Disruption of the LOV−Jα Helix Interaction Activates Phototropin Kinase Activity†

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ABSTRACT: Light plays a crucial role in activating phototropins, a class of plant photoreceptors that are sensitive to blue and UV-A wavelengths. Previous studies indicated that phototropin uses a bound flavin mononucleotide (FMN) within its light−oxygen−voltage (LOV) domain to generate a protein−flavin covalent bond under illumination. In the C-terminal LOV2 domain of *Avena sativa* phototropin 1, formation of this bond triggers a conformational change that results in unfolding of a helix external to this domain called Jα [Harper, S. M., et al. (2003) *Science 301*, 1541−1545]. Though the structural effects of illumination were characterized, it was unknown how these changes are coupled to kinase activation. To examine this, we made a series of point mutations along the Jα helix to disrupt its interaction with the LOV domain in a manner analogous to light activation. Using NMR spectroscopy and limited proteolysis, we demonstrate that several of these mutations displace the Jα helix from the LOV domain independently of illumination. When placed into the full-length phototropin protein, these point mutations display constitutive kinase activation, without illumination of the sample. These results indicate that unfolding of the Jα helix is the critical event in regulation of kinase signaling for the phototropin proteins.

Among the many aspects of plant growth and development affected by the sun, one of the most recognizable phenotypes is the characteristic phototropic response of plants bending toward light. Despite its being one of the first blue-light responses discovered in plants, the proteins controlling this process remained a mystery for years (*1*). Only recently have a family of proteins known as phototropins been identified as the photoreceptors responsible for phototropism and several other blue-light responses (*2*). Phototropins are light-activated serine/threonine kinases that contain two N-terminal light−oxygen−voltage (LOV)[1] domains, named LOV1 and LOV2.

LOV domains play a critical role in the phototropin activation process by both detecting incoming blue light and converting this into a biochemical signal that activates the C-terminal kinase domain. These domains are a subclass of the much larger family of PAS (Per−ARNT−Sim) protein−protein interaction domains (*3*), which participate in a diverse array of biological signaling pathways. Within this group, LOV domains can be identified by a series of conserved residues that surround an internally bound flavin, which acts as the chromophore for the protein. Spectroscopic studies of isolated LOV−FMN complexes have shown that blue light activates the FMN molecule, generating a covalent adduct between the isoalloxazine C4a position and a conserved cysteine residue within the LOV domain (*4*, *5*). This photoadduct is quite stable, spontaneously relaxing back to the noncovalent dark state on the order of tens to hundreds of seconds. In phototropins, formation of this adduct in the LOV2 domain proximal to the kinase is accompanied by a concomitant increase in autophosphorylation of the phototropin protein (*2*), and mutations that replace the LOV2 reactive cysteine prevent both adduct formation and kinase activation (*6*). While this photochemistry also occurs within LOV1 domains, its functional importance remains unclear at this time.

The structural mechanism by which the FMN−LOV2 adduct enhances phototropin kinase activity has remained an open question to this point. Crystal structures of isolated, consensus LOV domains from the *Adiantum capillus-veneris* phy3 and *Chlamydomonas reinhardtii* phototropin proteins have shown that they adopt a typical PAS domain structure, including a five-stranded, antiparallel β sheet flanked on one side by a series of α helices (*7*, *8*). These structural elements cradle a single molecule of FMN within the protein hydrophobic core, placing the conserved cysteine near the isoalloxazine C4a position. While blue-light illumination of these proteins leads to the photochemical formation of C4a−cysteine bonds within the crystalline lattice, this is accompanied by surprisingly minimal changes in the surrounding protein (*8*−*10*). In contrast, solution studies of longer LOV-containing constructs show significantly larger light-induced protein conformational changes (*11*−*14*). NMR

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[1] Abbreviations: LOV domain, light−oxygen−voltage domain; PAS domain, Per−ARNT−Sim domain; FMN, flavin mononucleotide; AsLOV2, fragment encoding the light oxygen voltage 2 domain plus adjacent Jα helix (residues 404−560) from *Avena sativa* phototropin 1; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence.

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studies of one such construct from the *Avena sativa* phototropin LOV2 domain (AsLOV2) indicate that this is due at least in part to a segment outside the consensus LOV domain. In this case, a segment immediately C-terminal to the LOV2 domain forms a novel α-helix (referred to as the Jα helix) that covers the typically solvent-exposed face of the LOV2 β-sheet (*13*). This interaction is broken upon illumination, with adduct formation triggering conformational changes within the LOV2 domain that disrupt the Jα helix, causing it to unfold in solution.

