the following charge recombination between Tyr8 and flavin stabilizes the altered H-bonding (*23*). The model assumes a photoreaction of BLUF proteins in a mechanism common with many other flavoproteins that undergo the photoinduced charge separation between flavin and nearby aromatic amino acid residues on a picosecond time scale (*44*). FTIR study also indicated the modification of H-bonds of Tyr8 upon photoconversion (*42*). Tyr8, thus, should be an indispensable player in the photoconversion according to the model published recently (*45*).

In the photoconversion of TePixD at low temperature, two types of intermediate states (I and J) were accumulated upon illumination (*19*, *20*). The I state that shows the 5 nm red shift was formed at 5 K, turned into J with an 11 nm red shift width above 50 K, and then turned into the signaling form (F) that has a broader bandwidth when warmed above 240 K in the dark. The flavin anion or the charge-separated state has, however, not been trapped as a stable intermediate state even at 5 K. This suggests either the very low activation energy of the charge separation−recombination reactions postulated in the model or the formation of the red-shifted forms by some other mechanism.

In this study, we assessed the photoreactions of wild-type (WT), Y8F, Y8A, Q50N, and Q50A mutatant proteins of TePixD at low temperature. Y8F and Y8A proteins exhibited the red shift at 80 K, but not at 283 K. Q50N and Q50A proteins did not exhibit the red shift at both 80 and 283 K. The results indicate that Tyr8 is not indispensable for the red shift of flavin at least at low temperature. We will discuss the role of the Tyr8 residue on the basis of the new findings.

## MATERIALS AND METHODS

*Cloning, Expression, and Purification.* The coding region of TePixD of *T. elongatus* was amplified from the genomic DNA by PCR with primers (5′-ACATATGGGACTACATCGCCTG-3′ and 5′-CAGATCTAGGATCCTTGACTCA-3′). PCR was performed with Pfu DNA polymerase (Stratagene), and the PCR products were cloned into *Srf*I-digested pPCR-Script (Stratagene). Site-directed mutagenesis

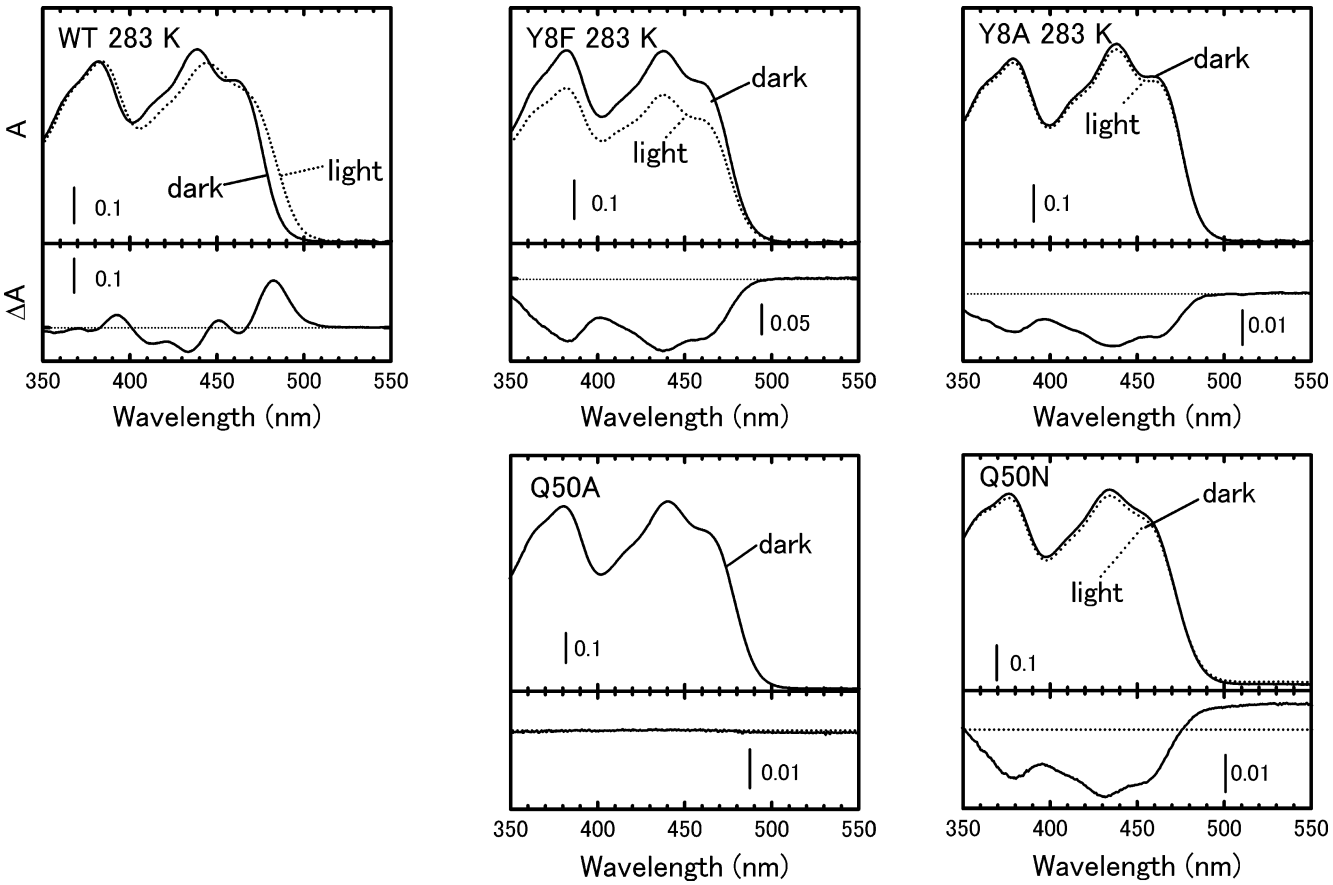



FIGURE 2: Absorption spectra (top panels) and light-minus-dark difference spectra (bottom panels) of WT, Y8F, Y8A, Q50A, and Q50N proteins at 283 K: (—) dark-adapted samples and (···) samples after a 50 s illumination by LED light centered at 455 nm.

