Biochemistry **2004,** *43,* 14979−14986

Structural Intermediate in the Photocycle of a BLUF (Sensor of Blue Light Using FAD) Protein Slr1694 in a Cyanobacterium *Synechocystis* sp. PCC6803[†]

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Received June 25, 2004; Revised Manuscript Received September 17, 2004

ABSTRACT: Slr1694 in *Synechocystis* sp. PCC6803 is a family of blue-light photoreceptors based on flavin adenine dinucleotide (FAD) called BLUF (sensor of blue light using FAD) proteins, which include AppA from *Rhodobacter sphaeroides* and PAC from *Euglena gracilis*. Illumination of dark-state Slr1694 at 15 °C reversibly induced a signaling light state characterized by the red shift in the UV−visible spectrum and by the light-induced Fourier transform infrared (FTIR) difference spectrum for structural changes of a bound flavin and apo protein. Illumination at the medium-low temperature (−35 °C) led to the red shift in the UV−visible spectrum despite some small difference in the light-induced changes. In contrast, the −35 °C illumination resulted in a completely different light-induced FTIR spectrum, in which almost all of the bands were suppressed with the exception of the bands for the change of C4═O bonding of the FAD isoalloxazine ring. The C4═O bands were induced at −35 °C with almost the same intensity, but the band frequency for the light state was upshifted by 6 cm^{-1}. The changes in frequency of the light-state C4═O band and in amplitude of other bands showed the same temperature dependence with a half-change temperature at approximately −20 °C. It was indicated that the light-induced structural changes of apo protein and FAD were inhibited at low temperature with the exception of the change in hydrogen bonding to the C4═O group. The light-induced formation of the FTIR bands was similarly inhibited by sample dehydration. We discussed the possibility that this constrained light state is a trapped intermediate state in the photocycle of Slr1694.

Slr1694 in the cyanobacterium *Synechocystis* sp. PCC6803 is a member of the BLUF[1] (sensor of blue light using FAD) proteins (*1*), which have been categorized by the characteristic flavin adenine dinucleotide (FAD)-binding domain, named BLUF (*2*, *3*). A computer-aided DNA database search has indicated that the putative BLUF proteins are distributed over at least 15 organisms, although they show no identifiable sequence similarity outside of the BLUF domain. This suggests that the BLUF protein is one of the major photoreceptor families that functions in nature and involved in the perception of a blue-light signal. Until now, four BLUF proteins have been characterized, which are AppA in the purple bacterium *Rhodobacter sphaeroides* (*3, 4*), PAC in the green alga *Euglena gracilis* (*5*), YcgF in *Escherichia coli* (*6*) and Slr1694 (*1*). AppA regulates the activity of a repressor, PpsR, for controlling photosynthesis-gene expression through a blue-light-dependent change in direct protein−protein interaction; PAC is involved in the photoavoidance behavior through a blue-light-dependent activation of an intramolecular adenylyl cyclase; and YcgF was suggested to modulate the cellular levels of the second messenger cyclic diguanylate (c-di-GMP) in a blue-light-dependent manner. Although the physiological function of Slr1694 has not been conclusively defined, disruptant of *slr1694* resulted in abnormal phototaxis behavior (*7*) and the possible involvement of Slr1694 in the blue-light cyclic AMP signal transduction system concerning the cell motility was proposed (*8*).

Upon illumination, AppA, Slr1694, and YcgF showed a relatively small but characteristic red shift in the UV−visible absorption of FAD, being converted into the light state, which has been assumed a signaling state (*1*, *4*, *6*). The light state was dark-relaxed to the ground state within a period of seconds to minutes. An NMR study of the BLUF domain of C-terminal truncated AppA suggested that alternations of $\pi-\pi$ stacking interactions between the FAD isoalloxazine ring and aromatic amino acid residues, including AppA Tyr21, are important for the red shift of FAD absorption (*9*). The light-induced Fourier transform infrared (FTIR) difference spectrum of the BLUF domain of the C-terminal truncated AppA showed the prominent bands for the C4═O stretching mode of an FAD isoalloxazine ring, but the other FAD bands had much smaller intensities (*10*). On the other hand, the light-induced FTIR difference spectrum of Slr1694 showed several prominent bands for FAD, indicating that

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[†] This work was supported by grants for the Frontier Research System and Special Postdoctoral Researchers Programs (to S.M. and K.H.) at RIKEN, and Grant-in-Aid for Young Scientists (B) (15770101) (to K.H.) and (16770046) (to S.M.) from MEXT of Japan.

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[1] Abbreviations: BLUF, sensor of blue light using FAD; FAD, flavin adenine dinucleotide; FTIR, Fourier transform infrared; LOV, light, oxygen, or voltage.

10.1021/bi048671n CCC: $27.50

light illumination predominantly weakened the C4═O and C2═O bonding and strengthened the N1C10a and/or C4aN5 bonding in the isoalloxazine ring but changed little in the skeletal vibrations of the ring (*1*). The weakened bonding at C4═O and C2═O suggested a light-dependent formation of hydrogen bonding(s) to C4═O and C2═O. Furthermore, light illumination induced distinct changes in the amide I modes of the peptide backbone, indicating light-dependent changes of the protein moieties of Slr1694 (*1*).

The light-induced structural changes of photoreceptor proteins are thought to be closely associated to their signaling state. The structural change is triggered by cis−trans configurational change of the chromophore in families including phytochromes, rhodopsins, and xanthopsins (reviewed in ref *11*). In several cases, the light excitation of the chromophore is followed by the formation of intermediate(s) that were detected by time-resolved measurement and/or trapped at low temperature and then convert into the final signaling state. In the light, oxygen, or voltage (LOV) domain of phototropins, the light absorption generates a triplet state of flavin (FMN) via intersystem crossing from an excited singlet state. The triplet-triggering photochemical reaction leads to the formation of covalent cysteinyl-adduct at flavin C(4a) with no apparent intermediate state (*12*−*14*). The formation of the signaling state proceeds even at 77 K, and the accompanied changes of protein backbone structure occur despite some modulation at lower temperatures (*15*). In cryptochromes, the singlet excited FAD induces an inter- or intraprotein electron transfer, which has been proposed to trigger conformational changes of cryptochromes as a signaling state (*16*), although structural changes of cryptochromes have not been experimentally proven.

