

A Q63E *Rhodobacter sphaeroides* AppA BLUF Domain Mutant Is Locked in a Pseudo-Light-Excited Signaling State[†]

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ABSTRACT: The AppA BLUF photoreceptor from *Rhodobacter sphaeroides* contains a conserved key residue, Gln63, that is thought to undergo a shift in hydrogen-bonding interactions when a bound flavin is light excited. In this study we have characterized two substitution mutants of Gln63 (Q63E, Q63L) in the context of two constructs of the BLUF domain that have differing lengths, AppA1–126 and AppA17–133. Q63L mutations in both constructs exhibit a blue-shifted flavin absorption spectrum as well as a loss of the photocycle. Altered fluorescence emission and fluorescence quenching of the Q63L mutant indicate significant perturbations of hydrogen bonding to the flavin and surrounding amino acids which is confirmed by ¹H–¹⁵N HSQC NMR spectroscopy. The Q63E substitution mutant is constitutively locked in a lit signaling state as evidenced by a permanent 3 nm red shift of the flavin absorption, quenching of flavin fluorescence emission, analysis of ¹H–¹⁵N HSQC spectra, and the inability of full-length AppA Q63E to bind to the PpsR repressor. The significance of these findings on the mechanism of light-induced output signaling is discussed.

AppA is a member of the “blue-light using FAD” (BLUF)¹ class of photoreceptors that is widely distributed among prokaryotes (1, 2). BLUF photoreceptors were initially identified based on their involvement in regulating well-known light-driven events such as phototaxis in cyanobacteria and algae (2–6) or, in case of AppA, the transcriptional control of genes required for photosynthesis (7, 8). However, there are many nonphotosynthetic species that contain BLUF photoreceptors of unknown function with well over 100 examples of BLUF-containing proteins in genome databases. A BLUF domain connected to an EAL domain in YcgF from *Escherichia coli* has been rather extensively studied (5, 9–13).

X-ray crystallographic studies have demonstrated that the BLUF domain is comprised of a well-conserved $\beta\alpha\beta\beta\alpha\beta$ fold. The major structural differences among different BLUF structures are a slight variation in the length of the $\beta 5$ strand and the conformation of the loop that connects the $\beta 4$ and $\beta 5$ strands. NMR studies on light- and dark-adapted BLUF proteins also demonstrate that the $\beta 4$ – $\beta 5$ loop is dynamic and undergoes a light-dependent conformational change (14, 15). One controversial feature is the precise positions of the side chains of conserved tryptophan and methionine residues in the $\beta 5$ strand that are found in distinct orientations in various BLUF structures (17–19). The Andersen et al. (19) structure of an AppA BLUF domain comprised of residues 17–133 (AppA17–133) has Trp104 buried near the flavin and hydrogen bonded to Glu63 while Met106 is swung away from the flavin. This is in contrast to the crystal structure of an AppA BLUF domain containing residues 1–124 (AppA1–124 C20S mutant) by Jung et al. (17) where Trp104 is swung away from the flavin and solvent is exposed while Met106 is located near the flavin and within hydrogen-bonding distance to

Gln63. The different orientations of Trp104 and Met106 lead Andersen et al. (19) to propose a model where excitation of the flavin results in a hydrogen bond rearrangement between Gln63, the flavin, and Trp104. This model has been studied using FTIR analyses, theoretical calculations, and fast spectroscopy (2, 16, 20–24). In this model, the dark state BLUF domain is characterized by a hydrogen bond between Trp104 N ϵ 1 indole proton and Gln63, with Gln63 also donating a hydrogen bond to N5 of FAD. Light excitation of the flavin initiates fast electron and proton transfer from a conserved tyrosine (Y21 in AppA) to the flavin. This is followed by Gln63 undergoing an $\sim 180^\circ$ rotation around the C γ –C δ bond breaking the hydrogen bond to Trp104 allowing Trp104 to swing away from Gln63. Gln63 is then proposed to form a new hydrogen bond with the carboxamide protons donating hydrogen bonds to both N5 and C4=O of FAD, resulting in a pronounced spectral shift in the FAD absorption and fluorescence emission spectrum. Another variant of this model proposes that Met106 is hydrogen bonded to Gln63 in the dark state while Trp104 is bound to Gln63 in the lit state (17, 22). Finally, there is also a model based on theoretical thermodynamic calculations that proposes that flavin light excitation promotes Gln tautomerization that subsequently allows Gln63 rotation and exchange of Trp104 with Met106 (23). Yet another model proposes Met106 and Trp104 exchange following Gln63 tautomerization without its rotation (24).

Analysis of the structural environment of Trp104 in solution was recently undertaken by Dragnea et al. (25) in the dark and lit states using a combination of NMR spectroscopy and absorption and steady-state fluorescence spectroscopy, as well as collisional quenching of tryptophan fluorescence. These results indicated that Trp104 is indeed buried and likely hydrogen bonded to Gln63 in the AppA17–133 domain as described by the crystal structure of Anderson et al. (19) and that an AppA domain comprised of residues 1–126 (AppA1–126) has Trp104 far from Q63 as shown in the structure by Jung et al. (17). Furthermore, it

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¹Abbreviations: BLUF, blue-light sensing using FAD; DTT, dithiothreitol.

was also demonstrated that Trp104 does not appreciably move when the flavin is light excited and that full-length wild-type AppA has Trp104 in a conformation far from Q63 as indicated by the structure of Jung et al. (17). These new findings call into question the relevance of the various models that invoke movement of Trp104 and therefore require a reassessment of the role of Gln63 in controlling a light-generated output signal in AppA.

In this study, we characterize Gln63 mutants in the context of both AppA17–133 and AppA1–126 to assess the role of Gln63 in controlling the characteristic light-mediated BLUF spectral shift and output signal. Using a combination of NMR, absorption, and fluorescence spectroscopies, we show that a Q63L mutant exhibits a permanent blue-shifted spectrum and that the structure takes on a non-native conformation independent of light conditions. This is in dramatic contrast to a Q63E mutant that is both spectrally and conformationally locked in a light-excited state. These results provide further evidence that Gln63 indeed has a critical role in conferring both the spectral properties and output signals of BLUF photoreceptors.

