Photoreaction Cycle of the Light, Oxygen, and Voltage Domain in FKF1 Determined by Low-Temperature Absorption Spectroscopy†

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ABSTRACT: Flavin-binding Kelch repeat F-box (FKF1) protein plays important roles in the photoregulation of flowering in *Arabidopsis*. FKF1 has a light, oxygen, and voltage (LOV) sensing domain binding a flavin mononucleotide (FMN) as a chromophore noncovalently. Photoreaction of the FKF1-LOV polypeptide was studied by low-temperature absorption spectroscopy. Upon blue light irradiation, a ground state, D_{450}, is converted to S_{390} known as a cysteinyl-flavin adduct intermediate in the photoreaction of phototropin. Below 150 K, bleaching of D_{450} was much reduced and a new photoproduct, Z_{370}, appeared as well as S_{390} formation. The calculated absorption spectrum for Z_{370} is very similar to those of flavoproteins in an anion radical state. On the basis of the results that S_{390} formation proceeds to Z_{370} formation and that Z_{370} formed at low temperatures reverts to D_{450} upon temperature increase, Z_{370} is concluded to be not an intermediate from D_{450} to S_{390}. Z_{370} is suggested to be formed from the biradical triplet-excited state after relaxing to the ground state with the FMN anion radical trapped at the low temperature, in which the SH of the cysteine is in the wrong position that is able to produce a radical pair but unable to form the cysteinyl-flavin adduct. The counter SH in the cationic radical state may revert to the ground state by extracting an electron from the unidentified amino acid residue. Interestingly, S_{390} that has been thought to be irreversible to D_{450} was revealed to revert to D_{450} very slowly with a half-life time of 62.5 h in solution at 298 K. The photoreaction mechanism is discussed in reference to the calculated activation energy of the reaction processes.

Plants have acquired three major photoreceptive pigments, a red/far-red photoreversible receptor, phytochrome, and two blue light receptors, cryptochrome and phototropin (phot),[1] to sense their environmental conditions during the evolutional processes. Phot (*1*, *2*) was first identified as a photoreceptor for tropic responses in *Arabidopsis thaliana* (*A. thaliana*) (*3*) and then proved to be a photoreceptor for chloroplast relocation (*4*, *5*) and stomata opening (*6*). Phot has two chromophoric domains in the N-terminal half named LOV (light, oxygen, and voltage) (*7*−*9*) that bind a FMN noncovalently. The LOV domains are known to be a subfamily of the PER-ARNT-SIM (PAS) superfamily thought to act as protein−protein interaction modules in eukaryotic cellular signaling (*10*). Phot has isoforms phot1 and phot2, in *A. thaliana*, that show different light sensitivity and share the regulatory functions (*11*).

In addition to the phot families, *A. thaliana* has three more unique proteins that have only one LOV domain, named ZTL (LKP1, ADO1) (*12*−*14*), LKP2 (ADO2) (*15*, *16*), and FKF1 (LKP3, ADO3) (*17*). All of them have three characteristic domains, a LOV domain, a F-box, and a Kelch repeat in this order from the N- to the C-termini. The function of the F-box is to interact with SKP proteins that are a component of the SCF (Skp/Cullin/F-box) class of E3 ubiquitin ligases that link the target substrates to the core ubiquitinating activity of the ligase complex (*18*, *19*). Kelch repeat, while, is known to act as a protein−protein interacting site (*16*, *20*). FKF1 has been shown to be involved in the light-regulated expression of *CONSTANS* (*CO*) (*17*), one of a key gene in day-length discrimination leading to flowering under long-day conditions through controlling the expression of a gene, *Flowering Locus T* (*FT*) (*21*−*23*), by enhancing degradation of CDF1, a repressor of *CO* expression (*24*) possibly through an ubiquitin−proteasome protein degradation pathway (*25*−*27*). The binding site between FKF1 and CDF1 is reported to be in the Kelch repeat region (*24*), and

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[1] Abbreviations: *A. thaliana*, *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; LKP, LOV Kelch protein; 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, phytochrome 3; ZTL, ZEITLUPE.

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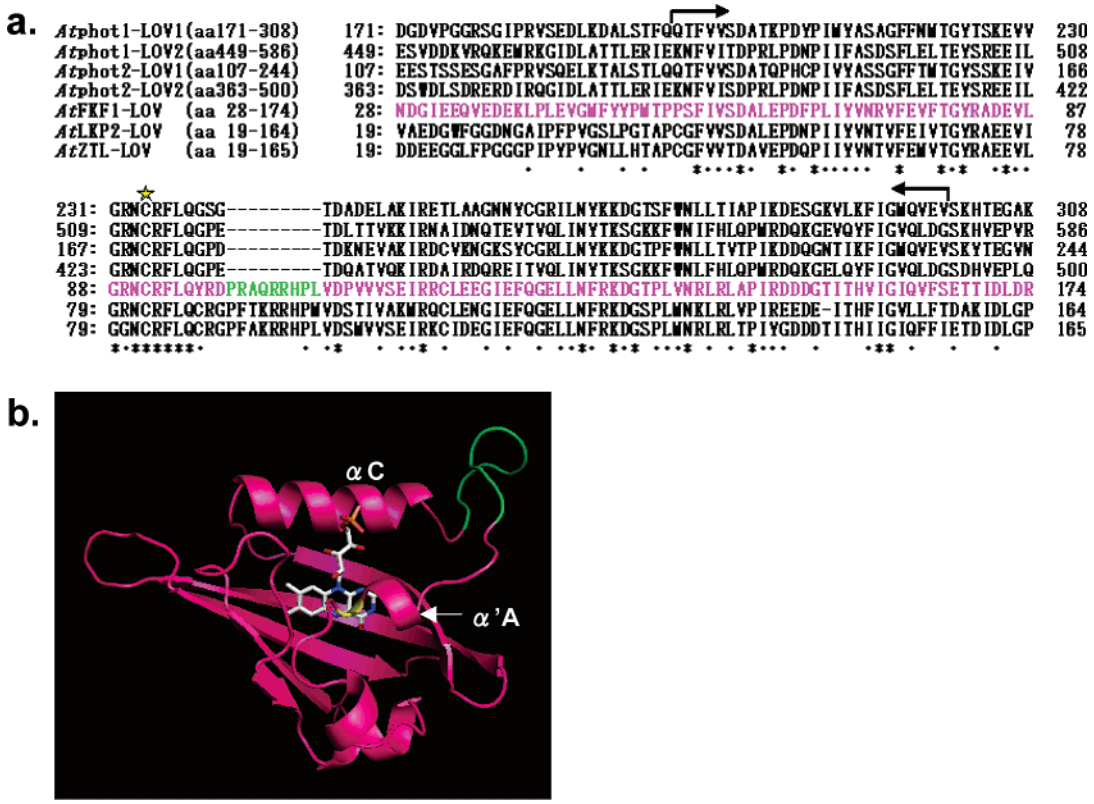

