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Blue-Light-Induced Unfolding of the Jα Helix Allows for the Dimerization of Aureochrome-LOV from the Diatom *Phaeodactylum tricornutum*

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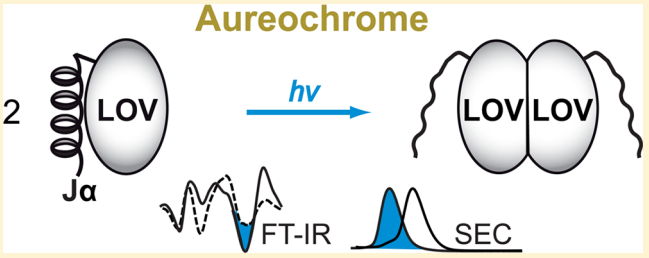
Supporting Information

ABSTRACT: Aureochromes have recently been shown to act as blue-light-regulated transcription factors in the stramenopile alga *Vaucheria frigida*. They comprise a light-, oxygen-, or voltage-sensitive (LOV) domain as a sensory module with flavin mononucleotide (FMN) as a chromophore and a basic region leucine zipper (bZIP) domain as an effector. Aureochromes are the only members of a large LOV protein family, where the effector domain is located N-terminal to the sensor domain. This domain inversion positions the linking Jα helix of other LOV proteins to the terminus, raising the question of the role of Jα in aureochrome signaling. In phototropins, signaling proceeds from LOV2 via dissociation and unwinding of the Jα helix to the C-terminal kinase effector domain. In contrast, other LOV proteins have been demonstrated to activate the effector without the unfolding of Jα. We investigated the LOV domain of aureochrome1a from the diatom *Phaeodactylum tricornutum* both with and without the Jα helix. Fourier transform infrared difference spectroscopy provides evidence that the Jα helix unfolds upon illumination. This unfolding is prerequisite for light-induced dimerization of LOV. Under illumination, full conversion to the dimer was observed by size exclusion chromatography. In the absence of the helix, a monomer was detected in the dark and in the light. As a further effect, the recovery of the dark state is 6-fold slower in LOV-Jα than LOV. We therefore postulate that the Jα helix plays an important role in aureochrome signaling.



LOV (light-, oxygen, or voltage-sensitive) proteins are a growing family of blue light receptors. LOV domains were first identified as the light-sensitive domains of phototropins in plants.[1] This discovery opened up the search for counterparts in fungi, i.e., White Collar1 and Vivid,[2,3] as well as in bacteria, i.e., Ytva[4] and many other candidates with various domain topologies.[5] The recently discovered aureochromes attracted attention as a new class of blue-light receptors.[6] Aureochromes act as blue-light-regulated transcription factors in *Vaucheria frigida*, a stramenopile alga.[6] They contain an untypical domain arrangement with an N-terminal DNA-binding bZIP (basic region leucine zipper) domain as effector and a C-terminal blue-light sensing LOV domain. This arrangement might offer new possibilities for the application of LOV domains in the field of optogenetics.[7] The domain topology is inverse to other characterized LOV-effector proteins, which consist of an N-terminal LOV domain and a C-terminal effector domain.[5,8] This inversion raises the question of how the signal is transmitted in aureochromes.

In phototropin, a Jα helix resides in the linker region between the N-terminal LOV2 domain and the C-terminal kinase domain and plays a decisive role in signal transfer to the kinase domain (Figure 1A). Signaling is initiated by activation of the LOV2 domain by blue light. Absorption of the light by the chromophore flavin mononucleotide (FMN) leads to adduct formation with a nearby cysteine residue (Figure 1B).[9] As a consequence, the Jα helix dissociates from the β-sheet surface as revealed by NMR spectroscopy,[10] which is a crucial step in signaling and enables autophosphorylation.[11] The dissociated helix unwinds, which has been demonstrated by transient grating[12] and Fourier transform infrared (FT-IR) spectroscopy.[13−15] In other LOV proteins than phototropins, it has been shown that the Jα helix does not necessarily dissociate and unwind upon illumination. The C-terminal helix of the bacterial Ytva-LOV is decoupled from the LOV domain surface and extends into the solvent both in the dark and in the light.[16] Similarly, the lit state structure of the bacterial LOV protein PpSB1 shows an extended and folded Jα helix.[17] In the bacterial DNA-binding protein EL222, the helix-turn-helix effector domain is released from the LOV surface upon illumination, but the linking Jα does not respond to this process.[18] Furthermore, light activation of the bacterial sensory histidine kinase LovK proceeds without unfolding of Jα.[19] The fungal Vivid lacks a Jα helix. There, signaling has been shown to

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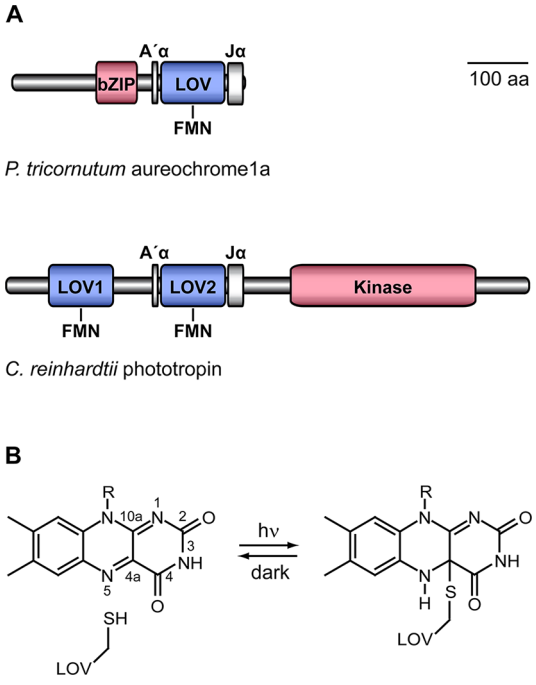

Figure 1. (A) Domain organization of aureochrome1a from *P. tricornutum* (top) and phototropin from *C. reinhardtii* (bottom). The aureochrome comprises a C-terminal LOV domain and an N-terminal bZIP effector domain. This domain topology is inversed to other characterized LOV-effector proteins such as phototropin. The core LOV2 domain of phototropin is flanked by an N-terminal A′α and a C-terminal Jα helix. In the aureochrome, these structural motifs are conserved, but the Jα helix does not reside in the linker region to the effector but at the C-terminus. (B) Chemical structure of the flavin chromophore in the LOV domain in its oxidized state in the dark. Illumination with blue light leads to the reversible formation of a covalent adduct between the flavin and an adjacent cysteine from the protein moiety.