METHODS

Mutant Construction and Protein Purification. Q63L and Q63E mutations were introduced into genes encoding AppA1–126 (plasmid pTY-AppA126) and AppA17–133 (plasmid pTY-AppA17–133), as well as in the AppA full-length protein (pTY-AppA) using a QuickChange kit (Stratagene), appropriate primers, and their complements. Carrying a chitin binding domain as a tag, all proteins were purified using Chitin beads (New England Biolabs). The chitin tag was removed during 50 mM DTT incubation on Chitin beads overnight, and proteins were further purified by size-exclusion chromatography on Superose 12 using the AKTA FPLC system in 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl.

Visible Spectral Analysis. Absorption spectra were recorded on a Beckman DU-640 spectrophotometer using a 1 cm path quartz cuvette. Fluorescence emission spectra from 310 to 570 nm were recorded on a PerkinElmer LS50B spectrofluorometer using dilute samples to avoid self-absorption ($A_{280} = 0.02$) that were excited at 295 nm. Fluorescence quenching experiments with acrylamide as a quencher were recorded on the same dilute samples as above with excitation at 295 nm and single point emission reading at 360 nm. With temperature controlled at 15 °C, 2 mL samples were stirred and quenched by the additions of 8 M acrylamide added at regular intervals up to 250 μ L (0.88 M acrylamide). For the light-excited protein, the strong white light was applied to the sample for ~ 30 s, and the fluorescence signal was read immediately following irradiation.

For proteins with homogeneous fluorophore, the fluorescence quenching by acrylamide can be described by Stern–Volmer relationship (26):

$$F_0/F = (1 + K_{sv}[Q])e^{V[Q]}$$

in which F_0 is the fluorescence intensity in the absence of quencher and F is the fluorescence intensity in the presence of various concentrations of quencher $[Q]$. K_{sv} and V are Stern–Volmer quenching constants used to describe dynamic and static quenching, respectively. The correction for sample dilution and acrylamide absorption was taken into account in the fit. Each quenching curve was measured at least three times for reproducibility.

NMR Chemical Shift Perturbation Experiments. All NMR spectra were recorded on a Varian Inova 500 MHz spectrometer equipped with an inverse probe at 35 °C. ^1H – ^{15}N HSQC spectra were recorded for WT, Q63E, and Q63L AppA1–126 and AppA17–133 with 160×512 t_1 and t_2 data points, respectively. All experiments were performed at ambient low light conditions, in which AppA WT does not get light-excited. The backbone assignments for WT AppA were obtained from previously published spectra (14). The majority of the resonance assignments ($>95\%$) for the Q63E mutant were obtained by inspection and comparison with spectra obtained for the analogous WT protein. Q63L mutants exhibited large chemical shifts when compared to AppA WT or Q63E, which prevented peak assignment.

Size-Exclusion Chromatography and Characterization of AppA WT or AppA Q63 Mutant Complexes with PpsR. PpsR was kindly purified and provided by Liang Yin in our laboratory. AppA WT, AppA Q63E, or AppA Q63L full-length proteins were eluted from chitin beads after overnight incubation with 50 mM DTT and mixed immediately in $10\times$ molar excess with PpsR. The mixture was incubated in the dark for at least 30 min prior to loading on a Sephacryl 200 size exclusion column (1.6×80 cm) connected to an AKTA FPLC filtration system. Chromatography was performed in the dark in a Tris-HCl buffer, pH 8.0, 400 mM NaCl, and 5 mM DTT, and eluted complexes were characterized by SDS–PAGE.

RESULTS

Spectroscopic Analysis of Q63E and Q63L AppA Mutants. We probed the involvement of Gln63 in the photocycle by constructing Glu and Leu substitutions of Gln63 (Q63E and Q63L, respectively) in two different structural constructs, AppA1–126 and AppA17–133. Leu occupies an occluded volume that is similar to that of Gln but is unable to donate or accept hydrogen bonds; Glu, on the other hand, is capable of hydrogen bonding but introduces an ionizable group in place of the neutral carboxamide functionality. Two AppA BLUF domain constructs were used as they have different hydrogen bonds to Gln63 (25). In AppA1–126 Met106 is hydrogen bonded to Gln63, and in AppA17–133 Trp104 is hydrogen bonded to Gln63 (17, 19, 25). Interestingly, these two BLUF domains exhibit photocycles that are similar to that of full-length AppA with each having an identical dark state spectrum and each showing a similar light-induced ~ 12 nm red shift of the flavin absorption spectrum and similar decay rates to the ground state (25) (Figure 1A).

Spectral analysis of the Q63E and Q63L substitution mutants in AppA1–126 and AppA17–133 reveals that each lacks a photocycle. In addition, these mutants exhibit distinct absorbance maxima that are not influenced by the nature of the parent construct. Overall, there is a locked 12.5 nm red shift in the flavin absorption maximum of the Q63E mutant ($\lambda_{\text{max}} = 449.5$ nm) relative to the Q63L mutant ($\lambda_{\text{max}} = 437$ nm) which also exhibits a locked spectrum (Table 1, Figure 1B). This is in comparison to wild-type AppA1–126 and AppA17–133 that are characterized by a dark state maximum of 446 nm and a light-excited maximum of 458 nm. Thus, substitution of Q63 with Leu results in a locked flavin spectrum that is blue shifted 9 nm relative to the dark spectrum of the wild-type AppA. In contrast, substitution of Q63 with Glu, which retains the ability to form hydrogen bonds to both N5 and C4=O of the flavin (as well as to other residues), results in a flavin absorption spectrum that is permanently shifted to a position between that of the dark- and light-adapted wild-type