FIGURE 1: (a) Multiple sequence alignment of phototropin and FKF1-type LOV domains in *A. thaliana*. LOV sequences in the alignment include the following: *A. thaliana* phot1 (AAC01753) and phot2 (AAC27293), FKF1 (AF216523), LKP2 (NP849983), and ZTL (AF252294). Asterisks indicate 100% identity, and dots indicate similarity. The LOV core region (*40*) and the conserved cysteine for FMN binding are indicated by arrows and a star, respectively. FKF1 is shown by pink letters, in which a nine amino acid insertion characteristic with the FKF1-type LOV is shown by green letters. The indicated amino acid sequence of FKF1 (Asp28−Arg174) is used in this experiment. (b) Cartoon modeling of the predicted three-dimensional (3D) structure of the FKF1-LOV core domain. 3D structure prediction for the FKF1-LOV was carried out using an automated comparative protein-modeling server, Swiss Model (http://swissmodel.expasy.org/). The nine amino acid insertion between the α′A-helix and αC-helix (helical connector) and the conserved photoactive cysteine are colored by green and yellow, respectively. FMN is shown by stick modeling. Carbon, nitrogen, oxygen, and phosphorus atoms in the FMN molecule are colored by white, blue, red, and orange, respectively.

the binding is thought to be controlled by light received by the LOV domain.

Photoreactions of the LOV domains in phot have been well established. Upon absorbing blue light, the LOV domains undergo a unique photochemical reaction cycle. The ground state of the FMN in the LOV domains shows typical absorption spectra of flavoproteins with an absorption maximum from 445 to 450 nm and named D_{450}, that is excited to a singlet excited state by blue light and then interconverted to a triplet excited state, L_{660} (*28*). FMN in the L_{660} forms an adduct with a nearby cysteine conserved in all of the LOV domains with a time constant of ca. 4 μs, that has an absorption maximum from 380 to 390 nm and is termed S_{390} (*28*−*30*). Recently, a new photointermediate state was detected that has the same electronic structure around the FMN chromophore, however, a different protein structure from those of the S_{390} (*30*−*33*), and is thought to be a signaling state. To discriminate the two S_{390}, we have proposed recently to name them as S_{390}I and S_{390}II (*34*). S_{390} reverts to D_{450} with time constants from several seconds to a few minutes depending on the species (*35*).

In contrast to the LOV domains of phot, photoreactions of the LOV domains in FKF1 families have been poorly understood. They form a photointermediate state alike to the S_{390} that are reported to be not reversible to D_{450} after 40 min (*17*) or 2 h (*36*). LOV domains of the FKF1 families have a nine amino acid insertion between the α′(A)-helix having the conserved cysteine and α(C)-helix (helical connector) as compared to those of phot families (Figure 1). The observed irreversibility may come from this structural difference and possibly be correlated with the different functions between them. Phot has Ser/Thr kinase in the C-terminal region, and the LOV domains act as a light-regulated molecular switch of the kinase in both in vivo autophosphorylation (*37*) and in vitro substrate phosphorylation (*38*). On the other hand, LOV domains of FKF1 families are involved in the formation of protein complexes, for example, FKF1 with CDF1(*24*), ZTL with TOC1 (*12*), and LKP2 with PRR1(TOC1) and PRR5 (*39*).

To understand the molecular bases underlying the different functions between the phot-type and the FKF1-type LOV domains, it is requisite to establish the photoreaction of the FKF1-type LOV domain. We, therefore, investigated the photoreaction of the LOV domain of FKF1 by low-temperature UV−visible absorption spectra and figured out the photoreaction including a novel photoproduct at low temperature. Furthermore, we found that the S_{390} of the FKF1-LOV also reverts to D_{450} at room temperature although its reversion is very slow. The photoreaction kinetics is

discussed on the basis of these data, together with the calculated activation energies of the reactions.

MATERIALS AND METHODS

Preparation of Recombinant LOV-Containing Polypeptide. A LOV domain-containing polypeptide of *A. thaliana* FKF1 protein was prepared by an overexpression system with *Escherichia coli* (*E. coli*). Using *Arabidopsis* cDNA as a template, a DNA fragment corresponding to the polypeptide from Asp28 to Arg174 was amplified by the PCR method with primers providing appropriate restriction sites. That has 26 and 8 extra amino acid sequences to the N- and C-termini, respectively, of the LOV core (*40*). The amplified DNA was isolated, digested, and cloned into a pGEX4T1 expression vector (Amersham Biosience) as a fusion protein with glutathione *S*-transferase (GST). Its nucleic acid sequences were verified by DNA sequencing with a CEQ2000XL DNA analysis system (Beckman Coulter). *E. coli* JM109 strain transformed by the expression vector was grown at 310 K in LB medium containing 50 μg mL^{-1} ampicillin until A_{600} comes to 0.3 and then incubated with 0.1 mM isopropyl β-D-thiogalactopyranoside for 20 h at 293 K in darkness. The following purification procedures were carried out at 273−277 K under dim red light. Bacteria were collected by centrifugation, washed with phosphate-buffered saline (PBS, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.4), and resuspended in PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The cells were lysed by sonication, and the supernatant was mixed with glutathione−Sepharose 4B (Amersham Bioscience). After the resin was washed with PBS, the LOV polypeptides were cleaved from the GST tag with thrombin that leaves two extra amino acid residues, Gly-Ser, to the N-terminus of the LOV-containing polypeptide. The dissociated polypeptides from the gel were purified further by gel filtration on a Sephacryl S-100 HR column (Amersham Bioscience) equilibrated and eluted with 100 mM NaCl, 25 mM Tris-HCl, and 1 mM Na_2EDTA (TBS, pH 7.8). The purity of the polypeptides was examined with Coomassie Blue staining of SDS−PAGE.

