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# Orientation of a Key Glutamine Residue in the BLUF Domain from AppA Revealed by Mutagenesis, Spectroscopy, and Quantum Chemical Calculations

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In many biological photoreceptors, such as rhodopsins, phytochromes, photoactive yellow protein, and phototropins, gross structural changes are photoinduced in the chromophore molecule, which triggers a series of protein structural changes that ultimately lead to a formation of a signaling state.[1] In contrast, flavin-containing BLUF (blue-light using flavin−adenine−dinucleotide (FAD))[2] proteins show a different photoactivation mechanism that is not accompanied with the prominent structural changes of the chromophore molecule.[3−5] In BLUF proteins, the signaling state is characterized by light-induced ∼10 nm red shift of the UV−visible absorption spectrum[3,6−11] and ∼16 cm$^{-1}$ downshift of the C4═O stretching mode of the flavin ring.[3−5] However, the photochemistry and structural dynamics underlying the signaling state formation are largely unknown.

To date, six BLUF proteins have been characterized, which are AppA[6,12] and BlrB[7] from *Rhodobacter sphaeroides*, PAC[13] from *Euglena gracilis*, Slr1694[3] from *Synechocystis* sp. PCC6803, YcgF[9] from *Escherichia coli*, and Tll0078[10] from *Thermosynechococcus elongatus* BP-1. AppA is the representative of BLUF proteins and regulates photosynthesis gene expression in a blue-light-dependent manner.[6] The crystal structure of the BLUF domain of AppA indicates that the flavin ring is involved in a hydrogen bond network with highly conserved residues, such as Tyr21, Asn45, and Gln63.[14] On the basis of the crystal structure as well as FTIR and Raman spectroscopic data,[3−5] Anderson et al.[14] proposed that the amine group of Gln63 forms a hydrogen bond with the flavin N5 atom in the dark state (Figure 1A), and this residue rotates by ∼180° to form a new hydrogen bond with the flavin C4═O group upon illumination (Figure 1B). Consistently, the replacement of the corresponding glutamine residue with another amino acid results in the lack of the light-induced red shift in Tll0078[15] and PAC.[8] However, reported crystal structures of Tll0078[15] and BlrB[7] showed that the glutamine residue arranges in a manner that corresponds to the signaling state structure of AppA to form a hydrogen bond with the C4═O oxygen in the dark state (Figure 1B). Therefore, it is crucial to determine the state of the hydrogen bonding between the side chain of Gln63 and the flavin ring in solution for understanding the mechanism of the signaling state formation. For this purpose, we characterize the effects of Gln63 → Leu (Q63L) mutation, which removes a putative hydrogen bond between the flavin ring and the Gln63 side chain, on Raman and UV−visible absorption spectra in the BLUF domain of AppA (AppA126). Furthermore, the observed spectral changes are theoretically evaluated by quantum chemical calculations based on density functional theory (DFT). The results demonstrate the Gln63-to-N5 hydrogen bond in the dark state (Figure 1A). This finding is compatible with the idea that the light-induced reorientation of the Gln63 side chain forms a new hydrogen bond to the C4═O position in the signaling state (Figure 1B).

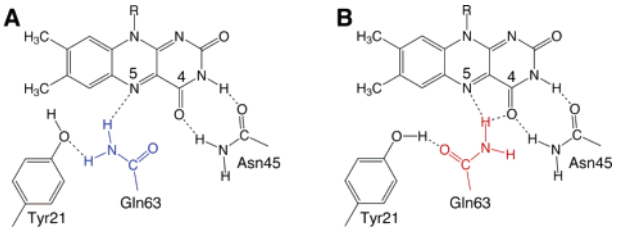


***Figure 1.*** Possible structures of the active site of the AppA BLUF domain in the dark (A) and signaling (B) states based on ref 14.

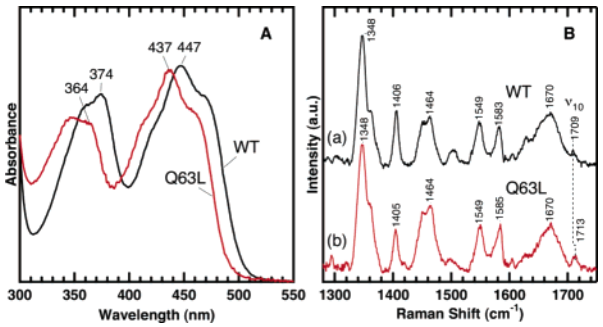


***Figure 2.*** Effects of the Q63L mutation on (A) UV−visible absorption and (B) Raman spectra of AppA126 in 50 mM Tris-HCl, 1 mM NaCl at pH 8.0.

Figure 2A displays the UV−visible absorption spectra of AppA126 (see Supporting Information for materials and methods). The Q63L mutation causes a ∼10 nm blue shift of the flavin absorption bands from 447 and 374 nm to 364 and 437 nm. Notably, the mutant shows no absorption red shift upon illumination as reported in the corresponding mutants in Tll0078[15] and PAC[8] (Figure S1; see Supporting Information for supplemental results and discussion), confirming a critical role of this glutamine residue in AppA. Figure 2B depicts the Raman spectra of the dark-adapted WT AppA126 and the Q63L mutant at 647.1 nm excitation. Most of the observed bands in the WT spectrum (trace a) have been assigned to vibrational modes for the FAD chromophore, and a shoulder at 1709 cm$^{-1}$ on a broad amide I band around 1670 cm$^{-1}$ can be assigned to the carbonyl C4═O stretching vibration $\nu_{10}$ of the isoalloxazine ring.[5] The spectrum of the mutant (trace b) is very similar to that of WT AppA126, and the $\nu_{10}$ frequency was slightly upshifted by 4 cm$^{-1}$. The small upshift (∼5 cm$^{-1}$) was similarly observed in $D_2O$ buffer (Figure S2). This small upshift