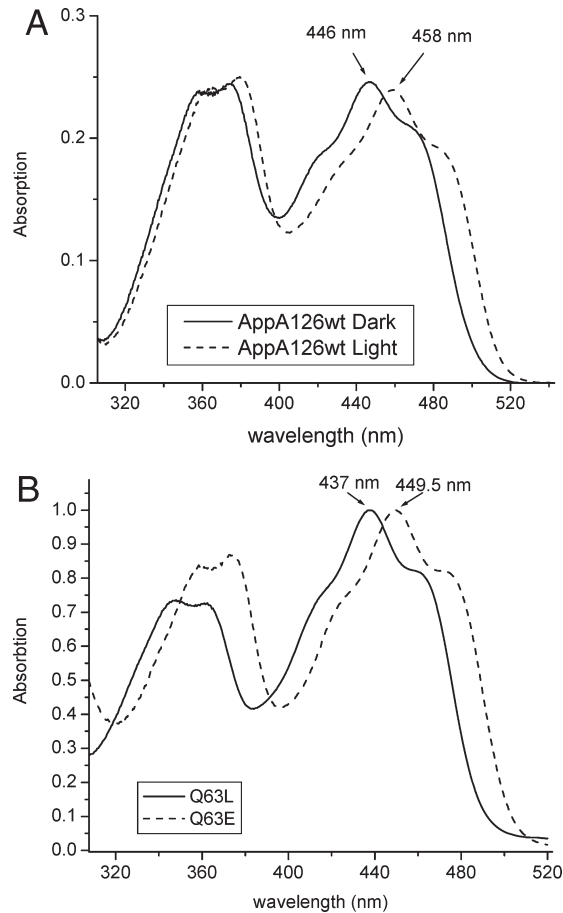


FIGURE 1: Absorption spectra of AppA1–126 WT at dark and light (A) and Q63E (B, dashed line) and Q63L mutants (B, solid line).

Table 1: Absorption Maxima and Photocycle Lifetimes of AppA and Its Various Clones and Mutants

mutant	flavin abs max (nm), dark (light) ± 0.5 nm	photocycle τ (s)
WT 126	446 (458)	948.5 ± 6.0
17–133	446 (458)	690.9 ± 3.1
Q63L 126	437	
Q63E 126	449.5	
Q63L 133	437	
Q63E 133	449.5	

AppA spectra. These spectral studies confirm that Q63 has a major role in the light-induced photocycle red shift, a finding consistent with models of the photocycle that invoke a change in the hydrogen bonding of the flavin to Gln63.

We next compared the fluorescence emission spectra of wild type, and the Q63E and Q63L mutants, in the AppA1–126 and AppA17–133 BLUF domains upon excitation of Trp at 295 nm. Wild-type AppA17–133 and AppA1–126 both exhibit higher flavin fluorescence emission intensity in the dark-adapted state than in the light-adapted state (Figure 2A,B). In addition, the intensity of the flavin fluorescence is much greater in wild-type AppA17–133 than in wild-type AppA1–126. Increased flavin fluorescence by AppA17–133 has been attributed to the fact that Trp104 is closer to the flavin in AppA17–133 and likely transfers more energy nonradiatively to FAD than does AppA1–126 where Trp104 is further away (25). For comparison, the insert in Figure 2A shows fluorescence emission of full-length wild-type AppA, which is similar to that of wild-type AppA1–126. It is

interesting to note that tryptophan fluorescence maxima are identical for full-length AppA and the separate BLUF domains in a dilute state, which is not the case for more concentrated samples. In dilute state, all samples possess Trp emission maxima at ~ 343 nm. In more concentrated samples, separate BLUF domains have tryptophan maxima blue shifted to ~ 333 – 336 nm (25).

Similar analysis of flavin fluorescence was undertaken for the Q63 mutants in the AppA17–133 and AppA1–126 constructs (Figure 2B,C). In addition to the absence of a photocycle, these mutants also show no change in their fluorescence spectrum when the samples were kept in the dark versus exposed to light prior to spectral analysis. Consequently, only the fluorescence spectra of dark-adapted samples are shown in Figure 2. The Q63E mutant in both AppA17–133 and AppA1–126 reveals low flavin emission intensity, much like that of light-adapted wild-type AppA17–133 and AppA1–126 (Figure 2C,D, dashed line). In contrast to Q63E, the Q63L mutants exhibit increased flavin emission coupled with a strong red shift of the flavin emission maximum to ~ 515 nm as well as a shoulder at ~ 485 nm (Figure 2C,D, solid line). Thus, at least two peaks seem to contribute to the Q63L flavin emission with the ratio of these two peaks varying with sample concentration and between different sample preparations (not shown). This is indicative of a non-native heterogeneous pocket surrounding the flavin in the Q63L mutants.

In a previous study, we demonstrated that acrylamide is an effective quencher of Trp fluorescence and can be used to detect light- versus dark-adapted structural changes in AppA1–126 and AppA17–133 (25). As previously observed, wild-type AppA1–126 is characterized by partial solvent exposure of Trp104 since it exhibits linear acrylamide quenching in both the dark- and light-adapted states with Stern–Volmer quenching constants of $K_{sv} = 2.06$ and 3.47 M^{-1} , respectively (Figure 3A). This is in contrast to wild-type AppA17–133 that exhibits no acrylamide quenching when in its dark state (K_{sv} close to 0) and only moderate quenching when in the light state ($K_{sv} = 0.77 \text{ M}^{-1}$) (Figure 3B). Indeed, partial light-driven Trp movement of wild-type AppA17–133 never reaches a degree of exposure of Trp to the quencher as is observed with the dark-adapted AppA1–126. These results, coupled with other techniques (25), led to the conclusion that AppA1–126 contains Trp104 partially solvent-exposed in both light and dark states while AppA17–133 has Trp104 buried near the flavin in the dark that only becomes slightly exposed in the light-adapted form (25).

