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Fate Determination of the Flavin Photoreceptions in the Cyanobacterial Blue Light Receptor TePixD (Tll0078)

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PixD (Tll0078, Slr1694) is a BLUF (sensor of blue light using FAD)-type blue light receptor protein of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 and the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803. BLUF protein is known to show light-induced ~10 nm red shift of flavin absorption that is coupled with strengthening of the hydrogen bond between the O(4) of the isoalloxazine ring and a certain amino acid residue. According to the 3D structure of TePixD we determined, O(4) of the ring is linked to Gln50 and Asn32. A survey of flavin-interacting residues by site-directed mutagenesis showed that Gln50 but not Asn32 is essential for the normal red-shifting photoreaction. Here, we further studied the role of Gln50 and its close neighbor Tyr8. All the mutated proteins of Gln50 and Tyr8 (Q50A, Q50N, Y8A and Y8F) lost the normal red-shifting photoreaction. Y8A, Y8F and Q50N, instead, showed a light-induced flavin triplet state and a low yield of subsequent flavin reduction that is analogous to the photocycle of the LOV (light-oxygen-voltage-sensing) domain of phototropins, while Q50A did not. Fourier-transform infrared (FT-IR) analysis of N32A showed that O(4) of the ring is hydrogen-bonded to Asn32 both in the light and dark. These results, together with the 3D structure, indicate that the hydrogen bond network of Tyr8-Gln50-O(4)/N(5) (flavin) is critical for the light reaction of the BLUF domain. Based on the structural and functional similarities of the BLUF and the LOV domain of phototropins, we propose that the interaction between apoprotein and N(5) of flavin determines the photoreaction of the flavin-binding sensors.

Keywords: blue light sensor; BLUF; cyanobacteria; flavin; photoreceptor

Introduction

Light perception is one of the important signal recognition processes in most living organisms both in phototrophs and non-phototrophs. Three distinct types of flavin-binding blue light receptor domains, sensor of blue light using FAD (BLUF), light oxygen voltage (LOV) (a subgroup of Period-ARNT-Sim (PAS) domain), and photolyase homology region (PHR) domains, are known to date.[1–3] The LOV domain of plant phototropins and bacterial counterparts, which binds FMN non-covalently, shows light-induced adduct formation of the isoalloxazine ring with a conserved Cys residue of apoprotein.[2,4] The PHR domain of cryptochromes, which binds FAD non-covalently, shows light-induced intra-protein electron transfer from aromatic amino acid residues to the isoalloxazine ring.[5] The BLUF domain of PixD and AppA shows a light-induced spectral shift of the absorption of flavin to the longer wavelength side by ~10 nm by some kind of unidentified interaction

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Abbreviations used: BLUF, blue light using FAD; LOV, light oxygen voltage; PAC, photoactivated adenylyl cyclase; FT-IR, Fourier-transform infrared.

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between flavin and apoprotein.[6,7] These photoreceptor proteins are totally unrelated to each other and employ distinct photoreactions of the flavin for recognition of the blue light signal. It can be assumed that one of the specific photoreaction pathways of flavin is selected by the feature of protein environment to induce further structural changes of proteins to achieve their function. The photic information is transmitted to the output domain activity, such as protein kinase, adenylyl cyclase and protein–protein interaction.

BLUF domain proteins are widely distributed in many bacteria and some unicellular eukaryotes. Photoactivated adenylyl cyclase (PAC) is a blue light receptor for the photophobic movement of *Euglena gracilis* cells.[8] PACα and β subunits contain two BLUF domains, each followed by an adenylyl cyclase domain. Blue light illumination induces activation of catalytic activities. AppA of a photosynthetic bacterium *Rhodobacter sphaeroides* mediates blue light-induced transcriptional suppression of photosynthesis genes. The N-terminal BLUF domain interacts with the C-terminal anti-repressor domain that binds to a transcriptional regulator PpsR.[6] SyPixD (*Synechocystis* positive phototaxis factor, Slr1694) is a blue light receptor for phototactic regulation of pili-dependent cell motility in *Synechocystis* sp. PCC 6803.[7,9] The light-dependent regulation may be mediated by protein–protein interaction with SyPixE. All these BLUF domains show a very similar red-shifting photoreaction irrespective of variation in the subsequent signaling.

BLUF domain proteins are known to show the reversible red-shift of the absorption spectrum upon light illumination that recovers in the subsequent dark-adaptation within seconds to minutes.[6,7,10–12] This photoreaction is only observed in BLUF domain protein. FT-IR analysis of AppA and SyPixD suggested the strengthening of the hydrogen bond between apoprotein and C(4)=O of the isoalloxazine ring of flavin coupled with the red-shift of absorption spectrum.[10,13,14]