proceed to an unusual N-terminal cap region,[20] which regulates dimerization.[21]

The sequence of the Jα helix is in general not well conserved (Figure S1 of the Supporting Information), but the presence of a Jα fold was recently demonstrated for the LOV domain of aureochrome1 from *V. frigida* by X-ray crystallography.[7] In this crystal structure, the Jα helix points to a different direction than those of phototropin or Ytva. In support of this remarkable finding, the linker region between the β-sheet of LOV and the Jα helix of aureochrome1 is significantly shorter than that of phototropin. However, only half of the possible helical fold of Jα was included in the construct for crystallization (Figure S1 of the Supporting Information). Most importantly, it remains unclear if this Jα helix dissociates and unfolds upon illumination because neither the light state structure of LOV-Jα[7] nor transient grating experiments[22] provided any indication for such an unwinding. A response to light was detected as a change in diffusion coefficient of LOV-Jα, which was interpreted as a concentration-dependent dimerization with a time constant of 140 ms.[22] Additionally, a limited loss of overall helicity of 6−7% was revealed by circular dichroism (CD) spectroscopy.[7,23]

To elucidate the role of the Jα helix in the photoreaction of aureochromes, we investigated the LOV domain of aureochrome1a from the diatom alga *Phaeodactylum tricornutum* (*Pt*AUREO1a). Diatoms contribute significantly to the global primary production.[24] The genome of *P. tricornutum* encodes four different aureochromes, three of class 1 and one of class 2.[25] *Pt*AUREO1a has been demonstrated to be involved in the light-dependent induction of cell division.[26] Furthermore, there are indications for an important role of *Pt*AUREO1a in photoacclimation from *in silico* binding site analysis of blue light-upregulated genes.[27] Its LOV domain is highly homologous to that of aureochrome1 from *V. frigida* (Figure S1 of the Supporting Information). Two constructs of this LOV domain with and without Jα helix were studied by UV−vis spectroscopy, FT-IR difference spectroscopy, and size exclusion chromatography. Both constructs contained additionally to the LOV core domain an N-terminal flanking A′α helix (Figure 1A). Although the Jα helix is located at the C-terminus and not in a linker region of *Pt*AUREO1a, we observed a strong influence of the Jα helix on the photoresponse of the LOV domain.

MATERIALS AND METHODS

Expression Constructs. The nucleotide sequences encoding LOV-Jα (amino acids 238−378) and LOV (amino acids 238−356) of *Phaeodactylum tricornutum* aureochrome1a (Protein-ID 49116, Joint Genome Institute (JGI), http://genome.jgi-psf.org/Phatr2/Phatr2.home.html) were optimized in codon usage for *E. coli* (GENEius software, Eurofins MWG/BioLink) and modified to include a *Nde*I restriction site at the 5′ end and a stop codon followed by a *Sal*I restriction site at the 3′ end. These sequences were *de novo* synthesized by Eurofins MWG and cloned into the *Nde*I and *Sal*I site of the pET28a (+) expression vector (Novagen) in such a way that the proteins carry a $(His)_6$-tag and a thrombin-cleavage site at the N-terminal end.

Protein Purification. LOV and LOV-Jα were expressed in *E. coli* BL21 (DE3) pLysE. The cells were grown at 37 °C until an OD_{600} of 0.5. Expression was induced with 10 μM IPTG at 27 °C and an OD_{600} of 0.8. Then, proteins were expressed at 18 °C for 20 h in the dark. Cell pellets were resuspended in 350 mM phosphate buffer at pH 8 containing 300 mM NaCl, 20 mM imidazole, and protease inhibitor (cOmplete EDTA-free, Roche) and were lysed via a french press (SLM Aminco) at 1000 psig. The supernatant obtained after centrifugation at 108.000*g* was loaded onto a Co^{2+}-enriched His-Bind resin (Novagen). After extensive washing at 4 °C with buffer containing 20 and 40 mM imidazole, respectively, elution was performed using 250 mM imidazole in 50 mM phosphate buffer at pH 8 and 300 mM NaCl. LOV was purified in the presence of 20% glycerol. Both eluted proteins were dialyzed three times against 400 volumes of 50 mM phosphate buffer at pH 8, 300 mM NaCl, and 20% glycerol. In the case of LOV, the dialysis buffer contained additionally 1 mM dithioerythritol (DTE). Proteins with a purity of >95% were obtained as determined from SDS−PAGE (Figure S2 of the Supporting Information). The chromophore occupancy was about 20%. Chromophore occupancy and protein concentration were determined by UV−vis spectroscopy using the extinction coefficients at 450 and 280 nm for free FMN[28] and calculated extinction coefficients for apoprotein at 280 nm with EXTCOEF (http://www.workbench.sdsc.edu).

UV−Vis Spectroscopy. UV−vis spectra were recorded on a Shimadzu UV-2450 spectrometer. Absorbance spectra were recorded in the dark and after illumination for 10 s with a blue LED (455 nm, 20 mW cm^{-2}, and 20 nm full width at half-maximum (fwhm), Philips Lumileds). Kinetic traces were

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recorded at 447 nm in the dark after illumination for 500 ms. The temperature of the sample was adjusted to 20 °C by a circulating water bath.

FT-IR Spectroscopy. For FT-IR experiments, samples were transferred into 50 mM phosphate buffer at pH 8 and 100 mM NaCl via repeated ultrafiltration using a Vivaspin 500 filter device with a 5 kDa cutoff (Sartorius). The sample was finally concentrated to ~2.4 mM with respect to the bound chromophore (OD_{448}~30). Two microliters of protein solution was applied on a BaF_2 window, and the sample was sealed by a second window without any drying. FT-IR spectra were recorded on Bruker IFS 66v and IFS 66/S spectrometers with a resolution of 2 cm^{-1} at 20 °C. Long wave pass filters (OCLI; Spectrogon) with a cut-on of 4.9 and 5.3 μm, respectively, were placed in front of the detector to block stray light and to improve the signal-to-noise ratio. For the difference spectra, 1024 scans were recorded before and after 2 s of illumination with a blue LED (455 nm, 20 mW cm^{-2}, 20 nm fwhm, Philips Lumileds). Several experiments on independent preparations were averaged to a total number of 18432 scans for LOV-Jα and 10240 scans for LOV. The spectra were scaled to the isolated C_4=O band of FMN at 1713 cm^{-1}. This scaling factor is supported by the complete overlap of several other isolated chromophore bands at 1251−1538 cm^{-1}. For time-resolved measurements, difference spectra with 1024 scans corresponding to 118 s of acquisition were recorded continuously after illumination over a time range of more than 30 min. The kinetics of chromophore recovery was analyzed by scaling the relative difference absorbance of chromophore bands in later spectra to those of the first difference spectrum. The reciprocal scaling factors s were fitted with a simple exponential function $s(t) = \exp(-t/\tau)$ to determine the time constant τ.