A comparative analysis of the Q63E mutant in AppA1–126 and in AppA17–133 reveals that this mutant is characterized by acrylamide quenching curves and K_{sv} values that mimic that of light state wild-type AppA1–126 and AppA17–133 ($K_{sv} \sim 3$ and 0.84 M^{-1} , respectively) (Figure 3C,D). Acrylamide quenching of these Q63E mutants is also nearly identical when performed at dark or after light illumination, indicating that the Q63E mutation locks these proteins in a lit conformation.

The Q63L mutant in AppA1–126 and AppA17–133 exhibits more complex behavior than that of the Q63E mutant. In AppA17–133 the Q63L mutant exhibits no fluorescence quenching under dark or lit conditions, indicating that tryptophans are buried under both conditions (Figure 3D). This mutant is also rather unstable with its F_0/F_c values often dropping in the negative range during the quenching experiment. When the Q63L mutation is present in AppA1–126, there is a slight quenching with values of $K_{sv} = 0.61$ and 0.56 under both dark

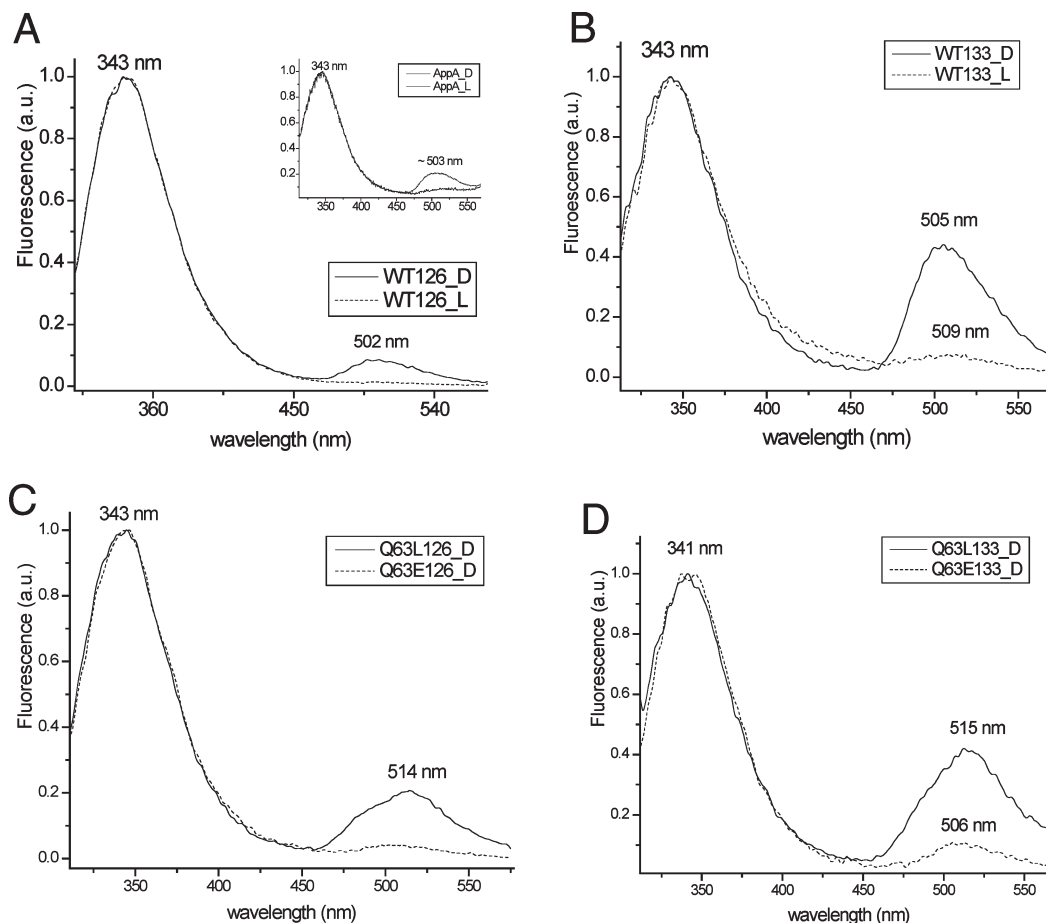


FIGURE 2: Normalized fluorescence emission spectra. (A) AppA1–126 WT (insert: AppA full length); (B) AppA17–133 WT; (C) AppA1–126 Q63E and Q63L; (D), AppA17–133 Q63E and Q63L. All samples were measured at dark (D) and light (L) conditions.

and light states, respectively (Figure 3C). These values do not reach the K_{sv} values of wild-type AppA1–126 under dark or light conditions, indicating a non-native conformation of this mutant.

NMR Spectral Analysis Confirms That Q63E in AppA1–126 and AppA17–133 Adopts a Lit-State Structure. We acquired ^1H – ^{15}N HSQC spectra of ^{15}N -labeled wild-type and Q63E AppA1–126 and AppA17–133 to confirm that Q63E substitution locks AppA into a lit-state structure in solution. Light- versus dark-state NMR structures of AppA5–125 by Grinstead et al. (14) reveal minor alterations of the overall structure of this domain upon light excitation of the flavin. Specifically, there was a reduction of cross-peak intensity for the backbone amide groups of C19–Y21 in the $\beta 1$ strand and Q63, W64, and E66 in the $\beta 3$ strand, as well as chemical shift perturbations for several additional cross-peaks, including the side chains of H44 (NH $\delta 1$), and W64 (NH $\epsilon 1$), and the backbone of F55, E94, R100, F101, G103, H105, Q107, L108, and S109 upon light excitation. ^1H – ^{15}N HSQC spectra of ^{15}N -labeled wild type versus Q63E mutant forms of AppA1–126 and AppA17–133 in the dark are shown in Figure 4A,C, with backbone chemical shift perturbation maps also shown for each AppA construct (Figure 4B,D). As can be seen in Figure 4, perturbation maps observed between the wild type and Q63E ^1H – ^{15}N HSQC spectra are virtually identical for both the AppA1–126 (Figure 4B) and AppA17–133 (Figure 4D) proteins, revealing that the changes in structure imposed by the Q63E substitution are immaterial to the BLUF construct to which it resides. Furthermore, the Q63E AppA perturbation map is strikingly similar to that observed for dark- versus light-adapted states of

