# Light Induced Structural Changes of a Full-length Protein and Its BLUF Domain in YcgF(Blrp), a Blue-Light Sensing Protein That Uses FAD (BLUF)†

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*Received September 9, 2005; Revised Manuscript Received January 24, 2006*

ABSTRACT: Blue-light sensing proteins that use FAD (BLUF) are members of a blue-light receptor family that is widely distributed among microorganisms. The *Escherichia coli* YcgF protein is a BLUF protein consisting of the *N*-terminal FAD-binding hold (BLUF domain) and the *C*-terminal EAL domain. The EAL domain of YcgF is predicted to have cyclic-di-GMP phosphodiesterase activity. Light-induced structural changes for the signaling state formation were studied using the light-induced Fourier transform infrared (FTIR) difference spectroscopy of both the full-length YcgF protein (YcgF-Full) and its BLUF domain (YcgF-BLUF). YcgF-Full and YcgF-BLUF showed identical UV−visible absorption spectra of flavin in the dark state and a light-induced absorption red shift for the signaling state, which relaxed to the dark state showing identical kinetics. The light-induced FTIR difference spectrum of YcgF-Full, however, was markedly different from that of YcgF-BLUF. The spectrum of YcgF-BLUF lacked most of the IR bands that were induced in the YcgF-Full spectrum. These bands were assigned to the light-induced structural changes of the protein. However, the bands for the C4═O stretching of a FAD isoalloxazine ring were induced at the same frequency with the same band intensity in the spectra for YcgF-Full and YcgF-BLUF. Furthermore, the YcgF-Full spectrum resembled that of the YcgF-BLUF when illuminated at medium-low temperatures because of the selective suppression of protein bands. The possibility that full-length-specific protein bands are predominantly ascribed to structural changes of the *C*-terminal EAL domain in the signaling state as a consequence of light excitation of the *N*-terminal BLUF domain is discussed.

Blue-light controls a wide variety of biological activities through various types of photoreceptors and signaling systems (*1*−*4*). BLUFs[1] (blue-light sensing proteins that use FAD) are members of the latest blue-light receptor family that has been identified that uses FAD as a chromophore. These proteins have been proposed to be implicated in blue-light-related cellular signaling processes in microorganisms (*5*, *6*). Light sensing is crucial to photosynthetic organisms for the maximization of the yield of photosynthesis and the avoidance of excess light-induced photodamage in response to environmental light conditions. Thus, BLUFs are well distributed in a number of photosynthetic microorganisms. Physiological functions and/or spectroscopic properties have been characterized in some BLUFs. Those that have been characterized include AppA (*7*−*15*) and BlrB (*16*) in the purple bacterium *Rhodobacter sphaeroides*, PAC in the alga *Euglena gracilis* (*17*), Slr1694 (PixD) (*18*−*21*) in the cyanobacterium *Synechocystis* sp. PCC6803, and Tll0078 (*21*−*23*) in the cyanobacterium *Thermosynechococcus elongatus* BP-1. Illumination of these proteins resulted in a very similar reversible redshift of the UV−visible absorption of FAD for the light-signaling state. This indicates that the change in the local environment of the FAD binding site upon illumination is very similar among BLUF proteins. This finding is in good agreement with the close similarity in the X-ray structures of the BLUF domains in AppA (*12*), Tll0078 (*23*), and BlrB (*16*).

The BLUF protein is composed of the *N*-terminal FAD-binding BLUF domain and the *C*-terminal domain whose function and size vary in each BLUF protein. The full-length AppA protein and the BLUF domain of AppA showed identical light-induced redshifts of the UV−visible absorptions for FAD (*8*). In many photoreceptors, the light absorbed by a chromophore induces structural changes in the chromophore and the apo-protein for further signaling (*4*). Therefore, the light-induced structural changes of apo-protein and FAD have been extensively studied using light-induced FTIR difference spectroscopy in the full-length Slr1694 protein (*18*−*20*) and the BLUF domain of AppA (*9*, *10*, *11*,

† This work was supported by grants for the Frontier Research System and Special Postdoctoral Researchers Programs at RIKEN to K.H. and Grant-in-Aid for Young Scientists (B) (17770102) to K.H. and (16770046) to S.M. from MEXT of Japan.

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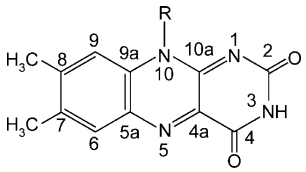
[1] Abbreviations: BLUF, blue-light sensing proteins that use FAD.; c-di-GMP, cyclic diguanosine monophosphate; FAD, flavin adenine dinucleotide; FTIR, Fourier transform infrared; IR, infrared; PCR, polymerase chain reaction; UV, ultraviolet.

10.1021/bi051820x CCC: $33.50 
Published on Web 02/23/2006

*13*). Although these revealed almost identical light-induced UV−visible absorption redshifts, their light-induced FTIR difference spectra were considerably different, with the exception of the bands for the C4═O stretches of the FAD isoalloxazine ring. Both spectra however showed small bands for the change in the skeletal structure of the isoalloxazine ring. The Slr1694 spectrum showed more bands from FAD



Isoalloxazine ring

and/or the apo-protein compared with the spectrum for the AppA BLUF domain. These differences may be ascribed to the difference in function of Slr1694 and AppA. However, it is also possible that these are due to the differences in the full-length protein and the BLUF domain of Slr1694 and AppA, respectively. The Slr1694 spectrum contains structural changes in the *C*-terminal domain that are transmitted from the BLUF domain as a signal. Further examination of the FTIR different spectra for both the full-length protein and the BLUF domain in the same BLUF protein is required to evaluate this possibility. So far, to our knowledge, this has not been accomplished for any BLUF protein.

The BLUF protein is distributed not only in photosynthetic, but also in nonphotosynthetic microorganisms (*5*, *6*). None of the physiological functions of these BLUF proteins have yet been defined. Furthermore, with the exception of YcgF from *Escherichia coli* (*24*, *25*), the spectroscopic properties of the isolated proteins have not been characterized. The YcgF protein is composed of an *N*-terminal BLUF domain and a *C*-terminal EAL domain. The EAL domain has been shown to encode the enzymatic activity involved in the hydrolysis of cyclic diguanosine monophosphate (c-di-GMP) (*26*, *27*), which is thought to function as a regulatory signaling molecule for various biological events (*28*−*30*). Upon illumination, the full-length YcgF protein showed a UV−visible absorption redshift for FAD that was characteristic of a BLUF protein (*24*, *25*). Therefore, it was proposed that YcgF functions as a blue-light regulated phosphodiesterase (Blrp) (*24*).

