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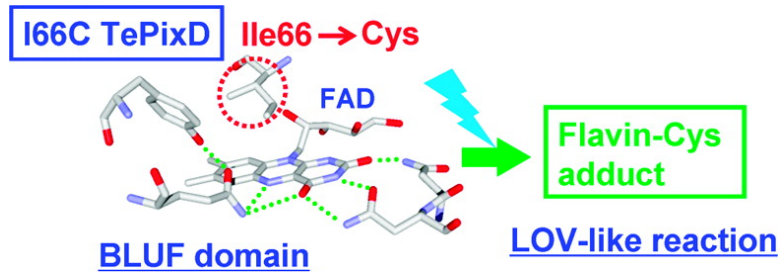


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# LOV-Like Flavin-Cys Adduct Formation by Introducing a Cys Residue in the BLUF Domain of TePixD

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Blue-light sensor proteins using flavin derivatives are involved in a wide range of organisms and regulate various photoresponses such as phototropism, stomatal opening, and photophobic movement.[1] The photoreceptor domains in these proteins are classified into three distinct families, i.e., PHR (photolyase homology region), LOV (light, oxygen, and voltage), and BLUF (blue light sensing using flavin adenine dinucleotide (FAD)) domains typically found in cryptochromes, phototropins, and PixD (positive phototaxis factor D), respecitvely.[1] They have no relationship at the sequence level, and the primary photochemistry of flavin is also different. The PHR domain performs the redox reactions of FAD, while the LOV domain forms a covalent adduct between the $C_{4a}$ of flavin mononucleotide (FMN) and the $S_\gamma$ atom of a Cys residue. The latter reaction has been revealed by a negative S−H band in Fourier transform infrared (FTIR) difference spectra upon formation of an intermediate S390[2] and its X-ray crystal structure.[3] In contrast, the photoreaction of the BLUF domain is characterized by H-bond rearrangement around the $C_4$═O of FAD, which induces ∼10-nm red shifts of visible absorption bands and a ∼15-$cm^{-1}$ downshift of the $C_4$═O band in FTIR spectra.[4] It has been shown that the Tyr-Gln-FAD H-bond network plays a crucial role in the BLUF photoreaction by various mutagenesis and spectroscopic studies with PixD and AppA.[4−6]

Such differences in the primary photochemistry between different domains naturally raise the following questions. What is the factor to determine the reaction type of flavin? Is the reaction controlled by the immediate interactions of the flavin with surrounding amino acids or by specific reaction fields produced by the distinct domain structures? To answer these questions, in this study we have modified the BLUF domain of PixD from *Thermosynechococcus elongatus* (TePixD) by site-directed mutagenesis to incorporate a Cys residue mimicking the LOV domain. When the X-ray structures of the BLUF[6,7] and LOV[3] domains were arranged to overlap the flavin isoalloxazine rings (Figure 1), Ile66 of TePixD is found at the position of the reactive Cys residue in the LOV domain. The distance between the $C_{\gamma1}$ atom of Ile66 and the $C_{4a}$ of flavin is 3.99 Å,[6] which is similar to the distance of 4.54 Å between the $S_\gamma$ atom of Cys450 and the $C_{4a}$ in the oat LOV2.[3b] Thus, we have replaced Ile66 with Cys and analyzed the photoreaction of the I66C mutant using light-induced FTIR difference spectroscopy.

Figure 2Aa shows a light-induced FTIR difference spectrum of the WT TePixD in the 1800−1200 $cm^{-1}$ region (Materials and Methods are described in the Supporting Information). Negative and positive peaks belong to the dark and light states, respectively. The most prominent and characteristic feature of the BLUF spectra is a downshift of the $C_4$═O peak from 1713 to 1699 $cm^{-1}$, which reflects the strengthened H-bond interaction at the $C_4$═O. The spectrum of the I66C TePixD (Figure 2Ab) showed significantly different features. The $C_4$═O peak at 1713 $cm^{-1}$ upshifted by 15 $cm^{-1}$ to 1728 $cm^{-1}$ contrary to WT. Also, a prominent signal appeared at 1663/1643 $cm^{-1}$ in the amide I region, representing a significant perturbation of the polypeptide chains. Another prominent signal at 1551/1534 $cm^{-1}$ and medium intensity peaks at 1581, 1449, 1418, 1374, 1351, 1313, and 1258 $cm^{-1}$