of this His-TePixD was performed using the PCR-based QuickChange site-directed mutagenesis kit (Stratagene) as described elsewhere (*1*, *31*). The sequenced DNAs were excised with *Nde*I and *Bgl*II and then inserted into pET28a (Novagen) to allow expression with an N-terminal $(His)_6$ tag. Plasmids carrying the desired amino acid substitutions were confirmed by nucleotide sequencing. Tagged proteins were expressed in *Escherichia coli* BL21(DE3) pLysS with these recombinant plasmids in the presence of 20 $\mu$g/mL kanamycin for 18 h at 37 °C. Cells were harvested, frozen at −80 °C, thawed at 4 °C, and then resuspended in a 20 mM HEPES-NaOH (pH 7.5) buffer containing 1 M NaCl. After being disrupted by sonication, samples were centrifuged at 48000*g* for 1 h at 4 °C. His-tagged fusion proteins were purified from the supernatants by nickel affinity column chromatography (HiTrap Chelating HP; Amersham Biosciences, Piscataway, NJ). A sample was loaded on a column, washed, and then eluted with a 20 mM HEPES-NaOH (pH 7.5) buffer containing 1 M NaCl with 500 mM imidazole.

*UV−Visible Spectroscopy.* UV−visible absorption spectra were measured with a split-beam double-monochromator spectrophotometer (UV-3100PC, Shimadzu, Kyoto, Japan). The purified BLUF proteins were dissolved in a reaction medium containing 20 mM HEPES (pH 7.5), 1 M NaCl, and 500 mM imidazole. The absorption spectra at cryogenic temperature were measured in a liquid nitrogen cryostat (DN1704, Oxford Instruments, Oxford, U.K.) with a temperature controller (ITC4, Oxford Instruments). Glycerol was added to the reaction medium to give a final concentration of 63% (v/v), and then 700 $\mu$L of a reaction medium in an acrylic cuvette (optical path length, 10 mm) was put into the cryostat and cooled to the desired temperatures. The light from a blue high-power LED centered at 455 nm was used as an actinic light source at an intensity of 4 mW/cm$^2$ on the cuvette surface (Luxeon star Hex LXHL-MRRC, Lumileds Lighting, San Jose, CA).

## RESULTS

*Absorption Changes Induced by Illumination of the BLUF Domain Proteins of WT, Y8F, and Y8A at 283 K.* The absorption spectrum of the WT-TePixD protein of *T. elongatus* was measured in its dark-adapted state (D form) at 283 K (Figure 2). It showed peaks at 438 ($S_0-S_1$ transition) and 383 nm ($S_0-S_2$). Upon illumination for 50 s, the flavin band showed the red shift as previously reported (*19*). The light-minus-dark difference spectrum showed positive (+) and negative (−) peaks at 483 (+), 462 (−), 452 (+), 434 (−), 422 (+), 413 (−), and 392 nm (+) (Figure 2). The peak positions were determined on the basis of the derivative spectra.

The absorption spectra of the dark-adapted Y8F and Y8A mutant proteins exhibited main peaks at similar wavelengths at 438($S_0-S_1$)/382($S_0-S_2$) and at 438($S_0-S_1$)/379($S_0-S_2$) nm, respectively, suggesting the similar environments of flavin inside the protein. The Y8F and Y8A mutant proteins did not show red shifts upon illumination at 283 K (Figure 2). Their light-minus-dark difference spectra measured after illumination for 50 s showed broad bleaching peaks at ∼438 and ∼380 nm as reported previously (*1*), suggesting the reduction of flavin. The bleaching was recovered in the dark