While these studies help to delineate the early events of photoactivation, it is still not understood how these conformational changes lead to kinase activation. The large light-induced changes in the Jα helix strongly implicate it as a potential participant in this process, leading us to further investigate its role in communicating photodetection events to the kinase. To this end, we have made a series of point mutations along the helix with the goal of artificially disrupting the interaction of the Jα helix with the core LOV2 domain. As assayed by NMR and biochemical methods, several of these point mutations disrupted interactions between Jα and the LOV domain, generating proteins that maintained a pseudo-lit-state structure in the absence of illumination. Upon testing these point mutations in the full-length phototropin protein, we found that the kinase became active in the absence of light. These results indicate that disruption of the Jα helix is sufficient for kinase activation in the absence of any light trigger within the LOV domain.

MATERIALS AND METHODS

Sample Preparation. A construct encoding the *Avena sativa* phototropin 1 LOV2 domain plus the Jα helix [residues 404−560, hereafter termed AsLOV2 (*13*)] was used for site-directed mutagenesis. Mutagenesis was done according to the Quick Change site-directed mutagenesis kit (Stratagene). Proteins were expressed in *Escherichia coli* BL21(DE3) cells grown in M9 medium supplemented with $^{15}NH_4Cl$ (1 g/L) and $^{13}C_6$-glucose (3 g/L) as needed. Proteins were purified as previously described (*13*) and concentrated for NMR experiments to a final concentration of 0.5 mM in a buffer containing 50 mM sodium phosphate, 100 mM NaCl, pH 6.0, 6 mM NaN_3, a protease inhibitor cocktail (Sigma), and 10% D_2O. Proteolysis experiments were done in 50 mM sodium phosphate buffer with 100 mM NaCl, pH 7.5, at a protein concentration of 0.1 mM.

In a similar manner, point mutations were generated in the *Arabidopsis thaliana* phototropin 1 full-length protein with the Quick Change approach, resulting in proteins containing the following changes: V601E, A605E, and I608E (corresponding to I532E, A536E, and I539E in AsLOV2). These proteins were expressed in insect cells and prepared as previously described (*2*).

Electrostatic Calculations. Electrostatic calculations were done on the LOV core and Jα helix by use of two programs, GROMACS and MEAD (*15, 16*). Using the previously described AsLOV2 model (*13*), we used GROMACS to convert our PDB coordinates into a MEAD-readable file. MEAD was then used to calculate the electrostatic surface of the proteins, assuming an inner dielectric of 2. All molecular structures were generated by use of the program PyMOL (*17*).

NMR Spectroscopy. All NMR experiments were performed on Varian Inova 500 and 600 MHz spectrometers as previously described (*13*). Lit-state HSQC spectra were generated with a 50 mW, 200 ms laser pulse at 472.7 nm during the 1 s relaxation delay between scans. Chemical shift differences between wild-type and mutant 3D HNCO spectra were quantitated by the minimum chemical shift difference method (*18*):

$$\Delta\delta_{min} = [\Delta\delta(^1H)^2 + 0.17\Delta\delta(^{15}N)^2 + 0.39\Delta\delta(^{13}C)^2]^{1/2}$$

Limited Proteolysis. Limited proteolysis experiments were conducted on the AsLOV2 I539E construct as previously described (*13*). Briefly, chymotrypsin was added to a final concentration of 20 μg/mL in a total reaction volume of 500 μL. Samples were incubated at room temperature, with aliquots removed after 0, 10, 30, and 60 min for SDS−PAGE analysis. Proteolysis of the lit state was done by use of the illumination setup described for the lit-state NMR experiments. Electrospray ionization mass spectrometry was used to analyze the proteolytic fragments (UT Southwestern Protein Chemistry Technology Center).