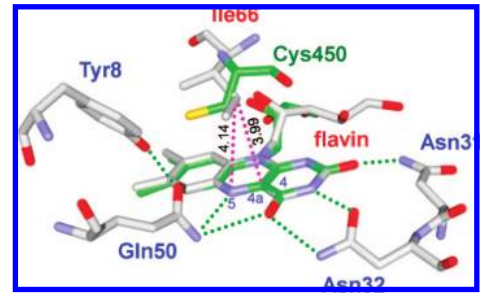


***Figure 1.*** X-ray crystallographic structure of the flavin binding site of the BLUF domain of TePixD[6] (gray sticks). The positions of the reactive Cys450 residue (in a major conformation) and the flavin in the oat LOV2 domain[3b] are also shown (green sticks) so as to overlap the isoalloxazine rings in the two domains.

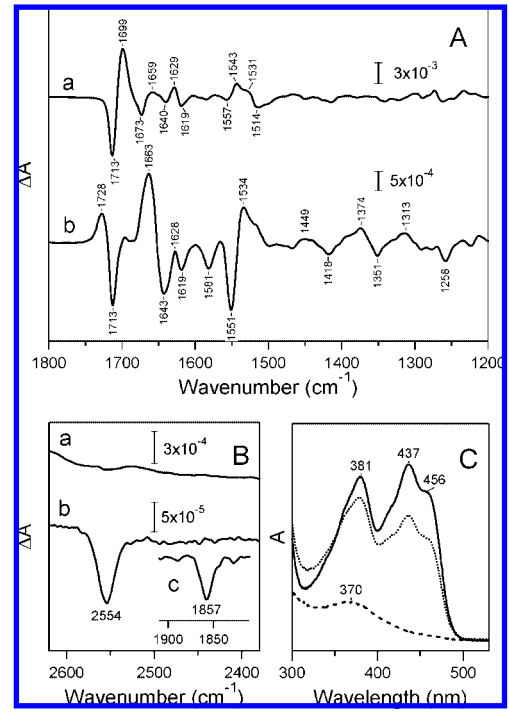


***Figure 2.*** (A) Light-induced FTIR difference spectra of WT (a) and I66C (b) TePixD in the 1800−1200 $cm^{-1}$ region. (B) The S−H stretching region of the FTIR difference spectra of WT (a) and I66C (b) TePixD, and the S-D region of I66C TePixD in a $D_2O$ buffer (c). (C) UV−vis absorption spectra of I66C TePixD before (solid line) and after (dotted line) illumination. The spectrum of a photoinduced intermediate (dashed line) was obtained by subtracting the spectrum before illumination from that after illumination with an appropriate factor.

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appeared. The relatively large intensities of these signals at 1600−1200 $cm^{-1}$, which probably arise from flavin vibrations,[2b,8,9] in contrast to the weak signals in this region of the WT spectrum suggest that drastic structural changes took place in the isoalloxazine ring in this mutant. These spectral features of the I66C TePixD are very similar to those of the LOV domains.[2b,8,9] For example, the LOV spectra showed the upshift of the $C_4$═O peak at ∼1712 $cm^{-1}$ by ∼15 $cm^{-1}$ and prominent signals assignable to the $C_{10a}$═$N_1$ stretch[9] at ∼1553/∼1538 $cm^{-1}$. In addition, medium intensity peaks corresponding to the above I66C peaks were detected at similar positions in the 1600−1200 $cm^{-1}$ region.

The reaction of the Cys side chain replacing Ile66 can be examined using a S−H stretching band in the 2600−2520 $cm^{-1}$ region, where no other vibrations from proteins and FAD overlap. Although the WT TePixD did not show bands in this region (Figure 2Ba), the I66C mutant exhibited a clear negative peak at 2554 $cm^{-1}$ without a positive counterpeak in the same region (Figure 2Bb). This negative peak is very similar to the S−H peak at 2572−2566 $cm^{-1}$ observed in the FTIR difference spectra of the LOV domains due to Cys adduct formation.[2] In a $D_2O$ buffer, the band at 2554 $cm^{-1}$ largely downshifted to 1857 $cm^{-1}$ (Figure 2Bc), analogously again to the deuteration shift of the S−H band to 1867 $cm^{-1}$ in the LOV spectra.[2b] Thus, it can be concluded that the band at 2554 $cm^{-1}$ arises from the S−H stretch of the Cys66 newly incorporated in the BLUF domain of TePixD.