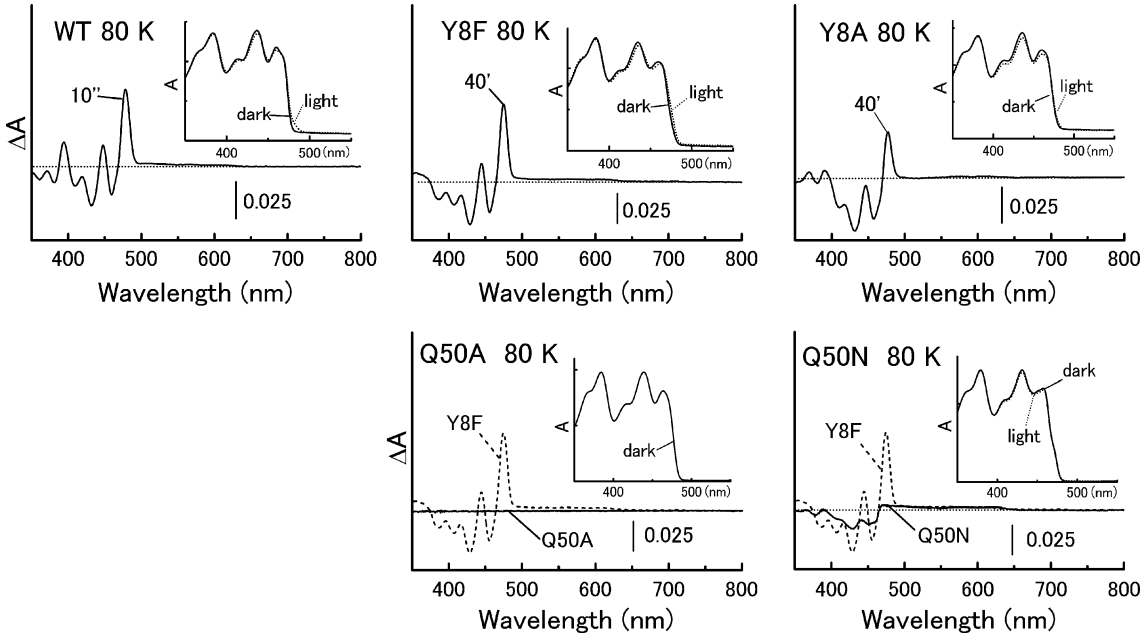



FIGURE 3: Absorption spectra (inset) and light-minus-dark difference spectra of the WT, Y8F, Y8A, Q50A, and Q50N proteins measured at 80 K. Difference absorption spectra after illumination for 10 s (WT) and for 40 min (mutant proteins) at 80 K are shown. The unit for the vertical axis gives the scale of absorbance change in each panel. The solid and dotted lines in the insets show the absorption spectra of the proteins before and after illumination, respectively. In the panels for Q50N and Q50A mutant proteins, the light-minus-dark difference spectrum of the Y8F protein was added as a control (- - -). The light from a 455 nm LED was used as the illumination light at an intensity of 4 mW/cm$^2$ at the cuvette surface. Absorption spectra were calculated as the average of 10 scans.

with very long time constants of 2000−3000 s. The results confirm that the photoconversion process is inactive in the Y8F- and Y8A-mutated PixD proteins at room temperature, as reported previously (*1*).

Absorption spectra of the Q50N and Q50A mutant proteins showed peaks at 440($S_0-S_1$)/381($S_0-S_2$) and 434($S_0-S_1$)/376($S_0-S_2$) nm, respectively, at room temperature. The peak wavelengths are a little different from those in the WT protein. Neither mutant protein exhibited a red shift by the 1 min illumination. A light-minus-dark difference spectrum of the Q50N mutant protein showed broad bleaching at ∼430 and ∼380 nm, suggesting the photoreduction of flavin as previously reported (*1*). The Q50A mutant protein, on the other hand, showed almost no absorption changes after illumination for 1 min.

*Absorption Changes Induced by Illumination at 80 K.* Figure 3 represents the absorption spectra and light-minus-dark difference spectra of the WT, Y8F, Y8A, Q50A, and Q50N proteins measured at 80 K. The dark-adapted WT protein exhibited absorption peaks at 471, 459, 439, 411, and 387 nm (Figure 3). After illumination for 10 s, the red-shifted form was accumulated, giving peaks of the light-minus-dark difference spectrum at 479 (+), 460 (−), 448 (+), 433 (−), 420 (+), 410 (−), and 394 nm (+) (Figure 3). The feature indicates the formation of the $J_{11}$ intermediate that exhibits the 11 nm red shift, as previously reported (*20*). The extent of the light-induced absorption change of the red-shifted form almost saturated after illumination for a few minutes (see Figure 4). With a longer illumination time, for example, after 40 min, bleaching at ∼430 nm became apparent in addition to the red shift (data not shown), suggesting the reduction of flavin as reported in the other BLUF proteins at room temperature after long illumination times (*12*−*14*).

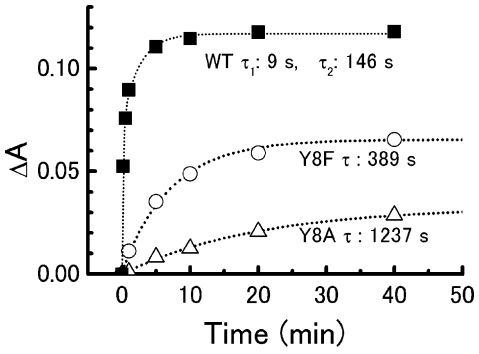



FIGURE 4: Time courses of accumulations of the red-shifted forms in the WT (monitored as the absorption change at 479 nm), Y8F (at 475 nm), and Y8A mutant proteins (at 477 nm) at 80 K. Data points were obtained from experiments similar to those depicted in Figure 3. Absorption spectra, which were obtained as the average of 10 scans, were measured during the dark period between each illumination period.