We have determined the 3D structure of TePixD (*Thermosynechococcus elongatus* BP-1 PixD, Tll0078) at 2.0 Å resolution by X-ray crystallography.[15] It consists of an N-terminal FAD-binding BLUF domain and a C-terminal extensional domain. The molecular structure of the BLUF domain consists of a sheet formed by five β-strands, and a cleft formed by two α-helices on one-side of the sheet. The isoalloxazine ring of flavin is embedded between the two helices-cleft. The 3D structure revealed that many side-chains of conserved amino acid residues exist in close contact around the isoalloxazine ring. As an initial study for the characterization of the functional role of those residues, we focused on three residues (Asn31, Asn32 and Gln50) that apparently interact with the hydrophilic side of the isoalloxazine ring by hydrogen bonds (Figure 1). We replaced those three residues with Ala and investigated its functional role. We found that only Gln50 is essential for the unique photocycle of flavin in the BLUF proteins, as judged from the result that N32A and N31A mutant proteins showed a red-shift of absorption as wild-type and Q50A did not. Taken together, we proposed that a hydrogen bond network of Tyr8-Gln50-O(4)/N(5) (flavin) is a critical and structural important factor for the BLUF-specific photoconversion mechanisms.[15]

Here, we further extended our studies of the site-directed mutagenesis to elucidate functional roles of Gln50 and Tyr8 that strongly interacts with the amide side-chain of Gln50. The result showed that both residues were essential for light-induced formation of the normal red-shift of flavin absorption at room temperature. Interestingly, instead of the normal photoreaction, the Tyr8 mutants and Q50N (Gln50-to-Asn) mutant formed a transient triplet state of flavin upon flash excitation and showed subsequent photo-bleaching. This photoreaction rather resembles that of other light-sensory flavoproteins, LOV domain, than that of BLUF wild-type. The results support that the hydrogen bond network of Tyr8-Gln50-O(4)/N(5) (flavin) is important for the photoconversion in the BLUF domain, and provide experimental evidence for fate determination of the flavin photocycle in relation to the FMN-binding phototropin.

Results and discussion

We replaced the conserved Tyr8 with Ala (Y8A) or Phe (Y8F) and the conserved Gln50 with Asn (Q50N), respectively. Q50A protein was prepared as reported.[15] SDS-PAGE showed that these proteins were purified into homogeneity. Based on the absorption ratio of A_{450}/A_{280}, we roughly estimated the flavin integration as follows: wild-type, 100%; Y8A, 100%; Y8F, 80%; Q50A, 60%; Q50N, 80%. We measured absorption spectra of mutant proteins under dark and continuous blue-light illumination (Figure 2(1)). The dark-adapted absorption spectra of these mutants were qualitatively similar to that of wild-type, suggesting that the mutations did not significantly affect the environment of the isoalloxazine ring in the dark.

The light-minus-dark difference spectrum of wild-type protein showed a red shift of flavin absorption as previously reported.[15] On the other hand, the light-minus-dark difference spectrum of Y8F showed a bleaching of the absorption bands of flavin in the wavelength range of 420–480 nm (Figure 2(1)). This bleaching was almost reverted to the original level after the dark adaptation for 30 min (Figure 2(2)). Y8A and Q50N proteins also showed similar bleaching of flavin as Y8F protein, although the kinetics and yield varied among the mutants. These spectral changes were very different from that of wild-type showing a red shift of absorption spectrum. A reduction of oxidized flavin is known to show a bleaching in the 350–500 nm range in the difference spectrum with the isosbestic point at approximately 350 nm.[16] Similar difference spectra were observed during photo-bleaching and dark reversion in the mutant proteins, suggestive of

