Kinetics of conformational changes of the FKF1-LOV domain upon photoexcitation

Yusuke Nakasone¹§, Kazunori Zikihara²§, Satoru Tokutomi² and Masahide Terazima¹*

¹Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
²Research Institute for Advanced Science and Technology, Department of Biological Science, Graduate School of Science, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan

* Corresponding author: phone number: +81-75-753-4026, fax number: +81-75-753-4026, email address: mterazima@kuchem.kyoto-u.ac.jp

§Y. Nakasone, K. Zikihara contributed equally to the work.

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Abbreviations: At, Arabidopsis thaliana; CO, CONSTANS; E. coli, Escherichia coli; FKF, flavin-binding Kelch repeat F-box; FMN, flavin mononucleotide; FT, Flowering Locus T; GST, glutathione S-transferase; LKP2, LOV Kelch protein 2; ZTL, ZEITLUPE; LOV, light, oxygen, and voltage; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PAS, PER-ARNT-SIM; phot, phototropin; phot1, phototropin 1; phot2, phototropin 2; phy3, phytochrome3; GI, GiGANTEA; VVD, Vivid; WC-1, White Collar 1.
Abstract

Photochemical reaction dynamics of a LOV domain from the blue light sensor protein, FKF1 (FKF1-LOV), was studied by the pulsed laser induced transient grating method. The observed absorption spectral changes upon photoexcitation were similar to the spectral changes observed for typical LOV domain proteins, e.g., phototropins. The adduct formation takes place with a time constant of 6 µs. Following this reaction, a significant conformational change with a time constant of 6 ms was observed as a change in the diffusion coefficient. An FKF1-LOV mutant without the conserved loop connecting helices E and F only present in the FKF1/LKP2/ZTL family did not show these slow phase dynamics. This result indicates that the conformational change in the loop region is a major change of the FKF1-LOV photoreaction.
Higher plants have several photosensors that are used to monitor light conditions.\textsuperscript{1-3} In particular, the photochemistry of proteins that consist of LOV (Light-Oxygen-Voltage sensing) domains have recently attracted significant attention. LOV domains bind a flavin mononucleotide (FMN) as a chromophore.\textsuperscript{4} Typical LOV photosensor proteins are phototropins (Phot1 and Phot2), which regulate phototropism, chloroplast relocations and stomatal openings.\textsuperscript{5-8} Both Phot1 and Phot2 are homologous flavoproteins that contain two LOV domains (LOV1 and LOV2), a typical serine/threonine kinase catalytic domain at the C terminus and one linker region connecting the LOV2 and the kinase domains.\textsuperscript{9,10} Phot1 and Phot2 therefore act as light-regulated protein kinases. The photochemistry of these two proteins has been studied extensively.

Besides Phot1 and Phot2, \textit{Arabidopsis} has other photosensors containing LOV domains: FKF1 (flavin-binding Kelch repeat F-box), ZTL (ZEITLUPE) and LKP2 (LOV Kelch protein 2).\textsuperscript{11-16} These proteins have one LOV domain, an F-box domain and Kelch repeat domain. The function of the F-box is to interact with SKP proteins, which is important for ubiquitinating target substrates.\textsuperscript{17,18} Kelch repeat is known to act as a protein-protein interacting site.\textsuperscript{15,19} FKF1 has been shown to be involved in the light-regulated expression of CONSTANS (CO), a key gene involved in day-length discrimination leading to flowering under long-day conditions.\textsuperscript{16} The photoreaction of FKF1-LOV observed by the light absorption technique is similar to that of phot-LOV.\textsuperscript{20,21} Upon blue light illumination, the ground state FKF1-LOV possessing the absorption maximum at 450 nm is converted to a species with a blue shifted absorption spectrum with a peak at 378 nm.\textsuperscript{20} Since the absorption spectrum change is similar to the phot-LOV reaction, the reaction of the chromophore should be similar to that of phot-LOV; i.e., the species should correspond to the FMN-cysteinyl adduct, in which the sulfur covalently binds to the C(4a) carbon of the isoalloxazine ring of FMN. The final product returns to the dark adapted state with a half-lifetime of 62.5 h in solution at 298 K, which is noticeably slower than the half-lifetime of phot-LOV.\textsuperscript{20} Recently, several mutagenesis studies have shown that active site residues are relevant to the significantly different lifetimes of LOV domains.\textsuperscript{21-25} In addition, a comparison of their structures with other LOV domains has shown that the slow-cycling class of LOV domains possesses an additional loop region;\textsuperscript{23} e.g. FKF1-LOV has a characteristic loop region between P99 and L107 as shown in Figure 1. From this, we may speculate that the existence of the loop region may affect the stability of light-adapted state. In other words, the conformational change of FKF1-LOV under the light condition could be different from that of phot-LOV, whereas the structural change around the chromophore should be similar. However, small angle X-ray scattering data indicate a very small change between the dark and light conditions.\textsuperscript{26} Consequently, to characterize this potential change, a more sensitive technique is required. Furthermore, it is preferable to study the reaction dynamics by a method with a high time-resolution. In this respect, we have previously shown that the transient grating (TG) technique is a unique and very powerful approach for revealing the photochemistry of photosensor proteins. In particular, this approach provides information on the changes in molecular volume and the molecular diffusion coefficient ($D$).\textsuperscript{27-32}

