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Photoexcitation of the Blue Light Using FAD Photoreceptor AppA Results in Ultrafast Changes to the Protein Matrix

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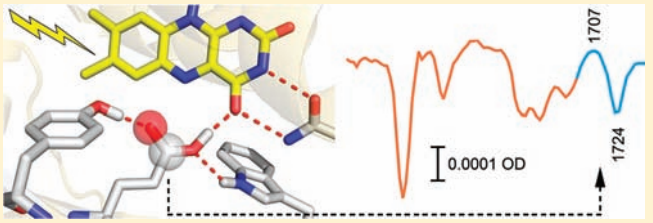
ABSTRACT: Photoexcitation of the flavin chromophore in the BLUF photosensor AppA results in a conformational change that leads to photosensor activation. This conformational change is mediated by a hydrogen-bonding network that surrounds the flavin, and photoexcitation is known to result in changes in the network that include a strengthening of hydrogen bonding to the flavin C4═O carbonyl group. Q63 is a key residue in the hydrogen-bonding network, and replacement of this residue with a glutamate results in a photoinactive mutant. While the ultrafast time-resolved infrared (TRIR) spectrum of Q63E $AppA_{BLUF}$ is characterized by flavin carbonyl modes at 1680 and 1650 cm^{-1}, which are similar in frequency to the analogous modes from the light activated state of the wild-type protein, a band is also observed in the TRIR spectrum at 1724 cm^{-1} that is unambiguously assigned to the Q63E carboxylic acid based on U-^{13}C labeling of the protein. Light absorption instantaneously (<100 fs) bleaches the 1724 cm^{-1} band leading to a transient absorption at 1707 cm^{-1}. Because Q63E is not part of the isoalloxazine electronic transition, the shift in frequency must arise from a sub picosecond perturbation to the flavin binding pocket. The light-induced change in the frequency of the Q63E side chain is assigned to an increase in hydrogen-bond strength of 3 kcal mol^{-1} caused by electronic reorganization of the isoalloxazine ring in the excited state, providing direct evidence that the protein matrix of AppA responds instantaneously to changes in the electronic structure of the chromophore and supporting a model for photoactivation of the wild-type protein that involves initial tautomerization of the Q63 side chain.



INTRODUCTION

Flavoproteins are a large family of proteins that contain a covalently or noncovalently bound flavin cofactor. Although the isoalloxazine ring of the flavin functions predominantly as an electron transfer intermediate in biochemical oxidation−reduction reactions, there are at least three subfamilies of flavoproteins that function as photoreceptors: the light-oxygen-voltage (LOV) domain proteins,[1,2] the cryptochromes,[3] and the Blue Light Using FAD (BLUF)-domain proteins.[4] The chromophores in traditional photoreceptors such as the rhodopsins, xanthopsins, and phytochromes undergo an isomerization when light is absorbed.[5] However, this is not the case for flavoprotein receptors, which cannot undergo significant structural changes and rely on neighboring protein residues to transform light absorption to a signaling event.[6] Thus there is substantial interest in understanding how absorption of light by the flavin is coupled to the conformational change(s) that lead to the signaling state. In the LOV domain proteins, which use FMN as their light-sensitive chromophore, a cysteinyl-flavin adduct is transiently formed in the signaling state. However, in the cryptochromes and BLUF proteins, the initial structural changes resulting from photoexcitation are less clear but in both cases may involve transient electron transfer to neighboring protein residues.[7,8]

The most extensively studied BLUF domain protein is AppA, an antirepressor involved in controlling photosystem biosynthesis in the photosynthetic bacterium *Rhodobacter sphaeroides*.[4,9,10] In the dark, AppA binds PpsR, a transcription factor, forming an AppA−$PpsR_2$ complex. When irradiated with high intensity blue light, the complex dissociates releasing PpsR and enabling PpsR to bind to DNA and repress photosystem biosynthesis.[9] Photoexcitation of AppA to form the signaling state is characterized by a 10 nm red shift in the 445 nm electronic transition of the isoalloxazine chromophore and is known to involve strengthening of hydrogen bonds between the C4═O group of the chromophore and the protein. However, the mechanism leading to these structural changes and how they relate to BLUF domain activity remains to be fully elucidated.[9,11,12] A plausible model for the BLUF photocycle has been proposed on the basis of ultrafast transient absorption spectroscopy of AppA and of the related BLUF domain protein Slr1694 from the cyanobacterium *Synechocystis*.[8,13−16] In this model, the light activated state of AppA (lAppA) is formed via an electron transfer that leads to formation of the anionic FAD semiquinone ($FAD^{\bullet-}$) followed

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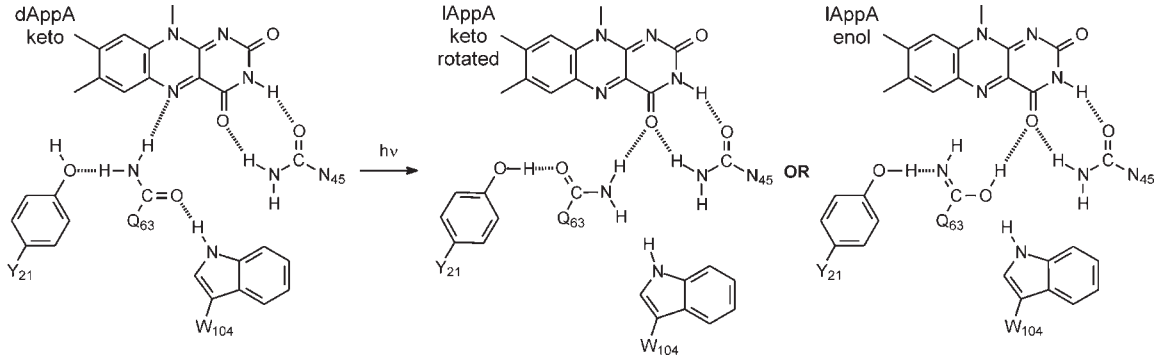

Figure 1. Hydrogen-bonding network in dAppA and lAppA. Hydrogen bonds are shown as dashed lines. Two structures are shown for lAppA that are based either on the rotation of Q63 or on the tautomerization of Q63. In both cases, this results in the formation of a new hydrogen bond with the flavin C4═O.

by the formation of the neutral semiquinone ($FADH^{\bullet}$), and finally lAppA in which the FAD has returned to its fully oxidized state. Subsequent photon absorption by lAppA is then proposed to lead to $FADH^{\bullet}$, which relaxes back to lAppA, explaining the absence of rapid photoreversibility.

