

Helical Contributions Mediate Light-Activated Conformational Change in the LOV2 Domain of *Avena sativa* Phototropin 1

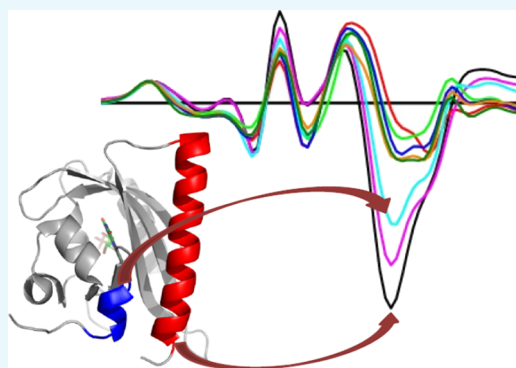
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ABSTRACT: Algae, plants, bacteria, and fungi contain flavin-binding light-oxygen-voltage (LOV) domains that function as blue light sensors to control cellular responses to light. In the second LOV domain of phototropins, called LOV2 domains, blue light illumination leads to covalent bond formation between protein and flavin that induces the dissociation and unfolding of a C-terminally attached α helix ($J\alpha$) and the N-terminal helix ($A'\alpha$). To date, the majority of studies on these domains have focused on versions that contain truncations in the termini, which creates difficulties when extrapolating to the much larger proteins that contain these domains. Here, we study the influence of deletions and extensions of the $A'\alpha$ helix of the LOV2 domain of *Avena sativa* phototropin 1 (AsLOV2) on the light-triggered structural response of the protein by Fourier-transform infrared difference spectroscopy. Deletion of the $A'\alpha$ helix abolishes the light-induced unfolding of $J\alpha$, whereas extensions of the $A'\alpha$ helix lead to an attenuated structural change of $J\alpha$. These results are different from shorter constructs, indicating that the conformational changes in full-length phototropin LOV domains might not be as large as previously assumed, and that the well-characterized full unfolding of the $J\alpha$ helix in AsLOV2 with short $A'\alpha$ helices may be considered a truncation artifact. It also suggests that the N- and C-terminal helices of phot-LOV2 domains are necessary for allosteric regulation of the phototropin kinase domain and may provide a basis for signal integration of LOV1 and LOV2 domains in phototropins.



INTRODUCTION

Many organisms depend on light for energy and tracking sunlight and have thus evolved light-sensitive proteins and signaling pathways. In algae and plants, Per-Arnt-Sim (PAS)-containing phototropins respond to light by activating signaling cascades that end in phototropism or chloroplast rearrangement.^{1,2} Phototropins are large proteins which contain a photoactivatable domain attached to a kinase domain.¹ They utilize a subclass of PAS domains termed the light-oxygen-voltage (LOV) domain that is activated by blue light.^{3–5} LOV domain activation occurs when the non-covalently bound flavin mononucleotide (FMN) or flavin adenine dinucleotide chromophore absorbs a photon that excites the chromophore into a singlet state. This state is converted into a triplet excited state with high probability,^{6–8} promoted by the heavy atom effect of the cysteine sulfur nucleus.^{6,9,10} The flavin forms a metastable covalent bond between its C4a atom and the sulfur of the neighboring cysteine predominantly from the triplet excited state^{11–13} and, in some cases, from the singlet excited state.^{13,14} During this process, the N5 atom of the flavin is protonated and reduced, receiving a donated hydrogen from the active site cysteine.^{8,15–18} This cysteinyl–flavin adduct spontaneously decays

in seconds–hours depending on solvent accessibility, steric interactions, and the local electrostatics.^{19–23}

Photon absorption by the flavin and the ensuing chemical reactions cause the major light-induced conformational changes in LOV proteins. The amino and carboxy termini of LOV domains are hotspots for structural rearrangement. The *Avena sativa* phototropin 1 LOV2 (AsLOV2) domain is a model protein used to study LOV function and allostery. Light absorption and adduct formation result in the unfolding of the amino-terminal $A'\alpha$ helix and the carboxy-terminal $J\alpha$ helix.^{24–27} The latter process occurs in multiple steps: the $J\alpha$ helix partly unfolds concomitant with adduct formation in microseconds, after which full unfolding ensues in hundreds of microseconds.^{28,29} Figure 1 (green) shows the X-ray structure of AsLOV2, with the $A'\alpha$ helix and the $J\alpha$ helix highlighted.³⁰ These helices have also been shown to be involved in activation of the attached kinase domain in the full-length phototropins.^{31,32} However, the extent of the conformational changes is not fully known as NMR and circular dichroism