Here, we investigated the photoreaction of FKF1-LOV by the TG method and observed two phases with a time constant of 6 $\mu$s and 6 ms. The former reaction was assigned to the adduct formation between the chromophore and the cysteine residue and the latter reaction was assigned to an intramolecular conformational change of the protein moiety. Interestingly, when we removed the characteristic loop region of FKF1-LOV, the conformational change associated with the 6 ms phase was not observed and the dark recovery rate was accelerated.
Therefore, we conclude that a major conformational change of FKF1-LOV occurs to the loop region. Contrary to this change, the CD spectrum did not depend on the light illumination as well as the previously reported small angle X-ray scattering profile. Consequently, the kinetic measurement of the intermolecular interaction is very sensitive in reflecting any protein conformational changes. We consider that this FKF1 is a unique photosensory protein that undergoes a key conformational change in the loop region.

**Experimental Procedures**

**Sample preparation**

The preparation of vector of the LOV domain (from Asp28 to Arg174) of the *Arabidopsis thaliana* (*At*) FKF1 protein is described in Supporting information (SI). In addition to the wild-type FKF1-LOV domain, a no loop-containing mutant (FKF1-LOV-NL) was also prepared. In the FKF1-LOV-NL, 13 residues (Tyr96–Val108) were removed and four residues of *At* phototropin2 LOV2 (Gly431–Thr434) were inserted. Both FKF1-LOV and FKF1-LOV-NL were expressed and purified in the same manner (The details are described in SI).

**Measurements**

The experimental setup for the TG measurements was similar to that reported previously. The principle of TG measurements is described in SI.27-32 A laser pulse from a XeCl excimer laser (Lambda Physik, Compex102) -pumped dye laser (Lumomics, HyperDye 300; 465 nm) was used as an excitation light source and a CW diode laser (Crysta Laser, 835 nm) as a probe light source. The excitation laser beam was split into two by a beam splitter and each beam was crossed inside a quartz sample cell with an optical path length of 2 mm. The probe laser beam was introduced to the crossing region under the Bragg condition. A part of the probe beam diffracted by the refractive index modulation (TG signal) was isolated from the excitation laser light with a glass filter and a pinhole, and then detected by a photomultiplier tube (Hamamatsu, R1477). Usually, 20–100 signals were averaged by a digital oscilloscope (Tektronix, TDS-7104) to improve the signal-to-noise ratio. The repetition rate of the excitation was usually 0.02 Hz and we stirred the sample solution each time after the incidence of one excitation pulse beam to avoid the excitation of the photo-product. The laser power for the excitation was set to be weak enough (< 10 μJ pulse⁻¹) to not excite the photoexcited protein twice by the laser pulse. The $q^2$ values at each experimental setup were determined from the decay rate of the thermal grating signal of the calorimetric reference (aqueous solution of bromocresol purple). The concentration of FKF1-LOV protein was ranged from 99 μM to 350 μM to measure the concentration dependence of TG signal. Other experiments were performed with the concentration of 350 μM for both FKF1-LOV and FKF1-LOV-NL. All TG measurements were carried out at room temperature.