Size Exclusion Chromatography. Size exclusion chromatography (SEC) was performed using an Äkta purifier (GE Healthcare) with a Superdex75 10/300 GL column (GE Healthcare) at 4 °C. For equilibration and elution, 50 mM phosphate buffer at pH 8 and 300 mM NaCl were used. Aliquots of 60 μL with a protein concentration of 3.7 mM for LOV-Jα and 1 mM for LOV were centrifuged at 21.400g for 10 min at 4 °C and loaded onto the column. SEC was performed either in the dark or under continuous illumination of the column using four blue LEDs (455 nm, 20 nm fwhm, Philips Lumileds).The elution profile was recorded using the flavin absorption at 448 and 390 nm to ensure that only protein with the bound chromophore contributes. Standard marker proteins (GE Healthcare) were used to determine the apparent molecular mass of the sample by calibration.

RESULTS

The core LOV domain with the N-terminal A′α helical element (referred to as LOV) and the core LOV domain with both N-terminal A′α and the C-terminal Jα helix (referred to as LOV-Jα) of *Pt*AUREO1a were obtained from the heterologous expression in *E. coli* as soluble and yellow proteins. The limits of both constructs were determined from sequence alignment with other LOV proteins (Figure S1 of the Supporting Information). UV−vis spectra of both proteins show the typical fine structured absorption of bound oxidized FMN with absorption maxima at 448, 375, and 358 nm in the dark (Figure 2). Illumination with blue light led to a bleaching of the chromophore absorption and to the formation of a new band at 390 nm, indicating the formation of the FMN-cysteine adduct.

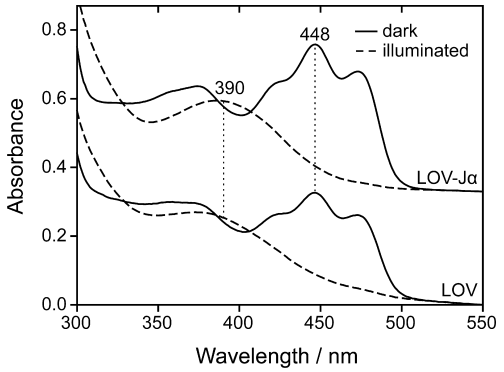

Figure 2. UV−vis spectra of LOV-Jα and LOV show the same absorption maximum at 448 nm in the dark and the typical bands of protein-bound oxidized flavin (black lines). After 10 s of blue-light illumination, the absorption maximum is shifted to 390 nm, evidencing that the flavin is completely converted into the adduct state (dashed lines). Spectra of LOV-Jα are offset for clarity.

After 10 s of illumination, the chromophores were completely converted from the oxidized state into the adduct state (Figure 2). The decay kinetics of the adduct state of both proteins were recorded at 447 nm in the dark after illumination with blue light for 500 ms (Figure 3). Traces were analyzed by fitting

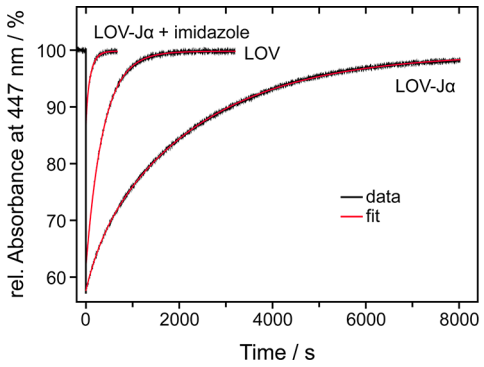

Figure 3. Recovery kinetics of LOV, LOV-Jα, and LOV-Jα in the presence of 1 mM imidazole recorded at 447 nm after illumination with blue light for 500 ms (black lines). Traces were analyzed by fitting exponential functions (red lines). For LOV, a monoexponential function was used, yielding a time constant of 370 s, whereas for LOV-Jα a biexponential function yielded time constants of 2290 s (86%) and 320 s (14%). The presence of the Jα helix slows down the recovery about 6-fold. The addition of 1 mM imidazole to LOV-Jα decreases both time constants of recovery about 20-fold to 105 s (78%) and 16 s (22%).

exponential functions. For LOV, a monoexponential function was used yielding a time constant of 370 s, whereas for LOV-Jα a biexponential function yielded time constants of 2290 s (86%) and 320 s (14%). The data are in excellent agreement with the respective exponential fit ($R^2 \geq 0.99995$). Time constants showed a maximum deviation of 3% within several experiments. The recovery kinetics of LOV is therefore strongly influenced by the presence of the Jα helix, which slows down the dominant recovery kinetics about 6-fold. Furthermore, a contribution of a second adduct species was observed in the presence of the Jα helix. An acceleration of the recovery kinetics of LOV domains by imidazole was reported before.[29] Accordingly, the presence of 1 mM imidazole decreased both the time constants of LOV-Jα about 20-fold to 105 s (78%) and

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16 s (22%) (Figure 3). To rule out any effect of residual imidazole from the purification procedure on the recovery kinetics, LOV and LOV-Jα were thoroughly dialyzed three times against 400 volumes of buffer.

Light-induced FT-IR difference spectroscopy was applied to both LOV and LOV-Jα to analyze by comparison potential Jα-dependent changes in secondary structure. In the difference spectra, only those bands are visible, which originate from light-induced structural changes of the chromophore and the protein moiety. Negative bands characterize the dark state, whereas positive bands originate from the light state. The difference spectra of both proteins show a good agreement in the position of bands below 1500 cm^{-1} (Figure 4A), which are typical for LOV domains and originate mainly from the chromophore.[30−32] Thus, the deletion of the Jα helix does not cause structural perturbation of the FMN binding pocket. Contributions from changes in secondary structure are limited to the amide I region ranging from 1615 to 1695 cm^{-1} and the amide II region between 1520 and 1570 cm^{-1}.[33] In these regions, the difference spectra of LOV and LOV-Jα disagree significantly, which indicate differences in the extent and origin of secondary structural changes upon illumination. A pronounced negative band is found at 1643 (−) cm^{-1} in the difference spectrum of LOV-Jα, whereas a significantly weaker contribution is present in the difference spectrum of LOV (Figure 4A). This spectral region is characteristic for α-helices.[33] The negative sign points to an unwinding of the helix. Similar signals at 1646, 1649, or 1650 cm^{-1} have been assigned to the unwinding of the Jα helix in FT-IR studies on LOV domains with and without the Jα helix on phototropins from *Chlamydomonas reinhardtii*[14] and *Arabidopsis thaliana*.[15,34] The weaker signal at 1643 cm^{-1} of *Pt*AUREO1a-LOV can be attributed to helical structural changes additional to those from Jα, similar to those identified before in the phototropin homologue neochrome1-LOV2 from *Adiantum capillus-veneris* without Jα.[31,34] Besides the strong negative band at 1643 cm^{-1} in the difference spectrum of *Pt*AUREO1a-LOV-Jα, a pronounced positive band can be found at 1630 cm^{-1}, which is also only weak in the difference spectrum of LOV (Figure 4A). In this spectral region, β-sheets absorb,[33] which points to concomitant Jα-dependent structural changes in β-sheets. Such a positive band was also found in the difference spectra of LOV2-Jα from *A. thaliana* phototropin1 and phototropin2 at 1625 cm^{-1} and was assigned similarly[15,34] in agreement with CD spectroscopy.[35] In the difference spectrum of the tandem construct LOV1−LOV2-Jα from *C. reinhardtii* phototropin and in LOV2 from *Avena sativa* phototropin1, however, this signal is missing.[13,14] This difference might be explained in the former case by a shielding of β-sheets by the LOV1−LOV2 interaction.