Because the FAD is fully oxidized in both the dark and the light adapted forms of AppA (dAppA and lAppA, respectively), transient changes in the electronic structure of the FAD isoalloxazine ring must trigger a metastable change in the protein environment that surrounds the cofactor and that distinguishes dAppA and lAppA. While there are several proposed structural changes involved in formation of the signaling state, all of them suggest an alteration of the Q63 hydrogen-bonding interactions with FAD.[17,18] On the basis of their structural data, Anderson and co-workers proposed a model where Q63 rotates upon light absorption resulting in cleavage of the hydrogen bond to the N5 of the flavin and formation of a new hydrogen bond to the flavin O4 carbonyl oxygen.[17] This rotation causes a rearrangement in hydrogen bonding, which includes the formation of a new hydrogen bond from the hydroxyl side chain of Y21 to the amide side chain of Q63 and disruption of the hydrogen bond to the nearby W104. On the basis of additional structural data and quantum chemical calculations, a more recent model proposes that Q63 tautomerizes after light absorption, resulting in the rearrangement in the hydrogen-bonding network around the flavin chromophore.[12,19,20] Both models for light activation of AppA are shown in Figure 1. In the present study, the photoinactive Q63E mutant has been generated in the N-terminal BLUF domain of AppA ($AppA_{BLUF}$) to alter the hydrogen-bonding network between this key glutamine residue and the flavin chromophore. The structure and kinetics of the Q63E mutant have been investigated by isotope-labeling and ultrafast transient IR and visible absorption spectroscopy. These studies provide new insight into the number and nature of hydrogen bonds required for photoactivity in the BLUF domain and demonstrate that communication of the flavin with the protein occurs essentially instantaneously (<100 fs) upon blue light absorption. The implications of this for the light activation mechanism are discussed.

EXPERIMENTAL SECTION

Materials. FAD (disodium salt) was from Sigma Aldrich. D_2O (99.9 atom %) and [U-$^{13}C_6$]-D-Glucose (99 atom %) were from Cambridge Isotope Laboratories. Ampicillin (disodium salt), 100x MEM vitamins, and Minimal media were from Fisher Scientific. Ni-NTA resin was from Novagen. ITPG was from GoldChemBio.

Protein Expression and Purification. The gene encoding the AppA BLUF domain ($AppA_{BLUF}$; residues 5−125) was inserted into a pet15b vector (Novagen) in frame with an N-terminal His-tag. $AppA_{BLUF}$ Q63E was constructed by site-directed mutagenesis using Pfu Turbo (Stratagene). Primer sequences for this mutant were 5′ GGC GTC TTC TTC GAG TGG CTC GAA GGC CGC 3′ (Forward) and 5′ GCG GCC TTC GAG CCA CTC GAA GAA GAC GCC 3′ (Reverse), and the mutant construct was verified by DNA sequencing. Protein expression was performed using BL21(DE3) *E. coli* cells. A single colony was used to inoculate 10 mL of Luria Broth (LB) media containing 0.5 mM ampicillin. This culture was incubated at 37 °C in a shaking water bath (250 rpm) overnight and was then used to inoculate 1 L of LB/ampicillin media in a 4 L flask. The 1 L culture was incubated at 30 °C until the OD_{600} reached ∼1.2 (5 h) after which the temperature was decreased to 18 °C for 30 min followed by addition of 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce protein expression. Both the expression of $AppA_{BLUF}$ and the subsequent purification were performed in the dark. The cells were harvested by centrifugation (5000 rpm, 4 °C) and stored at −20 °C. The cell pellet was then resuspended in 40 mL of buffer A (10 mM NaCl, 50 mM NaH_2PO_4 at pH 8) with an additional 200 μL of 50 mM phenylmethanesulphonylfluoride and 14 μL of β-mercaptoethanol. The cells were lysed by six rounds of sonication (15 s), in each case followed by 1 min recovery, and cell debris was removed by centrifugation (33 000 rpm, 90 min) after which the supernatant was incubated with a 300 mM excess of FAD for 45 min on ice in the dark. Subsequently, the supernatant was loaded onto a Ni-NTA (Qiagen) column (20 mL total column volume/10 mL of resin), which was then washed with buffer A (100 mL). After being washed with 100 mL of buffer A containing 20 mM imidazole, $AppA_{BLUF}$ was eluted using the same buffer containing 250 mM imidazole. The fractions containing $AppA_{BLUF}$ were pooled, giving a total volume of less than 10 mL, and then dialyzed against 3 L of buffer A overnight. After protein purity was checked by SDS-PAGE, protein samples were concentrated to 1 mM and aliquoted out into 1 mL samples, which were flash frozen in liquid N_2, lyophilized overnight, and reconstituted into an equal volume of D_2O for at least 5 h. Lyophilization followed by reconstitution with D_2O was repeated four times to ensure complete exchange after which the protein was lyophilized and stored at −80 °C.

Uniform ^{13}C protein labeling was performed by expressing $AppA_{BLUF}$ in BL21(DE3) *E. coli* cells that were grown on minimal media containing [U-$^{13}C_6$]-D-glucose as the sole carbon source. Single colonies containing plasmids for either wild-type or Q63E $AppA_{BLUF}$

that had been grown on LB/Amp plates were streaked on M9 minimal media/glucose/ampicillin plates containing 200 mg/mL ampicillin, 5 mg/mL glucose. This process is thought to acclimatize the cells to growth in minimal media, therefore leading to higher cell densities than if the cells had been transferred directly from LB media. A single colony from an M9 plate was used to inoculate 500 mL of M9/ampicillin minimal media in a 4 L flask that contained 4.0 g of glucose (dissolved in 10 mL of water and filtered through a 0.22 μm filter to sterilize) and 5 mL of 100x MEM vitamins (added after autoclaving). The cells were grown to an OD_{600} of approximately 0.5 at 30 °C, which took about 24 h. These cells were then pelleted and resuspended in fresh media with [U-$^{13}C_6$]glucose replacing the unlabeled glucose and incubated for 30 min at 18 °C. After 30 min, 0.8 mM IPTG was added to induce protein expression, and the culture was shaken in the dark for 24 h to maximize the yield of protein. [U-^{13}C]-$AppA_{BLUF}$ was then purified using the protocol described above.