In the photocycle of a BLUF protein, no state for a reaction intermediate was detected either during the formation of the light state or its decay to the dark state (*1*, *4*, *9*). In the present study, we found that illumination of Slr1694 at the medium-low temperature (−35 °C) or under dehydrated conditions induced a state that showed the similar UV−visible absorption changes of FAD but resulted in a completely different FTIR difference spectrum. On the basis of the results, it was inferred that a large part of the light-induced structural changes of apo protein and FAD are suppressed under these conditions. We discussed the possibility that this constrained light state is an intermediate state in the photocycle of Slr1694 trapped at the medium-low temperature or under dehydrated conditions.

MATERIALS AND METHODS

Sample Preparations. The Slr1694 protein of *Synechocystis* sp. PCC6803 was overexpressed in *E. coli* and purified as described previously (*1*). The protein at a concentration of 0.3 mM was dissolved in a H_2O (D_2O) medium containing 50 mM Tris/HCl (DCl) and 1 mM NaCl (pH/pD 8.0).

Spectroscopic Measurements. For FTIR, sample solutions (5−10 μL) applied to a BaF_2 disk were gently desiccated under N_2 gas for 5 min. The obtained sample film was subsequently sandwiched by another BaF_2 disk with a greased 0.5-mm thick Teflon spacer in the presence of a droplet of 1 μL of 40% glycerol/H_2O (v/v) (for hydration spectrum) or 40% glycerol/D_2O (v/v) (for deuteration spectrum) placed outside of the IR beam. The droplet of 40% glycerol solution was omitted for desiccation (dehydration or dedeutration) spectra. The IR sample was incubated at 15 °C for 3 h to equilibrate the water concentration in the sample and then relaxed to a dark state followed by further incubation at a designated temperature for 1 h. FTIR spectra were recorded using a Brucker IFS66v/s spectrophotometer equipped with a MCT detector at 4 cm^{-1} resolution by taking the average of 32 scans (20-s accumulation) as previously described (*1*). Ge band-pass filters (4000−800 cm^{-1}) (OCLI) were windowed on the exit hole of the cryostat to improve the signal−noise ratio. A light-minus-dark spectrum was obtained by subtracting the single-beam dark spectrum from that following illumination with continuous light (ME650, CABIN Industrial Co.) with ∼2000 μmol s^{-1} m^{-2} for 10 s (350−550 nm). After spectrum measurement at designated lower temperatures, the sample temperature was elevated to 15 °C and held for at least 1 h for full relaxation of the sample to the dark state. The sample was then incubated at the designated temperature for 1 h before further illumination. Two to nine spectra were collected to improve the signal−noise ratio by repeating this cycle. No sample damage was observed during measurements. The FTIR sample sandwiched between BaF_2 disks was directly applied to the measurements of UV−visible spectra on a SHIMADZU Multi Spec-1500 photodiode array spectrophotometer equipped with a homemade cryostat.

RESULTS

Figure 1 shows the UV−visible absorption spectra of dark-adapted (dark state) and illuminated (light state) Slr1694. As shown in A, illumination at 15 °C under hydrated conditions resulted in the red shift of flavin absorption with some spectral changes (a, red line), in which the peak at 468 nm broadened to be less prominent as reported previously (*1*). The dark-state spectrum at −35 °C under hydrated conditions (b, black line) was nearly identical to that at 15 °C, despite the more distinctive shoulder at 468 nm. The spectrum was similarly red-shifted by illumination at −35 °C, but the resulting light-state spectrum (b, blue line) was somewhat different from that induced at 15 °C. Consequently, the light-minus-dark spectrum at −35 °C shown in B (blue line) was changed as compared with that at 15 °C (red line); the positive band at 488 nm downshifted to 485 nm with little intensity change; and the positive band at 392 nm decreased in intensity with little peak shift. The effects of the medium-low temperature on the light-induced spectral changes of FAD were seen more clearly in the double difference spectrum shown in C, in which the light-minus-dark spectrum at −35 °C was subtracted from that at 15 °C. The resulting spectrum showed that the 499 and 464 nm peaks in the light-minus-dark difference spectrum at 15 °C were similarly downshifted by 19 and 15 nm in the −35 °C spectrum, respectively. Under dehydrated condition at 15 °C, the spectra for the dark (A, c, black line) and light (A, c, green line) states and therefore the light-minus-dark difference spectrum (B, green line) were rather similar to that of the hydrated Slr1694 at 15 °C, although some changes in the band intensity and the position were evident.

Figure 2 shows the effects of the medium-low temperature (−35 °C) and dehydration on the light-minus-dark FTIR difference spectrum of Slr1694. Illumination of the hydrated Slr1694 sample at 15 °C induced prominent bands at 1713−