Phosphorylation Assay. The autophosphorylation of full-length *A. thaliana* phot1 was performed as described previously (*2, 6*). Recombinant baculovirus encoding the *Arabidopsis PHOT1* cDNA carrying specific amino acid mutations was generated by use of the BaculoGold Transfection Kit (BD Biosciences Pharmingen) in accordance with the instructions of the supplier. Recombinant baculovirus was titered by end-point dilution and used to infect Sf9 (*Spodoptera frugiperda*) insect cells. Insect cells were grown in complete darkness and harvested under dim red light 3 days postinfection. Cells were centrifuged at 1000 rpm for 1 min and the cell pellet was resuspened in 100 μL of phosphorylation buffer [37.5 mM Tris, pH 7.5, 5.3 mM $MgSO_4$, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, and 1× protease inhibitor cocktail (Roche)]. Cells were sonicated and microfuged for 2 min and the crude soluble fraction was removed. Protein concentrations were determined by the Bradford protein assay (Bio-Rad) with BSA as standard, and a 10 μg protein sample was used for autophosphorylation analysis. ATP radiolabeled with γ-^{32}P (110 TBq/mmol; Amersham Pharmacia Biotech) was diluted 5-fold with unlabeled ATP (10 μM), and 1 μL was used for each phosphorylation reaction (in a total volume of 10 μL). Upon addition of radiolabeled ATP, phosphorylation reactions were carried out either under dim red light (dark) or in the presence of saturating white light (a total fluence of 30 000 μmol m^{-2} s^{-1}). Reactions were allowed to proceed for 2 min at room temperature and stopped by adding 10 μL of 2× SDS sample buffer (110 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.008% bromophenol blue). Experiments were repeated at least three times and the data shown are representative of the results obtained.

RESULTS

Interaction Surface of the Jα Helix with the LOV Core. Previously we identified a new structural component outside the canonical LOV core of the *Avena sativa* phototropin 1 LOV2 (AsLOV2) domain that we termed the Jα helix (*13*). Helical wheel analysis of Jα displays its amphipathic character, showing characteristic hydrophobic and polar faces

