Light-Induced Structural Changes of Apoprotein and Chromophore in the Sensor of Blue Light Using FAD (BLUF) Domain of AppA for a Signaling State†

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ABSTRACT: AppA is a new class blue-light receptor controlling photosynthesis gene expression in the purple bacterium *Rhodobacter sphaeroides* and retains a characteristic flavin adenine dinucleotide (FAD)-binding domain named the "sensor of blue light using FAD" (BLUF). AppA functions as an antirepressor controlling transcription of photosynthesis genes through the direct association with a transcriptional repressor PpsR in a blue-light-dependent manner [Masuda and Bauer (2002) *Cell 110,* 613−623]. Illumination of AppA induces a red shift in the UV−visible absorption of FAD, which results in a signaling state of AppA. Light-induced Fourier transform infrared (FTIR) difference spectrum of the AppA BLUF domain showed relatively simple features, which were mainly composed of two sets of derivative-shaped sharp bands at 1709(−)/1695(+) and 1632(+)/1619(−) cm^{-1}. We have developed an *in vitro* reconstitution method, by which a fully functional BLUF domain was reconstituted from free FAD and an apoprotein for the BLUF domain of AppA. An AppA BLUF domain that consisted of an apoprotein isotopically labeled with ^{13}C and unlabeled FAD was constituted using this method, and hydrated and deuterated samples were applied to FTIR spectroscopic analyses. When the spectra for the reconstituted domain were compared with those for uniformly ^{15}N- and ^{13}C-labeled or deuterated domains as well as for the unlabeled domain, the IR bands responsible for the light-induced changes in the FAD chromophore and apoprotein were identified. Unexpectedly, the light-induced spectrum of the unlabeled BLUF domain of AppA was predominantly composed of multiple apoprotein bands, while a C(4)═O stretching of an isoalloxazine ring was the only band exclusively assigned to FAD. The results showed that relatively large structural changes occur in the protein backbone of the BLUF domain of AppA upon illumination. These changes were discussed in relation to the mechanistic role of the BLUF domain in the process of blue-light perception by AppA.

AppA is a novel blue-light receptor with flavin adenine dinucleotide (FAD)[1] as a chromophore and regulates photosynthesis gene expression in response to blue light in the purple bacterium *Rhodobacter sphaeroides* (reviewed in ref *1*). AppA interacts with a transcriptional repressor PpsR to form a stable AppA−$(PpsR)_2$ complex in the dark or low-light conditions (*2*). Blue-light-excited AppA is unable to associate with PpsR and therefore allows PpsR to bind to the promoter regions of most photosynthesis genes in *R. sphaeroides* (*2*, *3*). AppA undergoes a photocycle reaction highlighted by an approximately 10-nm red shift in the ultraviolet (UV)−visible absorption of FAD upon illumination (*2*). The light-induced spectral red shift decays back to the ground state with a half-decay time of approximately 10 min (*2*). After illumination, the antirepressor activity of AppA was restored in accordance with the recovery of the dark-state spectrum (*2*), suggesting that the light-induced absorption change in FAD is closely correlated with the state for blue-light signaling in AppA. The N-terminal FAD-binding domain of AppA exhibits the same UV−visible absorption change as that in the full-length AppA (*4*, *5*). Blue-light-induced conformational and/or dynamic changes occurring in the N-terminal FAD-binding domain have been presumed to be responsible for controlling accessibility of PpsR to the central region of AppA (amino acid residues 210−380) (*4*), in which there exists an amino acid sequence that is similar to that found in another PpsR-interacting protein, AerR (*6*). In fact, it has been shown that the N-terminal domain can transmit the light signal via a truncated C-terminal domain even when they were expressed as separate domains in *R. sphaeroides* (*7*). This may suggest that the N-terminal FAD-binding domain is fully modular, and a direct interaction between these two domains is involved in the process of blue-light perception in AppA. However, there has been no evidence for the blue-light-induced structural changes in the N-terminal FAD-binding domain as well as the full-length AppA protein. Furthermore, the reported light-induced Fourier transform infrared (FTIR) difference spectrum of the

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[1] Abbreviations: BLUF, sensor of blue light using FAD; FAD, flavin adenine dinucleotide; FTIR, Fourier transform infrared; IR, infrared; LOV, light oxygen voltage; PCR, polymerase chain reaction; UV, ultraviolet.

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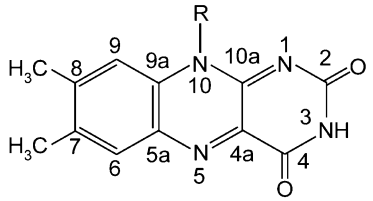
N-terminal FAD-binding domain of AppA showed few characteristic features with the exception of a pair of prominent derivative shape bands presumably because of C(4)═O of the isoalloxazine ring of FAD, suggesting almost no light-induced structural changes in this domain (*5*).



Isoalloxazine ring

Computer-aided similarity searches have indicated that the FAD-binding domain of AppA is present in various photosynthetic and nonphotosynthetic organisms (*1*, *8*). These proteins retain a homologous domain for FAD binding but show no homology outside of the FAD-binding domain, suggesting that this domain constitutes a new blue-light "input" domain used to control different cellular processes in response to blue light. The proteins retaining this class of flavin-binding domains have been proposed to be designated as "BLUF" proteins, standing for "sensor of blue light using FAD" (*8*). To date, three BLUF proteins other than AppA have been characterized, which are PAC in the green alga *Euglena gracilis* (*9*), Slr1694 in the cyanobacterium *Synechocystis* sp. PCC 6803 (*10*−*14*), and YcgF in *Escherichia coli* (*15*, *16*). PAC is involved in photoavoidance behavior, which occurs through blue-light-dependent activation of an intramolecular adenylyl cyclase (*9*), and Slr1694 is necessary for normal phototaxis behavior (*10*, *14*). The physiological function of YcgF is not clear, but the protein contains an EAL domain suggested to encode enzymatic activity for hydrolysis of the second messenger bis-3′-5′-cyclic diguanylate (c-di-GMP) (*17*−*19*), indicating that YcgF functions as a blue-light receptor modulating the cellular levels of this unusual cyclic nucleotide (*16*). All of the BLUF proteins showed red-shifted UV−visible spectral changes upon illumination so far tested (*4*, *11*, *16*), suggesting that the AppA-like photocycle reaction is characteristic to all BLUF proteins.

Our recent FTIR analyses of Slr1694 demonstrated that light excitation weakened C(4)═O and C(2)═O bonding and strengthened N1C10a and/or C4aN5 bonding of the isoalloxazine ring of bound FAD, concomitant with considerable changes in the protein structure (*11*, *12*). In Slr1694, it was indicated that the strengthen hydrogen bonding between amino acid(s) and the flavin C(4)═O group is mainly responsible for the red shift in the UV−visible absorption and the structural changes in the protein moiety are important for the formation of a light-signaling state (*12*). An NMR study on the BLUF domain of AppA suggested a light-dependent alternation of the $\pi-\pi$ stacking interaction between the flavin ring and Tyr21, which is conserved in all identified BLUF proteins (*4*). This change in the stacking interaction may be responsible for the light-induced changes of some skeletal modes of the isoalloxazine ring detected by FTIR in Slr1694 (*11*).