Raman Spectroscopy. Steady-state Raman spectra were recorded using a model 890 Ti:sapphire laser (Coherent, Santa Clara, CA), pumped by an Innova 308C argon ion laser (Coherent), providing 550 mW of 752 nm infrared excitation. The measurements were made by focusing the beam on the base of a quartz cuvette containing protein and collecting at 90° geometry. The Rayleigh scattered light was removed using a super notch plus holographic filter (Kaiser Optical Systems, Inc.). The beam was focused on a 2 mm × 2 mm quartz cell containing 70 μL of 500 mM protein and collecting data for 300 accumulations with an exposure time of 2 s. Raman spectra of the dialysis buffer were obtained without changing the optical setup directly after protein data were recorded with the same number of accumulations and exposure time. The buffer spectrum was then subtracted from the protein spectrum giving a protein spectrum, which was then calibrated using the Raman spectrum of cyclohexanone. The resolution of the system is 8 cm^{-1}.[21]

Time-Resolved Infrared Spectroscopy. Ultrafast transient IR measurements (TRIR) on dark adapted wild-type $AppA_{BLUF}$ ($dAppA_{BLUF}$), light adapted AppA ($lAppA_{BLUF}$), and the Q63E mutant were performed using an ultra stable 10 kHz amplified titanium sapphire laser pumping OPAs for UV and IR pulse generation, described in detail elsewhere.[22] The samples were excited with ~200 nJ linearly polarized pulses at 400 nm. To avoid degradation of the protein and the photoconversion of $dAppA_{BLUF}$, a raster sample mover was used in conjunction with a flow cell. In addition, to avoid contributions to the measured dynamics from orientational relaxation, the relative angle between the pump and the probe beams was set to the magic angle (54.7°) using a half wave plate.

Transient Absorption Spectroscopy. Ultrafast transient absorption measurements were performed using a Clark-MXR 1000 regenerative amplifier providing ~350 μJ pulses centered at 800 nm at a repetition rate of 1 kHz. The output of the amplifier was split in two (10/90%) where the pulse with the smaller energy was used for white light continuum generation in a CaF_2 crystal for use as the probe. The higher intensity pulse was frequency doubled to 400 nm and attenuated to ~200−400 nJ to act as the pump. Measurements were again made at the magic angle, and sample photodegradation was minimized using a Lissajous scanner and peristaltic pump. Absorption changes were measured with an Andor CCD, and data collection was performed with Labview data acquisition software.

RESULTS AND DISCUSSION

Q63 is a critical component of the hydrogen-bonding network that surrounds the flavin chromophore in AppA and which provides a mechanism for communication between the flavin chromophore and the protein matrix. Photoexcitation of the flavin results in a modulation in the protein−flavin interactions

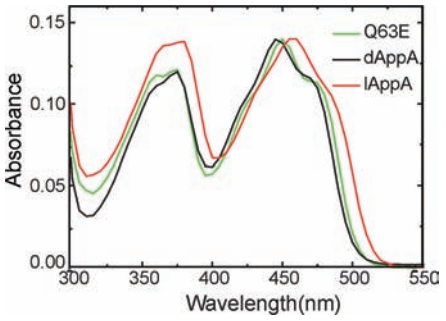

Figure 2. Absorption spectra of wild-type d and $lAppA_{BLUF}$ and Q63E $AppA_{BLUF}$. Steady-state absorption spectra of $dAppA_{BLUF}$ (black), $lAppA_{BLUF}$ (red), and Q63E $AppA_{BLUF}$ (green) bound to FAD. Protein concentration was 20 μM in phosphate buffer, pH 8.

that are mediated by this hydrogen-bond network and that lead ultimately to the light activated state of the protein. The importance of Q63 was previously shown through the Q63L mutation that renders the protein photoinactive.[23] More recently, Dragnea et al.[24] characterized the Q63E AppA mutant and proposed that this amino acid substitution locks the protein in the light activated state based primarily on NMR data, together with the observation that Q63E AppA was unable to bind the PpsR transcriptional repressor. In agreement with this observation, we observe that the vibrational spectrum of FAD in Q63E $AppA_{BLUF}$ resembles that of light activated wild-type $AppA_{BLUF}$ (see below). In addition, TRIR reveals a vibrational band assigned to the Q63E carboxylic acid side chain that alters hydrogen-bond strength within <100 fs of light absorption, indicating that the protein matrix responds instantaneously to flavin photoexcitation.

To evaluate the role of Q63 in the AppA photocycle, we used site-directed mutagenesis to replace Q63 with a glutamate in the AppA5−125 BLUF domain construct (Q63E $AppA_{BLUF}$). The UV−visible absorption spectrum of this AppA mutant is shown in Figure 2 where it can be seen that λ_{max} for the FAD absorption of Q63E $AppA_{BLUF}$ is red-shifted by 3 nm as compared to $dAppA_{BLUF}$, from 447 to 450 nm. On the basis of steady-state absorption measurements, this mutant was determined to be photoinactive because the λ_{max} at 450 nm does not shift when irradiated with 400 nm light as in the wild-type protein. As reported previously,[24] the difference in λ_{max} between Q63E $AppA_{BLUF}$ and $dAppA_{BLUF}$ is ~25% of the red-shift observed for wild-type $AppA_{BLUF}$ upon light activation (Figure 2; 447 to 458 nm). To explore changes in the ground-state structure of the flavin caused by the Q to E mutation, we used steady-state Raman spectroscopy to study Q63E $AppA_{BLUF}$. In the Raman spectrum of $dAppA_{BLUF}$, a weak band can be observed at 1710 cm^{-1}, which shifts to ~1690 cm^{-1} upon irradiation (Figure 3). This band has previously been assigned by isotope labeling on light minus dark FTIR difference spectra to a mode with a major contribution from the C4═O stretch of the flavin chromophore, and the observed ~20 cm^{-1} red shift (where a red shift implies a shift to lower wavenumber, and blue higher) in this band is indicative of a strengthening of hydrogen bonding to the C4═O group caused by light activation.[25] In Q63E $AppA_{BLUF}$, this weak band appears at about 1695 cm^{-1} (Figure 3). Thus, the absorption and Raman data indicate that the environment of the flavin in Q63E $AppA_{BLUF}$ is intermediate between that experienced by the chromophore in d and $lAppA_{BLUF}$.