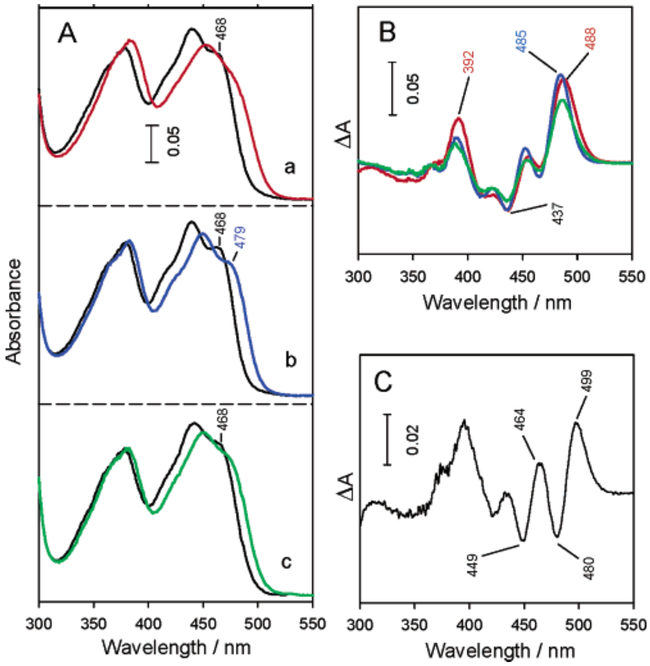

FIGURE 1: (A) UV−visible absorption spectra of dark state (black lines) and light state (colored lines) Slr1694. Hydrated (a and b) and dehydrated (c) samples were illuminated, and spectra were measured at 15 °C (a and c) and −35 °C (b), respectively. Spectra were measured using the same sample and were presented after normalization at the band intensity at 440 nm of the dark-state spectrum as compared with that in the hydrated spectrum at 15 °C. The samples was dehydrated after measuring the 15 and −35 °C spectra for spectrum c. (B) Light-minus-dark difference UV−visible spectra of hydrated Slr1694 at 15 °C (red line) and −35 °C (blue line) and dehydrated Slr1694 at 15 °C (green line). (C) Double difference UV−visible spectrum obtained by subtracting the light-minus-dark difference spectrum of hydrated Slr1694 at −35 °C from that at 15 °C.

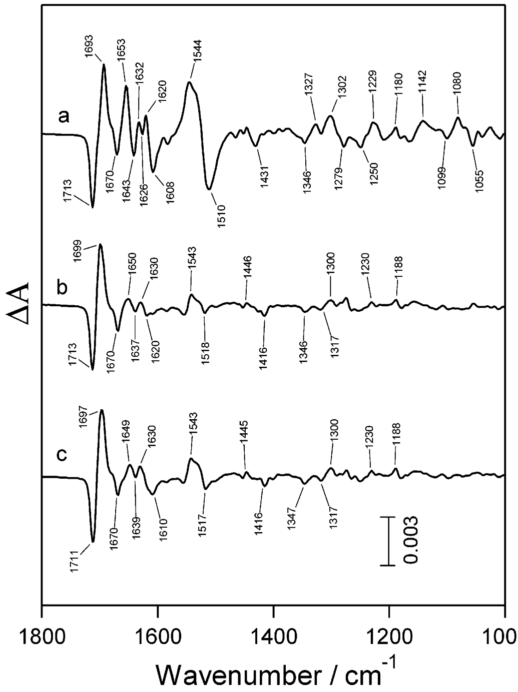

FIGURE 2: Light-minus-dark FTIR difference spectra of Slr1694. Hydrated samples were illuminated at 15 °C (a) and −35 °C (b), or dehydrated samples were illuminated at 15 °C (c), respectively.

1500 cm^{-1} and many smaller bands at 1500−1000 cm^{-1} (a). The positive and negative bands are ascribed to those for the light-induced and dark states, respectively. According to our previous isotopic-labeling study, the prominent bands at 1713(−)/1693(+) cm^{-1}, 1670(−)/1653(+) cm^{-1}, and 1544(+)/1510(−) cm^{-1} have been predominantly assigned to C4═O, C2═O, and C4aN5/N1C10a stretch vibrations of an isoalloxazine ring of the chromophore FAD, respectively, while the bands at 1643(−)/1632(+)/1626(−)/1620(+)/1608(−) cm^{-1} have been mainly ascribed to the amide I bands of the protein backbone (*1*). The relatively small bands at 1500−1000 cm^{-1} have been mainly ascribed to N3C4 stretch vibrations and CH_3 deformations/rockings of protein side groups and/or the isoalloxazine ring (*1*). These indicated that the light excitation induces structural changes in both FAD and the apo protein of Slr1694, although the changes in the skeletal vibrations of the isoalloxazine ring reported in the LOV2 domain of phototropins were not induced (*15*, *17*).

The light-minus-dark FTIR difference spectrum induced by illuminating the hydrated Slr1694 at −35 °C (b) was markedly different from that of the hydrated sample induced at 15 °C (a). The spectrum shows prominent derivative shape bands at 1713(−)/1699(+) cm^{-1} with intensity comparable to the corresponding 1713(−)/1693(+) cm^{-1} bands in the 15 °C spectrum. However, the other bands were largely suppressed or diminished almost completely in the −35 °C spectrum. The results indicate that most light-induced structural changes of FAD and apo protein are inhibited at −35 °C, leaving the change in the C4═O bond of the FAD isoalloxazine ring. The frequency of the positive band at 1699 cm^{-1} in the −35 °C spectrum was higher by 6 cm^{-1} than that of the control 15 °C spectrum, but the negative band appeared at the same frequency of 1713 cm^{-1} as that of the 15 °C spectrum. The results indicate that the interaction of the flavin C4═O group with the protein backbone and/or amino acid side groups in the light state weakens to some extent at −35 °C but that the low temperature does not influence the interaction in the dark state. Interestingly, the −35 °C spectrum strikingly resembled the light-minus-dark FTIR difference spectrum of the dehydrated Slr1694 at 15 °C (c). The insensitivity of the light-induced change of the C4═O group to the lowering temperature and dehydration indicates that either this change precedes the other light-induced structural changes in the apo protein and the isoalloxazine ring or proceeds in a independent manner. It is noteworthy in this context that the observed alterations of the light-induced spectra are not caused by some damage of the sample by the illumination under dehydrated or low-temperature conditions. This is because the normal light-minus-dark spectrum was restored when the illuminated samples were warmed to 15 °C or rehydrated and then reilluminated after dark incubation at 15 °C (data not shown).