In the present study, we measured the light-induced FTIR spectra of samples of uniformly ^{15}N- and ^{13}C-labeled, deuterated, or unlabeled BLUF domains of AppA to elucidate the role of the BLUF domain in the light perception process conducting by AppA. In particular, we have newly developed an *in vitro* reconstitution method, by which a fully active BLUF domain of AppA is successfully constituted from FAD and an apoprotein. By selective isotope labeling of the apoprotein, we were able to identify the infrared (IR) bands corresponding to the light-induced changes of FAD and the apoprotein from the light-induced FTIR spectrum. The results clearly showed that a large part of the light-induced FTIR spectrum was attributable to bands from the apoprotein with the exception of the flavin C(4)═O stretching mode.

MATERIALS AND METHODS

Construction of Expression Plasmid and Protein Purification. The expression plasmid for the BLUF domain of AppA (AppA126) was obtained as follows. First, the region corresponding to residues 1−126 of AppA was amplified by polymerase chain reaction (PCR) using up- and downstream oligonucleotide primers pET28-F (*4*) and AppA126-R, 5′-GGGAATTCTCAGATCTGCCGGCTCTCGGC-3′, respectively. An isolating pETAppA156 plasmid (*4*) was used as a PCR template for plasmid construction. The PCR-amplified fragment containing an *Nde*I restriction site at the start codon and an *Eco*RI site downstream of the stop codon (underlined) was digested with *Nde*I and *Eco*RI and then cloned into the *Nde*I−*Eco*RI restriction sites of pTYB12 (New England Biolabs) to generate the recombinant plasmid pTYAppA126. The obtained plasmid was transformed into the *E. coli* strain BL21(DE3) (Novagen), and the protein with a self-cleaved intein/chitin tag (New England Biolabs) was overexpressed by induction with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 18 °C for over 16 h in an M9 minimum medium. For uniform isotope labeling, the culture was grown using 18 mM $^{15}NH_4Cl$ (99.3% ^{15}N enrichment, Shoko Tsusho) for $^{15}NH_4Cl$ labeling or 11 mM glucose-U-^{13}C (98.3% ^{13}C enrichment, Shoko Tsusho) for ^{13}C labeling. Harvested cells suspended in a medium containing 0.5 M NaCl, 0.2 mM EDTA, and 20 mM Tris-HCl (pH 8.0) were ruptured with a chilled Bead-Beater (Biospec Products) for 2 min, and the soluble fraction was collected after centrifugation at 28000*g* for 30 min. The AppA126 protein was purified with a chitin-affinity column using the protocol described by the manufacturer (New England Biolabs). The purified protein was dialyzed against 0.3 M NH_4HCO_3 (pH 7.9), lyophilized, and then stored at −20 °C until use. No property change was observed during the storage. The purity of the proteins was estimated to be greater than 98% based on the densitometric intensity of the Coomassie-stained band of an SDS−PAGE gel (data not shown).

In Vitro Reconstitution of the BLUF Domain. The purified AppA126 was dissolved in PBS medium (10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1.8 mM KCl, and 140 mM NaCl at pH 7.3) supplemented with 6 M guanidine hydrochloride (WAKO) at a concentration of 30 μM protein and was dialyzed overnight at 4 °C against the same medium for the protein to be denatured and to completely release the bound FAD. For the reconstitution process, FAD disodium salt (Sigma) was added to the denatured apoproteins at a final concentration of 30 mM and then dialyzed against PBS medium supplemented with 3 M guanidine hydrochloride, 30 μM FAD, and 0.1 M arginine hydrochloride (Wako) for

6 h at 4 °C. The sample was further dialyzed against PBS medium supplemented with 1.5 M guanidine hydrochloride, 30 μM FAD, and 50 mM arginine hydrochloride for 6 h at 4 °C and then finally dialyzed against PBS medium overnight at 4 °C. The dialyzed samples were centrifuged at 18000*g* for 20 min to remove nonreconstituted denatured proteins. The reconstituted AppA BLUF domain in the supernatant was concentrated, and the solvent medium was replaced with a buffer medium containing 50 mM Tris-HCl and 1 mM NaCl (pH 8.0) by repeated concentration and dilution using Microcon YM-30 centrifugal filter devices (Millipore). The samples were finally concentrated to approximately 0.3 mM protein.

Spectroscopic Measurements. The lyophilized native AppA126 protein was dissolved in a H_2O (D_2O) medium containing 50 mM Tris-HCl (DCl) and 1 mM NaCl [pH (pD) 8.0] at a concentration of 0.3 mM protein and was incubated for 1 h at 4 °C. The sample solution (~5 μL) was applied to a BaF_2 disk and gently dried under N_2 gas for several minutes. For deuteration of the reconstituted AppA126, the sample solution (~5 μL) was applied to a BaF_2 disk and dried. The dried sample was dissolved in 10 μL of a D_2O, and the sample was dried again under N_2 gas. This process was repeated 4 times. No property change was detected in the native and reconstituted AppA126 during this deuteration process. A 1-μL droplet of 40% glycerol/H_2O (D_2O) (v/v) solution was placed outside the IR beam path on the BaF_2 disk for hydration (deuteration). The sample was subsequently sandwiched by the disks and was spaced with a 0.5-mm-thick Teflon spacer. The IR sample was incubated at 15 °C for 3 h in the dark to equilibrate the water present in the sample as described in a previous report (*11*). FTIR spectra were recorded using a Bruker IFS66v/s spectrophotometer with a MCT detector at 15 °C and 4 cm^{-1} resolution by averaging 32 scans (20 s accumulation). Ge band-pass filters (4000−800 cm^{-1}) (OCLI) were placed at the inlet and outlet holes of the measuring IR beam on a cryostat to improve the signal-to-noise ratio. The sample temperature was maintained using a homemade cryostat as described (*20*). A light-minus-dark spectrum was obtained by subtracting the single-beam dark spectrum from that acquired following illumination for 10 s with continuous light (350−550 nm) as described previously (*11*). Two to four spectra were collected by repeating this cycle with a ~2−3.5 h dark interval, during which the sample fully relaxed to its dark state. UV−visible absorption spectra of FTIR samples sandwiched between BaF_2 disks were recorded on a Shimadzu MultiSpec-1500 photodiode array spectrophotometer at 15 °C as described previously (*11*).