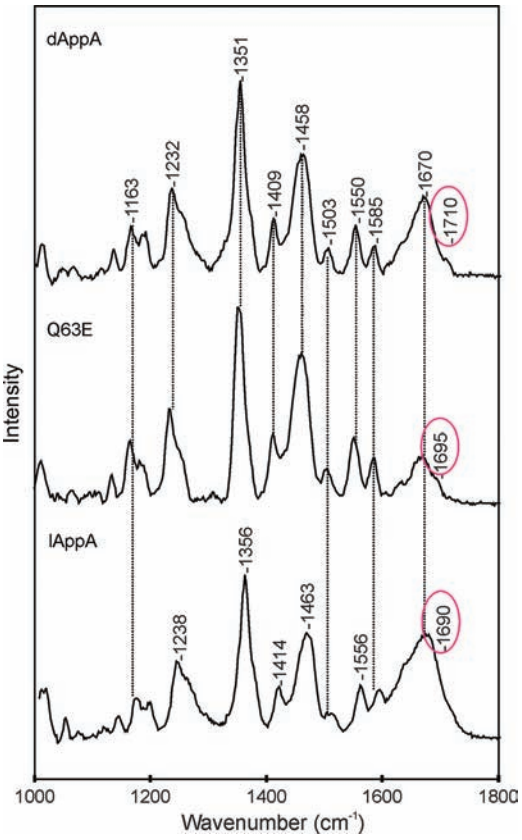

Figure 3. Raman spectra of wild-type d and lAppA$_{BLUF}$ and Q63E AppA$_{BLUF}$. Raman spectra of dAppA$_{BLUF}$ (top), lAppA$_{BLUF}$ (bottom), and Q63E$_{BLUF}$ AppA (middle) bound to FAD. Protein concentration was 1.5 mM in phosphate buffer, pD 8.

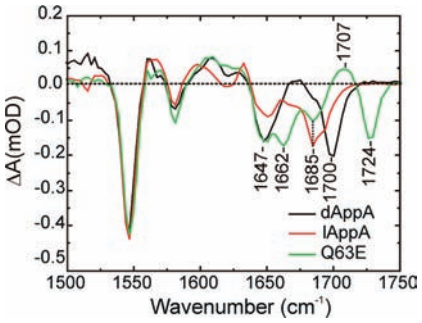

Figure 4. TRIR spectra of wild-type d and lAppA$_{BLUF}$ and Q63E AppA$_{BLUF}$ measured at 3 ps. TRIR spectra of dAppA$_{BLUF}$ (black), lAppA$_{BLUF}$ (red), and Q63E AppA$_{BLUF}$ (green) bound to FAD. Protein concentration was 2 mM in pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps. The spectra have been normalized to the intense FAD ring bleach mode at 1547 cm^{-1}.

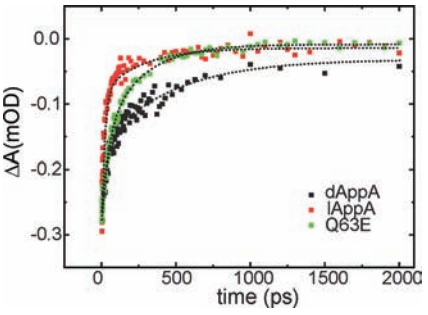

Figure 5. Kinetics of the ground-state recovery for wild-type d and lAppA$_{BLUF}$ and the Q63E AppA$_{BLUF}$ using TRIR spectroscopy measured at 1547 cm^{-1}. Comparison of kinetic measurements of dAppA$_{BLUF}$ (black), lAppA$_{BLUF}$ (red), and Q63E AppA$_{BLUF}$ (green) bound to FAD. Protein concentration was 2 mM in pD 8 phosphate buffer, and the ground-state recovery kinetics are measured at the intense bleach at 1547 cm^{-1}.

To investigate structural changes that occur rapidly upon absorption of 400 nm light, we used ultrafast TRIR spectroscopy to gain further insight into the structure of Q63E AppA$_{BLUF}$. The TRIR spectrum of Q63E AppA$_{BLUF}$ acquired 3 ps after photoexcitation is shown in Figure 4 together with the corresponding spectra of d and lAppA$_{BLUF}$. The most striking feature is the bleach/transient absorption observed at 1724/1707 cm^{-1} only in the Q63E mutant. We consider this new mode in detail below. In contrast, the lower wavenumber (<1600 cm^{-1}) portion of the Q63E AppA$_{BLUF}$ TRIR spectrum is very similar to that of d and lAppA$_{BLUF}$ and is characterized by major bleaches of the FAD chromophore at 1547 and 1581 cm^{-1}. Important differences exist however between the TRIR spectra of mutant and wild-type proteins between 1600 and 1750 cm^{-1}. The TRIR spectrum of dAppA$_{BLUF}$ is characterized by two major bleaches in this region at 1700 and 1647 cm^{-1} that are assigned to the flavin C4═O and C2═O carbonyl modes, respectively.[26−28] Upon irradiation of dAppA$_{BLUF}$ with 400 nm light to form the long-lived signaling state (lAppA$_{BLUF}$), the frequencies of the bands associated with bleaching of the two carbonyl modes change: the 1700 cm^{-1} band decreases in frequency and is replaced by a doublet at 1681/1690 cm^{-1}, while a shoulder appears on the high frequency side of the 1647 cm^{-1} bleach (lAppA$_{BLUF}$, Figure 4).[12] The TRIR spectrum of Q63E AppA$_{BLUF}$ also possesses a bleach at 1650 cm^{-1} together with well-resolved bleaches at 1662 and 1685 cm^{-1}. On the basis of comparison with the wild-type TRIR spectra together with the steady-state data described above, we assign the 1685 cm^{-1} bleach in Q63E AppA$_{BLUF}$ to the C4═O flavin carbonyl group and suggest that the 1662 cm^{-1} bleach has the same origin as the shoulder that appears on the 1647 cm^{-1} band in lAppA$_{BLUF}$. Thus, the Q63E AppA$_{BLUF}$ data are consistent with a protein environment that resembles the light activated form of the wild-type protein.