In the present investigation, we successfully expressed and isolated the full-length protein and the BLUF domain of YcgF. The full-length protein and the BLUF domain showed identical UV−visible absorption redshifts for FAD upon illumination. However, their light-induced FTIR difference spectra were markedly different. Also, the full-length spectrum resembled the BLUF domain spectrum when illuminated at a medium-low temperature. We discussed the possible contribution of the structural changes of the EAL domain to the full-length spectrum in which a light signal perceived by the BLUF domain is transmitted to the EAL domain inducing changes in the protein structure.

## MATERIALS AND METHODS

*Construction of Expression Plasmid and Protein Purification.* The *ycgF* gene of *E. coli* was amplified by PCR using the upstream and downstream oligonucleotide primers YcgF-F-NcoI: 5′-TATACCATGGTGCTTACCACCCTTATT-TATC-3′ and YcgF-R-HindIII: 5′-TATAAGCTTTTTCTC-TGGCCACGCTATG-3′ (NcoI and HindIII sites, respectively, are underlined) as well as an isolating pETYcgF plasmid (*25*) as a template. The amplified DNA fragment digested with NcoI and HindIII was cloned into the pET28-(a) vector (Novagen) at its NcoI and HindIII sites to construct the expression plasmid for the *C*-terminal His-tagged YcgF protein. The resulting plasmid was named pETYcgF2. To construct the expression plasmid for the *C*-terminal His-tagged BLUF domain of YcgF, the region corresponding to residues 1−148 of YcgF was amplified by PCR using upstream and downstream oligonucleotide primers pET28-F (*8*) and YcgF148-R-HindIII: 5′-TATAAAGCTTGTCT-TCGGCAGGGATCTC-3′ (the HindIII site is underlined). An isolating pETYcgF2 plasmid was used as the PCR template. The PCR-amplified fragment digested with NcoI and HindIII was cloned into the pET28(a) vector at its NcoI and HindIII sites to obtain the plasmid pETYcgF148C. The obtained plasmids were transformed into *E. coli* strain BL21-(DE3) (Novagen), and the histidine-tagged full-length YcgF protein (YcgF-Full) and the BLUF domain of YcgF (YcgF-BLUF) were overexpressed by induction with 1 mM isopropyl $\beta$-D-thiogalactopyranoside (IPTG) at 16 °C for 16 h in an LB or M9 minimum medium. To achieve uniform isotope labeling, the culture was grown in an M9 medium using 18 mM $^{15}NH_4Cl$ (99.3% $^{15}N$-enrichment, Shoko Tsusho) for $^{15}N$-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, 5 mM imidazole, and 20 mM Tris/HCl (pH 7.9) were ruptured using a chilled French press, and the soluble fraction was collected after centrifugation at 20 000g for 30 min. The full-length protein (YcgF-Full) and BLUF domain (YcgF-BLUF) of YcgF were purified using a His-Bind resin (Novagen) as described by the manufacturer and dialyzed against a buffer containing 2 mM NaCl, 2 mM $MgCl_2$, and 20 mM Tris/HCl (pH 8.0). Finally, the purified proteins were dissolved in an $H_2O$ ($D_2O$) medium containing 20 mM Tris/HCl (DCl), 2 mM NaCl and 2 mM $MgCl_2$ (pH/pD 8.0). SDS−PAGE analysis showed that the purity of YcgF-Full and YcgF-BLUF was estimated to be greater than 98% (data not shown). The AppA BLUF domain (AppA126) was prepared as previously described (*10*).

*Spectroscopic Measurements.* For FTIR, sample solutions (3−6 $\mu$L) applied to a $BaF_2$ disk were gently dried under $N_2$ gas for 5 min. For the deuteration spectrum, the sample solution was applied to a $BaF_2$ disk and dried. The dried sample was dissolved in 5 $\mu$L of $D_2O$ and dried again under $N_2$ gas. This process was repeated four times. The obtained sample film was subsequently sandwiched onto another $BaF_2$ disk with a greased 0.5-mm-thick Teflon spacer, after placing 1 $\mu$L of 20% glycerol/$H_2O$ (20% glycerol-d8/$D_2O$) (v/v) for the hydration spectrum (deuteration spectrum) as described previously (*18*). The IR sample was incubated at 15 °C for at least 4 h in the dark to equilibrate the water content of the sample. After equilibration, the sample absorptions for OH stretches and HOH deformations of water molecules at ∼3400 and ∼1600 $cm^{-1}$, respectively, were less than 0.7 to ensure a linear response of the detector as shown in Figure 1. FTIR spectra were recorded using a Bruker IFS66v/s spectrophotometer with a MCT detector with 4 $cm^{-1}$ resolution by averaging 32 scans (20 s accumulation). Ge

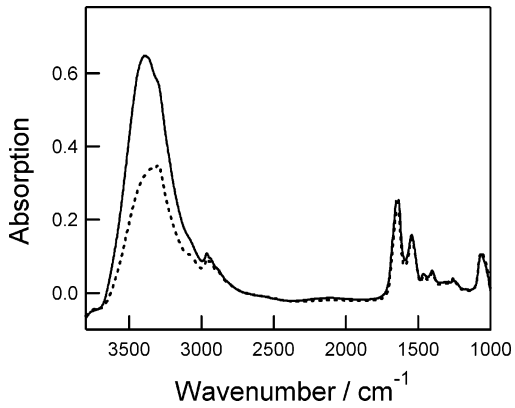



FIGURE 1: IR absorption spectra of hydrated (−−) and dried (.....) full-length YcgF protein samples in dark state.

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. 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). Two spectra were collected by repeating this cycle with a 3−4 h dark interval, during which the sample fully relaxed to its dark state. UV−visible absorption spectra were recorded on a Shimadzu MultiSpec-1500 photodiode array spectrophotometer as previously described (*18*).