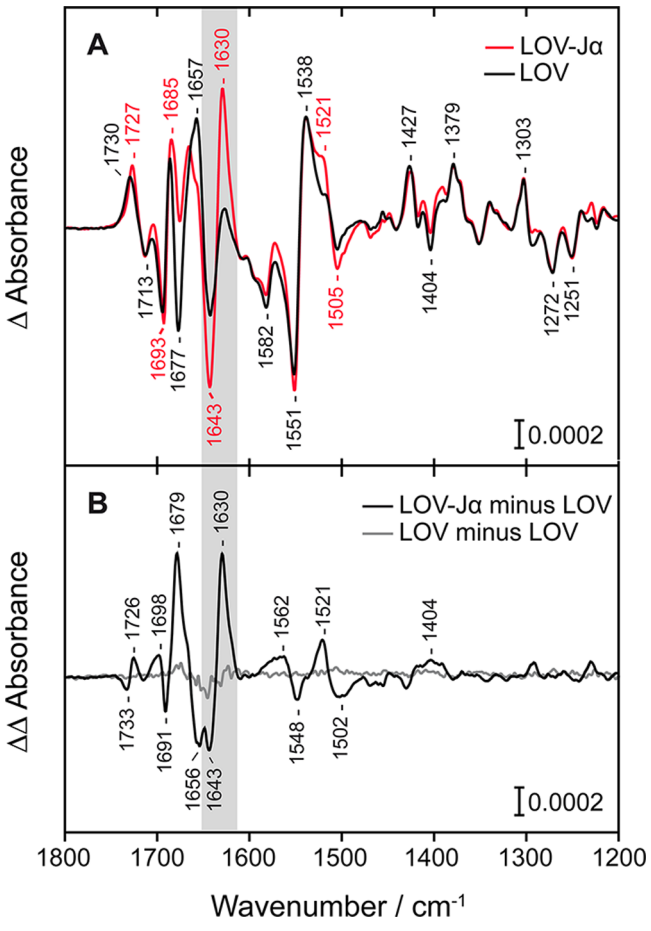

Figure 4. (A) Light-minus-dark FT-IR difference spectra of LOV-Jα (red line) and LOV (black line). The contribution of the Jα helix is evident at 1643 (−) cm^{-1}. The negative sign of the band provides evidence for a light-induced unwinding of the Jα helix. The positive band at 1630 cm^{-1} points to concomitant Jα-dependent structural changes in β-sheets. (B) The double difference spectrum obtained by subtraction of the difference spectrum of LOV from that of LOV-Jα. Prominent signals in the amide I and amide II regions point to further Jα-dependent secondary structural changes of the protein moiety. The double difference spectrum of LOV minus LOV illustrates the noise introduced by sample-to-sample variation of independent preparations.

To isolate more signals that are dependent on the presence of the Jα helix, a double difference spectrum was calculated by subtracting the difference spectrum of LOV from that of LOV-Jα (Figure 4B). Chromophore contributions cancel out so that only contributions of the protein moiety remain. In the amide I region, the already mentioned pronounced bands at 1643 (−) and 1630 (+) cm^{-1} are found. Further prominent bands at 1691 (−), 1679 (+), and 1656 (−) cm^{-1} in the amide I region and some other minor bands are evident. Studies are under way to assign these bands in the double difference spectrum and to reveal if the underlying processes are part of the signaling pathway. In summary, the presence of the Jα helix seems to have a strong influence on light-induced structural changes within the core LOV domain and/or the N-terminal flanking region.

Decay kinetics of the adduct states of LOV and LOV-Jα were also determined by FT-IR spectroscopy. Difference spectra were recorded until the signals were too small for analysis. The difference absorbance of flavin bands in the first difference spectrum after illumination was used as a reference value, and the decay was fitted with a monoexponential function with only a single parameter (Figure 5). Time constants of 450 and 1660 s were obtained for LOV and LOV-Jα, respectively, with a maximal deviation of 30% for LOV and 25% for LOV-Jα within several experiments of independent sample preparations. Considering these deviations, the time constants determined by FT-IR spectroscopy are in a reasonable agreement with those from UV−vis spectroscopy, although the protein concentration was about 80-fold higher in the FT-IR experiments. This finding evidences the maintenance of full hydration in the preparation of our FT-IR samples because decay kinetics of LOV domains are strongly influenced by the hydration level.[36]

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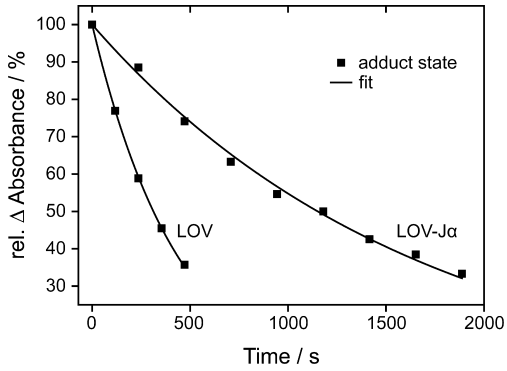

Figure 5. Decay kinetics of the flavin adduct of LOV-Jα and LOV obtained from time-resolved FT-IR difference experiments (black squares). The difference absorbance of flavin bands in the first difference spectrum after illumination was set to 100%. A monoexponential fit with only one parameter yielded time constants of 1660 s for LOV-Jα and 450 s for LOV (black lines).