All of the features in the TRIR spectrum of Q63E AppA$_{BLUF}$ appear within the time response of the instrument (~100 fs). In Figure 5, we plot the kinetics for the ground-state recovery of the intense 1547 cm^{-1} bleach to compare Q63E AppA$_{BLUF}$ to d and lAppA$_{BLUF}$ kinetics. As was observed for d and lAppA$_{BLUF}$, the data for the mutant can be fit with a sum of two exponential components. The ground state of lAppA$_{BLUF}$ is found to recover much more rapidly (mean recovery time of 45 ps) than the dAppA$_{BLUF}$ state (249 ps) (Table 1). The ground-state recovery kinetics for Q63E AppA$_{BLUF}$ are intermediate between these two states (159 ps).

Similar biexponential behavior is observed in the kinetics of the transient absorption spectra for mutant and wild-type AppA$_{BLUF}$. In Figure 6, we show the transient absorption spectra of the three proteins, which are each characterized by a combination of a broad bleach and stimulated emission at 550 nm.[29] Our visible transient absorption measurements are in good agreement with the data measured by Toh et al. where TA measurements

Table 1. Kinetics of $dAppA_{BLUF}$, $lAppA_{BLUF}$, and Q63E $AppA_{BLUF}$ Determined by TRIR and Transient Absorption Spectroscopy[a]

	transient absorption (450 nm)					TRIR (1547 cm^{-1})				
	a_1	τ_1/ps	a_2	τ_2/ps	$\langle\tau\rangle$/ps	a_1	τ_1/ps	a_2	τ_2/ps	$\langle\tau\rangle$/ps
dAppA	−0.25	10 ± 3	−0.75	311 ± 80	236	−0.51	34 ± 4	−0.49	473 ± 73	249
lAppA	−0.62	11 ± 1	−0.38	103 ± 14	46	−0.72	11 ± 1	−0.28	134 ± 24	45
Q63E	−0.17	8 ± 2	−0.83	162 ± 8	135	−0.45	47 ± 6	−0.55	252 ± 30	159

[a] The observed kinetics of dAppA, lAppA, and Q63E AppA derived from an analysis of the transient absorption (TA) and time-resolved IR (TRIR) spectra. The protein concentration was 500 μM and 2 mM in pD 8 phosphate buffer for the TA and TRIR experiments, respectively. The kinetics have been fit to two exponentials of the 450 nm TA and the 1547 cm^{-1} bleach in the TRIR spectra.

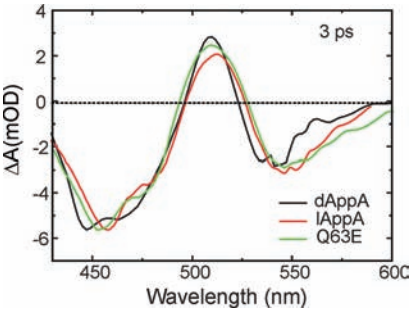

Figure 6. Transient absorption spectra of wild-type d and $lAppA_{BLUF}$ and the Q63E $AppA_{BLUF}$ measured at 3 ps. Transient absorption (TA) spectra of $dAppA_{BLUF}$ (black), $lAppA_{BLUF}$ (red), and Q63E $AppA_{BLUF}$ (green) bound to FAD. Protein concentration was 500 μM in pD 8 phosphate buffer, and the TA spectra were recorded with a time delay of 3 ps. The spectra have been normalized to the bleach at 450 nm.

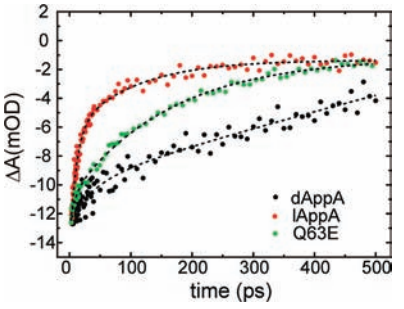

Figure 7. Kinetics of the ground-state recovery for wild-type d and $lAppA_{BLUF}$ and the Q63E $AppA_{BLUF}$ using transient absorption spectroscopy measured at 450 nm. Comparison of kinetic measurements of $dAppA_{BLUF}$ (black), $lAppA_{BLUF}$ (red), and Q63E $AppA_{BLUF}$ (green) bound to FAD. Protein concentration was 500 μM in pD 8 phosphate buffer, and the ground-state recovery kinetics are measured at the bleach at 450 nm.

were performed on d and lAppA.[15] Recovery of the bleach measured at 450 nm is plotted in Figure 7 for each protein, while the rate constants for the biexponential fitting and the mean recovery time are given in Table 1. The ground state recovery times are somewhat longer for TRIR than TA. However, the mean relaxation times are very similar. This behavior reflects the fact that the underlying kinetics are multiexponential rather than biexponential. The recovery of the major ground-state bleach of Q63E again occurs on a time scale between lAppA and dAppA (Figure 7). d and $lAppA_{BLUF}$, as well as Q63E $AppA_{BLUF}$ each have a short time component, which is the same within error for all three proteins (8−11 ps); however, significant differences arise from the long component. Q63E $AppA_{BLUF}$ has a slightly slower kinetic decay component (162 ± 8 ps) than $lAppA_{BLUF}$ (103 ± 14 ps) and approximately one-half of the long component of $dAppA_{BLUF}$ (311 ± 80 ps). Again, the spectrum of $lAppA_{BLUF}$ recovers more quickly than that of the dark adapted protein, while the response of Q63E $AppA_{BLUF}$ is intermediate between that of the two forms of the wild-type protein. Taken together, these kinetic data reinforce the conclusion that the chromophore environment in the Q63E mutant is intermediate between that of the dark and light states of wild-type AppA, and that the recovery kinetics depart markedly from single exponential behavior.[24]