Figure 3 shows the light-minus-dark FTIR difference spectra of Slr1694 in the high-frequency region (3100−2400 cm^{-1}) and the effects of the medium-low temperature (−35 °C) and dehydration on the spectra. The spectrum at 15 °C (a) showed prominent sharp bands at 3010−2800 cm^{-1}, which were attributed to CH stretch vibrations of CH_2 and/or CH_3 group(s) (*1*). The appearance of the bands suggests the light-induced structural changes of side groups of amino acid residues and/or the CH_3 group of FAD isoalloxazine ring I. The spectrum also showed relatively weakly bands at 2561(−)/2551(+) cm^{-1}. These bands were assigned to the SH stretch vibration of a cysteine residue based on the

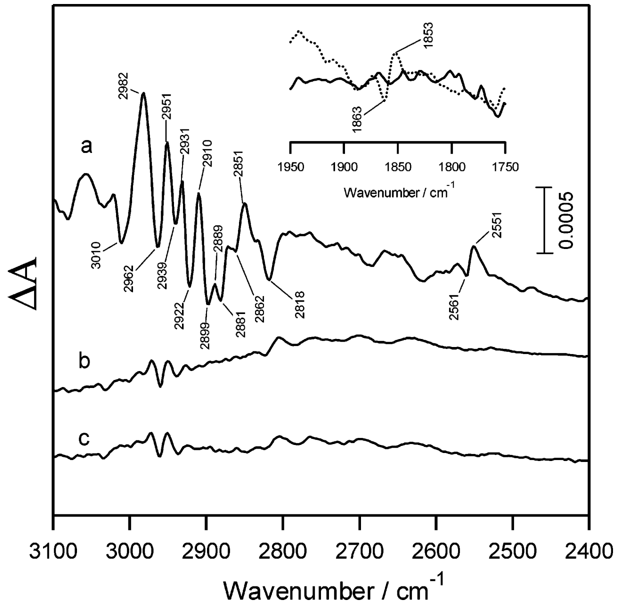

FIGURE 3: High-frequency region (3100−2400 cm^{-1}) of the light-minus-dark FTIR difference spectra of Slr1694. Hydrated samples were illuminated at 15 °C (a) and −35 °C (b), or dehydrated samples were illuminated (c), respectively. (Inset) Expanded view (×3) of the light-minus-dark FTIR difference spectra at 1950−1750 cm^{-1} in the hydrated (—) and deuterated (···) samples.

position and intensity (*18*) and a ~700 cm^{-1} of downshift by deuteration to 1863(−)/1853(+) cm^{-1} (inset, - - -) (*19*, *20*). The observed H/D exchangeability of the hydrogen atom of the SH group indicates that the putative cysteine residue is in a hydrophilic environment. The light-induced downshift of the SH (SD) stretch vibration by 10 cm^{-1} indicates that the SH (SD) bond was slightly weakened in light state (*18*). This implies that the hydrogen bonding(s) of the observed SH group(s) in the cysteine residue(s) is moderate in the dark state and strengthen in the light state. In the LOV domain of phototropins, the cysteine residue is directly involved in the mechanism of the light-induced structural change through the formation of a covalent flavin-cysteinyl adduct (*20*−*22*). However, the observed change may not reflect the specific change of a certain cysteine that is directly involved in the light perception mechanism in Slr1694 but rather attributes to the rearrangements of the hydrogen-bond network occurring in the apo protein because the BULF proteins have no conserved cysteine (*2*, *3*). Both the CH stretch bands at 3100−2800 cm^{-1} and the SH stretch bands at 2561(−)/2551(+) cm^{-1} were largely suppressed in the −35 °C (b) and dehydrated (c) spectra, which showed almost no bands with the exception of small residuals at 3000−2900 cm^{-1}. The results are consistent with the suppression of the midfrequency bands in the 1500−1000 cm^{-1} region at −35 °C and under dehydrated conditions as shown in Figure 2. Therefore, both the mid- and high-frequency results strongly indicate that the light-induced structural changes in FAD and apo protein are markedly and similarly constrained at −35 °C and under dehydrated conditions, although the light-induced changes in the UV−visible absorption proceed as shown in Figure 1.

Figure 4 shows the effects of the excitation temperature on the light-minus-dark FTIR difference spectrum. As shown in A, it is clearly seen that the light-induced spectral changes were markedly inhibited at medium-low temperatures with the exception of the 1713(−)/1693(+) cm^{-1} differential-shape band for the C4═O stretch vibration, which was induced with similar intensity even at −45 °C but with an upward shift of the frequency for the positive peak. The spectrum did not change much until ~0 °C, and the intensities of the bands decreased markedly below ~0 °C with the exception of the C4═O stretch band. Notably, the position and shape of the bands were changed little even though the intensities of the bands were markedly decreased at low temperatures. This suggests that the low temperature inhibits the reaction process commonly responsible for the formation of all of the temperature sensitive bands. Apparently, the 1713(−)/1693(+) cm^{-1} bands do not require this process for their formation. The temperature-dependent intensity change of the C4═O (●) and C4aN5 (■) stretch bands was evaluated quantitatively, and plotted in B with the frequency of the positive peak for the C4═O bands (○).