RESULTS

Reconstitution of the BLUF Domain with FAD Chromophore and Apoprotein. Figure 1A shows the light-induced changes in the UV−visible absorption of the native (a) and reconstituted (b) BLUF domain of AppA (AppA126). The proteins exhibit identical dark-adapted spectra with two broad flavin peaks centered at 370 and 443 nm and distinct shoulders at 355 and 466 nm (Figure 1A, black lines). Notably, the apoprotein before FAD reconstitution had no color (data not shown). Upon light excitation, the spectra of both proteins showed the same distinct red shift in the flavin peaks, resulting in new peaks at 378 and 456 nm with shoulders at 363 and 480 nm (Figure 1A, red lines). These features are largely compatible with those of full-length

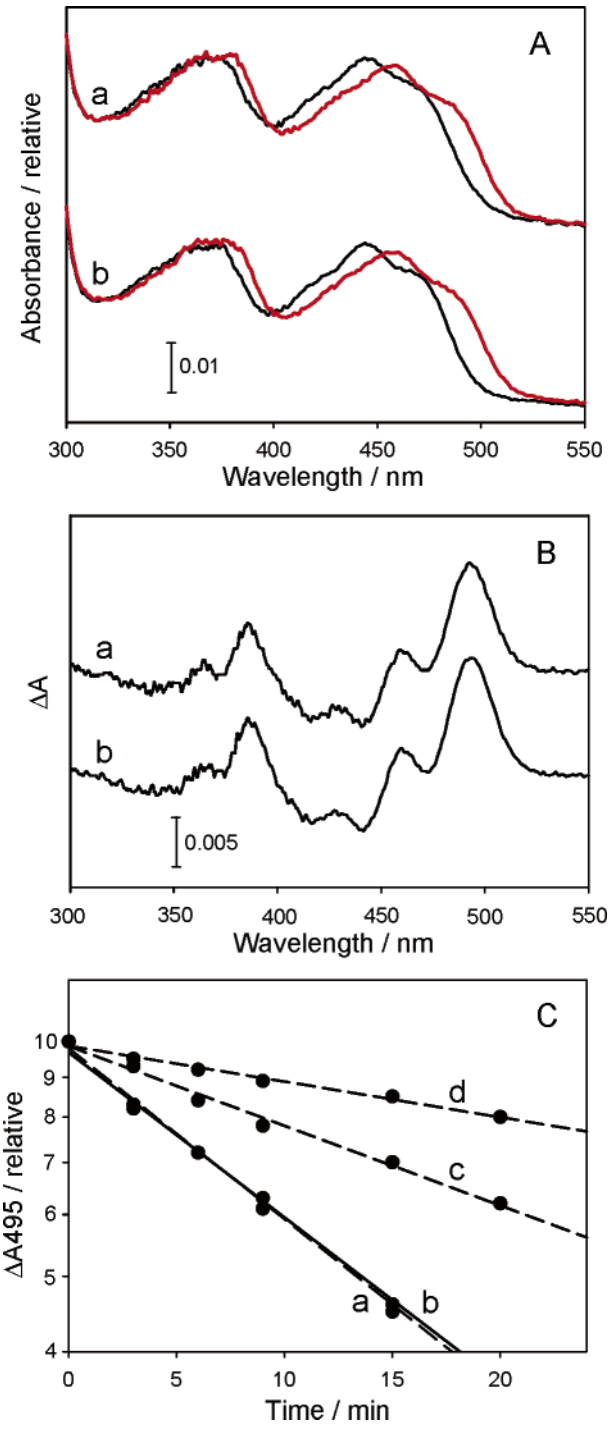


FIGURE 1: (A) UV−visible absorption spectra of the dark-adapted (black lines) and light-induced (red lines) native (a) and reconstituted (b) AppA BLUF domain (AppA126). (B) Light-minus-dark difference spectra of native (a) and reconstituted (b) AppA126. AppA126 samples in a H_2O medium containing 50 mM Tris-HCl and 1 mM NaCl (pH 8.0) were sandwiched between BaF_2 disks as described in the Materials and Methods. (C) Decay kinetics of the light-induced UV−visible absorption changes (recorded at 495 nm) in native (- - -) and reconstituted (—) AppA126. AppA126 was dissolved in a H_2O medium containing 50 mM Tris-HCl and 1 mM NaCl (pH 8.0) (a and b) or D_2O medium containing 50 mM Tris-DCl and 1 mM NaCl (pD 8.0) (c). Alternatively, AppA126 samples in a H_2O medium containing 50 mM Tris-HCl and 1 mM NaCl (pH 8.0) were sandwiched between BaF_2 disks as described in the Materials and Methods (d).

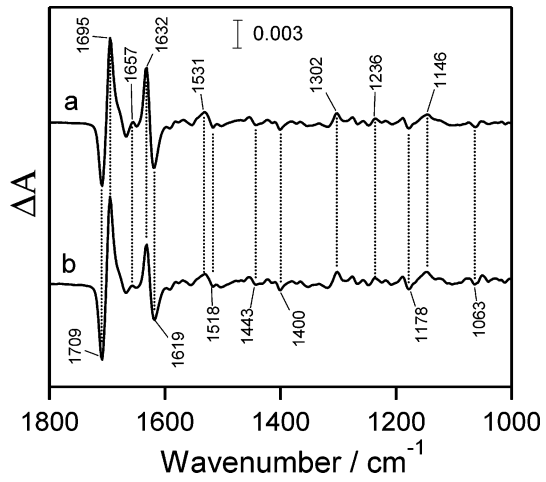


FIGURE 2: Light-minus-dark FTIR difference spectra of native (a) and reconstituted (b) AppA BLUF domain (AppA126). Spectra of hydrated samples are shown. The sample temperature was 15 °C. Two to four spectra were averaged to obtain the final data.

AppA and of a previously reported C-terminal truncated AppA (AppA156) (*2*, *4*), indicating that the photocycle proceeds equivalently in the BLUF domain in these three types of AppA proteins. As shown in Figure 1B, the light-minus-dark difference spectra of the native (a) and reconstituted (b) AppA126 are almost indistinguishable from each other. Furthermore, during the dark interval, the light-induced spectra of the native and reconstituted AppA126 decayed with very similar kinetics to dark-state spectra that were identical to those obtained before illumination (Figure 1C). We note in this context that the spectra presented in Figure 1 were for samples sandwiched between the BaF_2 disks required for FTIR measurement, but identical dark-adapted and light-induced spectra were observed in solution for both native and reconstituted AppA126, although the dark-decay kinetics of the BaF_2 disk sample ($t_{1/2} \sim 40$ min) were much slower than those of the solution sample ($t_{1/2} \sim 10$ min) (Figure 1C), as reported for Slr1694 (*11*). Furthermore, the dark decay in the deuterated AppA126 was approximately 2 times slower than in the hydrated AppA126 (Figure 1C).

Figure 2 shows the light-minus-dark FTIR difference spectra of the native (a) and reconstituted (b) AppA126. The positive and negative bands are attributed to the light-induced and dark states, respectively. It is clearly seen that, upon illumination, the same spectral changes were induced for the native and reconstituted AppA126, indicating that light-induced structural changes in the FAD chromophore and/or apoprotein of the reconstituted AppA126 are identical with those in the native proteins. Eventually, identical spectra were induced by illuminating the samples with higher water content, although the spectral quality became comparatively low because of the relatively large water band (data not shown). The UV−visible and IR results strongly indicate that AppA126 is functionally and structurally reconstituted.

Light-Induced FTIR Spectra in the Native BLUF Domain. Figure 3 shows the light-minus-dark FTIR difference spectra of unlabeled (a, black line; b−d, black dotted lines), uniformly ^{15}N-labeled (b, red line), uniformly ^{13}C-labeled (c, blue line), and deuterated (d, green line) AppA126. As the red-shifted UV−visible spectrum of AppA is correlated with its signaling state (*2*), the light-induced FTIR spectra are attributable to structural changes of AppA126 responsible for the formation of the signaling state. The most notable characteristics of the light-induced FTIR spectrum of unlabeled AppA126 are two prominent differential bands at 1709(−)/1695(+) and 1632(+)/1619(−) cm^{-1}. In addition, many bands appear at 1600−1000 cm^{-1} but with much smaller intensities. Because many skeleton vibrations of the FAD isoalloxazine ring have been observed at 1500−1000 cm^{-1} with intensities comparable to those at 1750−1500 cm^{-1} (*21*, *22*), the small IR bands below 1500 cm^{-1} could partly result from band shifts that are much smaller than their line widths and/or very small changes in the IR intensity of FAD. The present results indicate that no gross structural changes are induced in the FAD isoalloxazine ring upon illumination. The minimal light-induced change in the skeletal structure of the FAD isoalloxazine ring has been similarly observed in another BLUF protein, Slr1694, of the cyanobacterium *Synechocystis* sp. PCC 6803 (*11*−*13*), although the light-minus-dark FTIR difference spectrum of Slr1694 differs considerably from the present AppA126 spectrum in some aspects.