UV−Visible Absorption Spectra Measurement of the Solution Sample at Room Temperature. The purified FKF1-LOV polypeptide in TBS was concentrated to give a final concentration with $\lambda_{450} = 0.4$ using a Microcon YM-10 instrument (Millipore) at 277 K. Reversion from S_{390} to D_{450} of the polypeptide solutions in the dark was monitored with a UV-3310 spectrophotometer (Hitachi) at room temperature, 298 K. Blue light was supplied with a combination of a 1 kW projector (OLYMPUS) and a plastic filter sheet (λ_{max} of 480 nm and half-width of 40 nm; Nakagawa Chemicals) to activate the solution sample.

UV−Visible Absorption Spectra Measurement of the Hydrated Film Sample at Low Temperature. The purified FKF1-LOV polypeptide in TBS was concentrated to give a final concentration with $\lambda_{450} = 1.5$ using a Microcon YM-10 instrument (Millipore) and then dialyzed against 1 mM potassium phosphate (pH 7.0) at 277 K. Hydrated films were prepared after the procedure described before (*30*). Briefly, 70−80 μL of the solution was placed on a BaF2 window and then dried under reduced pressure. Dried films were hydrated by dropping H_2O next to the film on the plate and sealing it with one more plate and a silicon rubber gasket. UV−visible absorption spectra of the films were measured using V-550DS (JASCO) spectrophotometers. To measure low-temperature spectra, a cryostat (Optistat DN, Oxford) and a temperature controller (ITC 4, Oxford) were used with liquid nitrogen as coolant. The spectra were measured at eight different temperatures in which a new sample was used at each temperature. As a light source for activating the film samples, a combination of a 1 kW halogen−tungsten lamp and a long-pass filter (L42, Asahi Techno Glass) were used that gives a >400 nm light. In case to attenuate the light intensity, a 25% neutral density filter (ND-25, Asahi Techno Glass) was used.

RESULTS

Blue Light-Induced UV−Visible Absorption Spectral Changes in Hydrated Films Measured at 77−298 K. Blue light induced UV−visible absorption spectral changes of the FKF1-LOV containing polypeptide were measured at eight different temperatures, 298, 250, 200, 175, 150, 125, 100, and 77 K. Six of them are presented in the Figure 2. At 298 K, the polypeptide in the hydrated films prepared in the dark showed a typical spectrum of flavin in a protein pocket with λ_{max} at 450 nm (Figure 2a, 298 K, black line). Here, we call the dark state as D_{450} after the nomenclature of phot in the photocycle. The triplet peak bleached, and a new major peak and a broad shoulder appeared at 378 nm and in 300−320 nm, respectively, upon blue light irradiation (Figure 2a, 298 K, from green to brown lines). The absorption spectrum of this photointermediate state (Figure 2, 298 K, red line) is similar to those of S_{390} of oat phot1 LOV2 with λ_{max} at 390 (*28*) or 380 (*41*) nm, that we also name as S_{390}. At 250 (data not shown) and 200 K (Figure 2a, 200 K), similar light-induced absorption spectral changes were observed although prolonged irradiation is required in the latter case. At 175 K, however, even irradiation for 1 h could not complete S_{390} formation (data not shown) that showed spectral changes similar to those at 150 K (Figure 2a, 150 K). At 125 K, a new peak appeared at 370 nm as well as S_{390} formation (Figure 2a, 125 K) after 1 h irradiation, in which D_{450} bleaching was much reduced. The new peak became more prominent according with the decrease of the temperature. At 77 K, S_{390} formation almost disappeared and only the formation of the new species with λ_{max} at 370 nm was detected, which we propose to name Z_{370}. Formation of Z_{370} did not accompany the appearance of the shoulder at 300−320 nm observed with S_{390} formation (Figure 2a, 77 K).

Light minus dark absorption difference spectra revealed increased formation of Z_{370} at lower temperatures more clearly (Figure 2b). The S_{390} minus D_{450} spectrum obtained at 298 K has characteristic negative peaks at 370 and 450 nm and positive peaks at 310 and 390 nm, respectively. On the other hand, light minus dark spectra below 200 K showed an appearance of a peak at 370 nm that became recognizable as a positive peak at 150 K, comparable with the peak of S_{390} at 390 nm at 125 K, and dominant at 77 K. These results clearly show the formation of a novel photoproduct, Z_{370}, at the low temperatures.

Dependence of S_{390} and Z_{370} Formation on the Blue Light Irradiation Time at 150 K in Hydrated Films. The time course of the blue light-induced S_{390} and Z_{370} formation at