The absorption spectra and its dark reversion of the purified FKF1-LOV and FKF1-LOV-NL polypeptide were recorded with a spectrophotometer UV-3310 (Hitachi). For activating the sample, the protein solution in an optical cell was irradiated by LED illuminator (emission maximum at 467 nm, 150 μmol m⁻² s⁻¹ at the sample position). Dark reversion from light-adapted state to dark-adapted state of the polypeptide in solution was monitored at 298 K by controlling the temperature with a thermostable cell holder (Hitachi).

Circular Dichroism (CD) spectra of FKF1-LOV and FKF1-LOV-NL in PBS solution (0.4 mM Na2HPO4, 0.1 mM KH2PO4, 7 mM NaCl and 0.14 mM KCl, pH 7.4) were measured in the
far UV (180-260 nm) region at 298 K with a spectropolarimeter (J820, JASCO) equipped with an electric temperature-controlling system under flowing N2 gas. In order to eliminate the absorption of Tris-buffer at the UV region, PBS buffer was used instead. Optical pathway was 0.1 cm and the concentrations of the FKF1-LOV and FKF1-LOV-NL samples were determined by FMN absorbance (OD=0.05 at 450nm). For each measurement, 10 spectra were collected and averaged. Sample spectra were obtained by subtracting the spectra of the sample buffer. Setting a cuvette and measuring CD spectra were carried out in darkness. Measuring light from spectropolarimeter in the far UV region has little actinic effect on the UV-visible absorption spectra of FKF1-LOV and FKF1-LOV-NL solutions measured with a spectrophotometer (U-3310, Hitachi). The adduct form of the FKF1-LOV and FKF1-LOV-NL was induced by 1 min of blue light irradiation from a handmade LED illuminator (emission maximum at 467 nm) was used that was set beside the sample cuvette in the spectropolarimeter (100 μmol m⁻² s⁻¹ at the sample position).

Results and discussion

TG signal of FKF1-LOV

A typical TG signal of FKF1-LOV in the buffer at a concentration of 350 μM and at a grating wavenumber $q^2 = 1.8 \times 10^{10} \text{ m}^{-2}$ is shown in Fig. 2. The TG signal rose within the time response of our experimental system (~20 ns), decayed to the baseline and exhibited a rise-decay profile twice within a time range of 1 μs to 5 s. The initial decay-rise-decay profile was expressed well by a bi-exponential function with a constant background ($\delta n_{\text{spe}}$), which is a part of the later rise-decay peak.

$$I_{\text{TG}}(t) = \alpha \{ \delta n_i \exp(-k_1 t) + \delta n_{\text{th}} \exp(-D_{\text{th}} q^2 t) + \delta n_{\text{spe}} \}^2$$

The time constant of the first component ($k_1^{-1}$) was 6 μs, which was independent of $q^2$. This $q^2$ independence indicated that this phase represents a chemical reaction rather than a diffusion process. For the assignment of this component, we noted that the qualitative temporal profile is similar to that observed after the photoexcitation of the LOV domains of phototropins (phot1-LOV2, phot2-LOV2). Furthermore, the absorption spectrum of the light illuminated sample resembles that of the photoadduct state of phototropins. Therefore, this phase is attributed to the adduct formation between the C(4a) carbon of the isoalloxazine ring of FMN and the sulfur of the cysteine residue. Compared with the adduction formation rates of phototropins, e.g., phot1-LOV2 (1.9 μs) and phot2-LOV2 (0.9 μs), the FKF1 formation rate (6.0 μs) is relatively slow.

The time constant of the second decay component agreed with the signal from a calorimetric reference sample which converts all the absorbed photon energy to the thermal energy under the same condition. Consequently, this component is attributable to the thermal grating ($\delta n_{\text{th}}$) caused by the thermal energy released from the excited molecule and the enthalpy change of the reaction.