Upon uniform ^{13}C labeling (c, blue line), the major 1709(−)/1695(+) and 1632(+)/1619(−) cm^{-1} bands as well as the minor 1668(−)/1657(+)/1649(−) cm^{-1} bands observed in the unlabeled spectrum were coordinately downshifted by 39−42 cm^{-1}, a shift which is consistent with that of a C═O stretching mode, indicating that these bands can be ascribed to a C═O stretch. Notably, upon uniform ^{15}N labeling (b, red line), the 1709(−)/1695(+) and 1668(−)/1657(+)/1649(−) cm^{-1} bands but not the 1632(+)/1619(−) cm^{-1} bands were slightly downshifted by 2−6 cm^{-1}. Therefore, the downshifted bands can be ascribed to C═O stretches that include minor contributions from groups containing nitrogen atoms, but the nonshifted 1632(+)/1619(−) cm^{-1} bands must be assigned to isolated C═O stretching. The shoulder at 1679 cm^{-1} in the unlabeled spectrum is downshifted to 1630 cm^{-1} in the ^{13}C-labeled spectrum but similarly appeared in the ^{15}N-labeled spectrum at 1678 cm^{-1}. Therefore, the C═O stretch is responsible for this shoulder band. The bands below 1600 cm^{-1} were also affected by uniform ^{15}N or ^{13}C labeling, but manifestation of the effects is rather complicated because of extensively overlapped bands with low IR intensities. The bands at 1591−1400 cm^{-1} in the unlabeled spectrum were downshifted 10−20 cm^{-1} to 1547−1380 cm^{-1} with some changes in the band shape upon ^{13}C labeling (c, blue line) and were downshifted by 6−15 cm^{-1} to 1585−1396 cm^{-1} with little change in the band shape upon ^{15}N labeling (b, red line), indicating that these bands are ascribed to groups containing both carbon and nitrogen, with the exception of the 1454 cm^{-1} band, which is ascribed to a group containing only carbon because of its insensitivity to ^{15}N labeling. The bands at 1365−1319 and 1146−1000 cm^{-1} were downshifted upon ^{13}C labeling despite a complicate manner but were little affected upon ^{15}N labeling, indicating that these bands are attributable to groups containing carbon but not nitrogen. The bands at 1319−1178 cm^{-1} were downshifted by 10−21 cm^{-1} upon ^{15}N or ^{13}C labeling, indicating that the bands refer to groups containing both carbon and nitrogen.

As shown in Figure 3d (green line), several bands in the unlabeled/hydrated AppA spectrum (a) were affected upon deuteration. These deuteration-sensitive bands can be attributed to NH and OH groups with an exchangeable

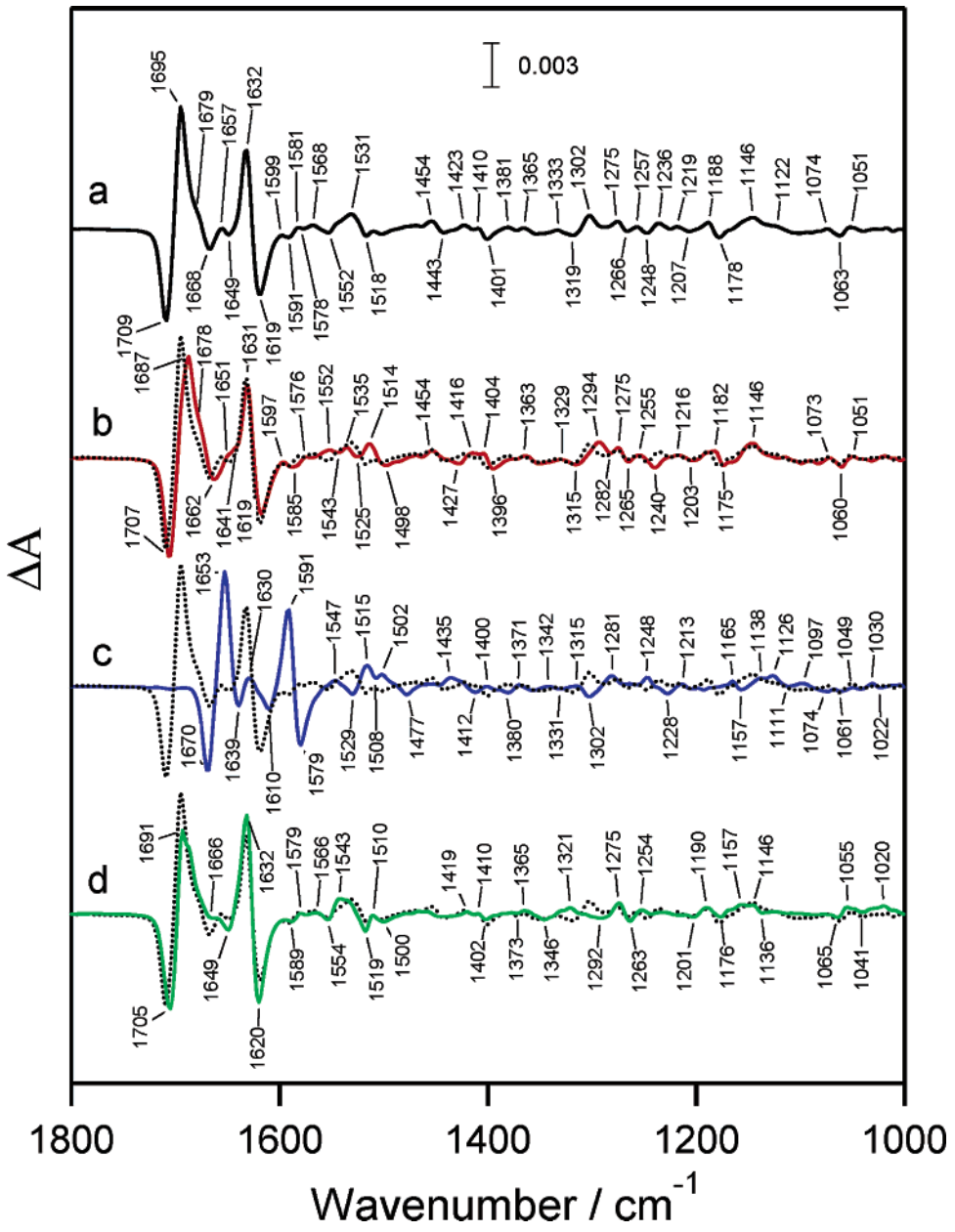


FIGURE 3: Effects of isotope labeling and deuteration on light-minus-dark FTIR difference spectra of the native AppA BLUF domain (AppA126). Unlabeled (a, black line ; b and c, black dotted line), uniformly ^{15}N-labeled (b, red line), uniformly ^{13}C-labeled (c, blue line), and deuterated (d, green line) spectra. The sample temperature was 15 °C. Two to four spectra were averaged to obtain the final data.