## RESULTS

*UV−Visible Spectra of the Full-Length Protein and BLUF Domain of YcgF.* Figure 2 shows the UV−visible absorption spectra of the dark-adapted (dotted line) and illuminated (solid line) full-length protein (YcgF-Full) (Figure 2A) and BLUF domain (YcgF-BLUF) (Figure 2B) of YcgF, sandwiched between $BaF_2$ disks for the FTIR measurements. YcgF-Full and YcgF-BLUF in the dark-adapted state showed almost the same spectra with two flavin peaks at 382 and 459 nm and a shoulder at 484 nm. Upon illumination, both YcgF-Full and YcgF-BLUF showed an identical red shift of flavin absorption by approximately 10 nm, and thus had nearly the same light-minus-dark difference spectra (inset). As shown in Figure 2C, the kinetics of the dark relaxation of the red-shifted absorption change was much slower in the FTIR samples (squares, $t_{1/2}$ = 649 s) than in the solution samples (circles, $t_{1/2}$ = 136 s), probably because of the lower water content in FTIR than in the solution samples (*10*, *18*). Furthermore, it showed distinct deuteration effects (triangles, $t_{1/2}$ = 251 s) consistently observed in other BLUF proteins (*18*, *20*). It is important to note, however, that these dark-decay behaviors were very similar for both YcgF-Full and YcgF-BLUF. These findings strongly indicate that the surroundings of the bound FAD and the light-induced changes are largely identical in both the BLUF domain in the full-length protein and the isolated BLUF domain in YcgF. The largely normal spectroscopic properties, including the dark-state spectrum and light-induced spectral changes as well as the dark-relaxation kinetics in YcgF-BLUF compared with those in YcgF-Full, strongly indicate that FAD is normally coordinated in the binding pocket and fully functional in the holo-enzyme of the isolated BLUF domain of YcgF. The relative population of the holo-enzyme, however, was estimated to be 30−40% on the basis of the intensity of the FAD absorption.

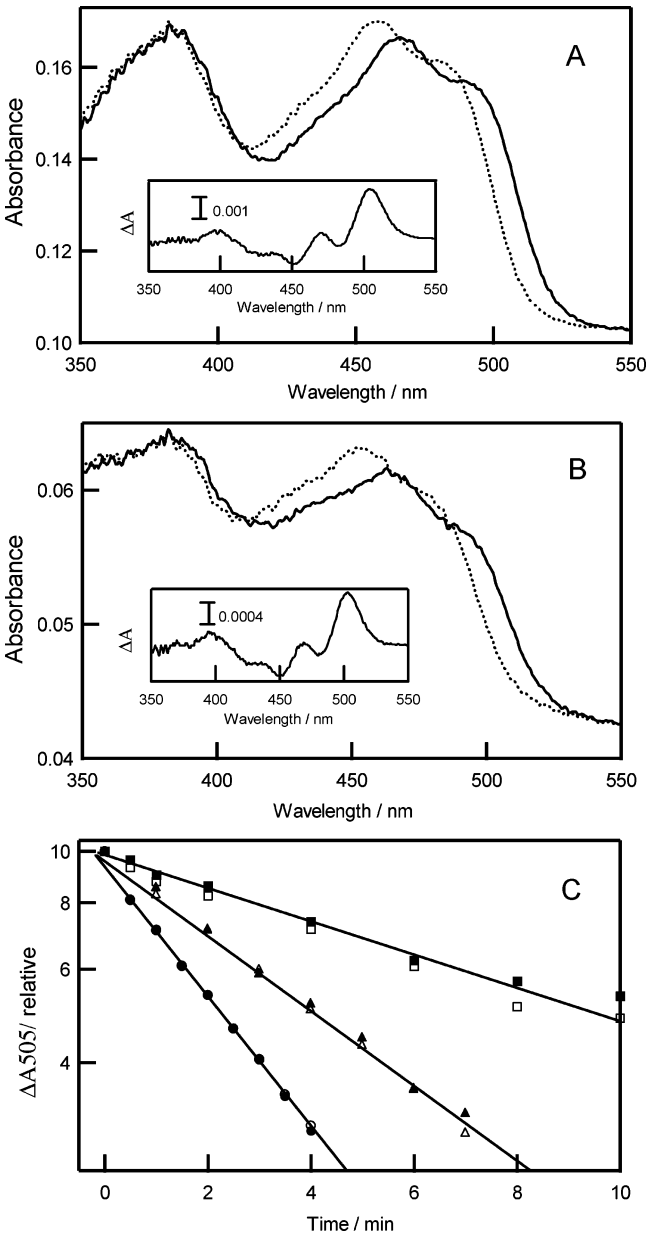



FIGURE 2: UV-visible absorption spectra of full-length YcgF protein (A) and isolated YcgF-BLUF domain (B) in the dark state (.....) and the light state (−−). The samples were sandwiched between $BaF_2$ disks as described in Materials and Methods. Light-minus-dark difference spectra are shown in inset figures. (C) Dark-decay kinetics of the light-induced UV−visible absorption changes recorded at 505 nm of full-length YcgF protein (●, ▲, ■) and isolated YcgF BLUF domain (○, △, □). Samples were dissolved in an $H_2O$ medium containing 20 mM Tris/HCl (pH 8.0), 2 mM NaCl, and 2 mM $MgCl_2$ (●, ○), a $D_2O$ medium containing 20 mM Tris/DCl (pD 8.0), 2 mM NaCl, and 2 mM $MgCl_2$ (▲, △), or sandwiched between $BaF_2$ disks and hydrated (■, □). The samples were illuminated at room temperature.

*Light-Induced FTIR Difference Spectra of Full-Length YcgF Protein.* Characteristic structural changes of FAD and apo-protein corresponding to the redshifted UV−visible absorption for the signaling state have been detected by means of light-induced FTIR difference spectroscopy in the BLUF domain of AppA (*9*, *10*, *11*, *13*) and full-length Slr1694 (*18*−*20*). Figure 3 shows the light-minus-dark FTIR difference spectra of YcgF-Full, unlabeled