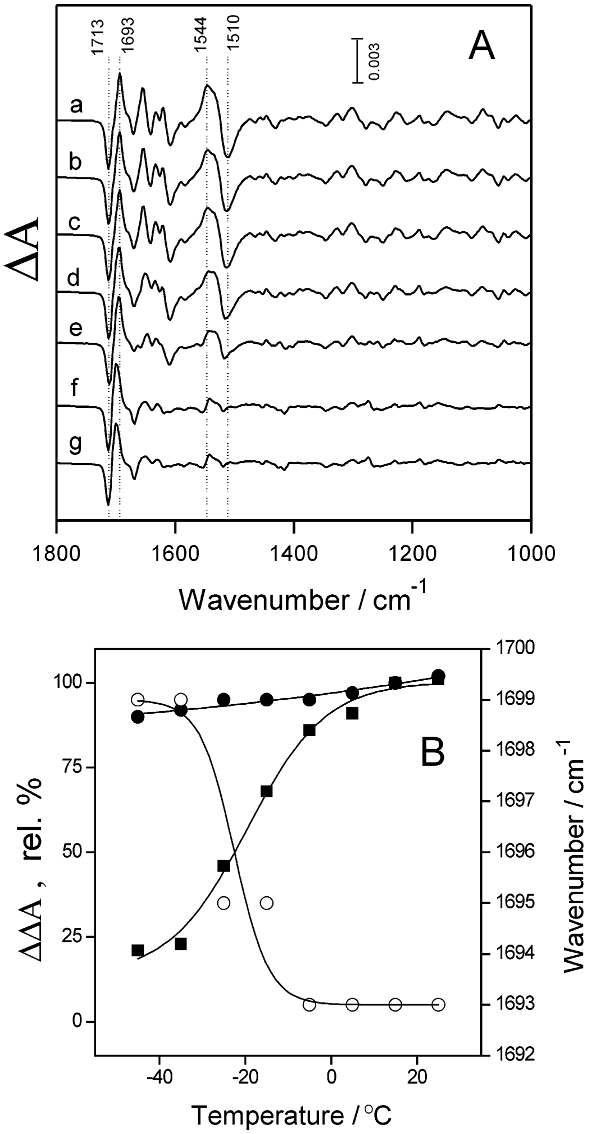

FIGURE 4: (A) Light-minus-dark FTIR difference spectra of hydrated Slr1694. Samples were illuminated, and spectra were measured at 15 °C (a), 5 °C (b), −5 °C (c), −15 °C (d), −25 °C (e), −35 °C (f), and −45 °C (g), respectively. (B) Temperature dependency of the intensities of derivative-shaped FTIR bands for the C4═O stretch at ~1713(−)/1693(+) cm^{-1} (●) and for the C4aN5/N1C10a stretch at ~1544(+)/1510(−) cm^{-1} (■) and the peak position of the light-state C4═O stretch band at ~1693(−) cm^{-1} (○). See text for details.

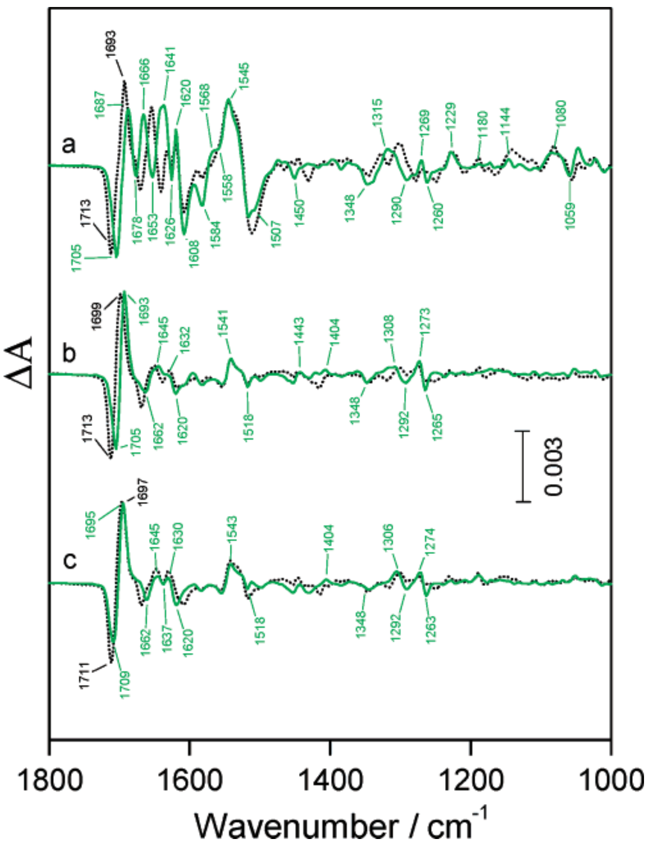

FIGURE 5: Effects of deuteration on light-minus-dark FTIR difference spectra of Slr1694. Samples were illuminated, and spectra were measured at 15 °C (a) and −35 °C (b) in the hydrated (···) and deuterated (green lines) samples, respectively. Hydrated and deuterated samples were desiccated, and de-deuterated spectra were measured at 15 °C (c).

These two modes appeared as prominent derivative shape bands with little overlapping by other bands, and therefore, the amplitude of the light-induced changes of these modes can be estimated quantitatively by measuring the peak(−)−peak(+) amplitude at ∼1713(−)/1693(+) cm^{-1} and ∼1544(+)/1510(−) cm^{-1}, respectively. The C4═O band intensity (●) was almost unchanged in the temperature range from 25 to −45 °C, while the C4aN5 intensity (■) decreased with lowering temperature with a half-inhibition temperature at approximately −20 °C. The frequency of the positive peak for the C4═O bands (○) increased with lowering temperature with a similar temperature dependency to that of the C4aN5 bands intensity. We note that the frequency of the negative peak for the C4═O bands (dark-state band) was insensitive to the change of temperature. The results indicate that the temperature-sensitive change and/or process responsible for the formation of the temperature-sensitive IR bands including the C4aN5 bands caused the frequency shift of the light-state C4═O band.

Figure 5 shows the effects of deuteration on the light-minus-dark FTIR difference spectra of Slr1694. Deuteration affects the vibrational modes in FAD and the apo protein with an exchangeable hydrogen atom, such as NH and/or OH groups. The hydrated spectrum measured at 15 °C (a, ···) was markedly affected upon deuteration (a, green line) and is consistent with our previous result (*1*). Both the C4═O bands at 1693(+) cm^{-1} for the light state and at 1713(−) cm^{-1} for the dark state in the hydrated spectrum were downshifted to 1687(+)/1705(−) cm^{-1} because of the decoupling of the contribution of the N3H bending mode of the flavin. However, the coupled N1C10a and/or C4aN5 bands at 1544(+)/1510(−) cm^{-1} were scarcely affected by deuteration (*1*). The band shapes of the amide I region at 1700−1600 cm^{-1} were complexly altered because of the overlapping of the deuteration-sensitive C2═O stretch vibration and the deuteration-insensitive amide I bands (*1*). The effects of deuteration were clearly seen also in the spectrum measured at −35 °C (b, green line), although the intensity of the bands were markedly suppressed with the exception of the C4═O bands, which were downshifted from 1713(−)/1699(+) to 1705(−)/1693(+) cm^{-1} upon deuteration. The 1645(+) and 1632(+) cm^{-1} bands that remained in the −35 °C spectrum were influenced little by deuteration, indicating that the formation of the deuteration-sensitive C2═O stretch band is suppressed almost completely at −35 °C. The spectrum of the sample that was deuterated and then desiccated with the same procedure for the dehydrated spectrum (de-deuterated spectrum) (c, green line) was very similar to the deuterated spectrum at −35 °C, although the downshift of the C4═O bands by deuteration (2 cm^{-1}) in the desiccated spectrum was relatively smaller than that found in the −35 °C spectrum. The frequency of the dark-state C4═O band in the dehydrated spectrum (1711 cm^{-1}) was slightly lower than the frequency in the hydrated and −35 °C spectra (1713 cm^{-1}), indicating that the hydrogen bonding of the C4═O group in the dehydrated Slr1694 is somewhat different from that in the hydrated or −35 °C Slr1694. Therefore, it may be possible that the change in the hydrogen bonding of the C4═O group affects the coupling of N3H bending mode and then influences the effects of deuteration on the C4═O bands in the de-deuterated Slr1694.