hydrogen atom. The prominent C═O bands at 1709(−)/1695(+) cm^{-1} in the unlabeled/hydrated spectrum were downshifted by 4 cm^{-1} to 1705(−)/1691(+) cm^{-1} upon deuteration. Considering the downshift of these bands upon ^{15}N or ^{13}C labeling, the 1709(−)/1695(+) cm^{-1} bands can be assigned to a C═O stretch that is coupled with an NH bending mode. The 1632(+)/1619(−) cm^{-1} bands were not affected by deuteration, which is consistent with the assignment of the bands to a C═O stretch without an NH bending contribution. The bands at 1600−1350 cm^{-1} were not greatly affected by deuteration with the exception of an upshift in the 1531(+)/1518(−) cm^{-1} bands to 1543(+)/1519(−) cm^{-1} and the disappearance of the 1454(+) and 1401(−) cm^{-1} bands. The amide II bands, which are a mixture of the CN stretches and NH bending vibrations of the protein backbone, appear at 1600−1500 or ∼1460 cm^{-1} upon deuteration (*23*, *24*). Therefore, little spectral change in these regions upon deuteration suggests that the amide II band did not contribute the obtained spectrum. Possible candidates for the bands at 1600−1350 cm^{-1} are CN stretches that are not coupled with an NH bending mode, although overlap of the CC stretch and CH_3 deformation bands cannot be excluded at 1500−1400 cm^{-1}. The bands at 1200−1000 cm^{-1} were insensitive to deuteration and ^{15}N labeling, suggesting that these are CC stretches, CH_3 rocking modes, and/or C—CH_3 stretches. The bands at 1319(−)/1302(+) and 1248(−)/1236(+) cm^{-1} were downshifted by 4−20 and 17−23 cm^{-1} upon ^{15}N and ^{13}C labeling, respectively, and were upshifted to 1346(−)/1321(+) and 1254(+)/1263(−) cm^{-1}, respectively, upon deuteration. These bands may be attributed to CC and/or CN stretches that are strongly coupled with NH bending.

Light-Induced FTIR Spectra in the Reconstituted BLUF Domain. Figure 4 shows the light-minus-dark FTIR difference spectra of hydrated (black lines) and deuterated (green

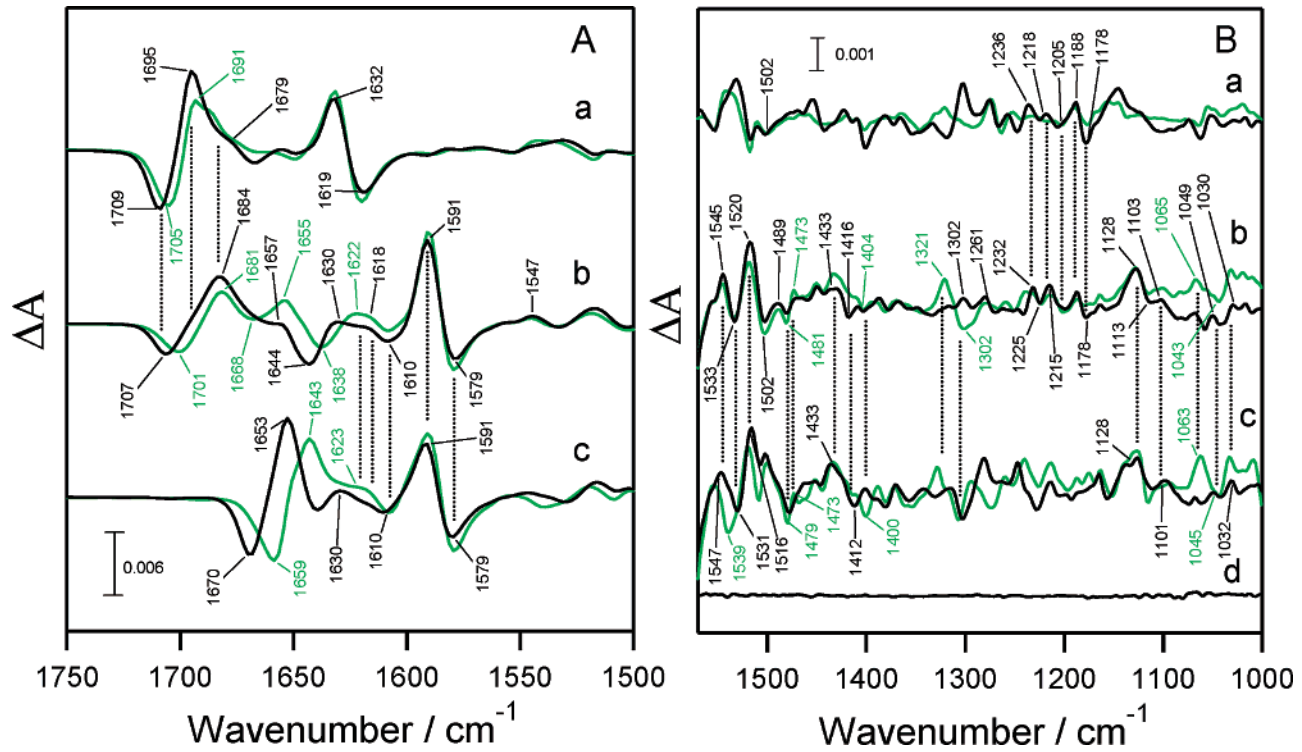


FIGURE 4: Light-minus-dark FTIR difference spectra of native (a and c) and reconstituted (b) AppA BLUF domain (AppA126) at 1750−1500 (A) and 1550−1000 (B) cm^{-1}. Hydrated (black lines) and deuterated (green lines) AppA BLUF domains that were unlabeled (^{12}C-FAD/^{12}C-apoprotein) (a), reconstituted with unlabeled FAD and uniformly ^{13}C-labeled apoprotein (^{12}C-FAD/^{13}C-apoprotein) (b), or uniformly ^{13}C-labeled (^{13}C-FAD/^{13}C-apoprotein) (c). Dark-minus-dark spectrum (d) shows a signal-to-noise level. The sample temperature was 15 °C. Two to four spectra were averaged to obtain the final data.

lines) AppA126, which were unlabeled (^{12}C-FAD/^{12}C-apo) (a), reconstituted from unlabeled FAD and uniformly ^{13}C-labeled apoprotein (^{12}C-FAD/^{13}C-apo) (b), and uniformly ^{13}C-labeled (^{13}C-FAD/^{13}C-apo) (c). Notably, the reconstituted BLUF domain showed identical light-induced UV−visible and FTIR difference spectra to those of the native BLUF domain as shown in Figure 2. Bands that are similarly induced in the ^{12}C-FAD/^{12}C-apo (a) and ^{12}C-FAD/^{13}C-apo (b) spectra arise from light-induced changes in FAD, whereas bands that are similarly induced in the ^{12}C-FAD/^{13}C-apo (b) and ^{13}C-FAD/^{13}C-apo (c) spectra arise from light-induced changes in the apoprotein. Figure 4A showed spectra in the 1750−1500 cm^{-1} region. The features of the reconstituted ^{12}C-FAD/^{13}C-apo spectra (b) at 1720−1680 cm^{-1} are similar to those of the ^{12}C-FAD/^{12}C-apo spectra (a), indicating that vibrations of FAD contribute to the bands in this region. Because the hydrated (black line) and deuterated (green line) ^{13}C-FAD/^{13}C-apo spectra (c) show no bands above 1680 and 1675 cm^{-1}, respectively, the 1707(−)/1684(+) (hydrated) and 1701(−)/1681(+) cm^{-1} (deuterated) bands in the reconstituted ^{12}C-FAD/^{13}C-apo spectra (b) can be assigned to C═O stretches of the FAD isoalloxazine ring of ^{12}C-FAD. Bands in the reconstituted ^{12}C-FAD/^{13}C-apo spectra (b) observed in the range of approximately 1675−1640 (hydrated) or 1665−1630 cm^{-1} (deuterated) are the consequence of overlap of both FAD and apoprotein bands, and these were unable to be isolated.