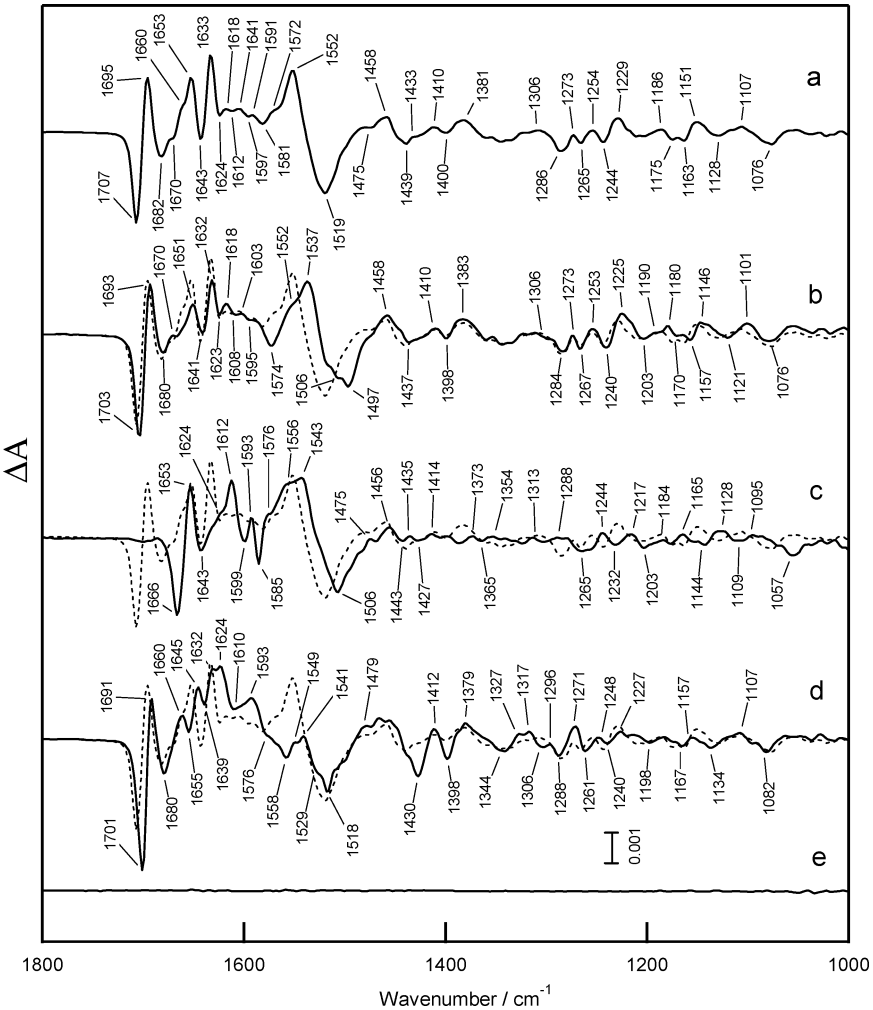



FIGURE 3: Light-minus-dark FTIR difference spectra of full-length YcgF protein and the effects of isotope labeling and deuteration. Unlabeled (a (−−) and b, c, and d (.....)), uniformly $^{15}$N-labeled (b (−−)), uniformly $^{13}$C-labeled (c (−−)), and deuterated (d (−−)) spectra. Dark-minus-dark spectrum (e) shows the signal-to-noise level. Sample temperature was 15 °C. Two spectra were averaged to obtain final data.

(a, solid line; b, c, and d, dotted lines), uniformly $^{15}$N-labeled (b, solid line), uniformly$^{13}$C-labeled (c, solid line), and deuterated (d, solid line). The positive and negative bands are attributed to the light-induced and dark states, respectively. The unlabeled spectrum (a) showed prominent bands at 1707−1500 cm$^{-1}$ and much smaller bands below 1500 cm$^{-1}$. The overall spectral features are comparable to those of Slr1694, despite the differences in the position, shape, and number of bands. The absence of strong bands below 1500 cm$^{-1}$ is a common spectral feature reported in the Slr1694 protein (*18*−*20*) and the AppA BLUF domain (*10*), indicating that the skeletal structure of chromophore FAD changes little upon illumination.

The bands in the light-minus-dark FTIR difference spectra were assigned on the basis of their positions, the effects of $^{13}$C- and $^{15}$N-labeling, and deuteration, as described previously (*10*, *18*). The bands at 1707−1591 cm$^{-1}$ were downshifted by 4−8 cm$^{-1}$ with little change in shape upon uniform $^{15}$N-labeling, indicating small contributions of the vibrational modes to these bands including nitrogen atoms. The 1707(−)/1695(+) cm$^{-1}$ bands in the unlabeled spectrum were downshifted to 1666(−)/1653(+) cm$^{-1}$ by 33−42 cm$^{-1}$ upon $^{13}$C-labeling and to 1701(−)/1691(+) by 2−4 cm$^{-1}$ upon deuteration. These bands were prominently observable and similarly affected by the isotopes in the spectra of full-length Slr1694 (1713(−)/1697(+) cm$^{-1}$) and the BLUF domain of AppA (1709(−)/1695(+) cm$^{-1}$), where these bands were assigned to C4═O stretches of a flavin isoalloxazine ring. Therefore, the 1707(−)/1695(+) cm$^{-1}$ bands were assigned to the vibration of C4═O stretches of FAD. The light-induced downshift of the C4═O stretch band indicates stronger hydrogen bonding to C4═O, which is responsible for the UV−visible absorption redshift. The frequency of the negative peak of the dark-state C4═O stretch band of YcgF (1707 cm$^{-1}$) is lower than that of Slr1694 (1713 cm$^{-1}$) and AppA (1709 cm$^{-1}$), indicating that hydrogen bonding to the C4═O group is stronger in YcgF than in Slr1694 and AppA in the dark state. This observation is consistent with the more redshifted position of the longer-wavelength peak of FAD in YcgF (459 nm) than that in AppA and Slr1694 (443 nm) because the peak position depends on the strength of the hydrogen bond to the FAD C4═O group (*20*).

The $^{15}$N-sensitive bands at 1682−1581 cm$^{-1}$ in the unlabeled spectrum were downshifted by 32−44 cm$^{-1}$ upon $^{13}$C labeling and 2−8 cm$^{-1}$ upon deuteration. Therefore, these bands were ascribed to the C═O stretches with small contributions from NH deformations in the amide I bands in the polypeptide backbone.

Table 1: Vibrational Assignments of Major IR Bands of the Light-Induced FTIR Difference Spectrum of Full-length YcgF Protein

| band positions ($cm^{-1}$) | predominant assignments |
|---|---|
| 1707(−)[a]s[b], 1695(+)s | C4═O stretch (FAD) |
| 1643(−)m, 1633(+)m | amide I |
| 1552(+)s, 1519(−)s | amide II |
| 1458(+)w, 1439(−)w | $CH_3$ deformation |
| 1244(−)w, 1229(+)w | CN/CC stretch, NH deformation |
| 1107(+)w, 1076(−)w | $CH_3$/$CH_2$ rocking |

[a] Sign of band. [b] Symbols s, m, and w indicate bands with relatively strong, medium, and weak intensities, respectively.