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activate phosphorylation in the downstream kinase domain, we created a number of AsLOV2 constructs that include truncations and additions with progressively longer LOV2 domains. We characterized their light-activated conformational changes using UV-vis and Fourier-transform infrared (FTIR) spectroscopy. Compared to the more frequently used constructs with short A'α helices, the constructs with longer A'α helices have different biophysical characteristics. The longer helix constructs have photocycle times that are approximately half that of the other constructs, possibly because of slight conformational shifts around the chromophore. We find that increasing the length of the amino terminus of AsLOV2 dampens the amount of light-activated conformational change as compared to the construct that is commonly used to study phototropins and for optogenetic engineering (AsLOV2 phot1 404–560). These findings emphasize that the effects of light on the LOV domain structure are context dependent, and hence, inferring the behavior of LOV domains in their biological context from the results on isolated domains should be done with caution.

RESULTS AND DISCUSSION

Recent work has begun to show the importance of interactions between two terminal helices in the LOV2 proteins.^{25,44} The phot1 AsLOV2 domain boundaries have commonly been demarcated at the start of the A'α helix (residue 404), and after the end of the Jα helix, near residues 545–560.^{24,30} Many studies of PAS domains have been on similarly truncated versions.^{45,46} The regions connecting AsLOV2 to the LOV1 and kinase domain are hypothesized to be mostly unstructured. This inference is in stark contrast to what is observed in the full-length structure of the LOV domain containing sensor histidine kinase YF1,⁴⁷ which contains only very small unstructured regions between the LOV and the kinase domain. Structures of other PAS-containing photoactive proteins such as phytochromes also contain long helical segments emitting from the amino and carboxy termini of the PAS domains that form long symmetrical interactions with a homodimerization partner.^{48,49} The only extended plant LOV2 domain structure of AtLOV2 shows helical regions extending from the A'α.⁴³ From this result, we hypothesized that AsLOV2 also might contain helical extensions (Figure 2). Secondary structure prediction of the LOV2 region of the full length *A. sativa* phot1 protein has two helical segments in the amino-terminal region that often have been truncated in constructs used in early studies of the LOV domain. In our study, taking the 404–560 construct²⁴ as a reference, we extend the A'α helix in four different constructs: A'α₅ (residues 399–560), A'α₁₅ (390–560), A'α₁₉ (385–560), and A'α₂₆ (378–560). The two longest constructs, A'α₁₉ and A'α₂₆ did not express well enough to be characterized. We also created AsLOV2 constructs that lacked the Jα helix (ΔJα, residues 404–524) or the A'α helix (ΔA'α, residues 409–560). Finally, we also created the 404–550 construct, which is similar to that used for the AsLOV2-Jα X-ray structure.³⁰

Structural Effects on the Stability of the Photoadduct. The time constant (τ_{FMN}) of the reversible photocycle of AsLOV2 can be measured by tracking the recovery of the absorbance of the FMN chromophore at ~450 nm. The photocycle process is controlled by interactions of protein's side chains with the chromophore, water molecules, or small bases.^{20,22,23,51–53} The generally used AsLOV2(404–560) construct has a photocycle τ_{FMN} of ~80 ± 2 s at 293 K. All

constructs tested that involved alterations of the A'α helix, including extension and truncations, speed up the photocycle (Table 1). Truncations of the Jα helix, however, do not have a

Table 1. Dark Reversion Time Constants of Constructs^a

construct	τ_{FMN}
404–560	81
404–550	80
404–560ΔJα (404–524)	82
404–560ΔA'α (409–560)	71
404–560ΔA'α/Δ; Jα (409–524)	83
A'α ₅ (399–560)	45
A'α ₁₅ (389–560)	52
A'α ₁₅ /Δ; Jα (389–524)	66
A'α ₅ /Δ; Jα (399–524)	71

^aFitted time constants have an error of ~2 s.

measurable effect on photocycle time, except for truncations of the Jα helix in the A'α helix extension background. The majority of mutations that have been made to AsLOV2^{21,25,53} has caused a slowing in the photocycle. Increasing the photocycle time was shown to be controlled by the solvent accessibility of the FMN,¹⁹ steric effects²³ or the presence of small bases.²⁰ These data are indicative of interactions between the protein and the chromophore being affected by the A'α helix and to a lesser extent by the Jα helix.