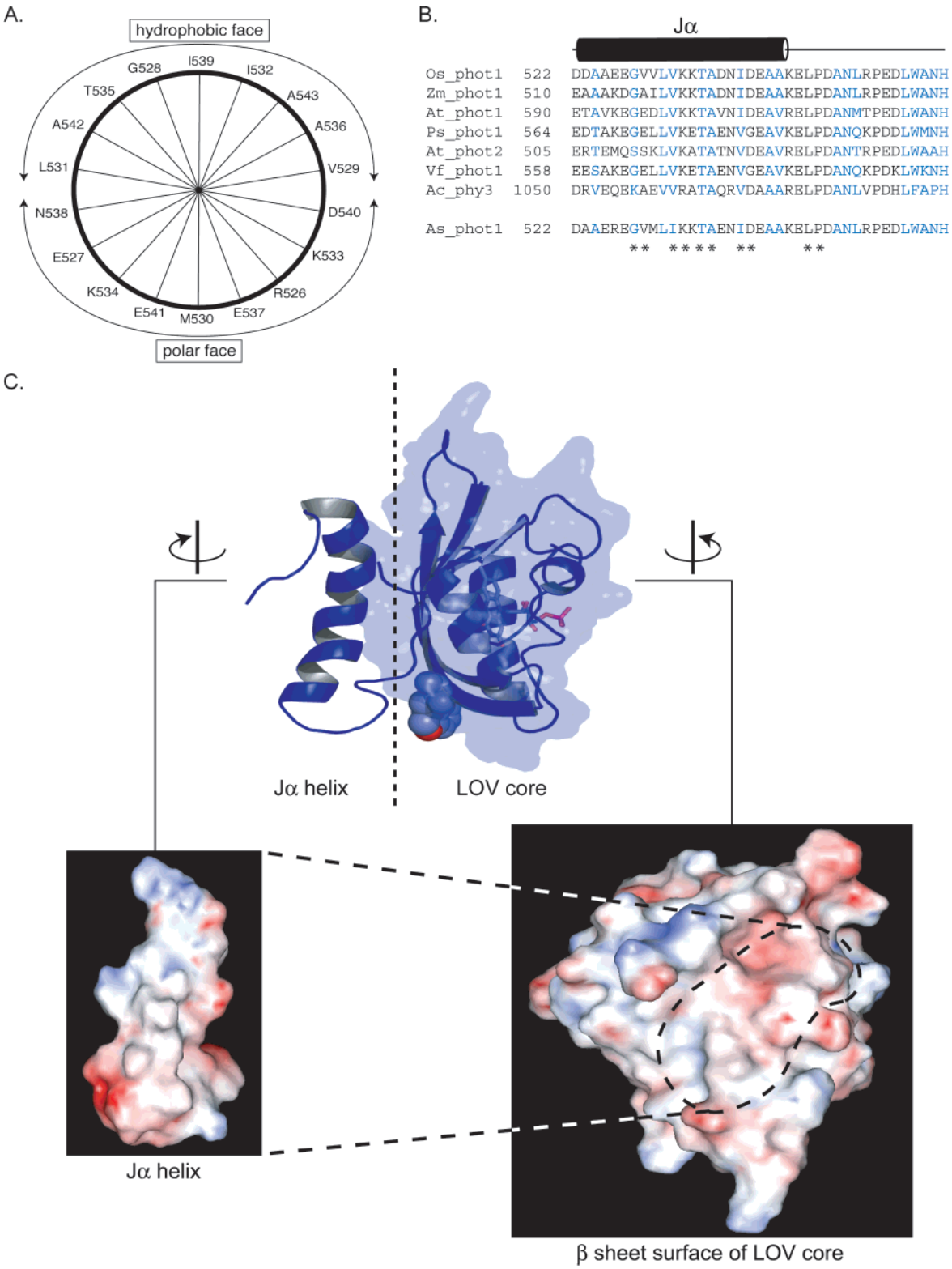

FIGURE 1: Conserved amphipathic pattern in Jα helix facilitates interactions between LOV core and Jα helix. (A) Helical wheel analysis of the Jα helix from AsLOV2, with residues 526−543 and a rotation of 100°/residue. (B) Sequence alignment of LOV2 domains from various phototropins: *Oryza sativa* phot1, *Zea mays* phot1, *Arabidopsis thaliana* phot1, *Pisum sativum* phot1, *Arabidopsis thaliana* phot2, *Vicia faba* phot1, *Adiantum capillus-veneris* phy3, and *Avena sativa* phot1. Conserved hydrophobic residues among the LOV2 domains are designated in blue, while those that were mutated in AsLOV2 for this study are marked with asterisks. Alignment was generated with MultAlin (*32*). (C) (Top) Overview of the AsLOV2 structure, showing the locations of the Jα helix and the LOV core. (Bottom) In this view, the Jα helix and LOV core were artificially separated and rotated by 90° in opposite directions to expose the interface between them. The electrostatic surface potential of each segment was calculated and mapped onto the surface, with red (blue) regions indicating areas of negative (positive) charge. Figures were generated by use of PyMOL (*17*).

on the helix (Figure 1A). The conservation of this patterning among other phototropin LOV2 domains (Figure 1B) suggested that this helix is a conserved element in phototropin signaling, especially given the importance of LOV2-mediated

photosensory function in kinase activation (*6*). NMR-based studies of the dark-state solution structure of AsLOV2 demonstrated that this helix docks onto the β-sheet surface of the LOV domain, covering several β-strands. Electrostatic calculations show that while most of the solvent-exposed surface of AsLOV2 is charged, the interface between the β-sheet of the LOV2 core and Jα helix is composed of hydrophobic residues that are buried from solvent by the sheet−helix interactions (Figure 1C).

Structural Effects of Jα Point Mutations. To artificially disrupt the "docked" dark-state conformation of AsLOV2 in a manner analogous to light activation, a series of point mutations were made along the sheet-binding face of the Jα helix (Figure 1B). Hydrophobic amino acids were individually changed to glutamate residues in hopes that the introduction of negative charge at the interface would prevent the docking of the Jα helix onto the LOV domain β-sheet surface. Additional mutations were made to assess the effects of breaking the Jα helix by introducing a single proline residue or reversing the charge of a single aspartate residue at the C-terminal end of the helix.

The structural effects of these point mutations were assayed by comparing dark-and lit-state $^{15}N-^{1}H$ HSQC spectra of the mutants to the corresponding data collected on wild-type AsLOV2 (Figure 2A). The wild-type protein has a dark-state spectrum with excellent chemical shift dispersion and fairly uniform peak line widths, both of which are hallmarks of well-folded proteins in single conformations. In contrast, the lit-state spectrum displays poorer peak dispersion in the ^{1}H dimension, the appearance of high-intensity peaks with ^{1}H shifts between 7.8 and 8.5 ppm (Figure 2A, green box), and peak disappearance due to conformational exchange (Figure 2A, blue box). These features are consistent with light-induced conformational changes that destabilize AsLOV2, causing the subsequent unfolding of the Jα helix (*13*, *14*). In addition to these changes in signals arising from backbone amides, AsLOV2 conformational changes are also reflected in significant alterations in peaks originating from two tryptophan side-chain indole Hε1/Nε1 positions. These include the side chain of Trp^{491}, located adjacent to the hinge region that links the Jα helix and LOV β sheet core. In $^{15}N-^{1}H$ spectra of AsLOV2 recorded in the dark, the two tryptophan signals are clearly separated (Figure 2B). Upon illumination, the two Trp peaks collapse toward a more central position, suggesting that their environments are less distinctive in the lit state as the Jα helix unfolds.