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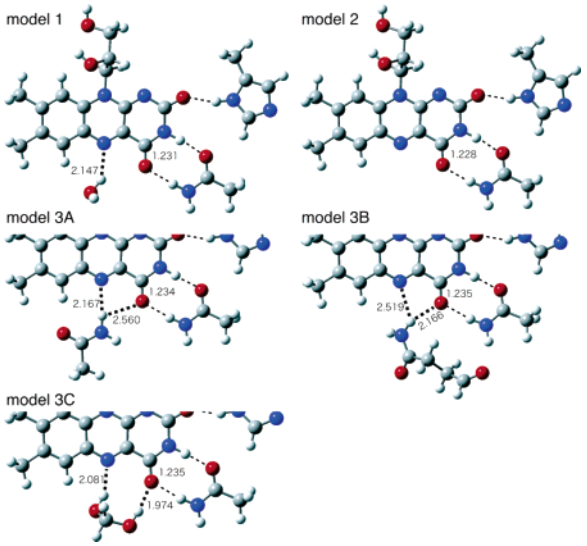


***Figure 3.*** Optimized structures for the active site models of AppA126. Distances (Å) for the important hydrogen bond as well as the C4═O bond are displayed in the figure. The upper parts of models 3A−C are not shown.

suggests that the state of hydrogen bonding at the C4═O position is largely unchanged in the mutant because the C═O stretching frequency is expected to be upshifted by approximately 30 $cm^{-1}$ if a hydrogen bond from the carbonyl oxygen was removed.[16,17] Therefore, the present Raman result strongly indicates no hydrogen bond between Gln63 and the flavin C4═O group in the dark state, being compatible with the structure A in Figure 1.

To evaluate this idea quantitatively, we have performed DFT calculations using Gaussian03.[18] We used the B3LYP functional with the 6-31G** basis set because of its high accuracy. For instance, the calculated frequencies usually deviate less than 10% from the experimental values.[19] For these calculations, 7,8-dimethyl-10-glycerylisoalloxazine (Figure 3) was employed as a chromophore model. In addition, water, acetamide, and 4-methylimidazole were included to reproduce hydrogen bonds with the active site residues of Gln63, Asn45, and His44 (see Figure 1A). The components were arranged on the basis of the crystal structure of AppA[14] and subsequently optimized to yield the structures illustrated in Figure 3 (model 1). Model 2 mimics the active site of the Q63L mutant, in which a water molecule was eliminated from model 1 to consider the removal of the hydrogen bond between Gln63 and the flavin ring. For the structure shown in Figure 1B, we used three optimized structures denoted as models 3A−C. These involve two hydrogen bonds at the N5 and C4═O positions with different distances. In models 3A and 3B, acetamide and 4-oxobutanamide mainly form a hydrogen bond with the N5 and the C4═O positions, respectively. On the other hand, methanediol in model 3C makes two hydrogen bonds with relatively short distances.

Next, we have calculated the C4═O stretching frequency, $\nu_{10}$, and UV−visible absorption maxima, $\lambda_{max}$, using the above structures. As can be seen in Table 1, a change from model 1 to 2 best explains the effects of the Q63L mutation, that is, the removal of the hydrogen bond at the N5 atom causes a shift of $\nu_{10}$ by +6 $cm^{-1}$ as well as blue shifts of $\lambda_{max}$ by 11 nm. In contrast, the other changes of model $3x \rightarrow 2$ ($x$ = A, B, and C) failed to reproduce the frequency shift and absorption shifts at the same time, indicating that model 1 is the most plausible structure of the dark state of AppA.

It is of note that the coupled C═N5/C−C stretching modes[5] of $\nu_{13}$ (∼1580 $cm^{-1}$) and $\nu_{14}/\nu_{15}$ (∼1550 $cm^{-1}$) may be sensitive to the hydrogen bond at the N5 atom. However, because these modes

***Table 1.*** Calculated Vibrational Frequency ($cm^{-1}$) and Absorption Maxima (nm) of the Active Site Models for BLUF Proteins[a]

| | model 1 | model 2 | model 3A | model 3B | model 3C |
|---|---|---|---|---|---|
| $\nu_{10}$ | 1704(6) | 1710 | 1699(11) | 1691(19) | 1691(19) |
| $\lambda_{max}(S_0 \rightarrow S_1)$ | 426(−11) | 415 | 432(−17) | 428(−13) | 437(−22) |
| $\lambda_{max}(S_0 \rightarrow S_2)$ | 344(−11) | 333 | 350(−17) | 340(−7) | 349(−16) |

[a] The numbers in the parentheses are the shifts of model 2 − model 1 or $3x$ ($x$ = A, B, or C). For the frequency calculations, the carbonyl C═O group other than the C4═O and the flavin C2 atom were labeled with $^{13}C$ or $^{18}O$ to drop their corresponding frequencies, thereby removing the interaction among the C═O stretching modes.

are delocalized over the flavin ring, the calculated shifts for model $1 \rightarrow 2$ are very small (Table S1) as experimentally observed (Figures 2 and S2). We also note that the models 3A−C are likely structures for the signaling state of AppA shown in Figure 1B, and the details will be discussed elsewhere.

In summary, the results reported here demonstrate the absence of a hydrogen bond between Gln63 and the flavin C4═O in the dark state AppA in solution. This is consistent with the proposal[14] that the Gln63 side chain rotates to make a new hydrogen bond with the C4═O position in the signaling state.

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**Supporting Information Available:** Materials and methods, supplemental results and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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