To investigate the light dependence of the oligomeric state of LOV and LOV-Jα, size exclusion chromatography (SEC) was performed in the dark and under continuous blue-light illumination (Figure 6). Absorbance was detected simultaneously at 448 nm and at 390 nm so that only protein with bound flavin contributed to the elution profile. Differences in absorbance at these two wavelengths result from the 2-fold lower absorbance coefficient of oxidized flavin at 390 nm than at 448 nm, whereas the flavin-cysteinyl adduct formed upon illumination absorbs mainly at 390 nm and only weakly (about 20%) at 448 nm. The majority of LOV-Jα eluted in the dark with an apparent mass of 24 kDa; only some higher oligomers (>75 kDa) and aggregates were detected (Figure 6A). Because of the agreement of the apparent mass with the theoretical molecular mass of LOV-Jα of 18.0 kDa, LOV-Jα is a monomer in the dark state. Under continuous blue-light illumination, LOV-Jα exhibits an apparent mass of 36 kDa, which corresponds to a dimer. LOV in the dark state shows an apparent mass of 13 kDa with a theoretical mass of 15.5 kDa (Figure 6B), which points to a monomer. Under continuous blue-light illumination, LOV shows an apparent mass of 15 kDa, consistent with a monomer in the light state. In conclusion, LOV is predominantly a monomer in both dark and light states, whereas most of LOV-Jα switches its oligomeric state upon light-activation from monomer to dimer. Hence, the presence of the Jα helix allows for a light-induced dimerization of the LOV domain.

DISCUSSION

The terminal localization of the Jα helix in aureochromes raises the question as to whether the Jα helix plays an active role in aureochrome signaling. Summarizing the presented results, we show that the Jα helix in aureochromes has multiple effects on the photoreaction (Figure 7):

(1) Upon illumination of the LOV domain, Jα unfolds in a manner similar to that of phototropin LOV2. (2) The unfolding is accompanied by further structural changes in the core LOV domain and/or A′α. (3) Jα unfolding and accompanying changes initiate a dimerization of LOV. (4) The recovery of the signaling adduct state is strongly delayed.

Site and Mechanism of Light-Induced Dimerization of LOV-Jα. SEC revealed LOV-Jα to be a monomer in the dark and to become dimeric upon illumination. Light-induced

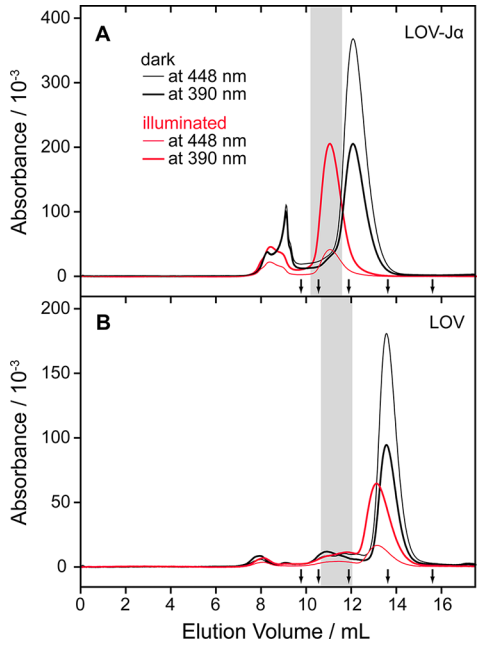

Figure 6. Size exclusion chromatography of LOV-Jα (3.7 mM) and LOV (1 mM) in the dark (black lines) and under continuous blue-light illumination (red lines). Only protein with bound flavin is detected simultaneously at 448 and 390 nm. (A) LOV-Jα elutes in the dark at 12.1 mL corresponding to an apparent mass of 24 kDa. With a theoretical molecular mass of 18.0 kDa, this is consistent with LOV-Jα being a monomer in the dark. Continuous blue-light illumination converts the flavin completely into the 390 nm-absorbing adduct state. Illuminated LOV-Jα elutes at 11.1 mL (36 kDa), which is consistent with the molecular mass of a dimer. (B) LOV elutes at 13.6 mL (13 kDa) and has a theoretical molecular mass of 15.5 kDa, which points to a monomer in the dark. Under blue-light illumination, LOV elutes at 13.1 mL (15 kDa), consistent with a monomer also in the light state. The presence of the Jα helix allows for a light-induced dimerization of the LOV domain. The void volume was 8 mL. The elution volumes of the protein standards used for the calibration curve are marked by arrows. From left to right: 1, conalbumin (75 kDa); 2, ovalbumin (44 kDa); 3, carbonic anhydrase (29 kDa); 4, ribonuclease A (13.7 kDa); 5, aprotinin (6.5 kDa).

dimerization of LOV-Jα is in agreement with results found by Toyooka et al. by transient grating measurements and SEC on LOV-Jα of aureochrome1 from *V. frigida*.[22] Other SEC experiments on the same protein showed only a limited light-induced shift interpreted as a change in secondary structure.[23] LOV in contrast is a monomer in both the dark and the light states (Figure 6). From these findings, it can be concluded that the presence of the Jα helix allows for a light-induced dimerization of LOV domains. Furthermore, the dimerization site is shielded in the dark and becomes accessible after conformational changes triggered by light in the presence of the Jα helix. It has been shown that the Jα helix covers a hydrophobic patch on the β-sheet surface in phototropin-LOV domains[10,37] and to a less extent also in the aureochrome,[7] which would be a good candidate for a dimerization site after Jα release. However, deletion of the Jα helix should then be equivalent to an unfolded Jα helix and generate a permanent lit-like state. Consequently, the LOV domain should be dimeric already in the dark, as the SEC of LOV was performed at high concentrations (1 mM). Instead, dimerization was suppressed in the dark and light in the absence of Jα, which excludes the hydrophobic patch beneath Jα as a dimerization site (Figure 7).