N-Terminal Helix A'α Modulates the Structural Response of the C-Terminal Jα Helix. To look more closely at what specific helical structures are being controlled by the extended A'α helix during light-activated conformational change, we performed FTIR difference spectroscopy on the different constructs. In light–dark difference spectra of phototropin LOV domains, undocking and unfolding of the Jα helix is typically observed as a large negative feature at around 1645 cm⁻¹ (Figure 3A).^{26–28} The signal and thus the photo-induced structural response of this part of the protein is sensitive to temperature and the hydration status of the protein.²⁶

In the 404–560 construct, the light-activated conformational change of the Jα helix is readily observed at 1645 cm⁻¹ where the difference signal has the largest amplitude (Figure 3A,B black). At low temperatures²⁶ and upon deletion of the Jα helix, this signal is lost (Figure 3B, red). Furthermore, when we delete the A'α helix, we also see a loss of the Jα response to light (Figure 3B, orange). This observation is consistent with the notion that upon deletion of the A'α helix, the Jα helix is constitutively unfolded in the dark²⁵ and hence will not give a signal upon illumination. Thus, the A'α helix is crucial for locking the Jα helix in its dark state and for inducing its undocking in the light state. In AsLOV2 that contain N-terminal extensions (A'α₅ and A'α₁₅), the Jα response is significantly reduced with the longer extension, A'α₁₅, having the greater effect (Figure 3B, magenta and cyan). These observations substantiate the crucial role of A'α in mediating a light-induced structural response in phot-LOV2 domains, as previously shown.²⁵

Helical change is a common theme in the function of PAS and LOV domain containing photoregulatory proteins.^{24,25,33,39,54} Of the LOV domains that have been studied extensively, most have an amino and/or carboxy terminal helix that regulates the function of the protein. Here we have shown that the increased length of the A'α helix decreases the amount

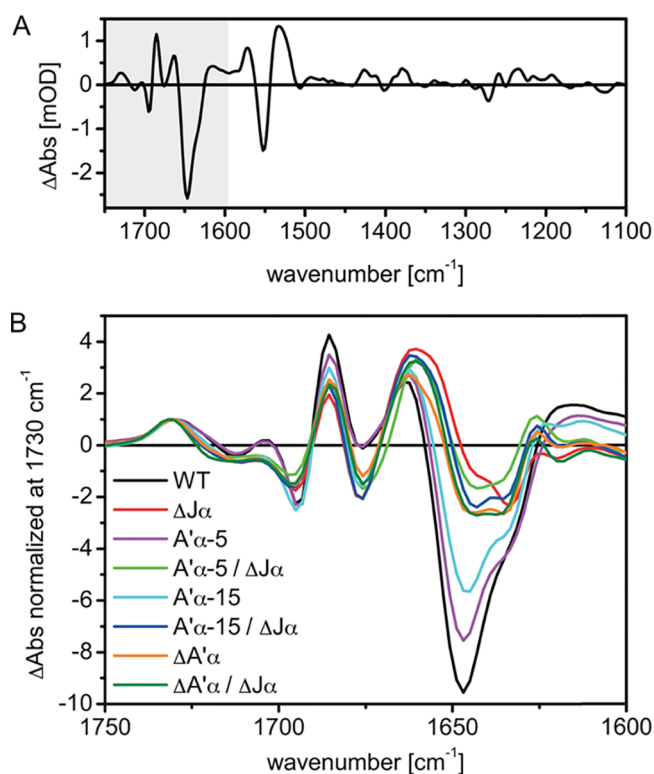


Figure 3. FTIR difference spectra of AsLOV2. Light-minus-dark difference spectra of the (A) wild type and (B) wild type and several N-/C-terminal extension/deletion constructs. The largest difference signal between 1750 and 1000 cm⁻¹ is found at around 1645 cm⁻¹ and is attributed to undocking and unfolding of the C-terminal $\text{J}\alpha$ helix, which is significantly influenced by N-/C-terminal extensions and deletions.

of conformational change, specifically in the $\text{J}\alpha$ helix, that was previously seen in shorter $\text{A}'\alpha$ helix LOV domain constructs.^{26,27} Our results are consistent with those reported for *Chlamydomonas* phototropin and LOV1-LOV2 tandem constructs,⁵⁵ where $\text{J}\alpha$ unfolding signals were observed with significantly smaller amplitude (4–5 fold less) than in the short $\text{A}'\alpha$ helix LOV domain constructs reported here and in ref 26. Also, in the LovK LOV-histidine kinase photoreceptor from *Caulobacter crescentus*, little loss of helical structure was observed upon photoactivation.^{40,56} These observations strongly suggest that the full $\text{J}\alpha$ helical unfolding that was so convincingly observed in the short $\text{A}'\alpha$ helix LOV domain constructs,^{24,26} is highly attenuated in the full-length LOV-based photoreceptors, and in essence may be regarded as an N-terminal truncation artifact. Interestingly, it is this nonnative photoinduced C-terminal helical unfolding that has enabled the widely successful application of AsLOV2 domain as an optogenetic engineering platform.^{57–62} The present results further provide an avenue to modulate the extent of AsLOV2 C-terminal structural change in engineered LOV2-based photoswitches by modifying the length of the $\text{A}'\alpha$ helix.