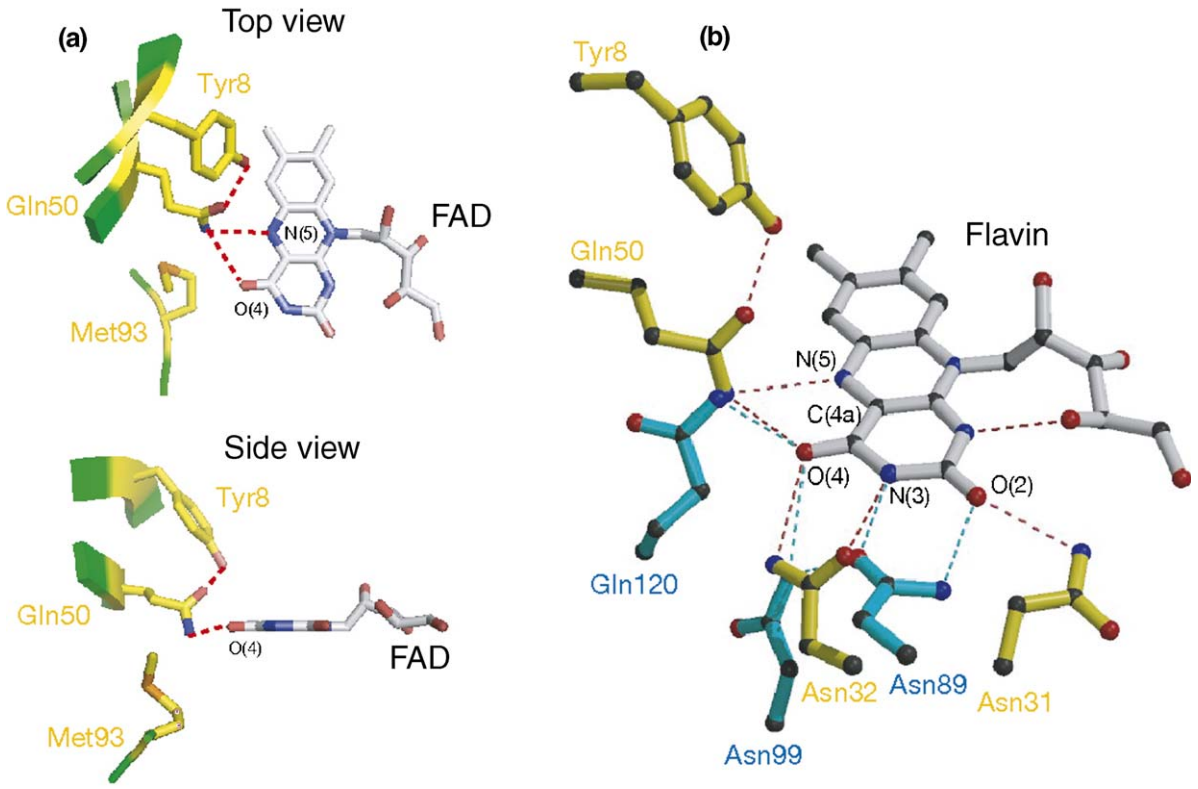


Figure 1. Structure of hydrogen bonds between flavin and side-chains. (a) The location of flavin, Tyr8, Gln50 and Met93 of TePixD. (b) The location of side-chains interacting with flavin in TePixD and Phot-LOV1. The flavin is colored in light gray. Tyr8, Asn31, Asn32 and Gln50 of TePixD are colored in yellow. Asn89, Asn99 and Gln120 of Phot-LOV1 are colored in blue. The flavin and side-chains are shown as a ball-and-stick drawing where the colors of the atoms are according to the CPK model. Dotted lines represent possible hydrogen bonds.

photoreduction of flavin. Further illumination of the mutant proteins for 30 min produced a new broad absorption increase above 600 nm (data not shown), and its spectral properties resemble that of neutral semiquinones. On the other hand, the Q50A mutant protein showed very little change in the absorption spectrum even after long illuminations, as reported.[15] Clearly, Q50A does not show any reaction like the normal red shift or the photoreduction. To eliminate the possible involvement of the His-tag, we expressed Y8F protein without the tag. The partially purified proteins showed more or less the same spectra (data not shown). These results suggest that side-chains of Tyr8 and Gln50 are critical for the primary reaction of the normal red shift.

The absorption change of mutant proteins, Y8F, Y8A, Q50N, and Q50A, induced by continuous illumination did not show an accumulation of a normal red-shifted form at room temperature. To investigate whether these mutant proteins show a formation of a red-shifted form in the faster time-region or not, we measured transient absorption changes induced by nanosecond laser flash excitation (Figure 3). The wild-type protein showed a formation of a red-shifted form in the difference spectrum at a 50 ns delay time. Absorption increases around 600–700 nm have not been observed, showing no formation of a triplet state of flavin. The result makes a clear contrast to the case with the AppA that formed a triplet state with an efficiency of 9%.[17] The red-shifted product disappeared in the dark with a slow half decay time ($t_{1/2}$) of 3.5 s. On the other hand, the Y8F mutant protein showed different absorption changes, compared to wild-type, with peaks around 400 nm (positive) and 440 nm (negative), and a positive broad band in the 500 nm–700 nm region at 50 ns. This transient product state almost disappeared at 100 μs. The transient broad band is similar to that of the flavin triplet state and is slightly different from that of the flavin semiquinone, although it is rather difficult to distinguish them clearly.[18,19] At the moment, we interpret the difference spectra at 50 ns to represent the transient diminution of flavin in the ground-state (400 nm–500 nm) and formation of the triplet state of flavin (500 nm–700 nm).[19] Similar difference spectra were obtained for Y8A and Q50N proteins, indicating a major formation of the triplet state at 50 ns as with the Y8F mutant protein. A triplet state of flavin is known to turn into a reduced form of flavin, such as an anion or neutral semiquinone form, in the presence of an appropriate electron donor. It is conceivable that a reduced form of Y8F, Y8A and Q50N observed in the difference spectra during continuous illumination (Figure 2(1)) was formed from the transient triplet state at low yield

(Figure 2(3)). In contrast, Q50A showed very little change in the absorption spectrum at 50 ns compared to the other mutant proteins.