The ^{13}C-FAD/^{13}C-apo spectra (c) and reconstituted ^{12}C-FAD/^{13}C-apo spectra (b) markedly resemble each other in the region of 1630−1550 cm^{-1}. Both spectra show bands at 1630(+), 1610(−), 1591(+), and 1579(−) cm^{-1} with similar intensities, indicating that the vibrations of the apoprotein are exclusively responsible for these bands. It is rational to consider that the deuteration insensitive 1632(+)/1619(−) cm^{-1} bands in the unlabeled spectra downshifted to 1591(+)/1579(−) cm^{-1} upon ^{13}C labeling. We may conclude that light-induced changes of the ^{12}C apoprotein of the BLUF domain are responsible for the 1632(+)/1619(−) cm^{-1} bands, although the 1630(+) and 1610(−) cm^{-1} ^{13}C-apoprotein bands were not resolved in the unlabeled spectra.

Figure 4B shows spectra in the 1550−1000 cm^{-1} region, in which assignments of FAD and apoprotein bands based on comparison among the reconstituted, unlabeled and uniformly ^{13}C-labeled spectra are rather obscure because of massive overlap of small bands. Nevertheless, several bands in the 1550−1520 and 1150−1100 cm^{-1} regions were similarly induced in the reconstituted ^{12}C-FAD/^{13}C-apo spectra (b) and ^{13}C-labeled (^{13}C-FAD/^{13}C-apo) spectra (c), suggesting that these bands can be mainly ascribed to vibrations of the apoprotein. However, bands in the 1240−1170 cm^{-1} region were similarly induced in the reconstituted ^{12}C-FAD/^{13}C-apo (b) and unlabeled (^{12}C-FAD/^{12}C-apo) (a) spectra, suggesting that these bands are attributable to vibrations of FAD.

Spectra Simulation with FAD and Apoprotein Bands. It is apparent that the positive peak positions for the FAD vibration in the hydrated (1695 cm^{-1}) and deuterated (1691 cm^{-1}) spectra of unlabeled (^{12}C-FAD/^{12}C-apo) AppA126 (Figure 4A, a) are considerably different from those (1684 and 1681 cm^{-1} for hydrated and deuterated spectum, respectively) of the ^{12}C-FAD bands in the ^{12}C-FAD/^{13}C-apo spectra (Figure 4A, b) in addition to the difference in intensity. These indicate that some protein bands overlap with the C═O stretch band of ^{12}C-FAD to form the composite

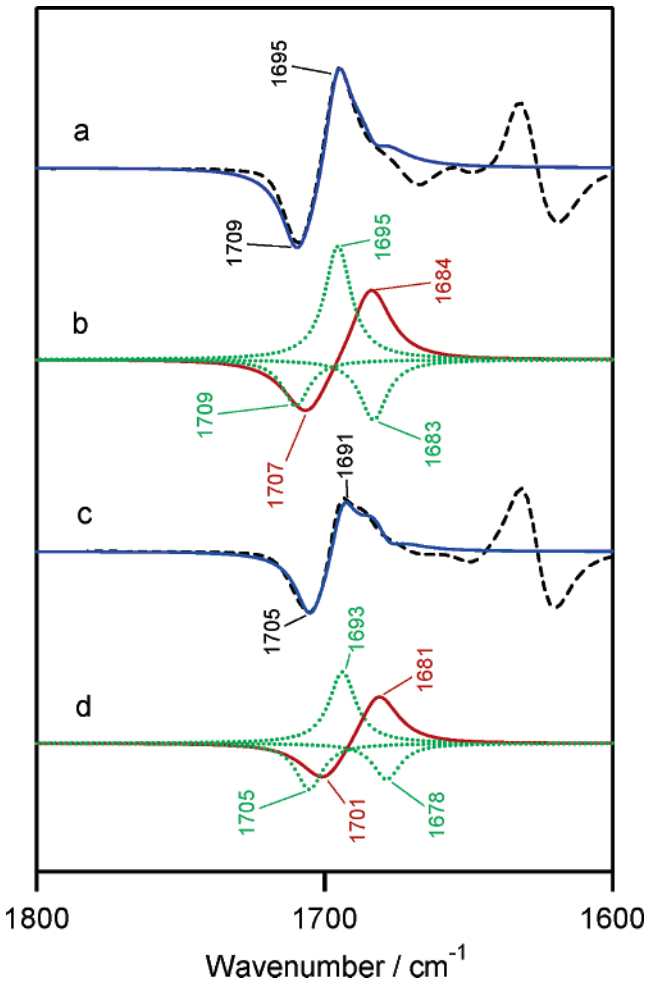


FIGURE 5: Simulation of the light-induced FTIR difference spectra of AppA BLUF domain (AppA126). Experimentally obtained (black dashed lines) and simulated (blue solid lines) spectra of hydrated (a) and deuterated (c) AppA126. The spectra were simulated by assuming bands from the C4═O stretch of the FAD isoalloxazine ring (red solid lines) and from the apoprotein (green dotted lines) under hydrated (b) and deuterated (d) conditions. See the text for other details.

1695(+) cm^{-1} band observed in the unlabeled hydrated spectrum. To assess the overlapped protein bands, the observed hydrated and deuterated bands for the FAD vibration of unlabeled AppA were simulated by the use of isolated ^{12}C-FAD bands from the reconstituted ^{12}C-FAD/^{13}C-apo spectra (Figure 4A, b) and assumed protein bands as shown in Figure 5. The observed bands in the hydrated spectra (a, dotted line) were well-reproduced by the hydrated ^{12}C-FAD bands at 1707(−)/1684(+) cm^{-1} (b, red line) and three protein bands at 1709(−), 1695(+), and 1683(−) cm^{-1} (b, green dotted lines). The observed bands in the deuterated spectra (c, dotted line) were also well-reproduced by the observed deuterated ^{12}C-FAD bands at 1701(−)/1681(+) cm^{-1} (d, red line) and the three protein bands that were the same set of bands used for simulating the hydrated ^{12}C-FAD bands but assuming downshifts of 2−5 cm^{-1} upon deuteration (d, green dotted lines). Although the simulation study did not provide information on the relationship among these protein bands, a possible interpretation is that the negative bands at 1709 and 1683 cm^{-1} for the dark-state AppA126 were down- and upshifted to form the composite 1695(+) cm^{-1} band in the light-state AppA126. Presumably, these protein bands downshifted upon ^{13}C labeling to be responsible for the bands at 1668−1644 cm^{-1} in the hydrated ^{12}C-FAD/^{13}C-apo spectrum (Figure 4A, b, black line).