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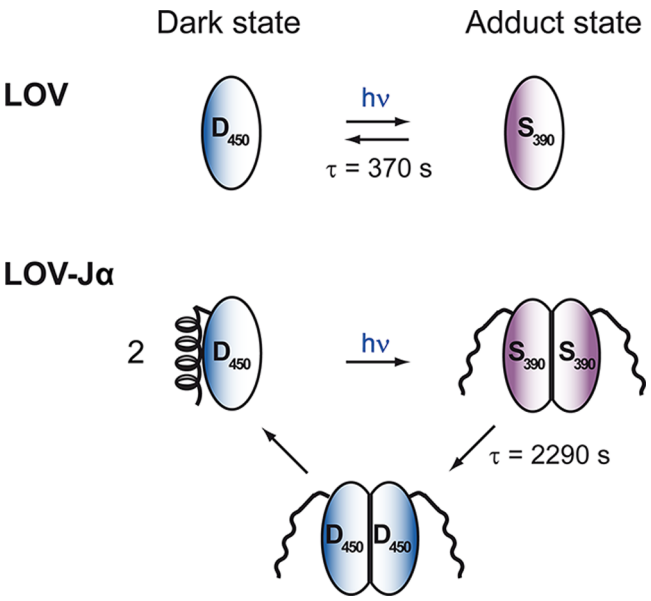

Figure 7. Schematic illustration of the photoreaction of *Pt*AUREO1a-LOV in the absence (top) and presence (bottom) of the Jα helix. Upon blue-light illumination, LOV forms the adduct state of flavin and cysteine, which decays in the dark with a time constant of 370 s. Blue-light illumination of LOV-Jα leads to the formation of the adduct state and to unfolding of the Jα helix, which triggers further conformational changes and therefore allows for a dimerization of two LOV domains. The dimerization site is not localized at the C-terminal site where the Jα helix folds against the β-sheet surface but might be exposed by a response of the N-terminal A′α helix by illumination. The adduct state of dimeric LOV-Jα shows a 6-fold slower recovery than that of LOV, most likely due to dimerization.

An alternative scenario would be that the Jα helix unfolding is accompanied by further secondary structural changes that expose the dimerization site to the bulk. Indications for such structural changes are evident in the FT-IR double difference spectrum of LOV-Jα *minus* LOV (Figure 4B). A second prominent negative band is found at 1656 cm^{-1} besides the one assigned to Jα unwinding. It is conceivable that the band at 1656 cm^{-1} might be due to an unwinding of the A′α helix, accompanying the unwinding of the Jα helix. It was recently found by CD and fluorescence spectroscopy on phototropin1-LOV2 from *Avena sativa* that the A′α helix also unwinds light-induced,[38] which is supported by a strong influence of these residues on the kinase activity of *Chlamydomonas* phototropin.[39] A possible role of the A′α helix in signaling of aureochromes has already been discussed before.[7] Our results point to a model where the N-terminal A′α helix covers the dimerization site, which then becomes accessible upon illumination.

Dissociation Kinetics of Dimeric LOV-Jα. The oligomerization state might have a direct influence on the kinetics of adduct decay because it was shown before that an interaction with a second LOV domain strongly alters these kinetics.[40] Therefore, the combination of results from SEC and spectroscopy might allow us to draw some conclusions on dissociation kinetics of dimeric LOV-Jα. In the simple case of LOV, SEC demonstrated the presence of a monomer in the dark and light independent of concentration (Figure 6B and Figure S3 of the Supporting Information). Congruently, even an 80-fold increase in concentration of LOV from UV−vis to FT-IR experiments did not strongly alter the time constant of adduct decay, determined to be 370 and 450 s, respectively (Figures 3 and 5). For LOV-Jα, a similar time constant might be expected because the unfolded Jα helix should not have any influence on the core LOV domain. An influence of Jα on the kinetics has been demonstrated for the dimeric LOV-Jα of bacterial LovK.[41] However, the latter Jα has been proposed to form a coiled coil structure,[41] whereas Jα of *Pt*AUREO1a unfolds. Indeed, similar recovery kinetics were found for LOV-Jα (320 s) as for LOV, but only as a minor component (Figure 3). The main time constant of recovery drastically slowed down 6-fold to 2290 s. The most likely reason for this change is the formation of a dimer upon illumination. The two recovery times might be rationalized by a slowly decaying adduct state in the dimeric LOV-Jα and a fast recovery in the monomeric state. The second monomeric component was not found in the SEC analysis of LOV-Jα because SEC was performed under illumination, whereas the adduct decay was monitored in the dark after a light pulse. Following this line of reasoning, dissociation of dimeric LOV-Jα is considered to be slow, and the equilibrium lies far on the side of the dimer. Otherwise, dissociation to the monomer would lead to a fast decay of the adduct, which was not observed. This interpretation is in agreement with results from SEC on LOV-Jα, where the elution profile of dimeric LOV-Jα does not show any tailing or broadening from any partial dissociation to the monomer (Figure 6A).

With regard to the concentration dependence, the behavior of LOV-Jα of *Pt*AUREO1a seems to differ from that of *V. frigida* aureochrome1. There, it was shown by transient grating measurements and SEC that LOV-Jα shows a concentration-dependent dimerization in the dark leading to a dimer at a concentration of 350 μM.[22] Furthermore, sedimentation velocity experiments on the same aureochrome but with different construct borders of LOV-Jα (Figure S1 of the Supporting Information) showed a dimer in the dark state at >4 μM concentration.[7] In the case of *Pt*AUREO1a, dimerization of LOV-Jα is only driven by light, which provokes a release of the dimerization site shielded in the dark (Figure 7).

Light-Induced Changes in Secondary Structure in the Presence and Absence of Jα. Further signals were detected in the FT-IR double difference spectrum (Figure 4B), which might be caused either by secondary structural changes in LOV and A′α accompanying Jα unfolding or by the Jα-induced dimerization. It is furthermore possible but considered unlikely that light-induced changes in the LOV domain are suppressed by the presence of Jα. Among the signals, a prominent positive band was found at 1679 cm^{-1} and a negative band at 1691 cm^{-1}, which are indicative of turn structural changes.[33,42] Similar bands were also identified in the double difference spectrum of LOV-Jα *minus* LOV of *A. thaliana* phototropin1.[15,34] Other bands at around 1550 cm^{-1} might be assigned to amide II bands, which always accompany amide I bands, and/or to changes in side chain vibrations.[33]

Interestingly, the LOV domain shows even in the absence of Jα prominent changes in secondary structure (Figure 4A) in agreement with previous assignments by infrared spectroscopy.[14,15,30,34,43,44] In support, the LOV domain shows a small but reproducible light-dependent increase in apparent molecular mass by 2 kDa in the SEC analysis at high concentrations (Figure 6B). The fact that a high salt concentration of 300 mM was applied in the SEC speaks against a shift by a decrease of interaction with the column. The observed increase in apparent mass is rather attributable to a change in secondary or tertiary

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structure that changes the effective hydrodynamic radius of the LOV domain. An alternative explanation would be a rapidly dissociating dimer.[45] Such a dimerization would lead to a concentration-dependent elution profile. However, the same shift was observed at a 6-fold lower concentration (Figure S3 of the Supporting Information). A similar shift by light has been demonstrated for another blue light receptor domain, the sensor of blue light using flavin adenine dinucleotide (BLUF),[46] but to our knowledge not for any other LOV domain without Jα helix.