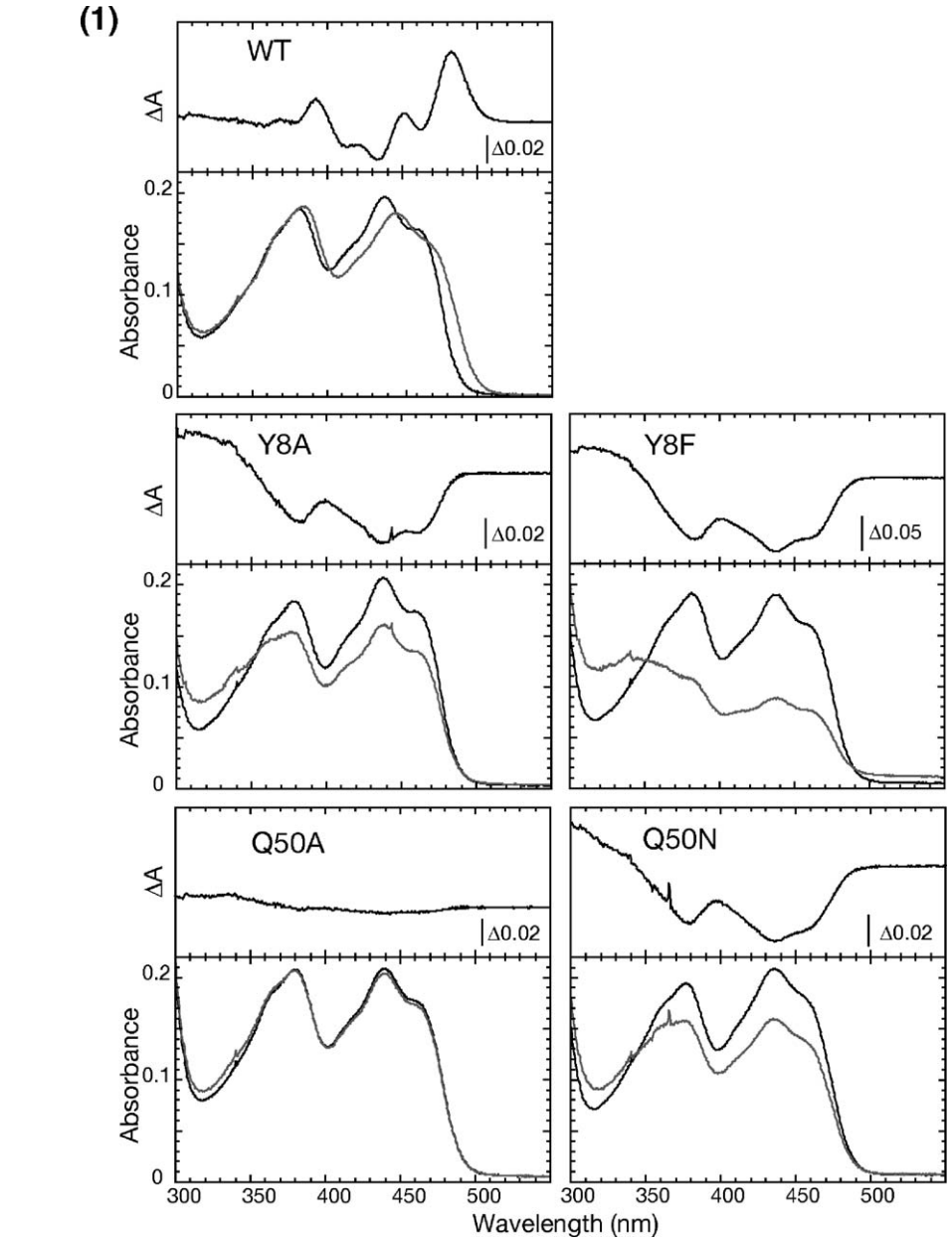
The formation of the triplet state followed by the reduction of flavin, seen in the Y8F, Y8A, and Q50N mutant proteins in this study, resembles the case in the LOV domain proteins, which form a Cys-adduct from the triplet state. The reaction never occurs in the photoreaction of wild-type BLUF protein that shows the 10 nm red shift of flavin absorption (Figure 3). The red-shifted form is the only photoproduct so far detected as a signaling state at room temperature (F form). On the other hand, we detected a 5 nm red-shifted intermediate form (I form) upon illumination below 50 K[20] that turns into a signaling state on warming (see also Figure 3). The normal photoreaction of BLUF proteins forms a red-shifted form from the singlet-excited state within about 100 ps after the blue light absorption accompanying the quenching of excited energy.[6,10,20] On the other hand, the Y8F, Y8A, and Q50N mutant proteins showed a formation of a triplet state, which was not observed in wild-type protein. The suppression of the fast competitive reaction path from the singlet excited state by mutation may elongate the singlet life time and seems to increase the quantum efficiency of a triplet formation followed by a subsequent reduction of flavin as seen in the LOV domain. We identified the key amino acid residues (Tyr8 and Gln50) that determine the fate of flavin photoreaction in TePixD.