wild-type AppA5–125 and also reported for other BLUF domains as studied by NMR (14, 27–29). These regions include elements in and around the flavin binding pocket (C19–Y21 in the $\beta 1$ strand, G52–L54 on $\beta 2$, and residues 62–65 in $\beta 3$), residues linked to E66 in the $\beta 3$ strand via hydrogen bonding (T51, R100, F101), H44 side chain (NH $\delta 1$), W64 side chain (NH $\epsilon 1$), and the C-terminal region including E94, G103, H105, and S109 on $\beta 5$. We can conclude from this analysis that the Q63E substitution mutants mimic the light-excited conformation of wild-type AppA in both AppA constructs.

For comparison, we also recorded the ^1H – ^{15}N HSQC spectra of ^{15}N -labeled Q63L mutants (Figure 5). Most of the cross-peaks exhibit large chemical shifts, and certain peaks disappear, when wild-type AppA are compared to Q63L mutant constructs which prevented the assignment of peaks for Q63L (Figure 5A,B). When comparing the Q63E and Q63L mutants (Figure 5C,D), it is obvious that Q63L mutants differ largely from the Q63E conformation as well. Therefore, we can conclude that Q63L mutation results in the BLUF domain folding in a non-native conformation that is different from the dark conformation of the wild-type and Q63E BLUF domains.

Complex Formation of AppA Q63 Full-Length Mutants with PpsR. The ability of Q63E and Q63L full-length AppA mutant proteins to form a complex with the PpsR repressor was also tested by size-exclusion chromatography. Since light disrupts the interaction between AppA and PpsR (8), analysis of complex formation was undertaken under dark reducing conditions. A 10-fold molar excess of AppA was incubated with PpsR and then subjected to chromatographic separation. As shown in Figure 6,

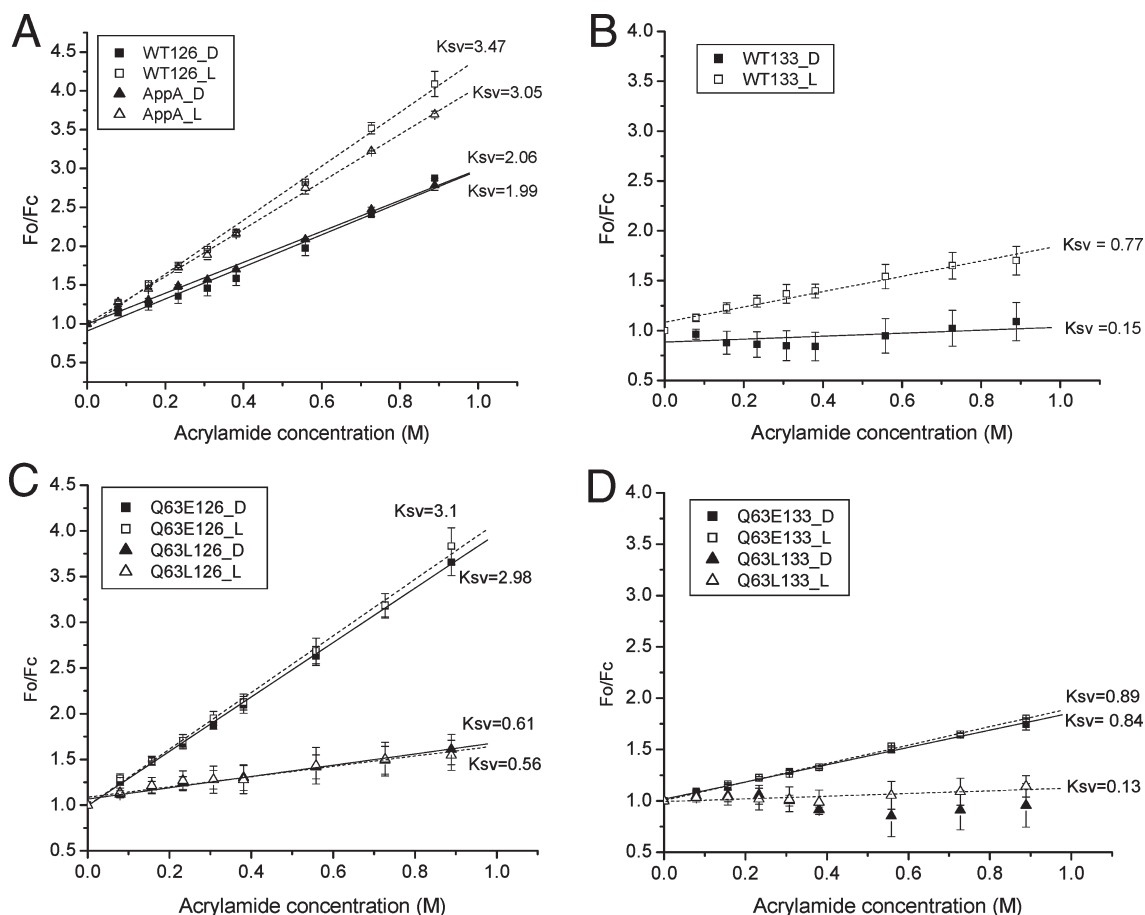


FIGURE 3: Quenching of tryptophan fluorescence with acrylamide. Samples are freshly purified and diluted to $A_{280} = 0.02$. (A) AppA1–126 WT and AppA full length; (B) AppA17–133 WT; (C) AppA1–126 Q63E and Q63L; (D) AppA17–133 Q63E and Q63L. All samples are measured at dark and light conditions. K_{sv} values are indicated.