CONCLUSIONS

The ability of LOV-Jα of aureochromes to dimerize upon illumination is exceptionally pronounced as compared to the many LOV domains of different light sensors studied. Only Vivid shows an increase in dimer upon illumination, which undergoes rapid dissociation.[21,47] The net oligomeric state of most LOV domains, especially from phototropins,[35] is hardly influenced by light, even if association and dissociation reactions could be detected.[48] To extrapolate the role of this special ability of light-induced dimerization to the full-length protein is somehow speculative, as bZIP-LOV-Jα has been shown to be a dimer in the dark.[22]

But, it is reasonable to conclude that the structural response of Jα to light demonstrated here will also take place in a full-length aureochrome. Therefore, aureochromes show in this regard more similarity to phototropins than to fungal or bacterial LOV proteins. The most pressing question remains as to how the structural change in Jα or concomitant changes might be relayed to the N-terminal bZIP domain.

ASSOCIATED CONTENT

Supporting Information

Sequence alignment of LOV domains, SDS−PAGE of expressed proteins, and size exclusion chromatography of LOV and LOV-Jα at lower concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

BLUF, sensor of blue light using flavin adenine dinucleotide; bZIP, basic region leucine zipper; CD, circular dichroism; DTE, dithioerythritol; FMN, flavin mononucleotide; FT-IR, Fourier transform infrared; LOV, light-, oxygen-, or voltage-sensitive; fwhm, full width at half-maximum; *Pt*AUREO1a, aureochrome1a from *Phaeodactylum tricornutum*; SEC, size exclusion chromatography

REFERENCES

(1) Christie, J. M., Salomon, M., Nozue, K., Wada, M., and Briggs, W. R. (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide. *Proc. Natl. Acad. Sci. U.S.A. 96*, 8779−8783.

(2) He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K. H., and Liu, Y. (2002) White collar-1, a DNA binding transcription factor and a light sensor. *Science 297*, 840−843.

(3) Heintzen, C., Loros, J. J., and Dunlap, J. C. (2001) The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. *Cell 104*, 453−464.

(4) Losi, A., Polverini, E., Quest, B., and Gärtner, W. (2002) First evidence for phototropin-related blue-light receptors in prokaryotes. *Biophys. J. 82*, 2627−2634.

(5) Losi, A., and Gärtner, W. (2008) Bacterial bilin- and flavin-binding photoreceptors. *Photochem. Photobiol. Sci. 7*, 1168−1178.

(6) Takahashi, F., Yamagata, D., Ishikawa, M., Fukamatsu, Y., Ogura, Y., Kasahara, M., Kiyosue, T., Kikuyama, M., Wada, M., and Kataoka, H. (2007) AUREOCHROME, a photoreceptor required for photomorphogenesis in stramenopiles. *Proc. Natl. Acad. Sci. U.S.A. 104*, 19625−19630.

(7) Mitra, D., Yang, X., and Moffat, K. (2012) Crystal structures of aureochrome1 LOV suggest new design strategies for optogenetics. *Structure 20*, 698−706.

(8) Crosson, S., Rajagopal, S., and Moffat, K. (2003) The LOV domain family: photoresponsive signaling modules coupled to diverse output domains. *Biochemistry 42*, 2−10.

(9) 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.

(10) Harper, S. M., Neil, L. C., and Gardner, K. H. (2003) Structural basis of a phototropin light switch. *Science 301*, 1541−1544.

(11) Harper, S. M., Christie, J. M., and Gardner, K. H. (2004) Disruption of the LOV-Jα helix interaction activates phototropin kinase activity. *Biochemistry 43*, 16184−16192.

(12) Nakasone, Y., Eitoku, T., Matsuoka, D., Tokutomi, S., and Terazima, M. (2007) Dynamics of conformational changes of *Arabidopsis* phototropin 1 LOV2 with the linker domain. *J. Mol. Biol. 367*, 432−442.

(13) Alexandre, M. T., van Grondelle, R., Hellingwerf, K. J., and Kennis, J. T. (2009) Conformational heterogeneity and propagation of structural changes in the LOV2/Jα domain from *Avena sativa* phototropin 1 as recorded by temperature-dependent FTIR spectroscopy. *Biophys. J. 97*, 238−247.

(14) Pfeifer, A., Mathes, T., Lu, Y., Hegemann, P., and Kottke, T. (2010) Blue light induces global and localized conformational changes in the kinase domain of full-length phototropin. *Biochemistry 49*, 1024−1032.

(15) Yamamoto, A., Iwata, T., Sato, Y., Matsuoka, D., Tokutomi, S., and Kandori, H. (2009) Light signal transduction pathway from flavin chromophore to the Jα helix of *Arabidopsis* phototropinl. *Biophys. J. 96*, 2771−2778.

(16) Möglich, A., and Moffat, K. (2007) Structural basis for light-dependent signaling in the dimeric LOV domain of the photosensor YtvA. *J. Mol. Biol. 373*, 112−126.

(17) Circolone, F., Granzin, J., Jentzsch, K., Drepper, T., Jaeger, K. E., Willbold, D., Krauss, U., and Batra-Safferling, R. (2012) Structural basis for the slow dark recovery of a full-length LOV protein from *Pseudomonas putida*. *J. Mol. Biol. 417*, 362−374.

(18) Nash, A. I., McNulty, R., Shillito, M. E., Swartz, T. E., Bogomolni, R. A., Luecke, H., and Gardner, K. H. (2011) Structural basis of photosensitivity in a bacterial light-oxygen-voltage/helix-turn-helix (LOV-HTH) DNA-binding protein. *Proc. Natl. Acad. Sci. U.S.A. 108*, 9449−9454.