The most striking difference between the TRIR spectra of wild-type and mutant $AppA_{BLUF}$ is the appearance of the new high frequency bleach at 1724 cm^{-1} in the Q63E $AppA_{BLUF}$ spectrum together with a transient absorption at 1707 cm^{-1} (Figure 4). These bands appear within 100 fs of excitation, and thus we initially considered whether the 1724 cm^{-1} bleach could be a ground-state mode of the flavin chromophore. However, no band is observed at this position in the steady-state Raman spectrum of Q63E $AppA_{BLUF}$, where modes arising from the flavin are selectively enhanced. In addition, if it arose from the flavin, then the most likely candidate is the C4═O carbonyl that has blue-shifted by 24 cm^{-1} upon replacing Q63 with a glutamate. Although Kim and Carey observe the C4═O of riboflavin at 1723 cm^{-1} when this molecule is bound to riboflavin binding protein in H_2O,[30] a large blue shift in the corresponding band in Q63E $AppA_{BLUF}$ seems unlikely given that other data are consistent with a red shift in this band: from 1700 cm^{-1} in $dAppA_{BLUF}$ to 1685 cm^{-1} in Q63E $AppA_{BLUF}$ (Figure 4). In addition, our data were obtained in D_2O, and we note that the C4═O of riboflavin bound to riboflavin binding protein is at 1710 cm^{-1} in D_2O.[30] Because the frequency of the 1724 cm^{-1} band is in the region expected for an unconjugated carboxylic acid,[31−33] we next investigated whether this band could arise from a protein mode that responds to flavin excitation on an ultrafast time scale. To test this hypothesis, we uniformly labeled both wild-type and mutant $AppA_{BLUF}$ with ^{13}C and reconstituted the proteins with unlabeled FAD. The resulting TRIR spectra show direct evidence that the vibrational mode observed at 1724 cm^{-1} is not the C4═O vibration of the flavin cofactor but a protein mode that shifts by ∼36 cm^{-1} upon ^{13}C uniform protein labeling (Figure 8). In comparison, the flavin carbonyl modes in ^{13}C labeled wild-type d and $lAppA_{BLUF}$ (Figure 9) are largely insensitive to labeling, although it is interesting to note that the transient absorption formed promptly at 1660 cm^{-1} in $dAppA_{BLUF}$ that was previously assigned to Q63 has been reduced in intensity by ^{13}C labeling, suggesting that it arises at least in part from a protein mode. The ^{13}C labeling also uncovers

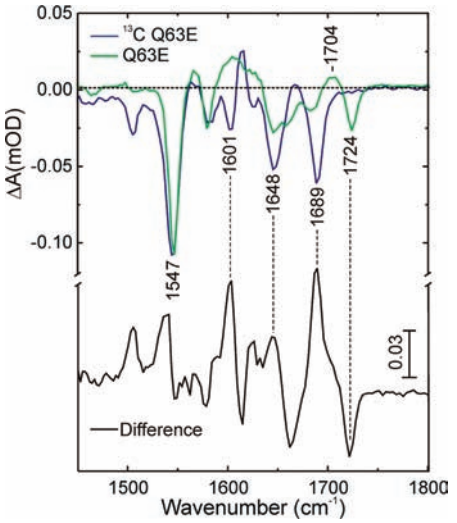

Figure 8. TRIR spectra of unlabeled and ^{13}C labeled Q63E AppA$_{BLUF}$ measured at 3 ps. TRIR spectra of unlabeled Q63E AppA$_{BLUF}$ (red) and ^{13}C labeled Q63E AppA$_{BLUF}$ (blue) bound to FAD. The difference spectrum is shown in the bottom panel (black). Protein concentration was 2 mM in pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps. The spectra have been normalized to the intense FAD ring bleach mode at 1547 cm^{-1}.

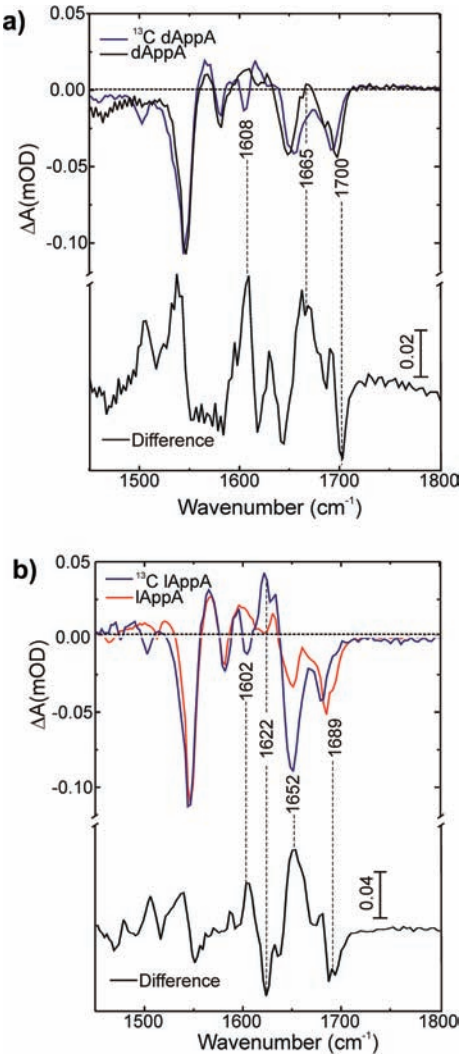

Figure 9. TRIR spectra of unlabeled and ^{13}C labeled d and lAppA$_{BLUF}$ measured at 3 ps. (a) TRIR spectra of unlabeled dAppA$_{BLUF}$ (red) and ^{13}C labeled dAppA$_{BLUF}$ (blue) bound to FAD. The difference spectrum is shown in the bottom panel (black). Protein concentration was 2 mM in pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps. The spectra have been normalized to the intense FAD ring bleach mode at 1547 cm^{-1}. (b) TRIR spectra of unlabeled lAppA$_{BLUF}$ (red) and ^{13}C labeled lAppA$_{BLUF}$ (blue) bound to FAD. The difference spectrum is shown in the bottom panel (black). Protein concentration was 2 mM in pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps. The spectra have been normalized to the intense FAD ring bleach mode at 1547 cm^{-1}.

additional protein modes perturbed by electronic excitation at 1601 and 1505 cm^{-1}.