Figure 3 depicts the TG signal measured at various $q^2$. The signal intensity was normalized by the thermal grating signal. It is apparent that the time range of the next rise-decay component depended on $q^2$. Hence, these dynamics were attributed to the protein diffusion process (diffusion peak). Since $\delta n_{\text{th}}$ is negative at this temperature, we determined the signs of $\delta n$ of the rise and decay components to be negative and positive, respectively. From these signs, the rise and decay components of the TG signal were attributed to the species grating due to the
reactant and the product, respectively. The slower rate constant of the product diffusion component indicates $D_P < D_R$; i.e., the diffusion coefficient decreases upon product formation. The presence of the $D$-change clearly indicated the presence of a conformational change and/or adduct state change. We will discuss the origin later.

It should be noted that not only the time range of the signal, but also the diffusion peak intensity was also dependent on $q^2$ (Fig. 3(a)). Since this diffusion peak intensity reflected the difference between $D_P$ and $D_R$, the gradual increase of the intensity with increasing observation time indicated that the difference in $D$ became larger with time. Furthermore, plotting the signals against $q^2t$, we found that the profiles were different from each other (Fig. 3(b)). These observations can be explained in terms of the time dependent $D$ of the product.

The temporal profile of the TG signal was analyzed using the reaction model of Scheme 1 of Supporting information (Eq. SI-3) with a time-dependent apparent $D$. To obtain a reliable rate constant of the $D$-change from the fitting, the number of adjustable parameters was reduced. The $q^2t$-plot (Fig. 3(b)) shows that the signals essentially overlap at low $q^2$-values. In other words, the time-dependence of $D_P$ almost completes in a slow time regime, e.g., 50 ms. Therefore, as $D_R$ and $D_P$ are constant in this slow time region, the diffusion signal after 50 ms should be expressed by a bi-exponential function (Eq. SI-2). By fitting the peak in a later time range by a bi-exponential function, $D_R$ and $D_P$ were determined to be $(8.6 \pm 0.1) \times 10^{-11}$ and $(8.1 \pm 0.1) \times 10^{-11}$ m$^2$/s, respectively. By using these parameters, the observed TG signal can be reproduced very well in a wide observation time range (100 μs to 10 s in Fig. 3(a)) at various $q^2$ values with only two adjustable parameters, i.e., $D_I$ and a reaction rate, $k$. The diffusion coefficient of the intermediate ($D_I$) and the time constant of the change determined from the fitting were $(8.4 \pm 0.1) \times 10^{-11}$ m$^2$/s and 6.0 ± 1.5 ms, respectively. The remarkable agreement between the fitted and the observed signals (Fig. 3a) supports the above model for describing this process.

The $D$ value of FKF1-LOV is smaller than proteins with similar molecular masses [e.g., phot2-LOV2 (monomer): 16 kDa, $D = 10.3 \times 10^{-11}$ m$^2$/s; lysozyme from human serum: 14.5 kDa, $D = 11.9 \times 10^{-11}$ m$^2$/s], but close to $D$ values of proteins with larger molecular masses [e.g., phot1-LOV2 (dimer): 34 kDa, $D = 8.0 \times 10^{-11}$ m$^2$/s; aminopeptidase from Aeromonas proteolytica: 35 kDa, $D = 8.5 \times 10^{-11}$ m$^2$/s]. This result indicates that FKF1-LOV exists as a dimer at this concentration. This is consistent with previous size-exclusion chromatography data and SAXS measurements even though the protein concentrations of these studies were lower than that of our sample (2.0 μM at the elution peak for the chromatography and 140 ~ 280 μM for the SAXS measurements). The dimeric forms have been reported for other LOV domains such as phot1LOV1 from Avena Sativa, phot1LOV1, phot2LOV1 from At and YtvA-LOV from Bacillus subtilis.

### Origin of the diffusion change

Since the diffusion of macromolecules such as proteins is considered to be mostly determined by the hydrodynamic friction because of the large molecular size, it was expected that a change in friction due to a change in conformation should be negligible and thus $D$ is rather insensitive to conformational changes. Such observations have been made previously, in which a change in $D$ was negligible for the ligand dissociation reaction of myoglobin, although conformation change is unavoidable by the ligand escape from a protein. However, contrary to this expectation, changes in $D$ have been frequently observed during reactions of photosensors. Mostly, the origin of the change in $D$ has been classified into two categories:
oligomer formation and conformational change [diffusion sensitive conformation change (DSCC)]. We may discriminate between these origins by the concentration dependence of the rate. If the protein undergoes conformational change, the reaction rate (and \( D \)) should be independent of protein concentration, and the profile of the TG signal in any timescale should not depend on concentration, except for the absolute intensity. On the other hand, if this \( D \)-change is caused by a multi-molecular process, the kinetics of the TG signal should be sensitive to the concentration.