(19) Alexandre, M. T., Purcell, E. B., van Grondelle, R., Robert, B., Kennis, J. T., and Crosson, S. (2010) Electronic and protein structural

dynamics of a photosensory histidine kinase. *Biochemistry 49*, 4752−4759.
(20) Zoltowski, B. D., Schwerdtfeger, C., Widom, J., Loros, J. J., Bilwes, A. M., Dunlap, J. C., and Crane, B. R. (2007) Conformational switching in the fungal light sensor Vivid. *Science 316*, 1054−1057.
(21) Zoltowski, B. D., and Crane, B. R. (2008) Light activation of the LOV protein Vivid generates a rapidly exchanging dimer. *Biochemistry 47*, 7012−7019.
(22) Toyooka, T., Hisatomi, O., Takahashi, F., Kataoka, H., and Terazima, M. (2011) Photoreactions of aureochrome-1. *Biophys. J. 100*, 2801−2809.
(23) Hisatomi, O., Takeuchi, K., Zikihara, K., Ookubo, Y., Nakatani, Y., Takahashi, F., Tokutomi, S., and Kataoka, H. (2012) Blue light-induced conformational changes in a light-regulated transcription factor, aureochrome-1. *Plant Cell Physiol. 54*, 93−106.
(24) Nelson, D. M., Treguer, P., Brzezinski, M. A., Leynaert, A., and Queguiner, B. (1995) Production and dissolution of biogenic silica in the ocean - revised global estimates, comparison with regional data and relationship to biogenic sedimentation. *Global Biogeochem. Cycles 9*, 359−372.
(25) Depauw, F. A., Rogato, A., Ribera d'Alcala, M., and Falciatore, A. (2012) Exploring the molecular basis of responses to light in marine diatoms. *J. Exp. Bot. 63*, 1575−1591.
(26) Huysman, M. J., Fortunato, A. E., Matthijs, M., Schellenberger Costa, B., Vanderhaeghen, R., Van den Daele, H., Sachse, M., Inze, D., Bowler, C., Kroth, P. G., Wilhelm, C., Falciatore, A., Vyverman, W., and De Veylder, L. (2013) AUREOCHROME1a-mediated induction of the diatom-specific cyclin dsCYC2 controls the onset of cell division in diatoms (*Phaeodactylum tricornutum*). *Plant Cell 25*, 215−228.
(27) Schellenberger Costa, B., Jungandreas, A., Jakob, T., Weisheit, W., Mittag, M., and Wilhelm, C. (2013) Blue light is essential for high light acclimation and photoprotection in the diatom *Phaeodactylum tricornutum*. *J. Exp. Bot. 64*, 483−493.
(28) Macheroux, P. (1999) UV-visible spectroscopy as a tool to study flavoproteins. *Methods Mol. Biol. 131*, 1−7.
(29) Alexandre, M. T., Arents, J. C., van Grondelle, R., Hellingwerf, K. J., and Kennis, J. T. (2007) A base-catalyzed mechanism for dark state recovery in the *Avena sativa* phototropin-1 LOV2 domain. *Biochemistry 46*, 3129−3137.
(30) 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.
(31) Iwata, T., Nozaki, D., Sato, Y., Sato, K., Nishina, Y., Shiga, K., Tokutomi, S., and Kandori, H. (2006) Identification of the C═O stretching vibrations of FMN and peptide backbone by ^{13}C-labeling of the LOV2 domain of *Adiantum* phytochrome3. *Biochemistry 45*, 15384−15391.
(32) Swartz, T. E., Wenzel, P. J., Corchnoy, S. B., Briggs, W. R., and Bogomolni, R. A. (2002) Vibration 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.
(33) Barth, A., and Zscherp, C. (2002) What vibrations tell us about proteins. *Q. Rev. Biophys. 35*, 369−430.
(34) Koyama, T., Iwata, T., Yamamoto, A., Sato, Y., Matsuoka, D., Tokutomi, S., and Kandori, H. (2009) Different role of the Jα helix in the light-induced activation of the LOV2 domains in various phototropins. *Biochemistry 48*, 7621−7628.
(35) Katsura, H., Zikihara, K., Okajima, K., Yoshihara, S., and Tokutomi, S. (2009) Oligomeric structure of LOV domains in Arabidopsis phototropin. *FEBS Lett. 583*, 526−530.
(36) Iwata, T., Yamamoto, A., Tokutomi, S., and Kandori, H. (2007) Hydration and temperature similarly affect light-induced protein structural changes in the chromophoric domain of phototropin. *Biochemistry 46*, 7016−7021.
(37) Halavaty, A. S., and Moffat, K. (2007) N- and C-terminal flanking regions modulate light-induced signal transduction in the LOV2 domain of the blue light sensor phototropin 1 from *Avena sativa*. *Biochemistry 46*, 14001−14009.
(38) Zayner, J. P., Antoniou, C., and Sosnick, T. R. (2012) The amino-terminal helix modulates light-activated conformational changes in *As*LOV2. *J. Mol. Biol. 419*, 61−74.
(39) Aihara, Y., Yamamoto, T., Okajima, K., Yamamoto, K., Suzuki, T., Tokutomi, S., Tanaka, K., and Nagatani, A. (2012) Mutations in N-terminal flanking region of blue light-sensing light-oxygen and voltage 2 (LOV2) domain disrupt its repressive activity on kinase domain in the *Chlamydomonas* phototropin. *J. Biol. Chem. 287*, 9901−9909.
(40) Guo, H., Kottke, T., Hegemann, P., and Dick, B. (2005) The phot LOV2 domain and its interaction with LOV1. *Biophys. J. 89*, 402−412.
(41) Purcell, E. B., McDonald, C. A., Palfey, B. A., and Crosson, S. (2010) An analysis of the solution structure and signaling mechanism of LovK, a sensor histidine kinase integrating light and redox signals. *Biochemistry 49*, 6761−6770.
(42) Krimm, S., and Bandekar, J. (1986) Vibrational spectroscopy and conformations of peptides, polypeptides, and proteins. *Adv. Protein Chem. 38*, 181−364.
(43) Bednarz, T., Losi, A., Gärtner, W., Hegemann, P., and Heberle, J. (2004) Functional variations among LOV domains as revealed by FT-IR difference spectroscopy. *Photochem. Photobiol. Sci. 3*, 575−579.
(44) Pfeifer, A., Majerus, T., Zikihara, K., Matsuoka, D., Tokutomi, S., Heberle, J., and Kottke, T. (2009) Time-resolved Fourier transform infrared study on photoadduct formation and secondary structural changes within the phototropin LOV domain. *Biophys. J. 96*, 1462−1470.
(45) Stevens, F. J. (1989) Analysis of protein-protein interaction by simulation of small-zone size exclusion chromatography. Stochastic formulation of kinetic rate contributions to observed high-performance liquid chromatography elution characteristics. *Biophys. J. 55*, 1155−1167.
(46) 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.
(47) Lamb, J. S., Zoltowski, B. D., Pabit, S. A., Li, L., Crane, B. R., and Pollack, L. (2009) Illuminating solution responses of a LOV domain protein with photocoupled small-angle X-ray scattering. *J. Mol. Biol. 393*, 909−919.
(48) Nakasone, Y., Eitoku, T., Matsuoka, D., Tokutomi, S., and Terazima, M. (2006) Kinetic measurement of transient dimerization and dissociation reactions of *Arabidopsis* phototropin 1 LOV2 domain. *Biophys. J. 91*, 645−653.