The dark-adapted Y8F mutant protein exhibited absorption peaks at 477, 467, 456, 436, 411, and 386 nm at 80 K (Figure 3). The peak positions were slightly different from those in the WT protein, although the differences were too small to be resolved at 283 K. Illumination of the protein at 80 K induced red shifts in the peak positions rather than the bleaching seen at 283 K. The light-minus-dark difference spectrum after illumination for 40 min showed peaks at 475 (+), 456 (−), 445 (+), 429 (−), 417 (+), 408 (−), 396 (+), and 387 nm (−) (Figure 3). The difference spectrum resembles that detected with the $J_{11}$ intermediate in the WT protein. However, the maximum amount of the red-shifted form was approximately half of that observed with the WT protein, judging from the peak height of the longest wavelength band [$\Delta A_{475}$(Y8F)/$\Delta A_{479}$(WT) ∼ 0.54]. A small increase in the absorption was also observed at 500−650 nm after illumination for 40 min at 80 K ($\Delta OD_{550}$ ∼ 0.002).

The spectral shape suggests it to be a photoreduced flavin. A larger extent of photoreduction of flavin was observed via illumination at the higher temperature, 150 K, and is discussed in the following section.

The difference spectrum obtained with the Y8A protein also indicated the photoaccumulation of the red-shifted form at 80 K, showing peaks at 477 (+), 457 (−), 446 (+), 432 (−), 417 (+), 410 (−), and 391 nm (+) (Figure 3). The maximum amount of the red-shifted form detected in the Y8A protein was less than a quarter of that detected in the WT protein judging from the peak heights at 477 nm, even after illumination for 40 min [$\Delta A_{477}$(Y8A)/$\Delta A_{479}$(WT) ∼ 0.24]. The red-shifted forms of both Y8F and Y8A mutants were stable and did not decay at 80 K. The Y8F and Y8A mutant proteins were estimated to be red-shifted by ∼8 nm (370 $cm^{-1}$) and 9 nm (380 $cm^{-1}$), respectively, by assuming simple red shifts of the absorption spectra of their dark-adapted forms. The extents of the red shifts are larger than that seen with the $I_5$ intermediate formed at 5 K in the WT protein and are closer to that of the $J_{11}$ intermediate that is formed above 80 K in the WT protein. The results suggest that WT and Tyr8 mutant proteins show similar conformational changes near flavin at 80 K.

The Q50A mutant protein showed no absorption changes even after illumination for 40 min at 80 K (Figure 3). The Q50N mutant protein did not exhibit the red shift and gave the broad absorption increase at 500−650 nm and a bleach around 430 nm, suggesting the accumulation of semiquinone or anion of flavin (Figure 3). Q50A and Q50N mutant proteins, therefore, cannot accumulate the red-shifted forms even at 80 K. It is, therefore, clear that Gln50 is indispensable for the photoconversion at any temperature. It suggests that the contact between flavin and Gln50 is a key for the photoconversion.

The WT protein accumulated the red-shifted form at 283 K, while the red-shifted forms, which resemble the J intermediates that have a slightly smaller shift width compared to the final signaling form, were trapped by the illumination at 80 K in the Y8F and Y8A proteins. The Q50A and Q50N proteins did not exhibit a red shift. The role of Tyr8 in the photoconversion is, therefore, different from that of Gln50.

*Accumulation Rates and Maximum Extents of the Red-Shifted Forms at 80 K.* The amounts of photoaccumulated red-shifted forms at 80 K are shown in Figure 4. In the WT protein, the level of the red-shifted form increased in two phases with time constants of 9 s (62% of the total extent) and 146 s (38%). The amount of the red-shifted form trapped at 80 K was estimated to be approximately one-quarter of the total TePixD proteins. In the Y8F and Y8A proteins, the levels of the red-shifted forms measured at 475 and 477 nm, respectively, increased slowly with time constants of 389 and 1237 s, which were 43 and 137 times longer, respectively, than that of the 9 s phase in the WT protein. If the quantum efficiency of the photoconversion at 80 K of the WT protein is similar to that at 283 K, i.e., 29% (*19*), we can assume the quantum efficiencies of 0.67 and 0.21% for the Y8F and Y8A mutant proteins, respectively. The maximum extents of the red-shifted forms were also smaller, approximately one-half and one-quarter, respectively, in the Y8F and Y8A proteins compared to that in the WT protein. The deletions of Tyr8, therefore, decreased both the quantum efficiency and the maximum extent of the photoconversion at 80 K.

*Effects of Dark Annealing at the Higher Temperature on the Red Shift at 80 K.* Illumination of the Y8F and Y8A proteins produced the red-shifted forms at 80 K but not at 283 K. We studied the effects of dark annealing in the Y8F protein at the higher temperatures to determine the stability of the red-shifted forms. The Y8F protein was illuminated for 40 min at 80 K at first to fully accumulate the red-shifted form (see Figure 4), and then, the absorption spectrum was recorded in the dark to calculate the light-minus-dark difference spectrum. Then the sample was warmed to 110 K and kept for 20 min in the dark. The sample was cooled to 80 K again to record the absorption spectrum after dark annealing at 110 K. Similar cycles of warming and cooling were repeated four times in the dark by changing the annealing temperature to 150, 240, and 280 K to record the effects of dark annealing on the light-minus-dark difference spectrum at 80 K.

The light-minus-dark difference spectrum of the Y8F mutant protein obtained after illumination for 40 min at 80 K indicated the formation of the red-shifted form (80KL). The difference spectrum after dark annealing at 110, 150, 240, and 280 K (designated 110KA, 150KA, 240KA, and 280KA, respectively) showed almost the same spectral pattern. The 240KA spectrum, however, exhibited smaller positive and negative peaks compared to those in the 80KL spectrum. It suggests a partial decay at 240 K. The 280KA spectrum exhibited a very small red-shifted form, suggesting its fast conversion into the dark-adapted form at 280 K. The spectrum showed weak broad negative bands around 350−470 nm (ΔOD ∼ 0.01) suggesting the formation of a small amount of reduced form or photodegradation products of flavin. This minor component may be related to the anion-like component formed by the 40 min illumination at 80 K.