Figure 4 shows the TG signals measured at various concentrations of FKF1-LOV. The signal intensity was normalized by the peak intensity of the diffusion signal. Since this signal appeared in a time range of the \( D \)-change kinetics, any concentration dependence of the rate of the \( D \)-change should be apparent as a different signal shape. The shape of the signal was observed to be almost identical. Hence, we concluded that the \( D \)-change is not due to the formation of a higher order species, but to an intramolecular conformation change (DSCC). Furthermore, the refractive index change of the diffusion signal was proportional to the concentration of FKF1-LOV. This result indicates that the reaction does not depend on the concentration; i.e., there is no oligomer formation and no dimer-monomer equilibrium, which was observed for phot1LOV2 from \( At \), photLOV1 from \( Chlamydomonas reinhardtii \) and Vivid-LOV from \( Neurospora crassa \).\(^{32,42-44}\) The SAXS measurements also reported that there was no light-dependent oligomeric transition for FKF1-LOV.\(^{26}\)

Conformation change of FKF1-LOV

An important question is; where is the region of the DSCC in the protein? To identify the region, we compared the dynamics of FKF1-LOV with those of the other LOV domains of phototropins. Previously, we investigated the DSCC of phot1-LOV1 (unpublished), phot1-LOV2, phot2-LOV1 and phot2-LOV2 by the TG method.\(^{27,29,31}\) Phot2-LOV1 and phot2-LOV2 showed very minor changes in \( D \).\(^{27,31}\) (Although \( D \) of phot1-LOV1 and phot1-LOV2 decreased upon the photoexcitation, the \( D \)-changes were mostly explained by the increase in the molecular sizes due to dimerization.\(^{29}\)) Hence, we concluded that the conformational change of the LOV domain of phototropin is small. In addition, it should be noted the unusually slow dark recovery rate of FKF1-LOV compared to phot-LOV domains. This fact implies the observed conformational change of FKF1-LOV could be relevant for the stabilization of light adapted state. A comparison of the structures of FKF1-LOV with that of phototropins shows that the FKF1-LOV protein possesses an additional loop region between P99 and L107 (Fig.1).\(^{20}\) In order to investigate the effect of this loop region, we measured the TG signal and the dark recovery rate of a FKF1-LOV without the loop region (FKF1-LOV-NL) (Fig. 6).

In the preparation of FKF1-LOV-NL construct, we removed 13 residues (Tyr96–Val108) from FKF1-LOV and inserted four residues of \( At \) phototropin2 LOV2 (Gly431–Thr434) to keep a stable binding of a chromophore. This extra insertion possibly affects the photoreaction itself. However, the molecular dynamics simulations of photLOV1 from \( Chlamydomonas reinhardtii \) and phy3-LOV2 from \( Adiantum capillus-veneris \) have shown that this inserted region did not show any remarkable motions at the dark state and the change of mobility upon photoexcitation was minor.\(^{46}\) Therefore, we exclude a possibility that the insertion of the additional four residues alters the fundamental photoreaction of FKF1-LOV-NL. Fig. 5-a shows the absorption spectra of dark- and light-adapted states of FKF1-LOV and FKF1-LOV-NL. The differences in the spectral shape between two constructs
were very minor, which clearly showed the covalent bond between chromophore and cysteine residue was formed upon light illumination for FKF1-LOV-NL as well as FKF1-LOV. The ratio of absorption peaks of dark-adapted species at 271 and 450 nm, 3.12 for FKF1-LOV, and 3.44 for FKF1-LOV-NL, allows us to estimate the fraction of apo-protein. The similarity of the value between these samples indicates that amount of the apo-protein for FKF1-LOV-NL is minor. Furthermore, the TG signal came from only the photoexcited protein by the chromophore. Hence, the effect of apo-protein on its reaction dynamics should be negligible.