The prominent bands at 1552(+)/1519(−) $cm^{-1}$ and the shoulder at 1572 $cm^{-1}$ in the unlabeled spectrum were downshifted to 1537(+)/1497 (−) $cm^{-1}$ and 1552(+) $cm^{-1}$ by 15−18 $cm^{-1}$ upon $^{15}N$-labeling, and to 1543(+)/1506(−) $cm^{-1}$ and 1556 $cm^{-1}$ by 9−13 $cm^{-1}$ upon $^{13}C$-labeling. Upon deuteration, the bands were largely suppressed leaving small bands at 1558(−), 1549(+), 1541(+), 1529(−), and 1518(−) $cm^{-1}$. The 1552(+)/1519(−) $cm^{-1}$ bands were thought to downshift by the decoupling of NH deformations to the 1480−1398 $cm^{-1}$ region where spectral features were considerably changed upon deuteration. Results indicated that the 1552(+)/1519(−) $cm^{-1}$ bands are primary attributed to amide II bands, which are due to the NH deformations and CN stretches of the polypeptide backbones. It is of note in this context that the 1552(+)/1519(−) $cm^{-1}$ bands are similarly observed at 1552(+)/1520-(−) $cm^{-1}$ in the light-induced FTIR difference spectrum of Slr1694 (*18*−*20*). The bands for Slr1694, however, were primary attributed to the FAD C4aN5/N1C10a stretches because they were insensitive to deuteration (*18*). However, the present results suggest that the Slr1694 bands were also attributable to the changes in the amide II band in the polypeptide backbone where the hydrogen is more resistant to deuteration.

The small bands at 1439−1254 $cm^{-1}$ in the unlabeled spectrum were insensitive to $^{15}N$-labeling but were changed in a complicated manner upon $^{13}C$-labeling. This observation indicates that the bands are ascribed to the vibrations that have only carbon-containing groups, such as a CC stretch or $CH_3$ deformation, although these band shapes are modified upon deuteration because of the overlap with the amide II′ bands. The bands at 1244−1107 $cm^{-1}$ were downshifted by 4−8 $cm^{-1}$ and 7−20 $cm^{-1}$upon $^{15}N$- and $^{13}C$-labeling, respectively, and were affected in shape upon deuteration. These indicate that CN and/or CC stretches coupling with NH deformation are responsible for these bands. The bands below 1100 $cm^{-1}$ were not affected by either $^{15}N$-labeling or deuteration, but were downshifted by 12−20 $cm^{-1}$ upon $^{13}C$-labeling, indicating that the bands are ascribed to the vibrations of only carbon-containing groups, namely, $CH_3$/$CH_2$ rocking and/or CC stretches. Table 1 summarizes the vibrational assignments for the FTIR difference spectrum of YcgF-Full.

*Light-Induced Structural Changes in the Full-Length Protein and BLUF Domain of YcgF.* Light-induced structural changes of FAD and apo-protein in YcgF-Full and YcgF-BLUF were compared. Figure 4 shows the light-minus-dark FTIR difference spectra (1800−1000 $cm^{-1}$) of YcgF-Full (a) and YcgF− BLUF (b) measured at 15 (A) and −35 °C

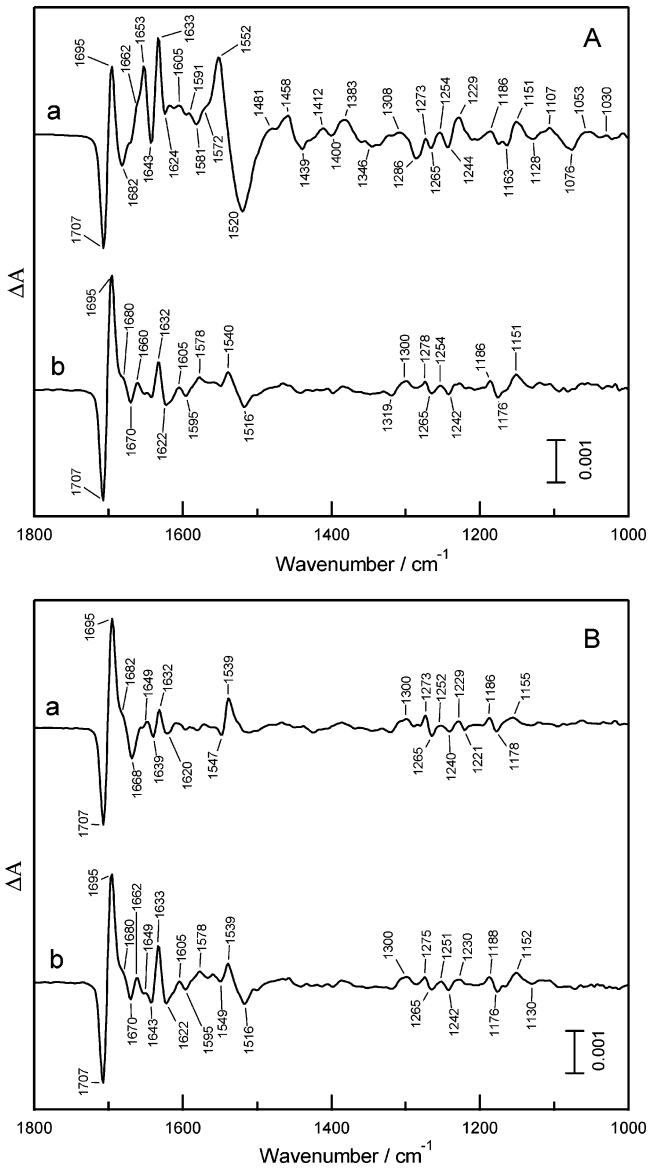



FIGURE 4: Light-minus-dark FTIR difference spectra of full-length YcgF protein (a) and isolated YcgF BLUF domain (b). Sample temperatures were 15 (A) and −35 °C (A). Two spectra were averaged to obtain final data. The BLUF domain spectrum is shown after normalization to the full-length spectrum on the basis of the amount of bound FAD determined from UV−visible absorption spectra.

(B), respectively. As shown in panel A, the illumination of YcgF-BLUF at 15 °C induced a markedly different light-minus-dark FTIR difference spectrum (b), in which the prominent 1707(−)/1695(+) $cm^{-1}$ bands assigned to the FAD C4═O stretch were induced as observed in YcgF-Full spectrum. However, other bands that were observed upon light-induced structural changes of the protein backbone were largely suppressed, leaving small residual bands at 1680-(+), 1670(−), 1632(+), 1622(−), 1578(−), 1516(−), 1300-(+), 1278(+), 1265(−), 1254(+), 1242(−), 1229(+), 1186-(+), 1176(−), and 1151(+) $cm^{-1}$. These results strongly indicate that most of the light-induced structural changes in the apo-protein detected in YcgF-Full do not occur in YcgF-BLUF. The marked difference in the light-induced structural changes detected by FTIR is in contrast to the very similar light-induced UV−visible absorption redshift between YcgF-Full and YcgF-BLUF shown in Figure 2.