*Anion Formation in the Y8F Mutant Protein via Illumination at 150 K.* Illumination at 80 K accumulated the red-shifted form in the Y8F and Y8A proteins, as shown above. On the other hand, illumination at 283 K accumulated no red-shifted forms and accumulated the reduced forms. The effects of annealing at 240 and 280 K (Figure 5) suggest that the red-shifted forms in the mutant proteins are very stable below 240 K and become unstable above 280 K. We, therefore, assumed that the apparent lack of the red-shifted forms after the illumination at 283 K came from the faster decay rate of the photoproducts at this temperature.

To investigate the reaction mechanisms to produce the red-shifted and reduced forms, we measured the effects of illumination at 150 K. At 150 K, the dark-adapted WT protein accumulated the red-shifted form ($J_{11}$) after illumination for 1 min (data not shown), as reported previously (*20*). At 150 K, the Y8F mutant protein accumulated a smaller extent of the red-shifted form compared to that at 80 K after illumination for 1 min (Figure 6). However, after the longer 20 min illumination, the light-minus-dark difference spectrum showed a bleaching peak at 430 nm together with the absorption increase at 360 nm. Flavin anion has been known to show a broad absorption band between 500 and 650 nm, a weaker band around 450 nm, and a stronger band around 350 nm. The difference spectrum induced by the illumination at 150 K showed this type of feature, suggesting the flavin anion to be a major photoproduct. The spectrum of a minor

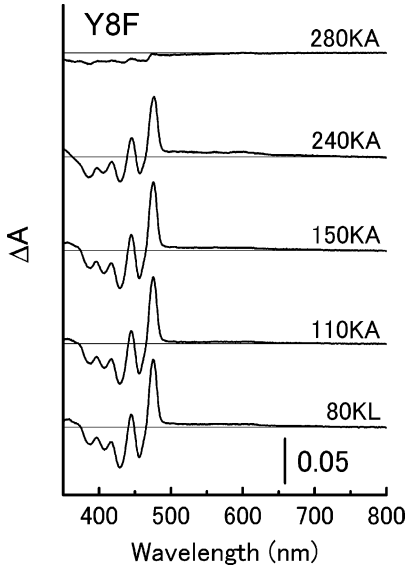



FIGURE 5: Effects of dark annealing at elevated temperatures on the red-shifted form of Y8F proteins that accumulated at 80 K. The Y8F protein was illuminated for 40 min at 80 K, and the spectrum was measured before and after the illumination to calculate the difference spectrum shown as 80KL. Then the protein was warmed to a higher temperature such as 110 K, incubated for 20 min, and cooled to 80 K again, and then, the spectrum was measured again to calculate the light-minus-dark difference spectrum after the annealing at 110 K (labeled as the 110KA difference spectrum in the figure). This procedure was repeated by changing the annealing temperature to obtain the 150KA, 240KA, and 280KA difference spectra as described in the text. After the annealing treatment, the baseline of each difference spectrum fluctuated a little so that we estimated the baseline by assuming the Δ*A* value between 750 and 800 nm was zero.

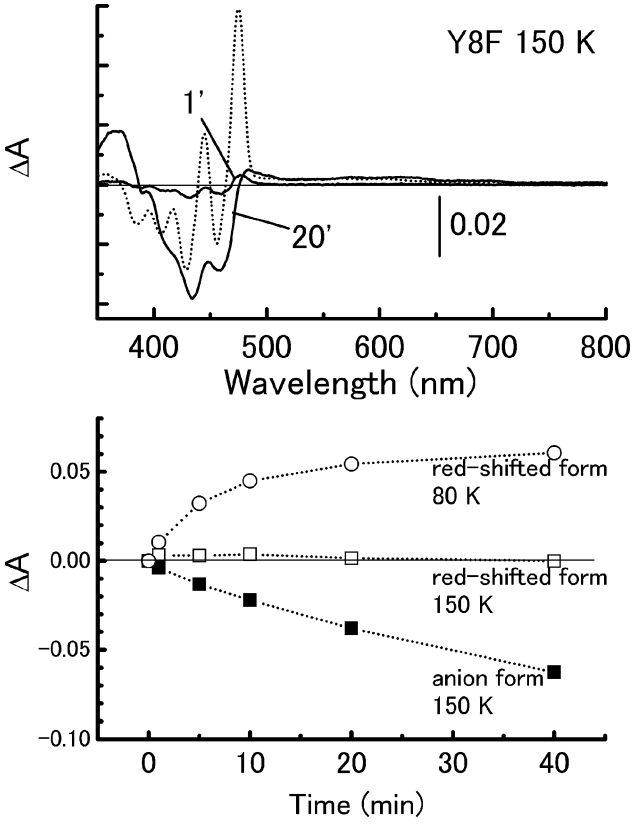
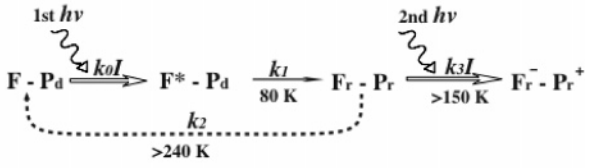