Fig. 5-b shows the time course of absorption change associated with the dark recovery reaction monitored at 450 nm. The signals were reproduced well by the single exponential function. The rate constants depended on the existence of the loop region and were determined to be 62.5 h and 20.9 h for FKF1-LOV and FKF1-LOV-NL, respectively. The accelerated dark recovery rate indicates the reaction of FKF1-LOV involves the conformational change of the conserved loop. However, even though the dark recovery rate was accelerated about 3-folds, the FKF1-LOV-NL is still classified into slow-cycling LOV family. Therefore, we conclude that the conformational rearrangement of vicinity of the chromophore also plays a key role in the stabilization of light-adapted state as reported by several site-directed mutagenesis studies.

The TG signal of FKF1-LOV-NL was similar to that of FKF1-LOV in a fast time region (< 5 ms). The time constant of the adduct formation is the same as that of FKF1-LOV (6 μs), indicating that the loop region does not affect the kinetics of the adduct formation. On the other hand, a notable difference was the weak diffusion peak of the FKF1-LOV-NL signal. Since this diffusion peak appeared by the decrease of $D$ of the photoproduct, the observed weak peak intensity indicates that the $D$-change of FKF1-LOV-NL is small. Hence, we concluded that the $D$-change comes from a conformational change of the loop region.

Moreover, the $q^2$ dependence of the diffusion peak (Fig. 6) was significantly different from that of FKF1-LOV. Contrary to the case of FKF1-LOV, the peak intensity did not depend on $q^2$, indicating that the $D$-change completed in a fast time regime. The temporal profile of the diffusion peak was reproduced well by a bi-exponential function (without reaction kinetics), and $D_R$ and $D_P$ were determined to be $(9.0 \pm 0.1) \times 10^{-11}$ and $(8.7 \pm 0.1) \times 10^{-11}$ m$^2$/s, respectively. The $D$ of the reactant of FKF1-LOV and FKF1-LOV-NL are very similar to each other. This similarity indicates that FKF1-LOV-NL also exists as a dimer. The absence of the elimination effect of the loop region suggests that the dimer is formed by an intermolecular interaction distal from the loop region and probably lies within the β-sheet region of the LOV domain.

The ratio $D_I/D_R$ of FKF1-LOV (0.98) and $D_P/D_R$ of FKF1-LOV-NL (0.97) are essentially identical. This similarity suggests that the conformation of the product of FKF1-LOV-NL is similar to that of the intermediate (not the final product) of FKF1-LOV. Since we did not detect any other kinetics in a fast time range, this $D$-change can be considered to have occurred during the adduct formation process and is mostly localized at the LOV domain. The rather localized nature of the conformational change is consistent with the above consideration.

On the basis of a number of previous experimental studies on protein diffusion, it is reasonable to consider that the observed $D$-change is due to an increase in the interaction between FKF1-LOV and the surrounding solvent. The loop interacting LOV core in the dark state may be exposed to the solvent and increase hydrogen bonding with the solvent in the light activated state. The amino acid sequence in the loop region is PRAQRRHPL. There are several
polar amino acids in this region. If these residues are re-oriented to enhance intermolecular interactions, the amount of friction should increase. Consequently, the D value was decreased and the light-adapted state could possibly be stabilized by the newly-formed weak interactions. For investigating changes in the amount of secondary structure, we measured CD spectra of FKF1-LOV and FKF1-LOV-NL in the dark and light states (Fig. 7). The loop deletion results in the minor change in its CD spectra, which indicates that the secondary structure of FKF1-LOV is conserved well upon the mutation. Additionally, there were no significant differences in their spectra between dark- and light-adapted state. Hence, the amount of secondary structure appears to have not changed upon light illumination. Moreover, a SAXS experiment showed that the FKF1-LOV polypeptide exhibits very small changes in the profile between the dark and light conditions. These results indicate that the D measurement using the TG method is a very sensitive approach to detect conformational changes. Such an approach can detect structural changes that cannot be detected by CD or SAXS techniques.