In the AtLOV2 structure with the extended $\text{A}'\alpha$ helix¹⁹ both the N and C termini are facing the same direction, making it likely that the parts of the phototropin structure usually termed “linker regions” could in fact be helical segments. Such structural arrangement is seen in many PAS domain-containing photoreceptors including phytochromes⁴⁸ and sensor histidine kinases.²² The N-terminal extensions in the $\text{A}'\alpha$ helix, which embed the LOV2 domain in a more native-like phototropin

environment, apparently modulate the extent of the structural response in the $\text{J}\alpha$ helix. This process might be used in phototropins to integrate the photoresponses of both LOV1 and LOV2 domains and also to transmit the signal to the downstream kinase domain. Analogous scenarios have been discussed for tandem-PAS domain proteins, for example, phytochrome photoreceptors or chemical sensors such as FixL, which form coiled coil connectors between their sensory domains.³⁹ The molecular arrangement may be different in phototropins and should be elucidated in further structural studies on full-length proteins. It is, however, known that discrete mutations in the $\text{A}'\alpha$ helix and its N-terminal extension greatly influence the light-activation of the kinase domain in *Chlamydomonas reinhardtii* phot.³² Overall, it is clear that extended helices from *A. sativa* phot1 LOV2 play a key role in the function of the protein.

■ MATERIALS AND METHODS

Cloning, Expression, and Purification. A clone of *A. sativa* phot1 LOV2(404–560) with a His₆-Gβ1 fusion was used.²⁴ Mutations were made using the quickchange site-directed mutagenesis strategy to add or delete residues. All proteins were expressed in *Escherichia coli* BL21(DE3) cells at 37 °C to an OD 600 nm of 0.6. Cultures were incubated for ~1 h at 18 °C and induced with 1 mM IPTG, then incubated at 18 °C overnight, pelleted, and frozen. Frozen pellets were resuspended in 50 mM Tris 100 mM NaCl and 0.01% sodium dodecyl sulfate, and cells were lysed using sonication and clarified with centrifugation at 10 000g for 45 min. The proteins were purified using metal affinity chromatography and exchanged into 50 mM Tris, 1 mM EDTA, and 5 mM DTT at pH 8.0. The His₆-Gβ1 tag was removed by incubating overnight at 20 °C with His₆-TEV protease. Metal affinity chromatography was used to remove His₆-Gβ1 and His₆-TEV protease from the solution. The final protein contains residues GEF on the amino terminus and G on the carboxy terminus as cloning artifacts. Proteins were run on a Sephadex S100 size exclusion column (GE Healthcare). Protein purity was assayed by comparing the absorbance ratio at 280 and 447 nm. If this ratio differed significantly from ~2.6 to 2.9, the proteins were further purified using anion exchange chromatography.

UV–Vis Spectroscopy. UV–vis spectra were acquired using Olis HP 8452 Diode Array (Bogart, GA) with a 1 cm pathlength cuvette at 22 °C. Kinetic traces were acquired after saturating illumination. Time-resolved spectra were taken every 1–3 s depending on the recovery rate at the FMN central peak maximum, usually 448 nm, until complete recovery of the signal was achieved.

FTIR Experiments. FTIR experiments were performed with Bruker IFS 66s as previously described.²⁶ Samples were concentrated to an absorbance of 50–70 units per cm and sandwiched between two CaF₂ wafers without spacer. Care was taken to avoid drying of the samples. The sample cell was additionally sealed with silicon grease. Light-minus-dark FTIR difference spectra were recorded between 1800 and 1100 cm⁻¹ by illuminating the sample using an LED light source (Luxeon 1 W 460 nm, effective intensity 20 mW/cm²). For each light-minus-dark spectrum, 100 scans of background and 100 scans under illumination were recorded. Ten such difference spectra were averaged for the final spectra displayed here. The FTIR spectra were normalized on the positive FMN C=O band near 1730 cm⁻¹ that is associated with covalent adduct

formation and cleanly separated from other vibrational signals.⁶³

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Author Contributions

J.P.Z. and T.M. contributed equally to the work. T.M. and J.P.Z. performed the experiments. T.M., J.P.Z., J.T.M.K., and T.R.S. analyzed the data and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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