DISCUSSION

The present results clearly demonstrated that illumination at the medium-low temperature (−35 °C) or under dehydrated conditions induces a photoinduced state, which is considerably different from the signaling light state induced by illuminating the hydrated Slr1694 at 15 °C. This state is referred to as a C4═O state hereafter because of the predominant appearance of the C4═O stretch vibration in the FTIR spectrum. In the C4═O state, almost all bands induced by illumination in the mid- and high-frequency FTIR difference spectra were largely suppressed with the exception of the bands for the C4═O group in the FAD isoalloxazine ring as shown in Figures 2 and 3. The 1670(−)/1653(+) and 1544(+)/1510(−) cm^{-1} bands in the FTIR difference spectrum were predominantly assigned to the C2═O stretch and coupled C4aN5/N1C10a stretch vibrations of the FAD isoalloxazine ring, respectively (*1*). The light-induced change of the band position indicated that the C2═O group is moderately hydrogen-bonded in the dark state and strongly hydrogen-bonded to the protein backbone and/or amino acid side groups in the light state and that the C4aN5 and/or N1C10a bonds are weak in the dark state and strengthen in the light state (*1*). The 1346(−)/1302(+)/1279(−)/1250(−) cm^{-1} bands have been primarily ascribed to the changes of N3C4/C4C4a/C4aC10 stretch vibrations of the FAD isoalloxazine ring (*1*). In contrast, the 1643(−)/1632(+)/1626(−)/1620(+)/1608(−) cm^{-1} bands have been mainly ascribed to the structural changes of the protein backbone (*1*). Therefore, the results indicate that most of the light-induced structural changes of the apo protein and FAD accompanying the light-state formation are inhibited in the C4═O state induced at low-temperature and dehydrated conditions. Furthermore, the concomitant suppression of these bands suggests that these changes in FAD are tightly coupled with the structural changes of the apo protein of Slr1694.

Close inspection of the C4═O stretch bands in the low-temperature/dehydration-induced C4═O state shown in parts a and b of Figure 2 revealed that distinct upshift was observed in the position of the light-induced (positive) band but not much in the dark-state (negative) band by lowering temperature and dehydration. These indicate that the microenvironments in the proximity of the C4═O group are not affected much in the dark state, but those after illumination (C4═O state) are changed by the lowering temperature or dehydration. This view is compatible with the observations that the lowering temperature or dehydration affected the dark-state UV−visible spectra little but induced changes in the features of the light-state spectra as shown in Figure 1. The downshift of the C4═O stretch vibration from 1713(−) to 1693(+) cm^{-1} upon the formation of the normal light state (Figure 2a) has been proposed to show that the C4═O group is moderately hydrogen-bonded in the dark state and strongly hydrogen-bonded in the light state (*1*). The appearance of the light-induced C4═O stretch vibration at higher frequency in the C4═O state shown in parts b and c of Figure 2 (1699(+) cm^{-1} for −35 °C and 1697(+) cm^{-1} for dehydration) than in the normally induced light state (Figure 2a, 1693(+) cm^{-1}) suggests the weaker hydrogen bonding of the C4═O group in the C4═O state than in the normal light state. Therefore, it is rational to assume that this difference is responsible for the changes in the light-induced UV−visible spectra at −35 °C or under dehydrated conditions shown in Figure 1.

As shown in Figure 4, the position of the light-induced C4═O stretch band was changed by temperature with the same dependence as the decrease in amplitude of the other bands by lowering the temperature. This indicates that the formation of the C4═O band is closely correlated with that of the other bands, although the C4═O bands are generated even at −35 °C or dehydrated conditions in contrast to the other bands. A strong possibility is that the light-induced change in hydrogen bonding at the C4═O group triggers rearrangements of the hydrogen-bonding network in Slr1694 leading to further alterations of the interaction between FAD and the protein moiety. Under low temperature or dehydrated conditions, these rearrangements are interrupted to lead the inhibition of the formation of other FTIR bands.

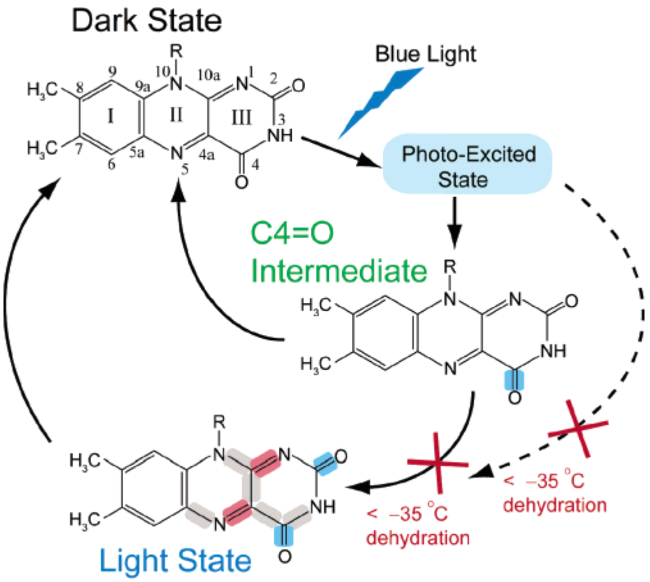

Figure 6: Schematic model of photocycle of Slr1694. Light-induced changes in the force constants in FAD isoalloxazine ring are shown. Bonds that are weakened or strengthened are highlighted in blue or red, respectively. Bonds with small strength changes are highlighted in gray. The C4═O intermediate represents a hypothetical reaction intermediate state between the photoexcited and the light states in the photocycle of Slr1694. See text for details.