**Role of FKF1-LOV**

The comparison of the photochemistry of FKF1-LOV and the LOV domain of other proteins would be interesting. The oligomeric form in the dark state, the photinduced changes in the oligomeric form and the conformational change that can be detected by the diffusion change (DSCC) of phot-LOV domains are summarized in Table 1. The results clearly show that the LOV1 domains favor dimer formation. This fact suggests that the phot1LOV1 domain is a dimerization site. This dimer of phot1LOV2 domain dissociates upon photoillumination, indicating that the intermolecular interaction is regulated by light. For the LOV2-linker sample, the LOV2 domain and the linker region interact in the dark, yet dissociate upon light illumination. We consider that these two reactions are related; in terms of the light regulation of the interprotein or interdomain interactions. Comparison of these features shows that FKF1-LOV forms a rather stable dimer and this binding does not dissociate by photoexcitation. By using the PSIPRED Protein Structure Prediction Server, the existence of helical structure was predicted both at the N-terminal (E24-K39) and the C-terminal (E167-C192) of LOV core in the full-length FKF1-protein. Therefore these regions might play a role in the signal-transduction. However, the FKF1-LOV-NL did not show significant D-change in spite of the existence of the N-terminal helix and a part of the C-terminal helix. This fact suggests that there is no remarkable conformational change in those helical structures. (We cannot completely exclude the possibility that the helices undergo structural change like other LOV2-linker samples, because our constructs contains only part of helices. This possibility should be examined in future.) Instead, we have clear evidence that the loop region of the FKF1-LOV is important in facilitating the changes in conformation which enhance intermolecular interactions. These observations suggest that the interdomain interaction between the FKF1-LOV and the other domains (F-box or Kelph repeat) is regulated through a conformational change of the loop in a light-dependent manner. Recently, Sawa et al. reported that the LOV domain of FKF1 interacts with a plant-specific protein called GIGANTEA (GI), which is a positive regulator of CO expression. This interaction occurs specifically in blue light irradiation of the LOV domain. As such, it is possible to consider that the light induced structural change of the loop region is responsible for the interprotein interaction between FKF1 and GI. The loop-depletion effect on the GI interaction should be a very interesting topic in future.

Since the LOV domains of Vivid (VVD) and White Collar 1 (WC-1) also have the
characteristic loop insertions like a FKF1-LOV, one may consider that they have similar mechanisms to regulate their signaling processes. However, it was reported that VVD showed a conformational change in its Ncap region and the formation of a rapidly exchanging homodimer upon photoexcitation, which are totally different from the reactions of FKF1-LOV. Here, we have to note that there is a significant difference in their nature, that is, VVD and WC-1 have an FAD as a chromophore instead of FMN and the loop regions seems to be important to stabilize their binding the chromophore by covering its adenine region. Therefore, the loop insertion of these proteins might have variant meaning in their functioning. In many cases, the loop regions of proteins are considered to be flexible and the conformation is not important for aiding the reactions. However, recently, the importance of protein flexibility in loop regions was reported for ribonuclease A by the NMR spectroscopy. This study found that shortening loop 1 results in a restriction in motion and conformational change of this loop, which are essential for a product release step. This indicates that the flexibility of this loop region is important for the efficiency of the ribonuclease A catalyzed reaction. In this study, we detected the conformational (and/or flexibility) change of the loop region and propose that this change may be important in a sensor protein reaction.

Summary

To identify any conformational change of the blue light sensor protein, FKF1 (FKF1-LOV), we used the pulsed laser induced transient grating method and detected both a volume and diffusion change in the time-domain. We found two phases with time constants of 6 μs and 6 ms. The faster reaction was assigned to the adduct formation between the chromophore and cysteine residue, whereas the slower reaction was assigned to a conformational change to the protein moiety. No conformational change with the 6 ms phase was observed for the mutant FKF1-LOV-NL protein. Consequently, we conclude that the major conformational change of FKF1-LOV involves the loop region. In other blue light sensor proteins with two LOV domains, e.g. phot1 and phot2: one LOV domain (LOV1) most likely plays a role in dimerization, whereas the other LOV domain (LOV2) plays a key role in regulating the biological function of the protein. Since FKF1 has only one LOV domain, we speculate that the LOV domain plays two functions. The dimerization site may be located within the β-sheet of the LOV domain like phot-LOV1 and YtvA-LOV, and the biological activity may involve the loop region which could be important for blue-light dependent FKF1/GI interaction. It would be interesting to test this postulate by monitoring the change in biological function upon deletion of the FKF1 loop in future.