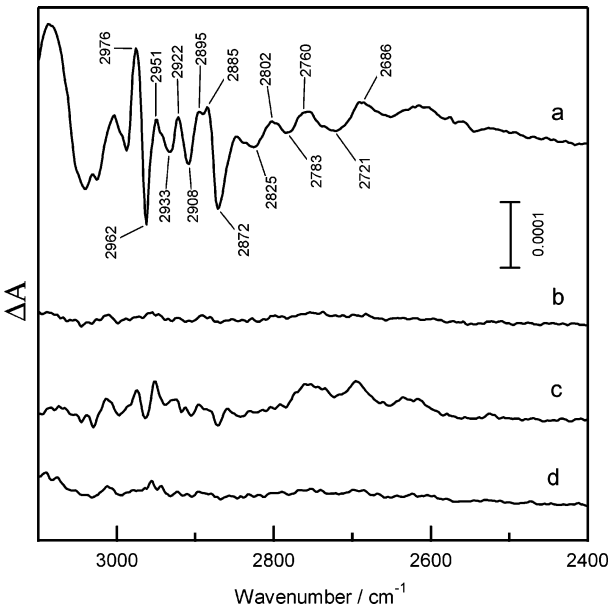



FIGURE 5: High-frequency region (3100−2400 $cm^{-1}$) of light-minus-dark FTIR difference spectra of the full-length YcgF protein (a, c) and the isolated YcgF BLUF domain (b, d). Sample temperatures were 15 (a, b) and −35 °C (c, d). Two spectra were averaged to obtain final data. Spectra were normalized on the basis of the amount of FAD determined from UV−visible absorption spectra.

As shown in Figure 4 panel B, most of the bands induced by illumination at 15 °C in the full-length protein (spectrum a in panel A) were not light-induced at −35 °C, leaving the 1707(−)/1695(+) $cm^{-1}$ bands intact. This observation indicates that most of the light-induced structural changes in YcgF-Full are inhibited at this medium-low temperature, with the exception of that of the FAD C4═O moiety. In contrast, the YcgF-BLUF spectrum induced at −35 °C (spectrum b in panel B) was almost identical to that induced at 15 °C (spectrum b in panel A). It is of note in this context that the full-length spectrum at −35 °C is extremely similar to the BLUF domain spectra at 15 °C and −35 °C.

Figure 5 shows the light-minus-dark FTIR difference spectra of YcgF-Full (a and c) and YcgF-BLUF (b and d) at 15 °C (a and b) and −35 °C (c and d), respectively, in the high-frequency region (3100−2400 $cm^{-1}$). The YcgF-Full spectrum at 15 °C showed prominent sharp bands at 3010−2800 $cm^{-1}$. These bands were attributable to the vibrational changes in $CH_3/CH_2$ stretches on the basis of their position. These bands are thought to form upon light-induced structural changes in the side groups of the amino acid residues. In addition to these bands, the spectrum showed smaller bands at 2850−2700 $cm^{-1}$. These bands were suppressed by deuteration (unpublished data), indicating that these bands are attributable to NH stretches. Interestingly, these bands were not induced in YcgF-BLUF (b and d) and YcgF-Full spectra when illuminated at −35 °C (c). This result is in good agreement with the absence of mid-frequency bands in the 1500−1400 and 1100−1000 $cm^{-1}$ regions in the YcgF-BLUF spectra and in the YcgF-Full spectrum at −35 °C. Therefore, the mid- and high-frequency results suggest that the light-induced structural changes observed in YcgF-Full at 15 °C are composed of those induced in the isolated BLUF domain, insensitive to the medium-low temperatures,

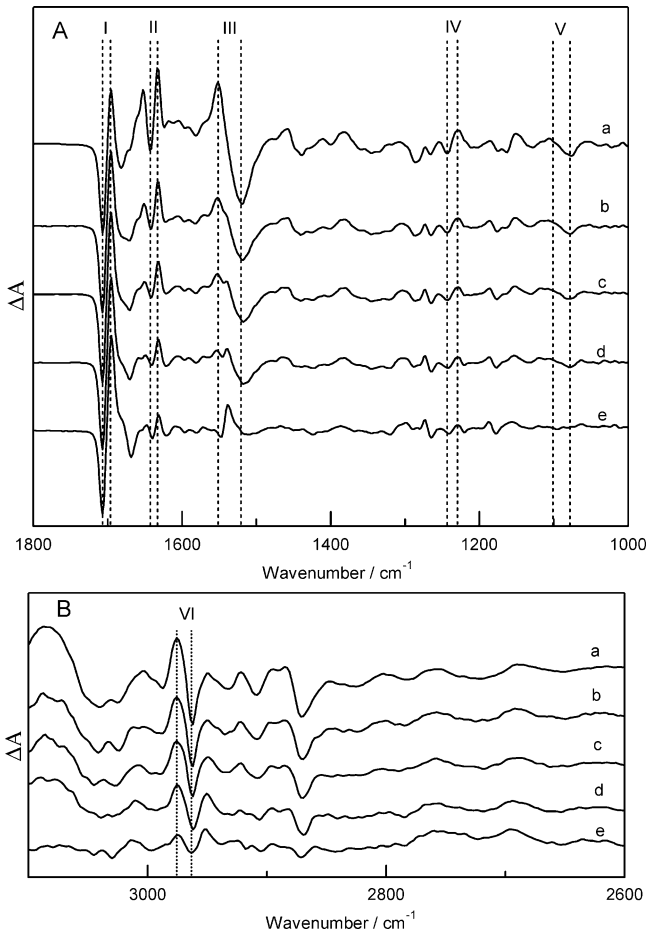



FIGURE 6: Mid-frequency (A: 1800−1000 $cm^{-1}$) and high-frequency (B: 3100−2600 $cm^{-1}$) regions of light-minus-dark FTIR difference spectra of full-length YcgF protein. Samples were illuminated, and the spectra were recorded at 15 (a), 5 (b), −5 (c), −15 (d), and −35 °C (e). Bands I, II, III, IV, V, and VI correspond to those at 1707(−)/1695(+), 1643(−)/1633(+), 1552(+)/1519(−), 1244(−)/1229(+), 1107(+)/1076(−), and 2976(+)/2962(−) $cm^{-1}$, respectively.

and those induced in the full-length protein, sensitive to the medium-low temperatures.