FT-IR spectroscopy of AppA and Slr1694 revealed that the light-induced weakening of C(4)=O stretching mode in the isoalloxazine ring in the signaling state.[14,21,22] A similar weakening of C(4)=O stretch vibrations in the FT-IR spectrum was also confirmed in TePixD (Figure 4). The downshift of the C(4)=O stretch from 1713 cm^{-1} to 1699 cm^{-1} was considered to represent a light-induced strengthening of the hydrogen bond at O(4) of the isoalloxazine ring. In the 3D structure, two conserved polar residues, Asn32 and Gln50, exist close to the O(4) of flavin (Figure 1). As previously reported, the N32A mutant showed a normal spectral red shift of flavin absorption upon illumination, whereas the Q50A mutant showed no apparent photoproduct even at a 50 ns delay time after flash excitation (see also Figure 2(3)). These results strongly suggest that the amide group of Gln50 but not Asn32 works as the hydrogen-bond donor for O(4) of the isoalloxazine ring in the signaling state. The Q50N, Y8F and Y8A mutant proteins also did not show the normal red shift in a time range from 50 ns to seconds after the laser excitation. This result suggests that the interaction between the hydroxyl group of Tyr8 and the amide oxygen atom of the side-chain of Gln50 is also essential for the primary photoreaction. Thus, the hydrogen bonds of Tyr8–Gln50-O(4) are critical for the normal red shift.

Then we examined the possibility of hydrogen bonding between the O(4) of the flavin and the amide side-chain of Asn32 by measuring the N32A mutant (Figure 4). Results showed that the overall spectrum of N32A was very similar to that of wild-type, excecpt that a slight shift of the prominent bands (1724 cm^{-1} (negative) and 1708 cm^{-1} (positive)). The shift in frequency was 11 cm^{-1} in the dark and 9 cm^{-1} in the light, when compared to the wild-type. These shifts to the higher wave number indicate that the O(4) of the flavin is also hydrogen-bonded to the side-chain of Asn32 both in the light and dark. Since the N32A mutant showed the normal red shift upon light excitation, the hydrogen bond to O(4) may not be sufficient to account for the normal light reaction.

The 3D structure of TePixD suggests that the amide of Gln50 is also linked to the N(5) of the isoalloxazine ring (Figure 1). N(5) of the isoalloxazine ring is a most reactive site for redox reactions generally. For example, when a free flavin in solution is sequentially reduced with two electrons upon illumination, the first electron reduces the double bond at N(5) and then the second electron comes to the N(1) position.[18] Taking this into consideration, a specific interaction of N(5) of the isoalloxazine ring with NH_2 of Gln50 may be essential for the formation of the signaling state. We demonstrated here that Q50N, Y8F and Y8A mutants acquired a new ability to form a triplet state and the subsequent flavin reduction. Site-directed mutants equivalent to Y8F of TePixD were also studied in AppA (Y21F) and Slr1694 (Y8F). Both mutant proteins lost the normal photoreaction activity as expected, although the triplet formation or flavin reduction were not mentioned.[13,22] It might have been rather difficult to detect the reduced flavin that is unstable under aerobic conditions. Recently, Laan *et al.*[23] mentioned that the illumination of the Y21F mutant of AppA accumulates a flavin semiquinone-like signal with a broad absorption band in a red-region. Moreover, wild-type AppA was shown to form the triplet state of flavin in addition to the normal red shift[17] in contrast to the wild-type TePixD that does not form any triplet state or semiquinone. It should also be noted that TePixD isolated from the thermophilic cyanobacterium is far more stable than SyPixD isolated from the mesophile.[7] Thus, TePixD appears to be more stringent than SyPixD or the truncated AppA. A rigidity of the apoprotein may affect the quantum efficiency of each reaction pathway, such as red shift, non-radiative transition and triplet state. Most likely, the specific interaction of N(5) of the ring and the amide NH_2 of Gln50 is critical for the determination of the photoreaction, thus highlighting the role of a hydrogen bond network of Tyr8-Gln50-O(4)/N(5) (Figure 1).

Another flavin-binding photoreceptor LOV domain of the phototropin is totally unrelated with the BLUF proteins at the primary and secondary structure levels. However, the overall 3D structures with five-stranded β-sheets and a few α-helices that hold the isoalloxazine ring are shared between the BLUF and the LOV domain as mentioned previously.[15] Furthermore, polar atoms in the isoalloxazine ring are hydrogen-bonded similarly:



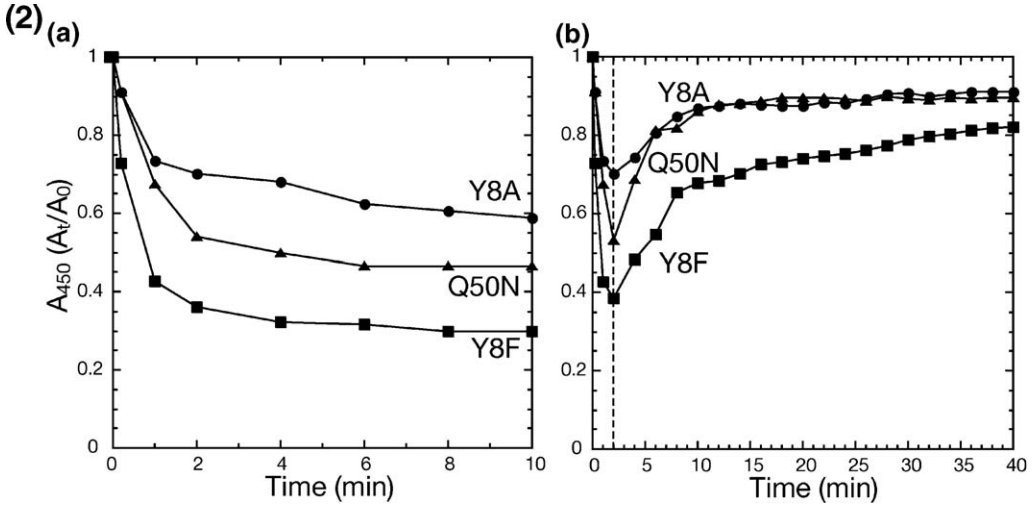


Figure 2 (*legend on next page*)