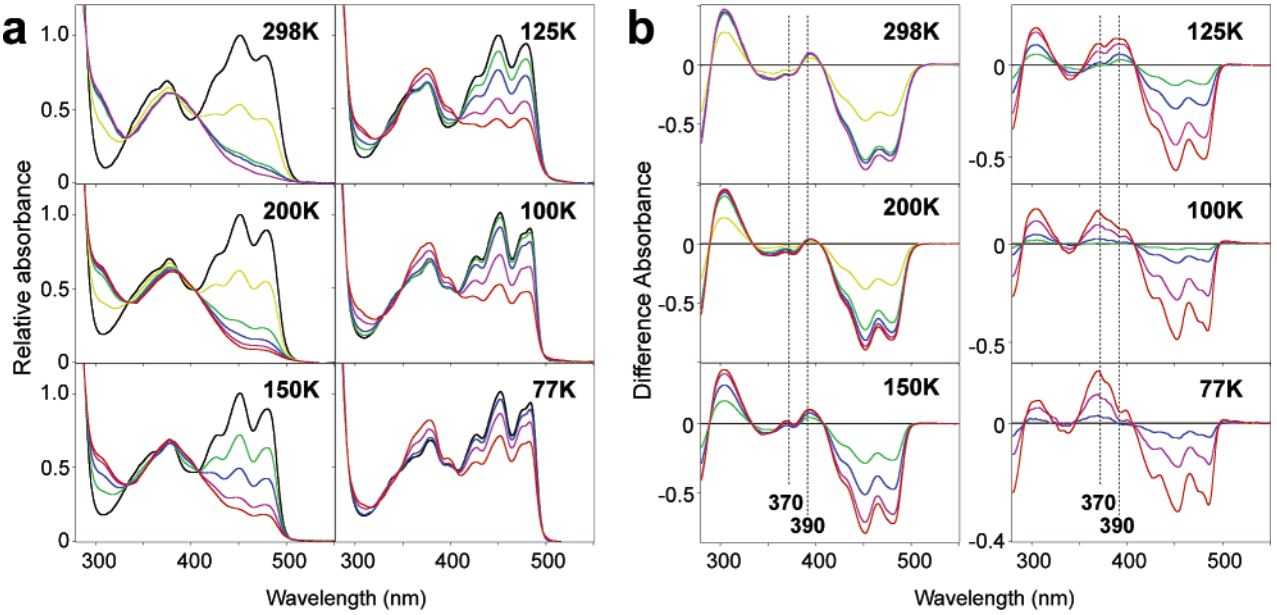

FIGURE 2: Blue light-induced absorption (a) and light-minus-dark absorption difference (b) spectra changes of *Arabidopsis* FKF1-LOV-containing polypeptide in a hydrate film measured at eight different temperatures from 298 to 77 K, where six results are presented with the measured temperatures. Irradiation times are 0 s (black), 2 s (yellow), 10 s (green), 1 min (blue), 10 min (purple), and 60 min (red).

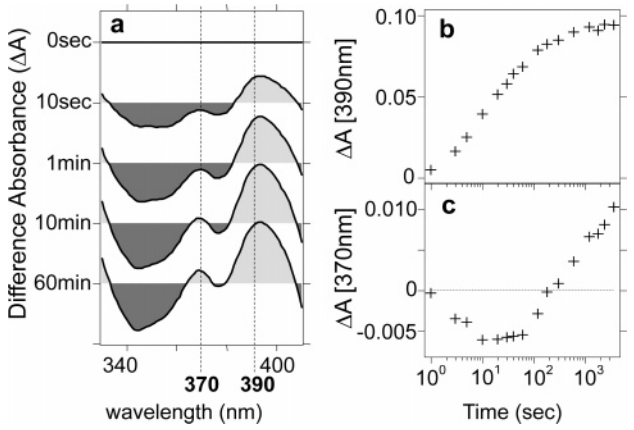

FIGURE 3: Blue light-induced light-minus-dark absorption difference spectra changes of *Arabidopsis* FKF1-LOV-containing polypeptide in a hydrate film measured at 150 K in the wavelength region from 330 to 410 nm (a) adapted from the spectra in Figure 2. Positive and negative areas are colored with light gray and dark gray, respectively. Time courses of the blue light-induced light-minus-dark absorption difference at 390 (b) and 370 (c) nm, where the time scale is logarithmic.

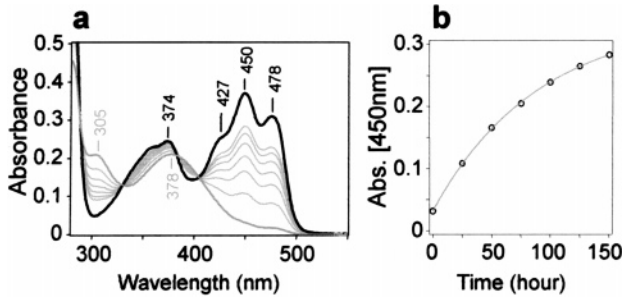

FIGURE 4: Absorption spectra of *Arabidopsis* FKF1-LOV-containing polypeptide in a 100 mM NaCl, 25 mM Tris-HCl, and 1 mM Na_2EDTA (pH 7.8) solution at 298 K before 1 min blue light irradiation (black) and immediately and every other 25 h after the irradiation (gray) (a) and the time course of absorption change at 450 nm (b) where the fitting curve was calculated by a method of nonlinear least squares.

150 K was analyzed using the data presented in Figure 2b. The absorption difference spectra in the wavelength region from 330 to 410 nm are magnified to trace the S_{390} and Z_{370} formation (Figure 3a). The absorbance increase at 390 nm representing S_{390} formation is almost in proportion to logarithmic irradiation time until 100 s and then gradually saturated (Figure 3b). On the other hand, the absorbance change at 370 nm showed a decrease until 20 s and then a linear increase until 60 min (Figure 3c). Since absorption at 370 nm decreases by S_{390} formation, the transient absorption decrease at 370 nm indicates delayed formation of Z_{370} to that of S_{390}. The delayed formation of Z_{370} to S_{390} formation excludes the possibility that Z_{370} is an intermediate between D_{450} and S_{390}. On the contrary, the delay raises the possibility that a part of S_{390} is photoconvertible to Z_{370} by prolonged irradiation of blue light as well as a simple interpretation that Z_{370} formation is delayed to S_{390} formation. However, the possibility can be excluded since Z_{370} formation decreased according to the temperature increase and disappeared at 298 K.

Reversion of S_{390} to D_{450} in the Dark in Solution at 298 K. S_{390} of the FKF1-LOV domain is reported not to revert to D_{450} in the dark; however, we found accidentally that S_{390} of our FKF1-LOV-containing polypeptide does revert to D_{450}. Then, the time course of the reversion was measured at room temperature, 298 K, in the TBS solution. Reverted spectra exhibited a vibrational structure in the main peak region (427, 450, and 478 nm) identical to that of D_{450} (Figure 4a). The time course of the reversion monitored at the absorption increase at 450 nm can be well simulated by a single exponential curve with a half-life time of 62.5 h (Figure 4b). These results indicate that S_{390} reverts to D_{450} through thermal processes very slowly at room temperature.