These considerations may lead to the view that the signaling light state is formed via the process that includes an intermediate similar to the C4═O state in the photocycle of Slr1694. Figure 6 illustrates a possible photocycle scheme of Slr1694 including this hypothetical intermediate state. In the normal photocycle of Slr1694, upon light absorption, the dark-state Slr1694 is converted to the photoexcited state, which may be a triplet or a singly excited state of FAD, although any photoexcited state of FAD has not been experimentally probed in the BLUF proteins. The photoexcited state is converted to an intermediate reaction state denoted C4═O intermediate. This state is analogous to the low-temperature/dehydration-induced C4═O state, in which the light-induced change is restricted in a close vicinity of the C4═O group. The change at the C4═O group subsequently triggers the changes in the force constants of FAD isoalloxiazine ring concomitant with structural changes of the apo protein to generate the signaling light state. The resulted light state then relaxes to the original dark state with no apparent intermediate. The C4═O intermediate is not stably accumulated at ambient temperature but accumulated as the C4═O state at −35 °C or under dehydrated conditions, which interrupt the conversion from the C4═O intermediate to the light state. The low-temperature/dehydration-induced C4═O state also relaxes to the dark state directly at −35 °C or under dehydrated conditions, because the amplitude of the FTIR difference spectrum for the C4═O state decreased to nearly zero with little change in spectral features during prolonged dark incubation and the C4═O state spectrum was identically induced upon reillumination (unpublished results).

In this scheme, the C4═O group triggering changes of the protein structure and in the force constant of the bondings in the FAD isoalloxazine ring are assumed to be directly responsible for transducing and/or transmitting the light signal, although the exact function of Slr1694 has remained yet to be defined. The effects of low temperature and dehydration on the SH bands in the high-frequency IR region (Figure 3) suggest that the rearrangement of the hydrogen-bonding network is involved in the putative structural change for the light-state formation. The inhibition of the light-state formation by dehydration may imply the involvement of exchangeable water molecules in the putative hydrogen-bonding network. This view is apparently compatible with the significant slowdown of the relaxation kinetics from the light state to the dark state by deuteration or dehydration (*1*). It is noteworthy in this context that the clear deuteration effects on the C4═O bands both in the dark- and light-state Slr1694 indicate the absence of a deprotonation event of the isoalloxazine ring upon illumination (Figure 5 and ref *1*).

The low-temperature/dehydration-induced C4═O state observed in this study is not exactly the same state as the hypothetical C4═O intermediate in Figure 6, because the C4═O state was trapped at low temperature or under dehydrated conditions, which may induce some constraint in the protein structure. In this context, at present, we cannot exclude the possibility that the C4═O state is a constrained light state that is adventitiously formed from the photoexcited state by preventing the formation of the normal light state as shown by the broken arrow in Figure 6 and is not related with the intermediate precursor state of the light state. However, it is noteworthy in this context that the FTIR difference spectrum of the present C4═O state is significantly similar to the reported light-induced FTIR difference spectrum of the hydrated BLUF domain of AppA measured at 20 °C (*10*). This may suggests that the C4═O state or C4═O-like state is a commonly induced principal state in the photocycle of BLUF. The presence of the C4═O intermediate may be evaluated by time-resolved FTIR spectroscopy in the future, if the reaction rate of the conversion from the photoexcited state to the C4═O intermediate is high enough as compared with the formation rate of the light state from the C4═O intermediate for accumulating the C4═O intermediate.

The formation of the C4═O state was accompanied with the red shift at the long wavelength end of the UV−visible spectrum to yield the difference spectrum that is similar to that for the formation of the normal light state as shown in Figure 1. Therefore, it may be rationally concluded that the light-induced change in the UV−visible spectrum is attributed to the strengthened hydrogen bonding at the C4═O group at least in the C4═O state. On the other hand, it was suggested from a NMR study of the BLUF domain of AppA that the enhanced $\pi-\pi$ stacking interaction between the phenol ring of the tyrosyl residue (Tyr21 of AppA) and the FAD isoalloxazine ring is important for the light-induced changes in AppA (*9*). The light-induced spectral changes in AppA are identical with those in Slr1694, and the tyrosyl residue corresponding to Tyr21 of AppA is well-conserved in all BLUF proteins. Therefore, the light-induced change in the UV−visible spectrum in Slr1694 may also be ascribed to the change in the putative $\pi-\pi$ stacking interaction. Our unpublished ab initio molecular orbital calculation for a flavin model showed that flavin absorption is expected to be red-shifted either by strengthened hydrogen bonding at the C4═O group or by enhanced $\pi-\pi$ stacking. On the basis of these, we propose that the UV−visible absorption change upon the formation of the normal light state can be mainly ascribed to the strengthen hydrogen bonding at the C4═O group but partly to the enhanced $\pi-\pi$ stacking interaction between the FAD isoalloxazine ring and phenol ring(s) of aromatic amino acid residue(s), including the putative tyrosyl residue. It is likely that structural changes required for the enhanced $\pi-\pi$ stacking interaction are interrupted by lowering temperature or by dehydration.

Slr1694 is a small protein with no other obvious functional domain and therefore may work by controlling the function of another protein through direct protein−protein interaction. Database search shows that many BLUF proteins, including AppA, contain another functional domain(s) in a molecule and may work by regulating the putative function of the domain (*2*, *3*). Therefore, the light signal absorbed by FAD must effect differently on the protein moiety depending on the functions of the BLUF proteins. The observed structural changes sensitive to low temperature or dehydration can be involved in the structural changes specific for the function of Slr1694. A comparison of light-induced FTIR difference spectra between various types of BLUF proteins will provide us with clues to understand the detailed mechanism of how blue light is perceived through the BLUF domain.