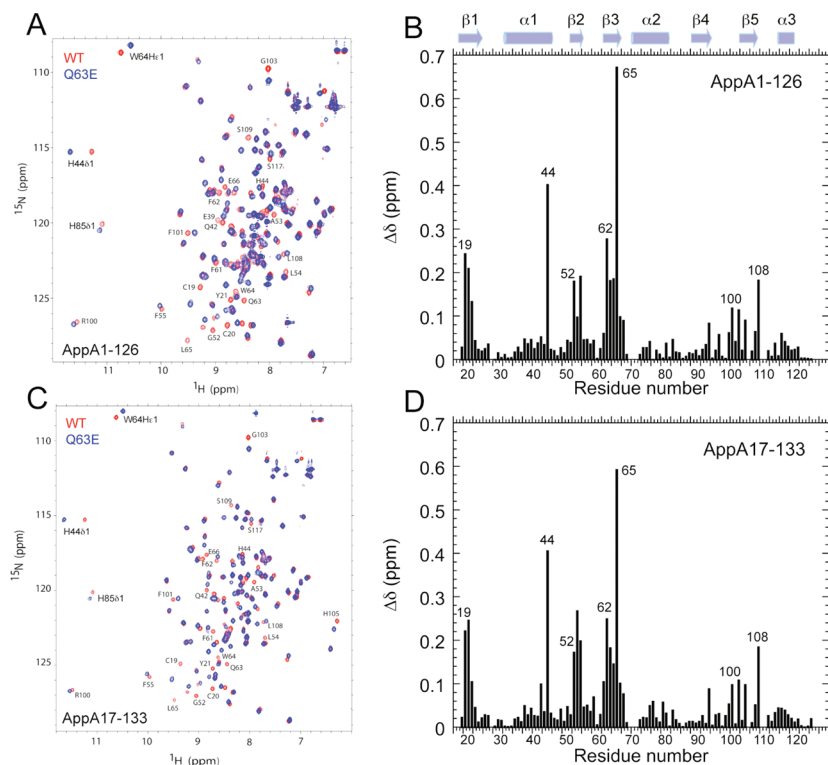


FIGURE 4: Superposition of the ^1H – ^{15}N HSQC spectra of wild-type (red cross-peaks) and Q63E (blue cross-peaks) AppA1–126 (A) and AppA17–133 (C). The chemical shift perturbation maps of AppA1–126 (B) and AppA17–133 Q63E (D) vs WT represent the combined ^1H and ^{15}N chemical shift, where $\Delta\delta$ (ppm) = $(\Delta\delta_{\text{H}}^2 + (\Delta\delta_{\text{N}}/7)^2)^{1/2}$ (36).

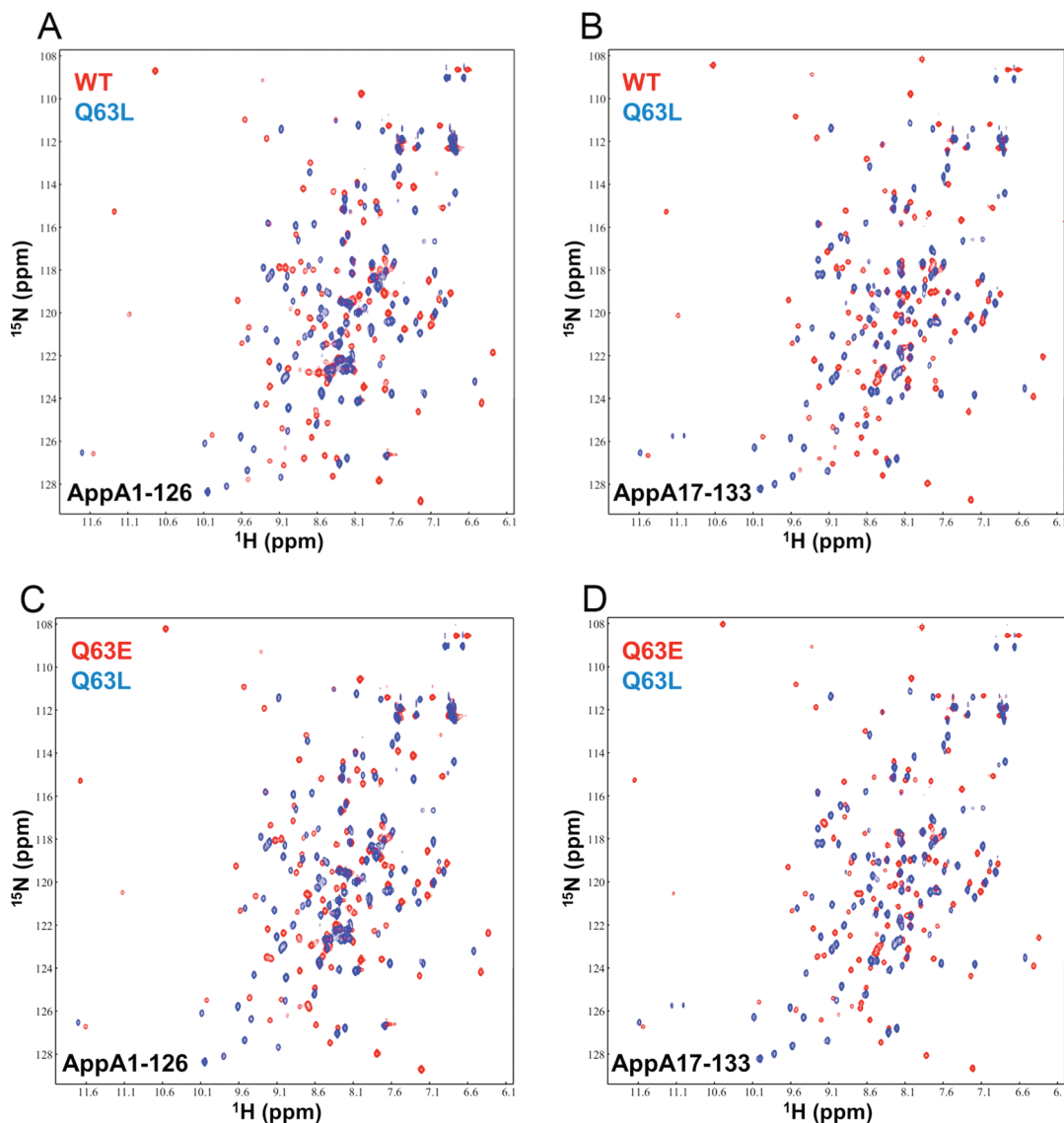


FIGURE 5: Superposition of the ^1H – ^{15}N HSQC spectra of the wild-type (red cross-peaks) and Q63L (blue cross-peaks) in AppA1–126 (A) and AppA17–133 (B); Q63L (blue) vs Q63E (red) in AppA1–126 (C) and AppA17–133 (D).