Calculation of an Absolute Absorption Spectrum of Z_{370} in Hydrated Films. An absolute absorption spectrum of Z_{370} was obtained by calculation from that in the photostationary state among Z_{370}, S_{390}, and D_{450} at 100 K, in which the other

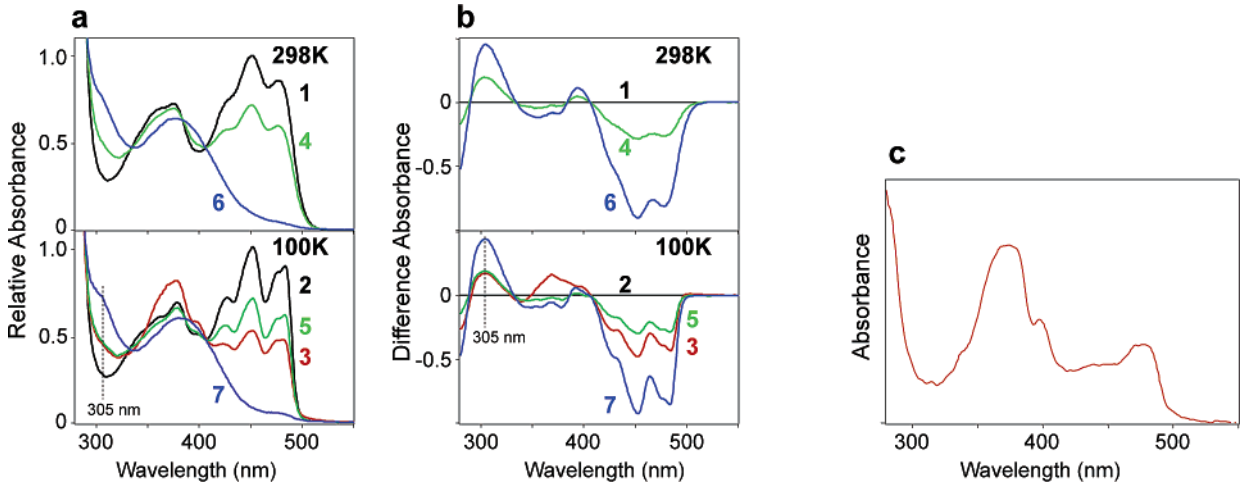

FIGURE 5: Absorption spectra (a) and light-minus-dark absorption difference spectra (b) changes of *Arabidopsis* FKF1-LOV-containing polypeptide in a hydrate film to calculate an absorption spectrum for Z_{370} (c). Absorption spectra were measured at either 298 K (upper) or 100 K (lower). The order of the measurements is indicated by the numbers attached to each spectrum. For details of the measurements, see the Results section.

populations can be negligible. At first, an absorption spectrum of D_{450} at 100 K was obtained that showed a more pronounced vibrational structure (Figure 5a, 2, 100 K) than that at 298 K (Figure 5a, 1, 298 K). One hour blue light irradiation at 100 K produced Z_{370} as well as S_{390} (Figure 5a, 3, 100 K) as described above. To obtain the absorption spectrum of S_{390}, the sample was then heated to 298 K (Figure 5a, 4, 298 K). Interestingly, the absorption peak at 370 nm disappeared, suggesting the thermal conversion of Z_{370} to D_{450} upon heating to 298 K. To verify this, the sample was again cooled to 100 K (Figure 5a, 5, 100 K). Comparison of the two spectra at 100 K between before and after the heating to 298 K revealed a marked decrease of the absorption around 370 nm and an increase in the main peak region of D_{450}, supporting the interpretation that Z_{370} was converted to D_{450} thermally upon heating. Next, the sample was heated to 298 K and then irradiated with blue light for 5 min that converted the sample completely to S_{390} (Figure 5a, 6, 298 K). Then the sample was cooled dawn to 100 K again and an absorption spectrum of S_{390} at 100 K was obtained (Figure 5a, 7, 100 K).

On assumption that the observed spectra at 100 K consist of the three spectra for D_{450}, S_{390}, and Z_{370} and that the reversion of S_{390} to D_{450} is negligible in this time range (Figure 4b), the absorption spectrum of Z_{370} was calculated using the following three spectra, where A and B are the photoconverted fraction of D_{450} to S_{390} and Z_{370}, respectively: D_{450} (Figure 5a, 2); $(1 - A - B)D_{450} + AS_{390} + BZ_{370}$ (Figure 5a, 3); $(1 - B)D_{450} + AS_{390}$ (Figure 5a, 5).

The spectrum of (Figure 5a, 2) minus (Figure 5a, 3) plus B(Figure 5a, 2) gives the spectrum of BZ_{370}. The spectrum of Z_{370} (Figure 5c) was obtained by dividing this spectrum by B that was determined to be 0.35 so as to cancel the negative main peaks of D_{450}. The spectrum has a major and a minor peak at 370 and 400 nm, and a broad peak at 460−480 nm, respectively. They are characteristic with the flavoproteins in the anion radical states (*42*−*46*). For example, the present spectrum is very similar to those in Figure 6, dashed line of ref *43*, or Figure 4A of ref *44*, although absorption peaks of our spectrum are much narrower and have less tailing due to sharpening at low temperature.

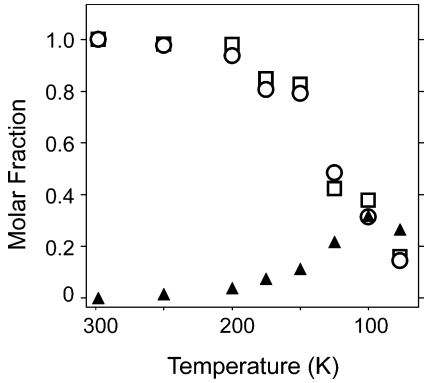

FIGURE 6: Molar fraction of D_{450} converted to S_{390} or Z_{370} by 1 h blue light irradiation at 298−77 K in hydrated films of *Arabidopsis* FKF1-LOV-containing polypeptide. (○), (□), and (△) represent the S_{390} fractions determined by cool-heating cyclic measurements at 450 nm, S_{390} fractions determined at 305 nm, and Z_{370} fractions. For calculation of the molar fraction, see the Results section.

The absorption spectrum of a film irradiated with blue light at 100 K, heated to 298 K, and then cooled and measured at 100 K minus that of D_{450} at 100 K has a shape similar to that of 100% S_{390} minus D_{450} measured at 100 K. This indicates that Z_{370} produced at 100 K by blue light was completely returned to D_{450} by the heating to 298 K.