Because the 1724 cm^{-1} bleach appears within 100 fs of excitation, this group either must be part of the electronic transition associated with light absorption (i.e., a flavin group) or must interact directly with the chromophore so that it responds instantaneously to light absorption. Importantly, the isotope labeling experiments allow us to confidently assign the high frequency 1724 cm^{-1} bleach and 1707 cm^{-1} transient in Q63E AppA$_{BLUF}$ to a protein residue. Given that the position of the 1724 cm^{-1} is in the region expected for a protonated carboxylic acid,[33−35] we assign this bleach to the COOH(D) group of Q63E. To respond instantaneously to photoexcitation, we propose that Q63E-COOH(D) is directly hydrogen bonded to the flavin chromophore, or possibly to a group that is itself in direct contact with the flavin such as Y21 or N45. It follows that the change in electronic structure of the flavin caused by photoexcitation leads to an instantaneous increase in the strength of the hydrogen bond between the chromophore and Q63E-COOH(D), leading to loss of the infrared absorption at 1724 cm^{-1} (bleach) and a new absorption at 1707 cm^{-1} (transient). On the basis of model studies that relate changes in carbonyl frequency and hydrogen bond strength, the 17 cm^{-1} decrease in wavenumber of the carboxylic acid group is consistent with an increase in hydrogen-bond enthalpy of ~3 kcal/mol.[36]

The TRIR spectra were acquired at a sample pD of 8, and thus the pK_a of the Q63E side chain must be at least 3 pH units more basic than the normal value for a carboxylic acid. Such changes in the pK_a values of buried protein side chains are not unprecedented: for example, the heme propionic acid group in cytochrome c has a pK_a of 9,[33] while E190 in the thermophilic F$_1$-ATPase has a pK_a of 7.[37] Because the frequency of the C4═O flavin carbonyl in Q63E AppA$_{BLUF}$ is similar to that in lAppA$_{BLUF}$, it is reasonable to propose that Q63E is hydrogen bonded directly to C4═O as proposed in the light activated state of the protein (Figure 10). This would require that Q63E COOH(D) is a hydrogen-bond donor to C4═O and a hydrogen-bond acceptor from Y21. Direct interaction of the Q63E-COOH(D) group with C4═O can also account for the instantaneous response of the Q63E side chain to light absorption because an increase in electron density on the C4═O oxygen in the flavin excited state will result in a strengthening of the hydrogen bond with the Q63E side chain (Figure 10). In one of the models for AppA activation (Figure 1), Q63 tautomerizes so that the amide nitrogen can accept a hydrogen bond from Y21 while the enol OH forms a hydrogen bond to the C4═O group.[12] Equivalent hydrogen-bond interactions are formed by Q63E in

Figure 10 together with a hydrogen bond to W104. Thus, one explanation for the loss of photoactivity in the mutant is that the Q63E side chain is an excellent isostere of the Q63 enol, thereby

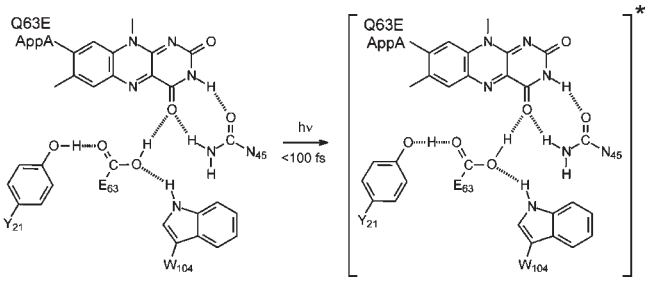

Figure 10. Proposed hydrogen-bonding structure of Q63E $AppA_{BLUF}$. Replacement of Q63 with a glutamate leads to a hydrogen-bond network in which the Q63E side chain mimics the enol form of Q63. The side chain of Q63E is hydrogen bonded to the flavin C4═O in the ground state, explaining the TRIR data in which the vibrational spectrum of the flavin resembles that of $lAppA_{BLUF}$. In the excited state, there is an increase in electron density on the C4═O oxygen, which strengthens the hydrogen bond with Q63E and results in polarization of the Q63E C═O and concomitant strengthening of the hydrogen bond between Y21 and Q63E. This change in electronic structure is shown schematically by lengthening of the Q63E C═O and O−H bonds and movement of the Y21 proton so that it is closer to the Q63E C═O group. Key bonds are colored red.

locking the protein in a state that resembles lAppA while maintaining a hydrogen bond to W104.[24] Returning to the wild-type protein, our observation thus suggests that the change in electronic structure of the flavin upon photoexcitation is a driving force for alterations in the hydrogen-bonding network, which lead ultimately to photoactivation. In addition, if the hydrogen-bonding network in the wild-type protein responds on the same time scale to that observed for Q63E AppA (<1 ps), then the TRIR data are consistent with an initial tautomerization of Q63,[12] because the proposed rotation of the Q63 side chain is expected to occur on a time scale of 10−100 ps.[38] However, while these data suggest that the transient stabilization of the Q63 enol in the AppA excited state may be a key step in photoactivation, the keto form of Q63 is expected to predominate in the lAppA ground state. This necessitates that Q63 must ultimately rotate for the keto form of the side chain to donate a hydrogen bond to the flavin C4═O, leading also to loss of the hydrogen bond with W104 (Figure 1). A model for AppA activation that encompasses both Q63 tautomerization and rotation is shown in Figure 11. In this new model, we propose that both keto and enol tautomers of Q63 are present in the ground state of dAppA, where the equilibrium favors the keto tautomer. Upon photoexcitation, the increase in electron density on the C4═O oxygen increases the relative stability of the enol tautomer that then leads ultimately to the light activated form of the protein in which Q63