wild-type AppA formed the previously defined 1:2 complex with PpsR eluting at ~ 160 kDa (Figure 6, peak WT1, solid line) while AppA elutes as a monomer at 60 kDa (Figure 6, peak WT2, solid line). When the AppA Q63E mutant was incubated with PpsR, no complex was observed, and instead PpsR eluted as a tetramer at ~ 200 kDa (Figure 6, peak M1, dashed line). Interestingly, AppA harboring the Q63E mutation elutes in two peaks, M2 at ~ 95 kDa and M3 at ~ 50 kDa, which likely represent dimer/monomer. This indicates that, unlike wild-type AppA, the AppA protein with the Q63E mutation exists as both dimer and monomer in solution and is not able to form a complex with PpsR, thereby confirming a lit-state conformation of this mutant.

AppA Q63L was also tested for complex formation with PpsR. If our conclusion about non-native conformation of this mutant is correct, we would expect AppA Q63L to not form a complex with PpsR and perhaps exhibit differences in chromatography profile when compared to the AppA Q63E mutant. This was, indeed, the case as AppA containing the Q63L mutation exhibits a similar chromatography profile as observed for the Q63E mutant with three major peaks but at slightly different positions (Figure 6, peaks L1, L2, and L3, dotted line). SDS–PAGE shows that no complex was formed with PpsR and that most of the

AppA Q63L eluted as dimer and monomer with the monomer peak (L3) containing both full protein and a slightly smaller degradation product that is often observed with AppA full-length preparations. The Q63L protein conformation must be somewhat different than that of the Q63E mutant as the positions of the peaks are not identical. An aberrant chromatography profile, coupled with an aberrant fluorescent emission and quenching profile of Q63L mutants, and finally the NMR ^1H – ^{15}N HSQC spectra suggest that the Leu substitution at this position results in a non-native conformation of the AppA BLUF domain.

DISCUSSION

The AppA1–126 and AppA17–133 BLUF domains are interesting AppA variants in that they possess similar spectral photocycles and yet are characterized by distinctly different hydrogen-bonding residues near the flavin-binding pocket (25). Crystallographic and spectroscopic studies have demonstrated that Gln63 is a key residue that in AppA17–133 forms a hydrogen bond to Tyr21, to N5 of the flavin, and to Trp104 (19, 25). However, in AppA1–126 there is a slight conformational change where Trp104 and Met106 swap positions allowing Gln63 to form a hydrogen bond to Tyr21, to N5 of the flavin, and

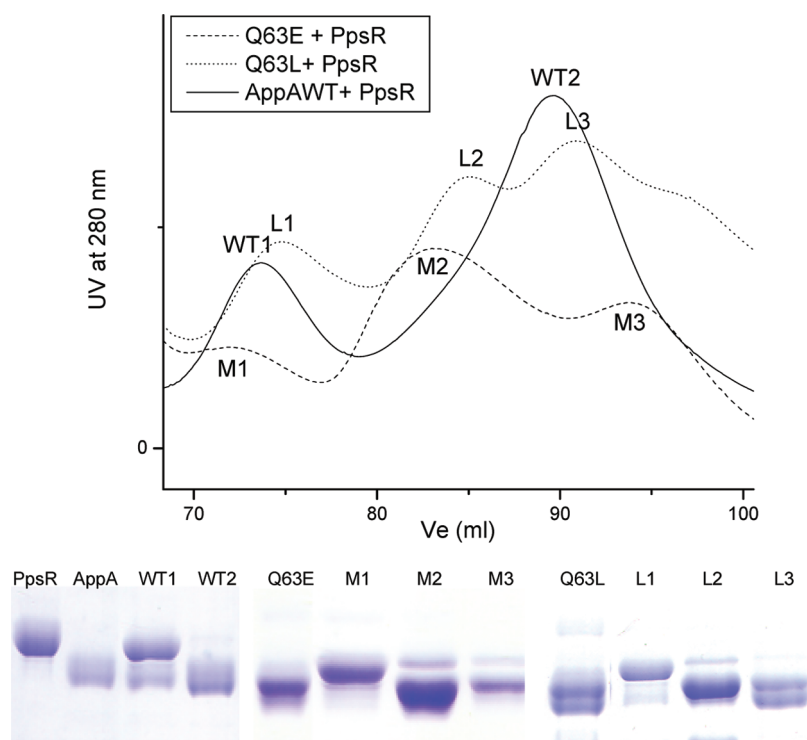


FIGURE 6: Size-exclusion chromatography profile (Sephacryl 200) of AppA WT-PpsR, AppA Q63L-PpsR, and AppA Q63E-PpsR, performed at dark and reducing conditions (5 mM DTT), with 10 $\times$ excess of AppA. Bottom: SDS-PAGE of chromatography peaks (as indicated) including PpsR, AppA WT, Q63L, and Q63E purified proteins as controls. AppA full-length protein often contains two bands as the unstable C-terminal (20–30 amino acids) gets easily degraded.

to Met106 (17). Despite these structural differences, these two BLUF domains exhibit nearly identical photocycles, which has led to an early proposal that light excitation of the flavin results in a hydrogen bond rearrangement where Trp104 and Met106 swap positions (18). However, this model is not supported by our recent study which shows that Met106 is close to Gln63 in the ground state and that light excitation of the flavin results in only limited movement of Trp104 (25).