Fraction of S_{390} and Z_{370} Formed in Hydrated Films at Different Temperatures. To determine the molar fraction of FKF1-LOV-containing polypeptide converted to S_{390}, the same cool-down and heat-up cycling measurements as shown in Figure 5 were performed at seven different temperatures as well as the measurement at 298 K. Molar fractions converted to S_{390} by 1 h blue light irradiation at temperature t, $F(t)$, is expressed as

$$F(t) = [\mathrm{At}(1) - \mathrm{At}(4)]/[\mathrm{At}(1) - \mathrm{At}(6)]$$

where At(1), At(4), and At(6) are the absorption at 450 nm of the spectra corresponding to 1, 4, and 6 of Figure 5a at temperature t. Calculated molar fractions are plotted against temperature (Figure 6, circle). At 298−200 K, D_{450} was almost completely phototransformed to S_{390}; however, the photoconvertible fraction decreased with the decrease of

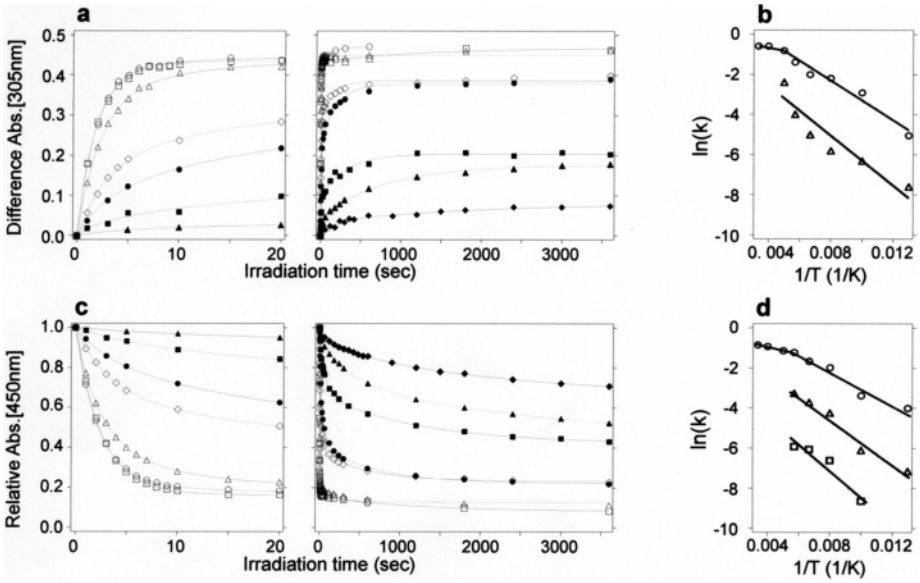

FIGURE 7: Time courses of blue light-induced absorption difference change at 305 nm (a), and absorption change at 450 nm (b), until 20 s (left) and 60 min (right) at 298 K (○), 250 K (□), 200 K (△), 175 K (◇), 150 K (●), 125 K (■), 100 K (▲), and 75 K (◆) of hydrated films of *Arabidopsis* FKF1-LOV-containing polypeptide. Fitting curves are obtained by a method of nonlinear least squares. For details of the fitting conditions, see the text. Arrhenius plots of the rate constants obtained from the time course shown in (a), (c) and in (b), (d). The straight lines were calculated by the method of least squares.

temperature below 200 K. At the liquid nitrogen temperature, only 15% was converted to S_{390}, that may be explained by either the increased population that cannot be converted to S_{390} or a decreased rate of the reaction at the low temperatures. The former possibility was discussed in LOV2 of *Adiantum* phytochrome3 (phy3) (*47*, *48*) on the basis of an unfavorable molecular position of the SH group to FMN to form a cysteinyl-flavin adduct revealed in the crystal structure of *Chlamydomonus* phot-LOV1 (*49*).

In addition to the absorption at 450 nm, that at 305 nm can be used to estimate the amount of S_{390} formed. As can be seen from the comparison of the absorption spectra in Figure 5a,b, the absorption at 305 nm of D_{450} was little affected by the presence of Z_{370}. The absorption change at 305 nm, thus, represents only S_{390} formation. Using this absorption, S_{390} formation can be quantified without the cool-down and heat-up cycling measurements. The result is indicated also in Figure 6 (box) that well coincides with the temperature-dependent curve determined by the absorption at 450 nm (circle). Together with the absorption spectrum of Z_{370} (Figure 5c), this confirms the absorption at 305 nm to be a good indicator for S_{390} formation.

Molar factions of Z_{370} formed by blue light irradiation were determined as follows. In comparison of the D_{450} spectrum of a film with its 100% S_{390} form, the relation, $A(450) = 1 - 0.90X$, was obtained where $A(450)$ is the absorption at 450 nm of a film after a molar fraction, X, is converted from D_{450} to S_{390}. By comparing the absorption spectra of D_{450} and Z_{370} normalized to the absorption at 305 nm based on the observation described above, the relation, $A(450) = 1 - 0.61Y$ was obtained, where $A(450)$ is the absorption at 450 nm of a film after a molar fraction, Y, is converted from D_{450} to Z_{370}. In combination of the two relations, $A(450)$ can be expressed as $1 - 0.90X - 0.61Y$. Y was calculated from the equation using the X determined by $A(450)$ in the previous section (Figure 6, circle) and shown also in Figure 6 (triangle). Z_{370} was formed below 200 K and increased according to the decrease of temperature until 100 K. At 77 K, in turn, it decreased.

Calculation of the Activation Energy for Photoreaction from D_{450} to S_{390} or Z_{370}. To calculate the activation energy of the photoreaction from D_{450} to S_{390}, the time course of the blue light-induced reaction was monitored by the absorption change at 305 nm as described in the previous section. The absorption changes are well simulated by a single exponential curve above 200 K and biexponential curves from 200 K to liquid nitrogen temperature (Figure 7a). An Arrhenius plot of the rate constants (Figure 7b) revealed an activation energy of 1.2 kJ/mol with the single component above 200 K and 3.8 and 4.8 kJ/mol with the two components below 200 K, respectively, indicating the presence of two different pathways from D_{450} to S_{390} at the lower temperatures. Furthermore, a discontinuity at 200 K in the first component suggests some conformational changes of the LOV polypeptide around 200 K.