*Effect of Illumination Temperature on the FTIR Spectrum of Full-Length YcgF Protein.* Figure 6 shows the effects of the illumination temperature on the light-minus-dark FTIR difference spectra of YcgF-Full in the mid- (A: 1800−1000 $cm^{-1}$) and high- (B: 3100−2600 $cm^{-1}$) frequency regions. The spectra did not change much until approximately 15 °C, where the band intensity started to decrease, despite almost no change in the frequency and shape of the bands. Although the FAD C4═O bands at 1707(−)/1695(+) $cm^{-1}$ were insensitive to lowering the temperature, the positive 1695-(+) $cm^{-1}$ band intensity tended to increase along with the appearance of a small shoulder at 1682 $cm^{-1}$ as the temperature decreased. Results suggest that the 1695(+) $cm^{-1}$ band at 15 °C is overlapped with a low-temperature-sensitive negative band around 1695 $cm^{-1}$. The detailed temperature-dependence of the bands at 1707(−)/1695(+) (C4═O stretch), 1643(−)/1633(+) (amide I), 1552(+)/1519-(−) (amide II), 1244(−)/1229(+) (CN stretch/NH bending), 1107(+)/1076(−) $cm^{-1}$ ($CH_3/CH_2$ rocking), and 2976(+)/2962 $cm^{-1}$ ($CH_3/CH_2$ stretch) were evaluated from the temperature-dependent spectral changes shown in Figure S1, and plotted in Figure 7. All structural changes showed similar

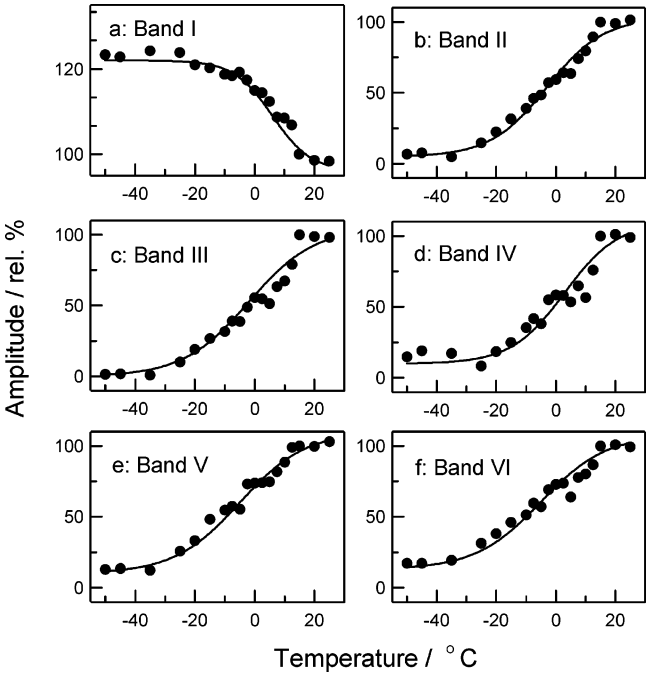



FIGURE 7: Temperature dependence of intensity of bands in light-minus-dark FTIR difference spectra of full-length YcgF protein. Peak-to-peak amplitude was determined for FAD C4═O bands at 1707(−)/1695(+) $cm^{-1}$ (a: Band I), amide I bands at 1643(−)/1633(+) $cm^{-1}$ (b: Band II), amide II bands at 1552(+)/1519(−) $cm^{-1}$ (c: Band III), CN stretch/NH bending bands at 1244(−)/1229(+) $cm^{-1}$ (d: Band IV), $CH_3/CH_2$ rocking bands at 1107(+)/1076(−) $cm^{-1}$ (e: Band V), and $CH_3/CH_2$ stretch bands at 2976(+)/2962(−) $cm^{-1}$ (f: Band VI).

temperature dependencies with the half-inhibition temperatures ranging from −4 to 5 °C.

*FTIR Spectrum of AppA BLUF Domain Is Insensitive to Low Temperatures.* The FTIR bands for the light-induced structural changes of YcgF-Full were largely suppressed at medium-low temperatures, whereas the YcgF-BLUF spectrum was insensitive to low temperatures (Figure 4). The full-length spectrum of Slr1694 (*18*−*20*) was similar to that of YcgF-Full, and the light-induced FTIR bands were also suppressed at low temperatures (*19*). In contrast, the BLUF domain spectrum of AppA (*10*, *11*, *13*) resembles that of YcgF-BLUF. Therefore, it is interesting to evaluate whether the light-induced spectrum of the AppA BLUF domain is sensitive to low temperatures. As clearly shown in Figure 8, nearly the same light-induced FTIR difference spectra were obtained in the *N*-terminal BLUF domain of AppA (AppA126) both at 15 and −35 °C. These results indicate that the light-induced FTIR bands are also insensitive to the low temperatures in the BLUF domain of AppA.

## DISCUSSION

The present study clearly demonstrates that the light-induced FTIR difference spectrum of YcgF-Full is considerably different from that of YcgF-BLUF. Most of the prominent bands in the full-length spectrum did not appear in the BLUF domain spectrum, with the exception of the C4═O stretch bands attributed to the FAD isoalloxazine ring. On the basis of the positions of the bands and the effects of $^{15}N$- and $^{13}C$-labeling and deuteration on the bands, the prominent bands specific for YcgF-Full were attributed to the amide I and II modes of the conformational changes in

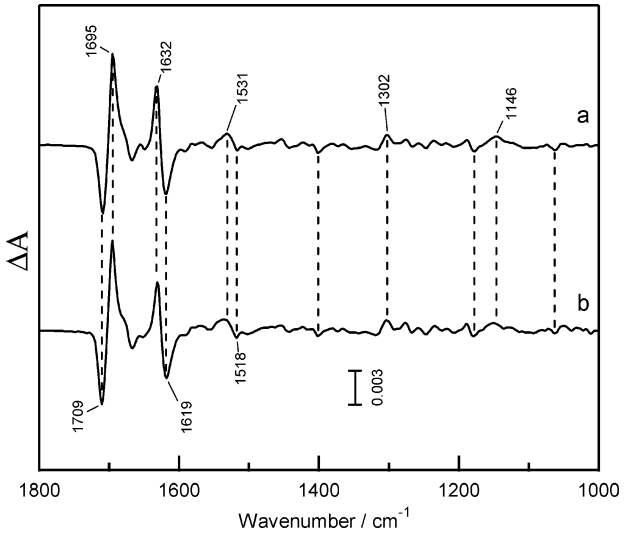



FIGURE 8: Light-minus-dark FTIR difference spectra of the AppA BLUF domain (AppA126). Sample temperatures were 15 (a) and −35 °C (b). Two spectra were averaged to obtain final data.

the polypeptide backbone as shown in Table 1. Therefore, the present results indicate that the prominent structural changes of apo-protein are induced in the full-length YcgF protein but not as much in the corresponding BLUF domain upon illumination. The BLUF domain is an integral part of the full-length protein, and therefore a simple and straightforward interpretation of the results is that the YcgF-Full specific protein bands are primarily due to the structural changes of the apo-protein in the *C*-terminal EAL domain, induced by the light excitation of FAD in the BLUF domain.