FIGURE 6: Light-minus-dark difference spectra (top) at 150 K of Y8F mutant proteins obtained after the 1 and 20 min illuminations at 150 K (—). The light-minus-dark difference spectrum of the Y8F protein measured at 80 K after illumination for 20 min was also plotted as the control (•••). Time courses of the absorption changes (bottom) due to the red shift of flavin measured at 475 nm (□) and at 433 nm due to the bleaching of flavin (■) induced by the illumination at 150 K. The time course of the accumulation of the red-shifted form measured at 80 K at 475 nm was also plotted as the control (○).

component accumulated at 80 K (Figure 3) with a broad 500−650 nm band also seems to represent the anion form. The result suggests that the photoreduction of flavin in the Y8F mutant protein becomes faster above 150 K as described in the Discussion.

At 150 K, the difference spectra measured at different illumination times did not show a clear isosbestic wavelength, suggesting the formation of multiple photoproducts (Figure 6). The extent of the red-shifted form accumulated after a 20 min illumination at 150 K was significantly smaller than that at 80 K. The time course at 80 K showed the saturation of the extent of the red-shifted form at a $\Delta OD_{475}$ value of 0.07 (bottom panel in Figure 6). On the other hand, the time course at 150 K indicates only a small extent of the red-shifted form just at the beginning of illumination. This is strange because the red-shifted form of the Y8F protein should not decay in the dark at 150 K, as seen in the previous section. The accumulation of the anion form at 150 K, on the other hand, showed some lag phase at the beginning of illumination and then increased slowly. The anion form, thus, does not seem to be the first photoproduct. It appears that the red-shifted form was produced by the illumination at 150 K at first, and then it turned into the anion form by the second photoexcitation during the prolonged illumination.

*Scheme 1*




*Scheme 2*

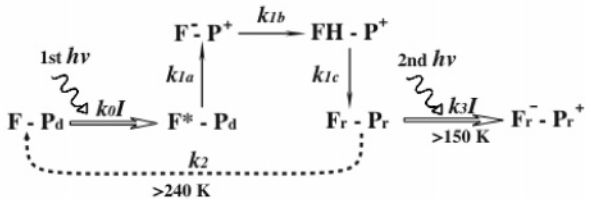



FIGURE 7: Reaction schemes for the photoconversion of BLUF protein at low temperature. Schemes 1 and 2 are essentially the same except for that the charge separation−recombination steps are inserted in Scheme 2. Empty, filled, and dashed arrows represent the photoexcitation steps, the dark-forward steps, and the dark-reversion steps, respectively. The second photoreactions (indicated as second h$\nu$) in both schemes represent the secondary photoreaction steps detected in theY8F protein, which became fast above 150 K.

## DISCUSSION

*Photoconversion of the Y8F and Y8A Mutant Proteins of TePixD of T. elongatus at Different Temperatures.* In the WT protein of TePixD of *T. elongatus*, the red shift of flavin was induced by the illumination at both 283 and 80 K. In the Y8F, Y8A, Q50A, and Q50N mutant proteins, situations were somewhat different. We can summarize the results in

this study as follows. (i) At 283 K, no red-shifted forms were accumulated by the illumination in all these mutant proteins as reported previously (*1*). (ii) The Q50A and Q50N mutant proteins did not accumulate the red-shifted form at 80 K. However, the red-shifted forms were accumulated in the Y8F and Y8A mutant proteins at 80 K. The difference spectra were almost comparable to that of intermediate state $J_{11}$ that was accumulated at 80 K in the WT protein (*20*). (iii) The formation rates of the red-shifted forms at 80 K were significantly lower in the Y8F and Y8A mutant proteins than in the WT protein. (iv) The maximum extents of the photoaccumulated red-shifted forms at 80 K in the Y8F and Y8A mutant proteins were approximately one-half and one-fourth compared to that in the WT protein. (v) The red-shifted form in the Y8F mutant protein was stable and unstable in the dark below and above 240 K, respectively. (vi) At 150 K, a small amount of red-shifted form was formed at first during the illumination in the Y8F mutant protein, and then, the accumulation of the anion form took over during the longer illumination.

The results in this study clearly indicate the light-induced accumulations of the red-shifted forms of flavin at 80 K in the Y8F and Y8A proteins. The red-shifted forms can be assumed to be formed either as the byproducts in the reaction that is different from the normal photoconversion process or as the intermediate states of the normal photoconversion. The result that the Gln50-mutated proteins did not form the red-shifted form at any temperature seems to support the second possibility, although this possibility should be tested more carefully as described below.