Photoreaction kinetics was also monitored at 450 nm that reflects the photoactivation of D_{450}. In contrast to those at 305 nm, the changes are well simulated by a single exponential curve above 175 K and triexponential curves from 175 K to liquid nitrogen temperature (Figure 7c). An Arrhenius plot of the rate constants (Figure 7d) gave an activation energy of 1.4 kJ/mol for the single component above 175 K that well agrees with the 1.2 kJ/mol obtained from 305 nm change although the temperature ranges differ slightly. The plot also showed 3.3, 4.7, and 5.4 kJ/mol for the three components, respectively. The activation energy of the former two, 3.8 and 4.8 kJ/mol, well correspond to those observed with S_{390} formation detected by the 305 nm change, suggesting that the two come from S_{390} formation. The third component with 5.4 kJ/mol, therefore, may be attributable to formation of Z_{370}. The populations of the three components were also calculated (data not shown). The first component decreased almost linearly with the decrease of temperature, whereas the second component showed a

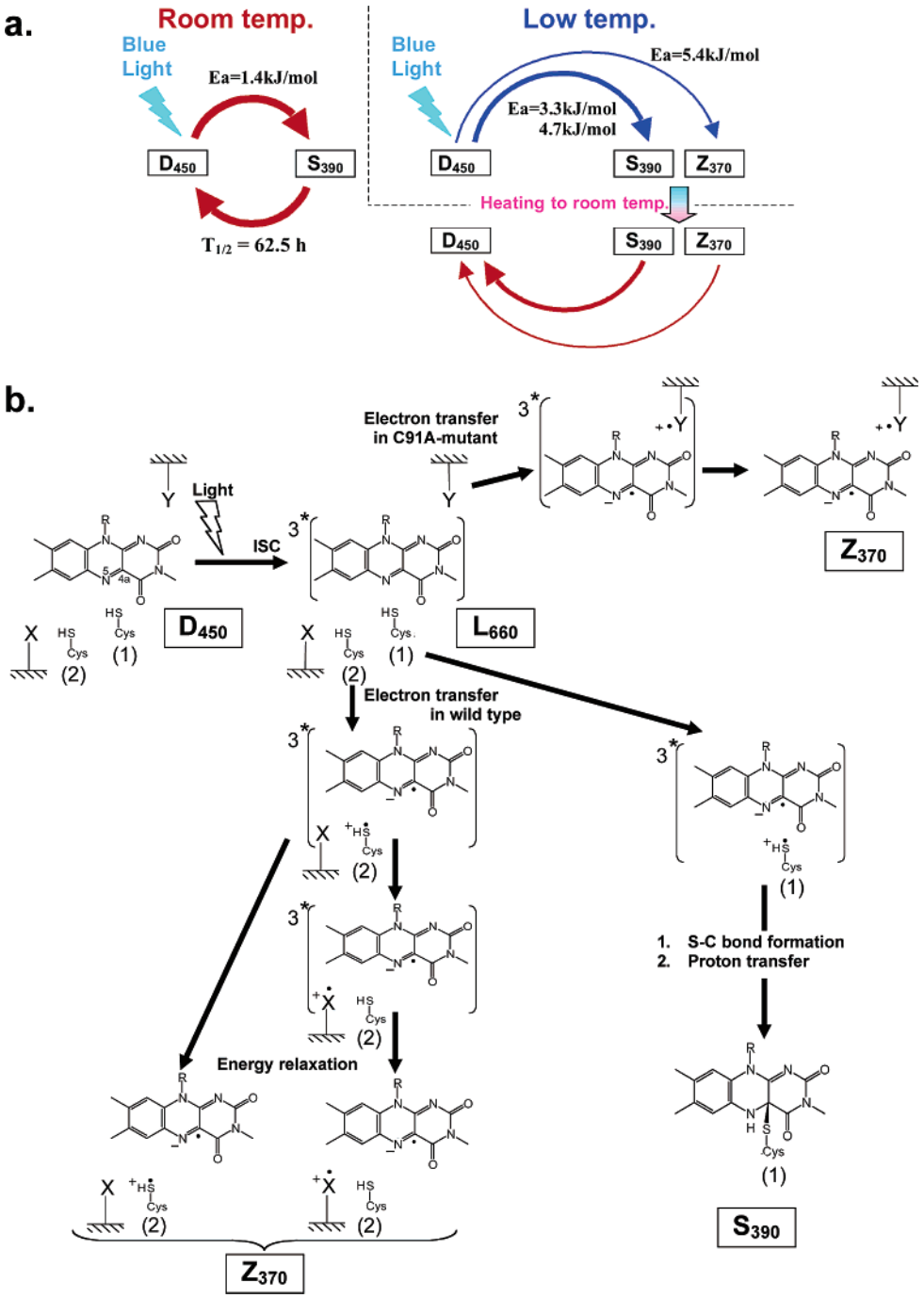

FIGURE 8: (a) Summary of the photoreaction cycle of the LOV-containing polypeptide of *Arabidopsis* FKF1. (b) Possible reaction pathways for Z_{370} formation at low temperature in the LOV-containing polypeptide of *Arabidopsis* FKF1. The upper right part is that for the C91A mutant of the polypeptide; the other part is for the wild-type polypeptide. (1) and (2) represent the SH group of the conserved cysteine in a right or a wrong position to form a cysteinyl-flavin adduct. 3* indicates the triplet-excited state. X and Y represent unidentified amino acid residues involved in the electron transfer reactions. X and Y cannot be distinguished at this moment. For details, see the Discussion.

maximum population around 200 K. The third component increased below 200 K that well agrees with the increased molar fraction of Z_{370} below 200 K (Figure 6, triangle), supporting the assignment of this component to Z_{370}.

DISCUSSION

Reversion of S_{390} to D_{450}. LOV domains of FKF1 families form a stable photointermediate state alike to S_{390} of phot. The S_{390} cannot be produced upon introduction of the mutation into the conserved cysteine (*17*), suggesting a cysteinyl-flavin adduct formation similar to that in S_{390} of phot (*28*−*30*, *50*, *51*). This S_{390} of FKF1 families has been thought to be irreversible to the ground state. In this study, we demonstrated clearly the reversion of S_{390} to D_{450} with a half-life time of 62.5 h (Figure 4). This is summarized in Figure 8a, left. This extremely slow reversion may derive from the nine amino acid insertion to the LOV core amino acid sequence of phot (Figure 1a). The insertion resides between the α′A-helix and αC-helix (helical connector). The former possesses the conserved photoactive cysteine, and the latter interacts with the ribitol and the phosphate groups of FMN (*47*, *49*) (Figure 1b). The NMR spectrum of the

phosphate group has been reported to change upon photoactivation (*52*). Since both of the helices are deeply involved in the photoreaction of phot LOV, the nine amino acid insertion could modify the dark reversion of FKF1-type LOV domains (Zikihara et al., manuscript in preparation). The slow reversion of LOV domains of FKF1 may have some role in its function, such as light-regulated formation of a stable protein complex that contributes to proper ubiquitination of target proteins.