Light absorption induces structural and/or conformational changes in an intrinsic chromophore as an initial signal in most of the known light sensory proteins (*4*). A chromophore undergoes trans-cis isomerization in rhodopsins, phytochromes, and photoactive-yellow protein, and a flavin chromophore forms a covalent adduct with an amino acid side group in phototropins. These local changes then trigger the structural change of the photoreceptor protein for the signaling state. In contrast, light absorption results in little structural changes in a flavin chromophore in the BLUF proteins. However, the strength of hydrogen bonding to the C4═O of FAD is strengthened. Therefore, it has been proposed that light-induced rearrangements of a hydrogen bond network with the FAD isoalloxazine ring trigger a structural change of the BLUF protein for the signaling state formation (*10*, *11*, *18*−*20*). The appearance of prominent C4═O stretch bands from the FAD chromophore at 1707(−)/1695(+) $cm^{-1}$ indicates that light-induced strengthening of a hydrogen bond with the FAD C4═O is induced upon illumination in the BLUF domain in YcgF, as is consistently observed in other BLUF proteins (*9*−*11*, *13*, *18*−*20*). These rearrangements in the BLUF domain are thought to be the primary cause of structural changes in the effector EAL domain. It has been reported that the full-length YcgF protein exists as a monomer, both in dark and light states, and displays no global structural changes upon illumination (*24*). Therefore, the light-induced changes of the protein bands in the YcgF-Full spectrum could not be attributed to changes in the global structure of the protein, which include changes in the oligomeric state and in the intramolecular structural association between the *N*-terminal BLUF and *C*-terminal

EAL domains. The latter, however, may be possible if it accompanies little change in the global structure of the protein. It is likely that the light-induced signal in the BLUF domain is transmitted to the area outside the domain through a hydrogen bond network, resulting in the changes in the specific structure of the EAL domain.

The EAL domain has been proposed to have the enzymatic activity of c-di-GMP phosphodiesterase; the enzyme degrades c-di-GMP, which acts as a second messenger to regulate cellular function in a diverse range of bacteria (*28*−*30*). Recent in vitro characterizations using purified enzymes showed that the EAL domain is sufficient for phosphodiesterase activity (*26*, *27*). Therefore, YcgF was assumed to be a putative blue-light regulated phosphodiesterase (Blrp) (*24*), despite the lack of biochemical evidence for c-di-GMP phosphodiesterase activity. It is of note in this context that the c-di-GMP phosphodiesterase activity of the EAL domain was allosterically activated by the binding of GTP to the neighboring GGDEF domain in CC3396, a GGDEF-EAL composite protein from *Caulobacter crescentus* (*31*). The GGDEF/EAL domain was also placed at the *C*-terminal of *Thermochromatium tepidum* Ppd (Photoactive yellow protein/phytochrome/diguanylate cyclase) protein, although enzymatic activity of the EAL domain was not clarified (*32*). Furthermore, it was suggested that the light-induced structural changes in the PAS domain are propagated outside the domain for functional regulation of the effector domain in PYP and photoropin (*33*, *34*). Therefore, an attractive interpretation of the present results is that the observed light-induced protein structural change is associated with the regulation of the putative c-di-GMP phosphodiesterase activity in the EAL domain. If this is the case, then a plausible scenario is that blue-light excitation induces a relatively small change of the apo-protein structure in the BLUF domain. This change is communicated to the EAL domain and induces specific structural changes in the EAL domain. The structural changes would thus regulate putative enzyme activity by changing the structure of the catalytic site, the accessibility of c-di-GMP to the catalytic site, or by interacting with another regulatory protein. The enzymatic activity of the YcgF EAL domain needs to be biochemically characterized to evaluate whether the observed structural change of the EAL domain is associated with activation of the enzymatic function of the *C*-terminal domain.

An alternative interpretation of the present results suggests that the BLUF domain and not the EAL domain is predominantly responsible for the full-length-specific protein bands. The BLUF domain is abnormally folded because of the absence of the EAL domain, and the capability of the light-induced structural change in the apo-protein is lost in YcgF-BLUF, or the BLUF domain is normally folded, but light-induced structural changes in the apo-protein only occur in the presence of the EAL domain. However, this interpretation is a less likely scenario, considering the fact that the rate of dark relaxation of the UV−visible absorption of redshifted FAD was largely the same for both full-length YcgF and the corresponding BLUF domain, as shown in Figure 2C. The dark decay of the redshifted UV−visible absorption is closely correlated with the rearrangement of the hydrogen bond network with the FAD C4═O group accompanied by the dark relaxation from light signaling to the dark state (*20*). Therefore, the lack of change in dark-decay kinetics suggests that the light-induced rearrangements of the hydrogen bond network occur identically for both YcgF-Full and YcgF-BLUF. This view is further supported by the finding that the peak frequencies of the C4═O bands in the YcgF-Full spectrum coincided with those in the YcgF-BLUF spectrum, indicating that the strength of the hydrogen bonds to the C4═O of FAD in full-length YcgF is identical to that in the isolated BLUF domain, both in the light-signaling and dark states. Furthermore, YcgF-Full at middle-low temperatures showed a light-induced FTIR difference spectrum that is very similar to that of YcgF-BLUF, as shown in Figure 4B. This indicates that the absence of protein bands is not due to abnormal polypeptide folding in YcgF-BLUF. These findings suggest that the BLUF domain undergoes very similar structural changes upon illumination both in the full-length protein and the isolated BLUF domain. Furthermore, structural changes in the EAL domain are primarily ascribed to the light-induced protein bands in the YcgF-Full spectrum.

The overall features of the light-induced FTIR difference spectrum in YcgF-Full resemble those of the Slr1694 spectrum (*18*−*20*), although the frequency position, amplitude, and shape of each band are different for the two spectra. Slr1694 is a small BLUF protein composed of the BLUF domain and the other small *C*-terminal domain containing two α-helices in which the function of the *C*-terminal domain is not defined. Therefore, light-induced structural changes of the *C*-terminal domain may be ascribed to the protein bands observed in the Slr1694 spectrum. Interestingly, the formation of these protein bands in Slr1694 was suppressed leaving the C4═O stretch bands when the protein was illuminated at medium-low temperatures, (*19*) as in YcgF-Full. This observation suggests that a similar mechanism is responsible for the formation of protein bands in the full-length YcgF and Slr1694 proteins.

## SUPPORTING INFORMATION AVAILABLE

Light-induced FTIR difference spectra of the full-length YcgF protein from 25 °C to −50 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI051820X