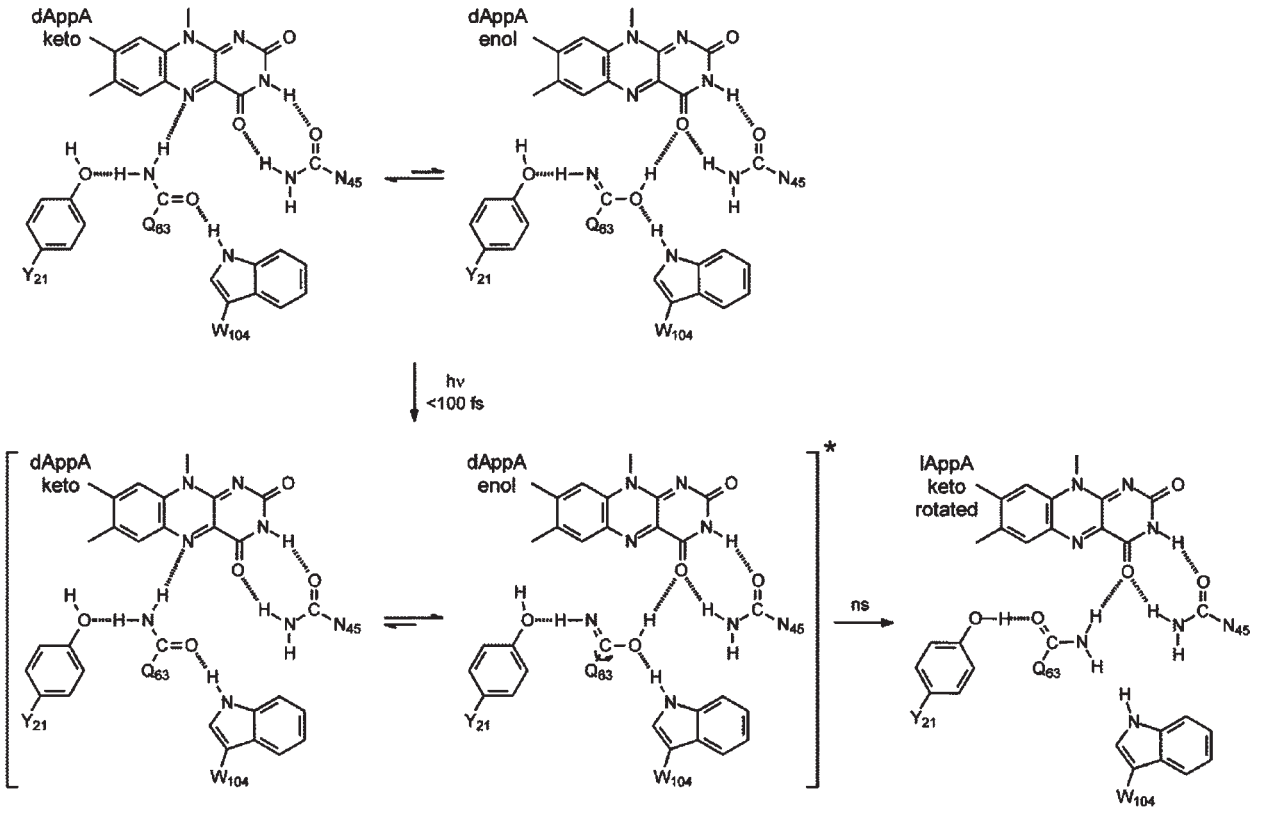

Figure 11. Proposed model for Formation of lAppA from dAppA. This model encapsulates both proposals for the conversion of dAppA to lAppA in Figure 1. In the ground state of dAppA, the Q63 side chain exists as an equilibrium mixture of keto and enol tautomers. In the enol tautomer, there is a hydrogen bond between Q63 and the flavin C4═O by analogy to the structure presented in Figure 10 for Q63E. However, as expected for an amide group, the position of the tautomeric equilibrium strongly favors the keto form, which is shown schematically by an equilibrium symbol with unequal half-arrows. Upon photoexcitation, there is an increase in electron density on the C4═O, which will increase the proportion of the enol tautomer, again shown by an equilibrium symbol with unequal half-arrows. We then propose that this enol tautomer leads to the lAppA ground state in which Q63 has rotated so that the bond to W104 has been broken.

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has rotated. We are currently investigating this model with TRIR spectroscopy and other photoactive and inactive mutants of AppA.

CONCLUSION

The mechanism of AppA photoactivation involves the reorganization of a hydrogen-bonding network that connects the chromophore to the protein. Q63 is a critical component of this network, and to improve our understanding of the role of this residue in photoactivation we replaced Q63 with a glutamate. While the Q63E $AppA_{BLUF}$ is not photoactive, steady-state and ultrafast spectroscopy indicates that this mutation has trapped the protein in a state that is intermediate between the dark and photoactivated states of $AppA_{BLUF}$ in which the flavin C4═O is more strongly hydrogen bonded than in $dAppA_{BLUF}$. Importantly, a new vibrational band is observed in the TRIR spectra of Q63E $AppA_{BLUF}$, which is assigned to the protonated carboxylic acid side chain of Q63E. This mode appears instantaneously upon photoexcitation, indicating that the carboxyl side chain is hydrogen bonded directly to the chromophore. We propose that Q63E is hydrogen bonded to the flavin C4═O and that excitation of the flavin leads to an increase in electron density on the C4═O oxygen, which strengthens the interaction with Q63E by ~3 kcal/mol. These data provide conclusive evidence that the hydrogen-bond network in the protein environment responds on the ultrafast time scale to light absorption by the flavin chromophore. Thus, independent of any other driving force, electronic excitation of the flavin influences the strength of H-bonds in the network linking the critical Q63 residue to the flavin.

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