Table 1: Vibrational Frequencies of FTIR Bands in AppA Protein and Assignments

band positions (cm^{-1})		assignments
observed	simulated	origin (modes)
1709(−)[a] [−4][b]	1709(−) [−4]	apoprotein (amide I?)
	1707(−) [−6]	FAD (C4═O stretch)
1695(+)[a] [−4]	1695(+) [−2]	apoprotein (amide I)
	1684(+) [−3]	FAD (C4═O stretch)
	1683(−) [−5]	apoprotein (amide I?)
1632(+)[a] [0]		apoprotein (amide I)
1619(−)[a] [0]		apoprotein (amide I)
1630(+)[c] [−8]		apoprotein (amide I)
1618(+)[c] [0]		apoprotein (amide I)
1610(−)[c] [0]		apoprotein (amide I)

[a] Frequencies for hydrated and unlabeled AppA126. [b] Shifts upon deuteration. [c] Frequencies for reconstituted (^{12}C-FAD/^{13}C-apoprotein) AppA126.

DISCUSSION

Assignments of FTIR Bands. The results of analyses on the main bands observed in the light-minus-dark FTIR difference spectrum in the unlabeled AppA126 are summarized in Table 1, which includes assignments of the observed and simulated bands to FAD and the apoprotein and possible modes for the bands. The observed 1709(−)/1695(+) cm^{-1} band in the unlabeled hydrated spectrum consists of C═O stretch bands of FAD at 1707(−)/1684(+) cm^{-1} and apoprotein bands at 1709(−), 1695(+), and 1683(−) cm^{-1}. FAD has C═O groups at positions C2 and C4. The C4═O stretch of flavin is observed at 1725−1690 cm^{-1}, while the C2═O stretch appears at a lower frequency (1690−1650 cm^{-1}) because of intermolecular hydrogen bonding of the C2═O group with N3H (*21*, *25*, *26*). Deuteration of the N3H group of the isoalloxazine ring results in a larger downshift for the C2═O stretch (12−30 cm^{-1}) than for the C4═O stretch (1−13 cm^{-1}) (*21*, *27*, *28*). Because the 1707(−)/1684(+) cm^{-1} FAD bands were reasonably assumed to downshift by 3−6 cm^{-1} upon deuteration, these were assigned to the C4═O stretches of the FAD isoalloxazine ring. It is noteworthy that the positive 1684(+) cm^{-1} C(4)═O band was not resolved in the observed unlabeled hydrated spectrum and may be attributed to the positive shoulder at 1679 cm^{-1} (Figures 3a and 4A, a).

The 1695(+) cm^{-1} apoprotein bands (Figure 5b) are predominantly responsible for the observed band at 1695(+) cm^{-1} in the unlabeled hydrated spectrum (Figure 5a). On the basis of the shift of this band upon ^{13}C and ^{15}N labeling and upon deuteration as shown in Figure 3, these apoprotein bands are ascribed to a C═O stretch coupled with an NH bending mode. As such, possible candidates are the C═O stretches of Asn/Gln or amide I bands. Asn and Gln residues show ^{15}N-sensitive CN stretch bands at 1420−1400 cm^{-1} and NH_2 deformation bands at 1630−1600 cm^{-1} (*29*); however, only very small band intensities are observed at 1420−1400 cm^{-1}, and there are no ^{15}N-sensitive effects on the spectrum at 1630−1600 cm^{-1}. These results suggest that C═O stretches of the protein backbone (amide I band) are responsible for the 1695(+) cm^{-1} apoprotein band. On the other hand, the origin of apoprotein bands at 1709(−) and

1683(−) cm^{-1} was equivocal because these bands were introduced by the simulation study and were not visible in the experimental spectra.

The derivative shape 1591(+)/1579(−) cm^{-1} bands in the hydrated ^{12}C-FAD/^{13}C-apo spectrum (Figure 4A, b, black line) are predominantly ascribed to ^{13}C-apoprotein bands. Notably, the frequency difference between the positive and negative peaks of the 1591(+)/1579(−) cm^{-1} bands is 12 cm^{-1}, which coincides with that of the 1632(+)/1619(−) cm^{-1} bands in the unlabeled hydrated spectrum shown in Figure 4A, a (black line). Furthermore, these bands were insensitive to deuteration. Therefore, it is rational to consider that the 1632(+)/1619(−) cm^{-1} bands in the unlabeled spectrum can be predominantly assigned to apoprotein bands, which downshifted by 41 cm^{-1} upon ^{13}C labeling. Only a slight shift upon ^{15}N labeling clearly reveal that the bands can be ascribed to a C═O stretch. Because C═O stretch bands for amino acid side groups have not been observed at 1630−1620 cm^{-1} (*29*), the observed bands are assigned to the C═O stretch of the protein backbone, possibly because of β-sheet structures based on the position (*24*).

Light-Induced Structural Change of the AppA BLUF Domain. The present reconstitution results in combination with isotope labeling clearly demonstrate that marked structural changes are induced in the apoprotein of the AppA BLUF domain upon illumination, which was rather unexpected considering the relative simplicity of its light-induced FTIR difference spectrum even when compared with that of another BLUF protein, Slr1694 (*11*). On the basis of the vibrational properties of the observed bands from the apoprotein, we may characterize some notable aspects of the light-induced structural changes in the AppA apoprotein and FAD.

The position of the protein backbone bands at 1632(+)/1619(−) cm^{-1} in the unlabeled hydrated spectrum (Figure 4A, a) are in accordance with C═O stretches of a β-sheet structure (*24*). The light-induced upshift indicates that the putative C═O group in the BLUF domain of the dark-state AppA is hydrogen-bonded but that the bonding is weakened upon illumination. However, the limited effects of uniform ^{15}N labeling and deuteration on the 1632(+)/1619(−) cm^{-1} bands suggest that coupling between the C═O stretch and NH bending mode is very small unlike general amide I bands, which are usually downshifted upon deuteration or ^{15}N labeling because of the coupling of the NH bending mode (*23*). At present, it is not clear how this specific situation is achieved, but some possibilities are that an NH group strongly interacts with some other group and is decoupled from the C═O stretch or a C═O group locates in some specific environment and/or state, in which coupling between the C═O stretching and NH bending mode is very small. The specific ^{13}C labeling of the AppA apoprotein revealed that ^{13}C protein attributed to the 1630(+), 1618(+), and 1610(−) cm^{-1} bands. When the theoretical downshift of the C═O stretching frequency upon ^{13}C labeling is taken into account, these three bands are expected to exist at ∼1667(+), ∼1655(+), and ∼1646(−) cm^{-1} in the unlabeled spectrum, although they were not resolved as distinct bands because of the overlap with other bands. Apparently, the ^{13}C protein bands were downshifted upon deuteration as shown in Figure 4A, b, and the 1700−1640 cm^{-1} region in the unlabeled spectrum was affected by uniform ^{15}N labeling as shown in Figure 3b. Therefore, it is likely that these bands are caused by the changes in structure for general amide I of the protein backbone. The position (1709 cm^{-1}) of the negative protein band assigned to C═O stretching of the protein backbone for the dark-state AppA126 by the simulation study (Figure 5) was even higher than that for a free C═O, indicating that the putative C═O bond is constrained and thus shortened in the dark-state AppA BLUF domain and then becomes free or weakly hydrogen bonds in the light-induced state.