In this study, we have used a variety of spectroscopic and structural approaches to analyze Leu and Glu mutants at position 63 to study their effect on the photocycle and on the structure of the BLUF domain. A Gln to Leu mutation at position 63 in both AppA1–126 and AppA17–133 spectrally locks each BLUF domain in a blue-shifted state. The Q63L mutants also appear to adopt at least two different non-native conformations based on the presence of a heterogeneous flavin fluorescence emission peak that is not seen in the wild-type protein or other mutations. The flavin is thought to be dynamic even in the dark ground state where it apparently samples several different orientations (29). Thus, it is perhaps not surprising that a Q63L substitution which disrupts hydrogen bonds to N5 of FAD and –OH of Tyr21 results in a heterogeneous and non-native microenvironment around the flavin. Indeed, the likelihood of a non-native conformation of the Q63L mutants is supported by our observation that full-length AppA Q63L does not interact with PpsR *in vitro* and has an aberrant chromatographic profile and its NMR ^1H – ^{15}N HSQC spectra are very different from either WT or Q63E proteins. In addition, Masuda et al. (30) have also reported that an AppA Q63L mutant is not able to derepress the activity of PpsR *in vivo*.

In contrast to a blue shift of the Q63L mutant, visible spectral analysis of the Q63E mutants shows that this substitution locks AppA in a red-shifted state. Previous NMR and FTIR studies of

AppA BLUF domains suggest that light excitation of the flavin results in stronger H-bonds between Tyr21 and Gln63 (29), as well as a stronger hydrogen bond between C4=O of FAD to Gln63 (31). We suspect that the Q63E mutation also establishes a stronger H-bonding pattern to both Tyr21 and C4=O of FAD, driving the protein into a rather rigid conformation, closely mimicking the lit state. The red shift observed in the flavin absorption spectrum of Q63E AppA mutants is only 3–3.5 nm instead of the normal 12 nm shift observed upon flavin excitation of wild-type AppA. This indicates that the H-bond to N5 of flavin is either missing or significantly weakened in the Q63E mutant. Further characterization of Q63E mutants by steady-state fluorescence, acrylamide quenching, and comparative analysis of the ^1H – ^{15}N HSQC spectra shows clear characteristics of lit spectral and conformational states. Indeed, our results are in agreement with a generally accepted idea that light excitation of the flavin results in numerous small changes in the whole BLUF domain (Figure 7) that represent a complex set of minor structural perturbations extending from residues on the β 3 strand (Phe62 through Gly67) to residues on the adjacent β 2 strand (Gly52, Ala53, Leu54). We suspect that structural changes caused by light excitation of the flavin propagate from the β 2– β 3 region to the β 4 and β 5 strands, the β 4– β 5 loop, and the downstream C-terminal helical region. Our conclusions seem to be in close agreement with recently reported NMR light-excited changes in BlrP1 from *Klebsiella pneumoniae* (15) and its crystal structure (32) and by collection of diffraction data on an AppA BLUF domain crystal (C20S AppA1–124) in the dark as well as after light excitation of the crystal (17). Light-induced changes of the AppA1–124 C20S crystal were assigned to the backbone of the loop before and the end of the β 4 sheet (residues 82–86 and 90–95) as well as minor changes in Tyr21, Leu54, Gln63, Asp82, His85, Arg83, Arg84, and Met106. Alterations in

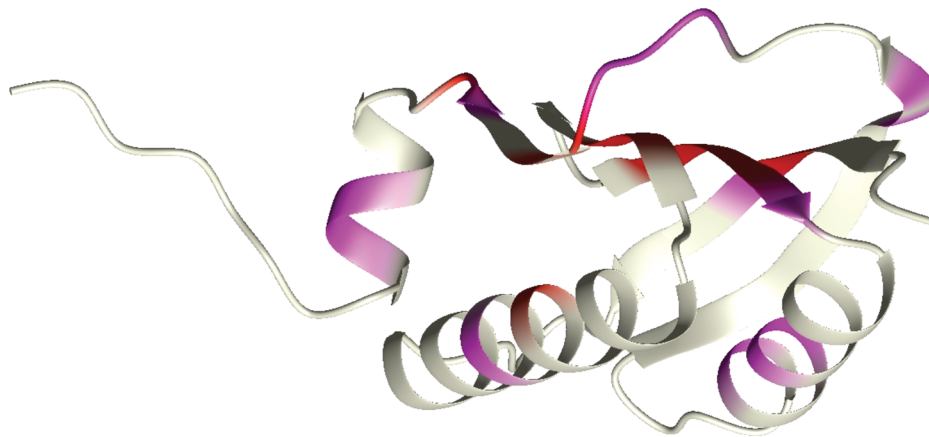


FIGURE 7: Ribbon views of the structural differences between wild-type and Q63E AppA17–133 from NMR chemical shift perturbations. Colors are ramped on a gray ribbon diagram of the structure of AppA17–133 based on the $\Delta\delta$ ppm as follows: magenta, $0.05 < \Delta\delta < 0.1$, and red, $0.1 < \Delta\delta < 0.60$ (see Figure 4).

Tyr21, Leu54, and Gln63 are observed in our NMR spectra of Q63E, as well as changes in the $\beta 4$ and $\beta 5$ sheets.

While our study does not directly address the exact mechanism of the events that occur at Q63 after light excitation of the flavin, our analysis of Q63 mutants does confirm that this residue is critical for the spectral and light-excited properties of BLUF domains. The small structural changes observed here suggest that the BLUF domain can be distinguished as a light sensor different from other blue light absorbing photoreceptors such as the LOV domain (33, 34) and PYP photoreceptors, which either undergo a covalent reaction with the chromophore or chromophore isomerization during the photocycle, respectively (35).

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