REFERENCES

1. Masuda, S., Hasegawa, K., Ishii, A., and Ono, T. (2004) Light-induced structural changes in a putative blue-light receptor with a novel FAD binding fold sensor of blue-light using FAD (BLUF); Slr1694 of *Synechocystis* sp. PCC6803, *Biochemistry 43*, 5304−5313.
2. Gomelsky, M., and Klug, G. (2002) BULF: A novel FAD-binding domain involved in sensory transduction in microorganisms, *Trends Biol. Sci. 27*, 497−500.
3. Masuda, S., and Bauer, C. E. (2005) The antirepressor AppA uses the novel flavin-binding BLUF domain as blue-light-absorbing photoreceptor to control photosystem synthesis, in *Handbook of Photosensory Receptors* (Briggs, W. R., and Spudich, J., Eds.) Wiley−VCH Publishing, Weinheim, Germany, in press.
4. Masuda, S., and Bauer, C. E. (2002) AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in *Rhodobacter sphaeroides*, *Cell 110*, 613−623.
5. Iseki, M., Matsunaga, S., Murakami, A., Ohno, K., Shiga, K., Yoshida, K., Sugai, M., Takahashi, T., Hori, T., and Watanabe, M. (2002) A blue-light-activated adenylyl cyclase mediates photoaviodance in *Euglena gracilis*, *Nature 415*, 1047−1051.
6. Masuda, S., and Ono, T. (2004) Comparative spectroscopic studies of sensor of blue-light using FAD (BLUF) proteins, AppA of *Rhodobacter sphaeroides* and YcgF of *Escherichia coli*, in *Photosynthesis: Fundamental Aspects to Global Perspectives* (van der Est, A., and Bruce, D., Eds.) Allen Press, KA, in press.
7. Okajima, K., Yoshihara, S., Geng, X., Katayama, M., and Ikeuchi, M. (2003) Structural and functional analysis of a novel flavoprotein in cyanobacteria, *Plant Cell Physiol. 44* suppl, 162.
8. Terauchi, K., and Ohmori, M. (2004) Blue light stimulates cyanobacterial motility via a cAMP signal transduction system, *Mol. Microbiol. 52*, 303−309.
9. Kraft, B. J., Masuda, S., Kikuchi, J., Dragnea, V., Tollin, G., Zaleski, J. M., and Bauer, C. E. (2003) Spectroscopic and mutational analysis of the blue-light photoreceptor AppA: A novel photocycle involving flavin stacking with an aromatic amino acid, *Biochemistry 42*, 6726−6734.
10. Laan, W., van der Host, M. A., van Stokkum, I. H., and Hellingwerf, K. J. (2003) Initial characterisation of the primary photochemistry of AppA, a BLUF-domain containing transcriptional anti-repressor protein from *Rhodobacter sphaeroides*: A key role for reversible intra-molecular proton transfer from the FAD chromophore to a conserved tyrosine? *Photochem. Photobiol. 78*, 290−297.
11. van der Horst, M. A., and Hellingwerf, K. J. (2004) Photoreceptor proteins, “star actors of modern times”: A review of the functional dynamics in the structure of representative members of six different photoreceptor families, *Acc. Chem. Res. 37*, 13−20.
12. Swartz, T. E., Corchnoy, S. B., Christie, J. M., Lewis, J. M., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2001) The photocycle of a flavin-binding domain of the blue light photoreceptor phototropin, *J. Biol. Chem. 276*, 36493−36500.
13. Kennis, J. T. M., Crosson, S., Gauden, M., van Stokkum, I. H. M., Moffat, K., and van Grondelle, R. (2003) Primary reactions of the LOV2 domain of phototropin, a plant blue-light photoreceptor, *Biochemistry 42*, 3385−3392.
14. Kottke, T., Heberle, J., Hehn, D., Dick, B., and Hegemann, P. (2003) Phot-LOV1: Photocycle of a blue-light receptor domain from the green alga *Chlamydomonas reinhardtii*, *Biophys. J. 84*, 1192−1201.
15. Iwata, T., Nozaki, D., Tokutomi, S., Kagawa, T., Wada, M., and Kandori, H. (2003) Light-induced structural changes in the LOV2 domain of *Adiantum* Phytochrome3 studied by low-temperature FTIR and UV−visible spectroscopy, *Biochemistry 42*, 8183−8191.

16. Giovani, B., Byrdin, M., Ahmad, M., and Brettel, K. (2003) Light-induced electron transfer in a cryptochrome blue-light photoreceptor, *Nat. Struct. Biol. 10*, 489−490.
17. Swartz, T. E., Wenzel, P. J., Corchnoy, S. B., Briggs, W. R., and Bogomolni, R. A. (2002) Vibrational spectroscopy reveals light-induced chromophore and protein structural changes in the LOV2 domain of the plant blue-light receptor phototropin 1, *Biochemistry 41*, 7183−7189.
18. Socrates, G. (2001) *Infrared and Raman Characteristic Group Frequencies*, 3rd ed., John Wiley and Sons, U.K.
19. Ataka, K., Hegemann, P., and Heberle, J. (2003) Vibrational spectroscopy of an algal phot-LOV1 domain probes the molecular changes associated with blue-light reception, *Biophys. J. 84*, 466−474.
20. Iwata, T., Tokutomi, S., and Kandori, H. (2002) Photoreaction of the cysteine S−H group in the LOV2 domain of *Adiantum* Phytochrom3, *J. Am. Chem. Soc. 124*, 11840−11841.
21. Salomon, M., Christie, J. M., Knieb, E., Lempert, U., and Briggs, W. R. (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin, *Biochemistry 39*, 9401−9410.
22. Crosson, S., and Moffat, K. (2002) Photoexcited structure of a plant photoreceptor domain reveals a light-driven molecular switch, *Plant Cell 14*, 1067−1075.

BI048671N