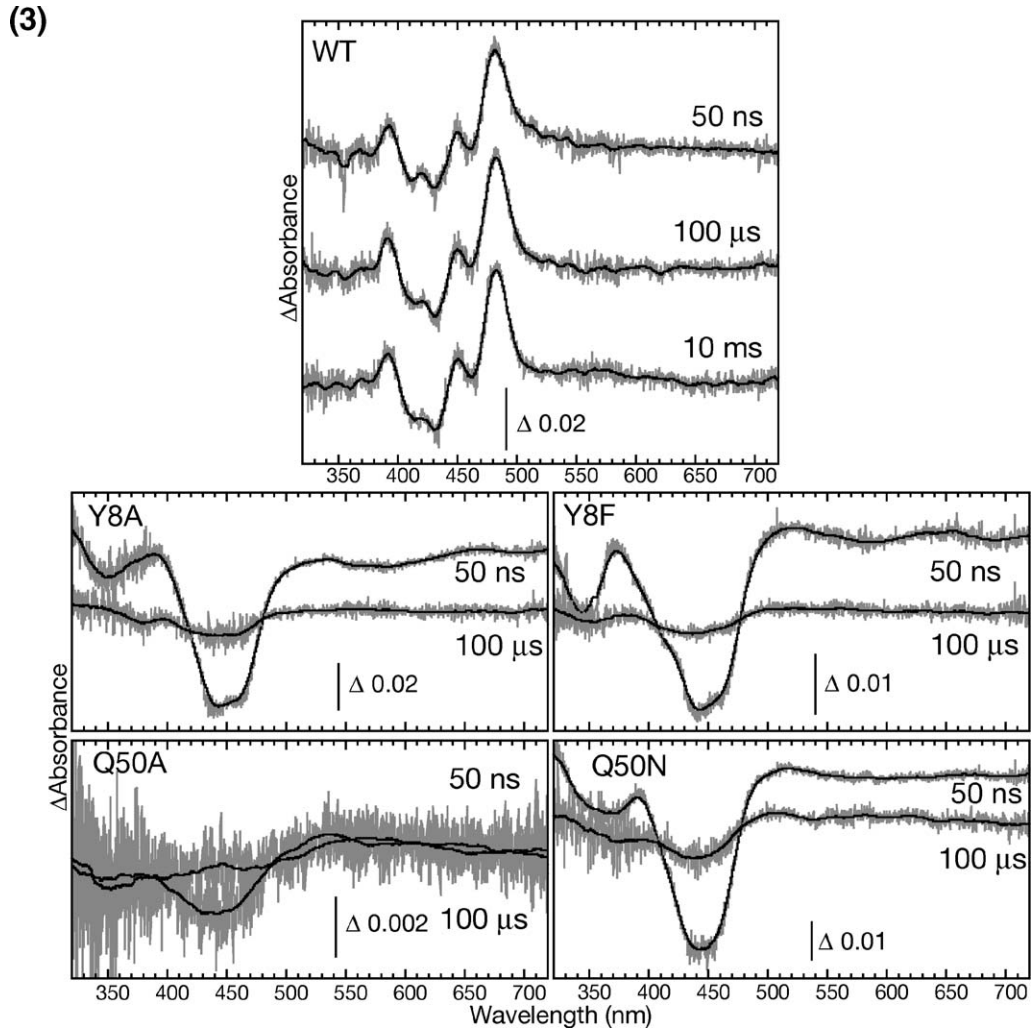


Figure 2. (1) Absorption spectra (lower panel) and light minus dark difference spectra (upper panel) of TePixD mutated proteins. Dark-adapted spectra are indicated with a black line and illuminated spectra are indicated with gray line. A polyhistidine-tagged TePixD (His-TePixD) was expressed with pET28a vectors in *Escherichia coli* BL21 (DE3), as described.[15] Site-directed mutagenesis of His-TePixD were performed using the PCR-based QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with primers (Y8A sense, 5′-CATCGCCTGATTgcTCTCAGTTGCGCTAC-3′; Y8A antisense, 5′-GTAGCGCAACTGAGAgcAATCAGGCGATG-3′; Y8F sense, 5′-CATCGCCTGATTTtTCTCAGTTG-CGCTAC-3′; Y8F antisense, 5′-GTAGCGCAACTGAGAaAAATCAGGCGATG-3′; Q50N sense, 5′-GGCATGTTTCTGaA-cACCCTTGAGGGC-3′; Q50N antisense, 5′-GCCCTCAAGGGTgTtCAGAAACATGCC-3′). Plasmids carrying the desired amino acid substitutions were confirmed by nucleotide sequencing with the BigDye terminator fluorescence detection method (Applied Biosystems, Foster City, USA) and a capillary sequencer (PRISM 310 Genetic Analyzer; Applied Biosystems). Mutated proteins were overexpressed in *E. coli* BL21 (DE3). Cells were cultured at 37 °C in LB medium containing 20 μg ml^{-1} kanamycin for 12 h, harvested, and frozen at −80 °C. Cells were suspended in 20 mM Hepes-NaOH (pH 7.5) buffer containing 1 M NaCl. After sonication for disruption, the homogenate was centrifuged at 100,000*g* for 30 min at 4 °C. His-tagged fusion proteins were purified from the supernatant by nickel affinity column chromatography (HiTrap Chelating HP; Amersham Biosciences, Piscataway, NJ, USA). The column was loaded with the supernatant, washed extensively, and then eluted using 20 mM Hepes-NaOH (pH 7.5) buffer containing 1 M NaCl and 500 mM imidazole. For spectral measurement, the purified proteins were aged for more than two days at 4 °C to avoid light-induced slow aggregation.[7] Ultraviolet and visible absorption spectra were recorded with a spectrophotometer (model UV-2400 PC; Shimadzu, Kyoto, Japan). Flavin was excited with a blue LED (500 μmol m^{-2} s^{-1}) for continuous illumination. Samples were incubated in the dark for at least 2 min for dark-adapted spectra. For light-excited spectra, samples were pre-illuminated for a few seconds and absorption spectra were recorded during the further illumination. The spectral measurements were done at room temperature. (2)Time course of photo-bleaching (a) and dark reversion (b) of Y8A, Y8F and Q50N mutants, as monitored at 450 nm. Light illumination was done with a blue laser.[20] In the dark reversion experiments, proteins were illuminated for 2 min and then incubated in the dark. (3) Transient absorption difference spectra of TePixD mutated proteins. The data were collected at 50 ns, 100 μs and 10 ms after the flash for wild-type and at 50 ns and 100 μs after the flash for mutants. Transient absorption spectra after 10 ns laser excitation were measured with a home built spectrophotometer.[20]