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References


Table 1. Comparison of the photoinduced changes and oligomeric states of FKF1-LOV and phot-LOVs.

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<td>phot2-LOV1</td>
<td>dimer</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>phot2-LOV2</td>
<td>monomer</td>
<td>a small conformation change</td>
<td>27, 31</td>
</tr>
<tr>
<td>phot2-LOV2-linker</td>
<td>monomer</td>
<td>dissociation of the linker (and unfolding of the linker)</td>
<td>27, 31</td>
</tr>
</tbody>
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Figure captions

Fig. 1: (a) Ribbon structures of simulated FKF1-LOV (magenta) and phy3-LOV domain (cyan). 3D structure prediction for the FKF1-LOV was carried out using an automated comparative protein-modeling server, Swiss Model (http://swissmodel.expasy.org/). The characteristic loop (P99 to L107) is shown in yellow. (b) Multiple sequence alignment of several LOV domains. LOV sequences in the alignment include A. thaliana FKF1 (AF216523), phot2 (AAC27293), Adiantum phy3 (BAA36192). Asterisks indicate 100% identity, and dots indicate similarity. The LOV core region is indicated by arrows. Characteristic loop region of FKF1-LOV is shown by red letters. The additional removed part of FKF1-LOV and the inserted 4 amino acid residues of phot2LOV2 are shown by purple and blue, respectively. The indicated amino acid sequence of FKF1 (Asp28-Arg174) is used in this experiment.

Fig. 2: A TG signal (broken line) of FKF1-LOV at 350 μM and $q^2 = 1.8 \times 10^{10}$ m$^{-2}$. The best fitted curve using Eq. (1) and (SI-3) is shown by the solid line which essentially overlaps with the observed signal.

Fig. 3: (a) TG signals (gray lines) of FKF1-LOV at $q^2$-values of (1) $1.1 \times 10^{13}$, (2) $1.4 \times 10^{12}$, (3) $1.5 \times 10^{11}$ and (4) $1.8 \times 10^{10}$ m$^{-2}$. The signals representing the molecular diffusion processes are shown and these signals are normalized in the initial part of the diffusion signal. The best-fitted curves to the observed TG signals by Eq. (SI-3) are shown by the solid black lines. The signals are reproduced well by the fitted curves. (b) The signals are normalized at the peak intensity and plotted against $q^2t$. The times of the diffusion peaks are 1.8, 13, 100 and 870 ms, respectively, and are indicated in the figure.

Fig. 4: Concentration dependence of the TG signal (dotted line) of FKF1-LOV measured at $q^2 = 2.8 \times 10^{12}$ m$^{-2}$. The concentrations were 350 (red), 250 (yellow), 160 (green) and 99 μM (blue). The signal intensities are normalized in the initial part of the diffusion signal.

Fig. 5: (a) The absorption spectra of dark- and light-adapted states of FKF1-LOV (blue line) and FKF1-LOV-NL (red line). (b) The absorption change associated with the dark recovery process of FKF1-LOV (open square) and FKF1-LOV-NL (open circle) monitored at 450 nm. The signals are reproduced well by single exponential function (black lines are fitting curves).

Fig. 6: (a) A TG signal (broken gray line) of the FKF1-LOV-NL mutant measured at $q^2$ of $9.6 \times 10^{10}$ m$^{-2}$. The best fitted curve to the observed TG signal (Eq. (2)) is shown by the solid black line. The lower part of the figure shows the magnified signal at the diffusion peak. (b) A $q^2$ dependence of the TG signal of the FKF1-LOV-NL mutant measured at $q^2$ of $8.1 \times 10^{11}$ m$^{-2}$ and $9.6 \times 10^{10}$ m$^{-2}$.

Fig. 7: CD spectra of FKF1-LOV and FKF-LOV-NL before (blue line for FKF1-LOV and black line for FKF1-LOV-NL) and after (light-blue line for FKF1-LOV and red line for FKF1-LOV-NL) light illumination.
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