In the photoreaction of TePixD protein, intermediate states (I and J) trapped by the illumination at low temperature turned into the signaling states just by the dark warming (*20*). FTIR and Raman spectroscopy demonstrated that the rearrangement of H-bonds around the flavin molecule occurs upon the photoconversion of the BLUF domain proteins (*8*, *9*, *21*, *33*−*39*). The change in the H-bond between the flavin carbonyl group (C4═O) and the Gln50 residue has been postulated to be coupled to the red shift of the absorption band. Tyr8 was assumed to be indirectly connected to flavin via the H-bond with Gln50 and to respond to the photoconversion (*42*). The illumination at 238 K modified the FTIR spectrum in the flavin moiety to an extent similar to that at 288 K. The light-induced changes in the protein moieties, on the other hand, were smaller at 238 K compared to those detected at 288 K (*21*). The intermediate state detected by FTIR at 238 K seems to correspond to the J intermediate measured by the optical study (*20*). A small structural change around flavin, which is initially formed by the illumination or is formed at low temperature, seems to trigger the larger movements of the protein structure that can be detected at the physiological temperatures. Redistribution of the heterogeneous conformations, which can be triggered by the photon absorption, might interpret the small absorption changes at 80 K, as studied by hole burning spectroscopy (*48*). However, if the simple redistribution produces the apparent "red shift" at 80 K, it seems rather difficult to interpret the different photoresponses of the mutant proteins at 80 K. On the other hand, the mutant proteins seem to have different structural heterogeneities compared to that in the WT protein based on the smaller maximum extents of the red shift formed at 80 K. The smaller shift widths seen in the Y8-mutated proteins resemble the case in a His44-mutated AppA protein (*45*) which showed almost normal photoconversion with the smaller shift width, although the counterpart to this residue is not conserved in TePixD.

*Schemes for the Photoreactions at Low Temperature.* The red shift detected at 80 K in the WT, Y8F, and Y8A proteins seems to be interpreted well by Scheme 1, where $P_d$ and $P_r$ represent the protein conformation in the dark-adapted photoactive and light-induced red-shifted forms, respectively. F* and $F_r$ represent the singlet excited state of flavin and the red-shifted ground state of flavin, respectively. $k_0$, $k_1$, and $k_2$ represent the rate constants of the photoexcitation of flavin in the [F−$P_d$] state at light intensity *I*, the rate of conversion from the [F*−$P_d$] state to the red-shifted [$F_r$−$P_r$] state, and the relaxation of this state to the dark-adapted [F−$P_d$] state, respectively.

$k_0I$ should be almost the same for both the WT and mutant proteins judging from their similar absorption spectra (Figure 3). However, the apparent rates were found to decrease in the following order: WT > Y8F > Y8A [judging from the different initial rates (Figure 4)]. The apparent $k_1$ values in Scheme 1 thus are expected to decrease in the following order: WT > Y8F > Y8A ≫ Q50A and Q50N (though the mechanism is not clear yet). The different $k_1$ values, however, do not explain the smaller maximum amounts of the red-shifted forms accumulated at 80 K in the mutant proteins. It seems to require some other effects like a decrease in the amount of $P_d$ due to the higher structural heterogeneities or the difference in the dominant structure in the mutant proteins.

The $k_2$ values are expected to be negligible at 80−240 K in all the proteins because we detected no decay of the red-shifted forms both in WT and in the mutant proteins below 240 K. Above 240 K, $k_2$ increased significantly in the Y8F protein but not in the WT protein as shown in Figure 5.

*Accumulation of the Anion Form in the Y8F Mutant Protein at 150 K.* The Y8F protein responded to the illumination at 150 K (Figure 6) differently from the WT protein as noted below. (i) The illumination at 150 K accumulated only a small amount of red-shifted form, although the annealing experiment in Figure 5 suggested the $k_2$ value to be low at this temperature. The anion form of flavin, which was not detected at 80 K, was slowly photoaccumulated at 150 K. The secondary photoreaction seems to interpret this result. We can assume that the [$F_r$−$P_r$] state is converted to the [$F_r^-$−$P_r^+$] state by the light-induced electron transfer from an amino acid residue in $P_r$ to $F_r$. Scheme 1 assumes the [$F_r$−$P_r$] state either to decay to the [F−$P_d$] state in the dark with a rate constant $k_2$ or to be photoconverted to the radical pair [$F_r^-$−$P_r^+$] state with a rate constant $k_3$. It is expected that $k_3$, which is low at 80 K, becomes high above 150 K in the Y8F mutant protein. The amino acid residue that donates the electron to flavin is not clear yet. However, it cannot be Tyr8 that is missing in the Y8F protein. Scheme 1 interprets the experimental results in the Y8F mutant proteins well.

*Testing the Charge Separation Mechanism.* The picosecond spectroscopy studies have suggested the fast charge separation and recombination between flavin and the protein amino acid residue trigger the photoconversion in the BLUF domain (*22*, *23*, *25*). It was proposed that F* is reduced to $F^-$ by the electron from Tyr21 in AppA and that the

rearrangements of the H-bonds between the flavin C=O group and nearby amino acid residues take place as discussed recently (*45*). The suppression of the photoconversion at room temperature by the deletion of Tyr8 (or its counterparts in the other BLUF proteins) strongly supported the proposal that Tyr8 serves as the electron donor for transient photoreduction of flavin. We tested this type of mechanism on the experimental results obtained in this study.

Scheme 1 can be modified to Scheme 2 by including the charge separation, proton transfer, and charge recombination processes that lead to the red shift of flavin as proposed recently (*45*), with rate constants $k_{1a}$, $k_{1b}$, and $k_{1c}$, respectively, where the $[F^{-}-P^{+}]$ state represents the transient charge-separated state between flavin and the protein and is different from the stable $[F_r^{-}-P_r^{+}]$ state formed by the secondary photoreaction above 150 K in Scheme 1. The protein conformation or the cation residue might be different between these two states. Tyr8 has been postulated to be the most probable candidate of the electron donors in the reaction to form the $[F^{-}-P^{+}]$ state because it is located the shortest distance from flavin, and its modification has led to the complete loss of the photoconversion activity at room temperature (*1*, *23*, *26*).