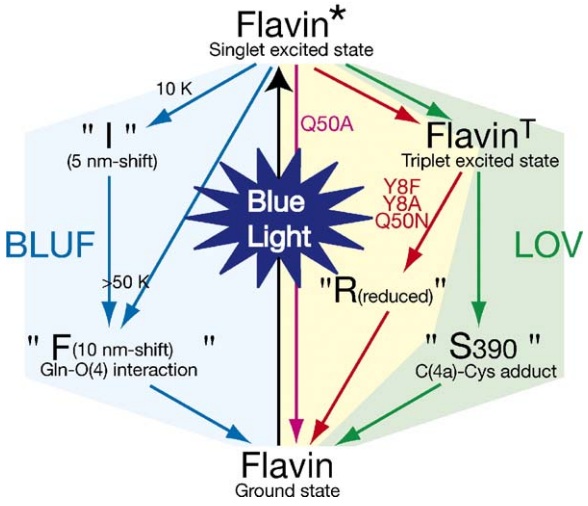


Figure 3. The reaction scheme of flavin blue light receptors. A singlet excited state of flavin was formed by blue-light absorption in both BLUF (wild-type and mutant proteins) and LOV domains. In the wild-type (blue arrows), the excited flavin was converted to the 10 nm red shift form (F form). At 10 K, the excited flavin was converted to a 5 nm shifted intermediate.[20] In the Q50A mutant (pink arrow), the excited flavin rapidly returned to the ground state. In Y8F, Y8A, and Q50N mutant proteins (red arrows), the triplet state of flavin was formed and then converted to a reduced form (R form) at low yield. In the LOV domain (green arrows), the singlet excited flavin turned to the triplet state and then formed the Cys adduct.

O(2) with Asn, O(4) with Asn and O(4)/N(5) with Gln residues (Figure 1).[24,25] The flavin in the LOV domain forms a triplet state and subsequently the adduct with a conserved Cys residue.[19] The photocycle is quite different from that of the BLUF domain. In the BLUF domain of TePixD, Gln50 interacts with the O(4) and N(5) atoms of the ring. Upon illumination, a strong hydrogen bond was formed between Gln50 and the O(4) with no formation of a triplet state. The results here indicate that the mutants of Tyr8 that strongly interact with Gln50, show a triplet state of flavin as reported in the LOV domain (Figure 2). It is known that the N(5) of the isoalloxazine ring is one of the most reactive sites in the flavin.[18,26] The interaction of the side-chain of Gln with N(5), therefore, seems to determine the fate of a singlet excited state of flavin either to form a triplet state (LOV-type reaction) or to modify the hydrogen-bonding of Gln50 to O(4) (BLUF-type reaction) as shown schematically in Figure 3. The critical differences between LOV domain proteins and PixD mutant proteins of Y8F, Y8A and Q50N is an adduct formation between Cys residues (Cys57 in Phot-LOV1 of *Chlamydomonas reinhardtii*) and C(4a) of the ring from the triplet state. There is no such Cys residue in BLUF proteins. The site-directed mutagenesis to introduce a Cys residue at conserved residue Ile66, which is located just above the C(4a) of the ring in TePixD, demonstrated the formation of Cys-flavin adduct (H. S., K. O., M. I. and T. N., unpublished data).