In contrast to the protein bands, C4═O bands of the isoalloxazine ring of FAD at 1707(−)/1684(+) cm^{-1} (Figures 4A and 5) were the only bands exclusively assignable to the FAD bands in AppA126, although we cannot completely exclude the possibility that other FAD bands exists but are unresolved because of the overlap with protein bands. Because the frequency of the C4═O band depends on the extent of hydrogen bonding (*21*, *27*, *28*), the results indicate that the C4═O bond of FAD is moderately hydrogen-bonded with other amino acid residues and/or the protein backbone in the dark state, and is strongly hydrogen-bonded in the light state. Of note is that the light-minus-dark FTIR difference spectrum had no prominent bands below 1500 cm^{-1}, where many skeletal bands of FAD appeared with comparable or higher intensities than the bands above 1500 cm^{-1} when the skeletal structure of the FAD isoalloxazine ring was changed as reported, through covalent association with other amino acid residues and/or the protein backbone in the phototropin light-oxygen-voltage (LOV) domain (*30*, *31*). The absence of prominent sketetal bands below 1500 cm^{-1} strongly indicates that the change in the skeletal structure in the FAD isoalloxazine ring does not occur in the AppA BLUF domain upon illumination. Furthermore, no deprotonation of an N3H group in the isoalloxazine ring in the dark- and light-signaling states was indicated by the definite downshift (3−4 cm^{-1}) of the C4═O bands upon deuteration because the downshift is caused by the decoupling of the C4═O stretching mode with N3H bending upon deuteration.

It has been proposed in a cyanobacterial BLUF protein Slr1694 that light excitation of FAD initiates a process that involves a proton transfer between amino acid residues and consequently strengthens hydrogen bonding to the C4═O group of the FAD isoalloxazine ring (*11*−*13*). This change is responsible for the light-induced change in the UV−visible spectrum and triggers other structural changes in FAD and apoprotein in Slr1694. Therefore, it may be conceivable that light-induced formation of strong hydrogen bonding at C4═O of the FAD isoalloxazine ring triggers structural changes in the protein backbone of the AppA BLUF domain with a similar mechanism to that proposed in Slr1694.

Light-induced FTIR difference spectrum for the BLUF domain of AppA was reported by Laan et al. (*5*). The reported spectrum completely lacked the protein bands and only showed prominent bands presumably for C4═O bands of FAD. At present, it is rather difficult to compromise the difference between the present and reported spectra because no isotope-labeling study required for the band assignment was conducted in ref *5*. However, it may be presumed that the light-induced structural changes in the apoprotein are sensitive to some sample treatment and were suppressed under the reported conditions for FTIR.

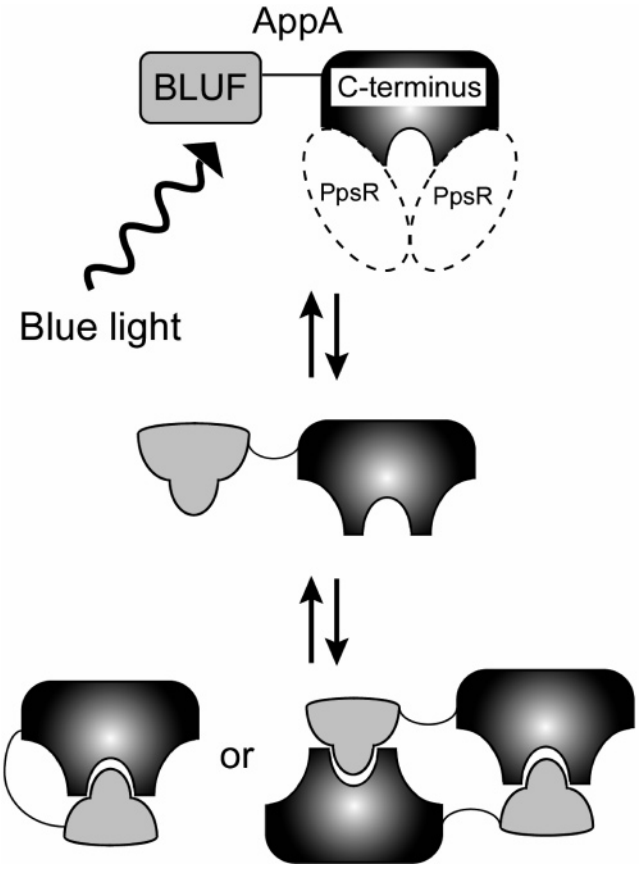


FIGURE 6: Model of the control of AppA−PpsR interaction by a light-induced structural change of the apoprotein in the AppA BLUF domain. The BLUF domain in the light-state structure is able to be bound to the C-terminal domain of AppA to prevent the binding of PpsR in a steric manner through intra- (left) or inter- (right) molecular interaction. See the text for other details.

Implication of Protein Structural Changes in Blue-Light Signaling. It may deserve special emphasis that the illumination induced marked structural changes in the apoprotein of the BLUF domain of AppA. We note in this context that a full-length BLUF protein Slr1694 of cyanobacterium *Synechocystis* sp. PCC 6803 also shows considerable light-induced structural changes in the protein backbone (*11*, *12*). Slr1694 shows a light-induced change in UV−visible absorption spectrum of FAD that is almost the same as that of AppA (*11*) and related to a phototactic behavior of this bacterium (*10*, *14*). Because Slr1694 is a small protein with no apparent functional domain without a BLUF domain, it is conceivable that the BLUF domain is directly responsible for the observed light-induced structural changes in full-length Slr1694. Therefore, it is likely that the BLUF domain is a module functioning as a transducer responsible for conversion of the light signal to the change in the protein structure for further signal transmission.

Several genetic and biochemical analyses have indicated that the AppA BLUF domain does not participate in the complex formation with PpsR to inhibit the repressor activity of PpsR under the dark conditions but is necessary for light-induced dissociation of the complex (*2*, *3*, *15*). Interestingly, the AppA BLUF domain and the C-terminal domain of AppA regulates the blue-light-dependent expression of the photosynthetic genes even when they were expressed as a separate domain in *R. sphaeroides*, of which the *appA* gene was genetically disrupted (*7*). The results strongly indicate that the light-state BLUF domain associates directly, through inter- or intramolecular interaction, with the corresponding C-terminal domain of AppA and consequently inhibits the association of the AppA C-terminal domain with PpsR. Therefore, it is rational to consider that the light-induced structural change of the AppA BLUF domain allow the domain to be bound to the C-terminal region. At present, it is not clear how the association leads to disjunction of the AppA−$(PpsR)_2$ complex. However, our unpublished preliminary result suggested that only a subtle structural change is induced in the AppA C-terminal region upon illumination. Therefore, a good possibility is that the binding of the BLUF domain to the C-terminal region simply interferes with the association of the C terminus with PpsR in a steric manner. This hypothesized mechanism was summarized and illustrated in Figure 6, in which the BLUF domain works as a signal transducer and photoreceptor module.

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