In phot, the reversion takes place with the half-life times from several seconds to a few minutes at ambient temperatures (*35*). At the low temperatures where S_{390} is stable, a photoconvertible fraction of D_{450} to S_{390} becomes smaller. These make the spectroscopic or structural studies on S_{390} difficult in phot families. In contrast, S_{390} of FKF1-LOV is very stable and can serve as an useful sample for these kinds of studies, especially for crystallization of LOV domains in a real S_{390} state. The LOV domain of FKF1 families has no tryptophan in contrast to the presence of one tryptophan in phot LOV. This makes the light-induced spectral changes in the UV region clearer. Actually, the absorption spectrum of S_{390} of FKF1-LOV showed a minor peak at 305 nm as well as a main peak at 378 nm, which is not seen in the spectrum of phot LOV due to large absorption of the tryptophan overlapping the absorption of FMN in this region. Accordingly, FKF1-LOV is a good sample to study the photointermediate states of LOV domains.

A Novel Photoproduct Z_{370}. The present low-temperature spectroscopic study detected a new photoproduct, Z_{370}, that appears below 200 K and dominated at liquid nitrogen temperature. The possibility that Z_{370} is an intermediate from D_{450} to S_{390} trapped at low temperature can be excluded on the basis of the following evidence. First, if so, molar factions of S_{390} produced at low temperatures will increase upon heating due to the conversion of Z_{370} to S_{390}. However, the population of S_{390} did not increase; instead, Z_{370} reverted to D_{450} (Figure 5a,b and Figure 8a, right). Second, evidence indicates that S_{390} formation proceeds Z_{370} formation (Figure 3). Then, a question arises, what does Z_{370} come from? FMN in Z_{370} is very likely to be an anion radical state judging from its absorption spectrum as mentioned in the Results. It has been proposed that a radical pair is formed between FMN and cysteine in the triplet state that makes FMN an anion radical (state C, in Figure 7 of ref *41*). Therefore, Z_{370} is suggested to be formed from this biradical triplet excited state after relaxing to the ground state with the FMN anion radical trapped at the low temperature. For the fate of the counter SH cation radical, there may be two possibilities. The SH cation radical may also be trapped at the ground state in case that the distance between the radical pair is enough to prevent the back-electron transfer. The second possibility is that the cation radical accepts an electron from an unidentified redox-active amino acid residue to revert to the ground state. Since the C91A mutant of the FKF1-LOV domain also showed formation of Z_{370} at 77 K (data not shown) indicating the presence of a redox-active amino acid residue other than the cysteine near the FMN isoalloxazine ring, the second case is very probable. Similar electron transfer from the other amino acid than the cysteine has been reported with the oat phot1 LOV2 C450A mutant (*50*). EPR study on Z_{370} will clear these points. These schemes are illustrated schematically in Figure 8b.

A probable origin for Z_{370} formation is discussed below. The photoconverted fraction of D_{450} to S_{390} decreased according to the temperature decrease (Figure 6). Similar decreases in the photoconvertible population at low temperatures were reported with *Adiantum* phy3-LOV2 (*30*, *54*) and suggested to derive from fixation of an unfavorable SH position relative to FMN to form a cysteinyl-flavin adduct at lower temperatures. Actually, the crystal structure of *Chlamydomonus* phot-LOV1 has revealed the presence of two, the right and the wrong, SH positions (*49*). Accordingly, the SH in the wrong position that is able to produce a radical pair but unable to form the adduct may originate Z_{370}. A slightly different molecular structure of FKF1-LOV from those of phot-LOV possibly due to the nine amino acid insertion etc. may enable the formation of this particular molecular species. The situation is also illustrated in Figure 8b. This scheme supports the proposal that electron migration proceeds proton transfer from the photoreactive cysteine to C(4a) of the FMN isoalloxazine ring (*41*).

It has been proposed that only a zwiterionic intermediate adduct without proton transfer (state D in Figure 7 of ref *41*) is formed at 80 K but S_{390} is not in oat phot1-LOV2. However, the red-shifted species corresponding to this state was undetectable in the FKF1-LOV samples at 77 K. Low-temperature FTIR and UV−visible spectra studies on *Adiantum* phy3-LOV have shown deprotonation of the SH and S_{390} formation at 77 K, that is marked in LOV2 but obscure in LOV1 (*30*, *51*, *53*). In FKF1-LOV, S_{390} formation was also observed at 77 K.

Activation Energy of the Photoreactions. The activation energy of S_{390} formation was calculated to be 1.4 kJ/mol above 200 K and 3.3 and 4.7 kJ/mol below 200 K, indicating a discontinuity around 200 K. Recently, the activation energy of S_{390} formation in *Adiantum* phy3-LOV2 was reported to be 0.67 and 1.04 kJ/mol below 200 K (*54*) that are comparable, however somewhat smaller than those of FKF1-LOV. In phy3-LOV2, blue light irradiation for a few minutes converted 64% and 36% of D_{450} to S_{390} at 100 and 77 K, respectively (*30*, *53*, *54*), while 1 h irradiation induced only 35% and 15% photoconversion to S_{390} at 100 and 77 K, respectively, in FKF1-LOV (Figure 6). These suggest lower quantum efficiency of the photoreaction in FKF1-LOV and may explain the larger activation energy in FKF1-LOV.

The activation energy of Z_{370} formation was calculated to be 5.4 kJ/mol that is larger than those of S_{390}, although the reaction may not include proton migration since SH of the cysteine is reported to be protonated in the triplet excited state (*55*). The larger activation energy may partly come from the larger energy barrier of electron migration from the wrong SH position than that of the right position.

Concluding Remarks. Cyclic photoreaction of the FKF1-LOV domain is established by the present study that includes a different pathway from S_{390} formation (Figure 8). The photoreaction cycle together with the activation energy provided useful information to figure out the molecular mechanism underlying the photoreaction of FKF1-LOV. To understand more precisely the photoreaction mechanism, the role of the nine amino acid insertion is under investigation.

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