3D structures of the BLUF domains that have been determined so far for TePixD, AppA and BlrB are only in the dark adapted form.[15,27,28] The full-length TePixD and another short BLUF protein, BlrB, exhibit very similar structure with interactions of Gln (Gln50 of TePixD and Gln51 of BlrB) with O(4) and N(5) of the flavin ring. The 3D structure of the C-terminally truncated AppA was determined by X-ray crystallography and NMR.[29] The structure of truncated AppA is similar to that of TePixD/BlrB except that the C=O of the amide side-chain of Gln63 was placed near the O(4) and N(5) of the ring in the dark, eliminating the possibility of their hydrogen bonding. The side-chain of Gln63 was proposed to rotate to form a hydrogen bond between the amide NH_2 of Gln63 and O(4)/N(5) upon light excitation. However, the current resolution (2.0 Å for TePixD, 1.9 Å for BlrB, 2.3 Å for AppA) is not sufficient to distinguish the N atom from the O atom of the Gln side-chain. To

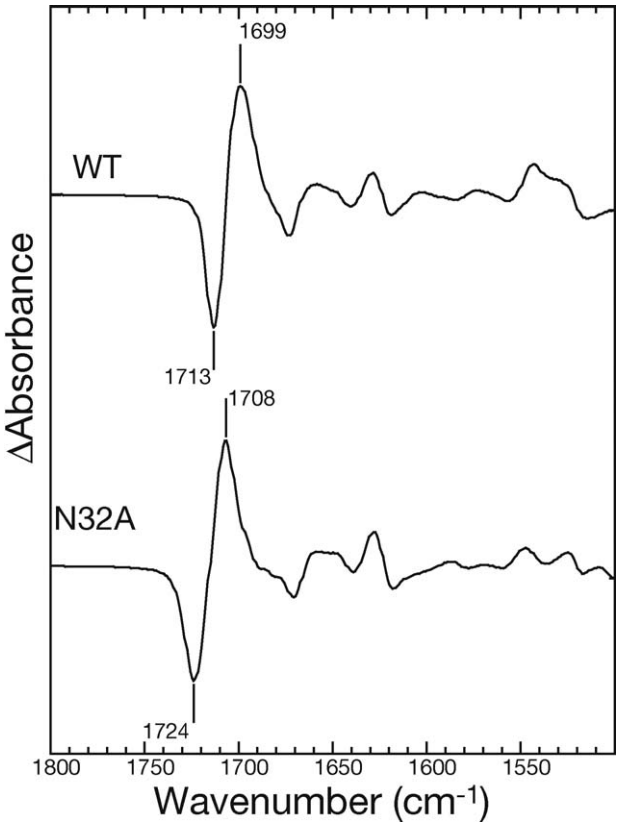


Figure 4. Light-minus-dark FT-IR difference spectra of wild-type and N32A mutant. FT-IR spectra were measured using a Bruker IFS-66/S spectrophotometer equipped with an MCT detector (InfraRed D316/8) at 4 cm^{-1} resolution. The sample was applied on a CaF_2 plate, and gently dried by N_2 gas and then hydrated by humidity control using a 40% glycerol/water solution placed in an IR cell without touching the sample. The sample temperature was adjusted to 10 °C by circulating cold water in a copper holder. The light-minus-dark difference spectrum was obtained by recording single-beam spectra for 10 s (20 scans) before and after white-light illumination for 25 s. The measurements were repeated 12 times at intervals of 5 min between illumination and the spectra were averaged.

clarify the situation, we are now studying the light-induced changes in the hydrogen bond between Gln50 and Tyr8 by FT-IR spectroscopy using TePixD that is labeled with a [4-^{13}C]Tyr isotope.[30]

Residues that directly interact with Gln50 would be important not only for the regulation of the flavin photocycle but also for signal transduction from the flavin to an output domain. In this context, there is another conflict between PixD/BlrB and AppA. Namely, next to the conserved Gln residue a conserved Met residue (Met93 for TePixD and Met94 for BlrB) is found in the former (Figure 1), while a conserved Trp residue is found in the latter. This is very unusual, since both side-chains of the corresponding Trp in PixD/BlrB (Trp91 for TePixD and Trp92 for BlrB) protrude outwards of the flavin pocket of BLUF. A recent FT-IR and site-directed mutagenesis study of the truncated AppA suggested that Trp moves during the photocycle and is somehow important for dark reversion from the red-shifted flavin absorption.[31] On the other hand, we are studying a role of Met93 in SyPixD. In *Synechocystis*, we previously reported that SyPixD interacts with a bacterial signal transduction response regulator SyPixE by yeast two-hybrid screening.[7] We recently found that Met93 is dispensable for the red-shifting photocycle of the flavin but is essential for the light-induced regulation of the protein–protein interaction with SyPixE (K. O. and M. I., unpublished results).

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