The results in this study have shown that the red shift of flavin occurs even without Tyr8 by the illumination at 80 K. The results suggest either a residue other than Tyr8 functions as the electron donor according to the charge separation−recombination reactions in Scheme 2 (i) or the red shifts are produced by a mechanism other than Scheme 2 in the mutant proteins (ii-a), or both in mutant and WT proteins (ii-b). To test the possibility (i), we calculated the edge-to-edge distance between flavin and the amino acid residues that can be the electron donor: Tyr8 (3.34 Å from flavin and in chain A of TePixD in structure 1X0P), Phe48 (3.52 Å), His72 (3.81 Å), and Tyr43 (7.14 Å). It is clear that Tyr8 is the residue closest to flavin and is the best candidate to be the electron donor. Phe48 and His72 are also close to flavin and may also serve as the donor to flavin in the major reaction or in the place of Tyr8 in the Y8F and Y8A proteins. However, it is not predictable because the electron transfer rate depends not only on the distance but also on the mutual orientation of reactant molecules and on the free energy difference of the reaction (*46*, *47*). We also require some additional mechanisms to stabilize the light-produced red-shifted form at the higher temperature in the WT protein.

Cases ii-a and ii-b above might also be possible. Then, a mechanism other than Scheme 2 is required to produce the red shift in either case. One possible mechanism might be the direct conversion from the [F*−P] state to the red-shifted $[F_r-P_r]$ state without the charge separation−recombination steps. It might be enabled by the rearrangement of the H-bonds between flavin and Gln50 in response to the change in the electronic structure of the flavin isoalloxazine ring upon the light excitation of flavin. In this mechanism, the deletion of Tyr8 is expected to simply modify the interaction between flavin and Gln50 and to destabilize the red-shifted form at room temperature. However, the mechanism is rather contradictory to the results obtained by ultrafast spectroscopy (*22*, *23*). More work is required to improve our understanding of the photoconversion mechanism and of the role of Tyr8.

*Interaction between Protein and Flavin.* We can assume similar conformational changes around Gln50 in the red-shifted forms in the WT, Y8F, and Y8A proteins for the following reasons. Y8F or Y8A mutant protein exhibited an absorption spectrum similar to that of flavin and WT protein. They showed the red shift at 80 K, with a shift width similar to that of the $J_{11}$ (or $I_5$) intermediate state in the WT protein (*20*). The Gln50 mutant protein did not accumulate the red-shifted form even at 80 K. These results suggest that the conformation changes around the Gln50 residue upon photoconversion in the Y8F and Y8A proteins are similar to those in the WT protein.

Pigment−protein interactions sometimes exhibit inhomogeneity in the photoreaction at low temperature due to the freezing in the multiple heterogeneous configurations. Light-induced redistributions of absorption bands in the pigment−protein complexes have been observed at low temperature by hole burning spectroscopy (*48*). The red shift formed at low temperature in the BLUF protein might also be interpreted as the light-induced redistribution between heterogeneous configurations. The redistribution, however, should be directly coupled to the protein function itself in this case because the red-shifted intermediates formed at 80 K were transformed to fully red-shifted signaling forms on warming (*19*, *20*). The illumination at 238 K induced the changes in the protein FTIR signal smaller than that at 288 K (*21*), suggesting that the light-induced local conformation change around flavin propagates to the whole protein region by the changes in the long hydrogen bond network (*9*, *21*, *35*−*38*, *42*). One important role of Tyr8, then, can be to fix the configuration of the amide group of Gln50 by H-bonding. The deletion of Tyr8 may decrease the population of proteins that has the proper conformation around Gln50 required for the efficient photoconversion (decrease $P_d$ in Scheme 1). This type of mechanism also interprets the low maximum extents of the red-shifted forms accumulated at 80 K in the Y8-mutated proteins.

## CONCLUSION

The Gln50 and Tyr8 residues in the BLUF domain proteins (or their counterparts in other BLUF proteins, such as Gln63 and Tyr21 in AppA) have been assumed to be indispensable for their photoconversion activities in the formation of the red-shifted signaling form, based on the effects of site-directed mutagenesis (*1*, *7*, *22*, *34*, *43*, *45*). The results in this study indicate that the loss of the Gln50 abolishes the red shift at both 80 and 283 K. On the other hand, the replacement of Tyr8 with phenylalanine or alanine did not stop the formation of the red-shifted form at 80 K, although the mutations decreased the efficiency and maximum extents of photoconversion at 80 K and abolished the photoconversion at 283 K. The replacement of Tyr8 destabilized the red-shifted form above 240 K and enhanced the secondary photoreaction to form reduced flavin above 150 K. We conclude that Tyr8 is not indispensable for the formation of the red-shifted form at least at low temperature, although it is required for the normal photoconversion at physiological temperatures. The results suggested two possibilities. (1) The role of Tyr8 that was postulated (*22*−*24*, *45*) to function as the electron donor to flavin in the initial step of the photoconversion can be replaced by other aromatic residues near flavin, or (2) Tyr8 works only to stabilize the photo-

conversion product even in the WT protein. Further work is required to determine the exact mechanism of the photoconversion.

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