The appearance of this negative S−H peak together with the close spectral similarity at 1800−1200 $cm^{-1}$ to the LOV spectra strongly suggests that the Cys66 in the I66C TePixD forms a Cys adduct with the $C_{4a}$ of FAD upon illumination. UV−vis spectra of the I66C TePixD showed that the FAD absorption is bleached upon illumination (Figure 2C, dotted line) concomitant with the formation of an intermediate with a broadband centered at 370 nm (Figure 2C, dashed line) with a comparable intensity as that for the decrease at 437 nm. This photoinduced intermediate probably corresponds to S390 of LOV domains; the band center blue-shifted by 20 nm is consistent with the observation that the flavin peaks of TePixD (0−1 peak: 437 nm in I66C and 439 nm in WT) are found at ∼20 nm bluer positions than those of LOV domains. This result strongly supports the view that a Cys-adduct intermediate similar to S390 is formed in the I66C TePixD. The presence of a large differential signal at 1663/1643 $cm^{-1}$ (Figure 2Ab), which is ascribable to the amide I bands of α-helical chains, is also consistent with the Cys-adduct formation, because Ile66 in TePixD (thus Cys66 in the mutant) is located on the α2-helix region,[6] which should experience a significant perturbation upon adduct formation. The quantum yield of the intermediate formation in I66C was estimated to be ∼3% that is much lower than ∼29%[4e] of WT, and the lifetime was ∼40 s, slightly shorter than that of WT (∼70 s), in a hydrated film.

It is known that the Cys adduct in the LOV domain is formed via the excited triplet state of flavin in ∼4 μs,[10] while the red-shifted light state of the BLUF domain is formed on a subnanosecond time scale via the excited singlet state.[5b,11] The absence of the photoinduced downshift of the $C_4$═O peak (Figure 2Aa) and of the red-shift of visible absorption (Figure 2C) indicates that the H-bond rearrangement around the $C_4$═O typical of a BLUF reaction was completely inhibited in the I66C mutant and the Cys adduct is formed via the triplet state of FAD. This is similar to the previous observations of photoreduction of FAD via its triplet state in Y8F, Y8A, and Q50N TePixD.[4d] The S−H frequency of 2554 $cm^{-1}$ in I66C TePixD (Figure 2Bb) is ∼15 $cm^{-1}$ lower than that in LOV domains,[2] indicating that the S−H of Cys66 in the BLUF domain forms a relatively strong H-bond. It is possible that the H-bond is formed with the C═O of Gln50, causing inhibition of the normal BLUF reaction by interferring with the Tyr8-Gln50-FAD H-bonded network. H-bonding to the Tyr8 OH is unlikely because the I66C/Y8F double mutant also formed a Cys adduct and showed a S−H band at an even lower frequency of 2541 $cm^{-1}$ (Figure S1). A slightly broader feature of the S−H(D) band on a higher-frequency side (Figure 2Bb,c) could indicate the presence of a reactive conformation having a S−H weakly H-bonded with the flavin $N_5$. Such multiple conformations of the S−H interactions have been revealed also in the LOV domains.[2c,3b]

In conclusion, we have reproduced the LOV-like photoreaction, i.e., flavin-Cys adduct formation, in the BLUF domain by introducing a Cys residue. This result indicates that different types of flavin photoreactions can be realized using the same domain if key amino acid residues are appropriately arranged around the flavin. Thus, the domain structure itself is not a crucial factor to determine the photoreaction type, and hence it may play a role in signal transduction processes rather than providing a specific reaction field. These results will provide an important aspect in protein engineering to design new functional proteins using natural domain structures.

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**Supporting Information Available:** Materials and Methods, and the FTIR difference spectrum of I66C/Y8F TePixD. This material is available free of charge via the Internet at http://pubs.acs.org.

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