Most of the point mutants assayed, including the D540R variant, gave similar dark- and lit-state $^{15}N-^{1}H$ HSQC spectra to wild type, indicating that these changes were unable to disrupt the Jα−LOV core interaction. However, a subset of the mutants showed altered dark-state spectra, as exemplified by the I539E mutant (Figure 2A). Proteins within this group had dark- and lit-state $^{15}N-^{1}H$ HSQC spectra that displayed the characteristic poor chemical shift dispersion, intermediate exchange, and intense peaks in the central 7.8−8.5 ppm portion of the ^{1}H spectrum observed in the wild-type lit spectrum. As well, the two peaks from the tryptophan indole resonances are poorly resolved to the point of overlapping in both spectra (Figure 2B). This suggests that these mutant proteins adopt a "pseudo-lit-state" conformation with the Jα helix dissociated from the LOV core despite the absence of the cysteine−FMN bond in the dark-state spectrum. As such, these proteins still show the characteristic UV−visible absorbance changes associated with adduct formation upon illumination, with comparable kinetics to wild-type protein for the spontaneous regeneration of the noncovalent dark state (data not shown).

Of the 10 point mutations made, four mutations at a total of three residues showed this altered mutant phenotype: I532, A536, and I539 (Figure 2C). These residues are located along three consecutive turns of the Jα helix, placing side chains into the center of the interaction surface with the LOV2 core. Mutations introducing either charged glutamate or helix-disrupting proline residues were able to disrupt the core−helix interaction. Interestingly, although all of these residues are located along the helical surface, changes at other residues along the same surface were not sufficient to trigger this change (e.g., V529E). This suggests that there is an uneven distribution of binding energetics among the residues at the Jα helix−β-sheet interface, consistent with observations of "hot spots" at protein−protein interfaces (*19*). These mutants demonstrate that maintenance of the hydrophobic character at the interface of the Jα helix−LOV core is important and introduction of a single charge triggers Jα displacement and unfolding.

Structural Analysis of I539E. To obtain more insight into the pseudo-lit-state conformation generated by these mutations, we conducted further analyses on the I539E replacement. One way to quantitate the structural changes caused by this mutation is to use a minimum chemical shift analysis of 3D HNCO spectra recorded on the wild-type and I539E mutant proteins in their dark-state conformations. By this approach, peak locations from these spectra were compared to obtain the minimum chemical shift changes for each wild-type peak to its nearest neighbor in the I539E data set. Although this method provides only a conservative estimate of these changes (*18*), this analysis clearly revealed that the I539E point mutation introduces structural perturbations localized to the Jα helix and β sheet of the LOV core (Figure 3A). Therefore, the overall conformation of the AsLOV2 structure is maintained despite mutation, and the observed structural differences result from disassociation of Jα from the LOV β-sheet.

As an independent way to evaluate dark- and lit-state structures of the I539E point mutant, we utilized limited proteolysis. By use of chymotrypsin, digestion of the point mutant was monitored under dark- and lit-state conditions (Figure 3B). In both cases, proteolysis resulted in a truncated fragment produced by cleavage after M530, a residue located in the middle of the Jα helix. In contrast to the wild-type protein, where proteolysis takes place slowly in the dark and is significantly accelerated in the light (*13*), the point mutant was cleaved similarly under both conditions. This indicates that the I539E point mutant has certain lit-state structural properties in the absence of illumination, provided solely by disrupting the Jα helix−LOV core interaction.

Kinase Activity of Full-Length Phototropin 1 Containing Jα Point Mutations. Our data indicated that AsLOV2 containing point mutations, I532E, A536E, or I539E, has key structural features of the lit state of the wild-type protein, most notably with the Jα helix dissociated from the LOV core. If this dissociation is essential to the photoinduced signaling process, we reasoned that proteins containing

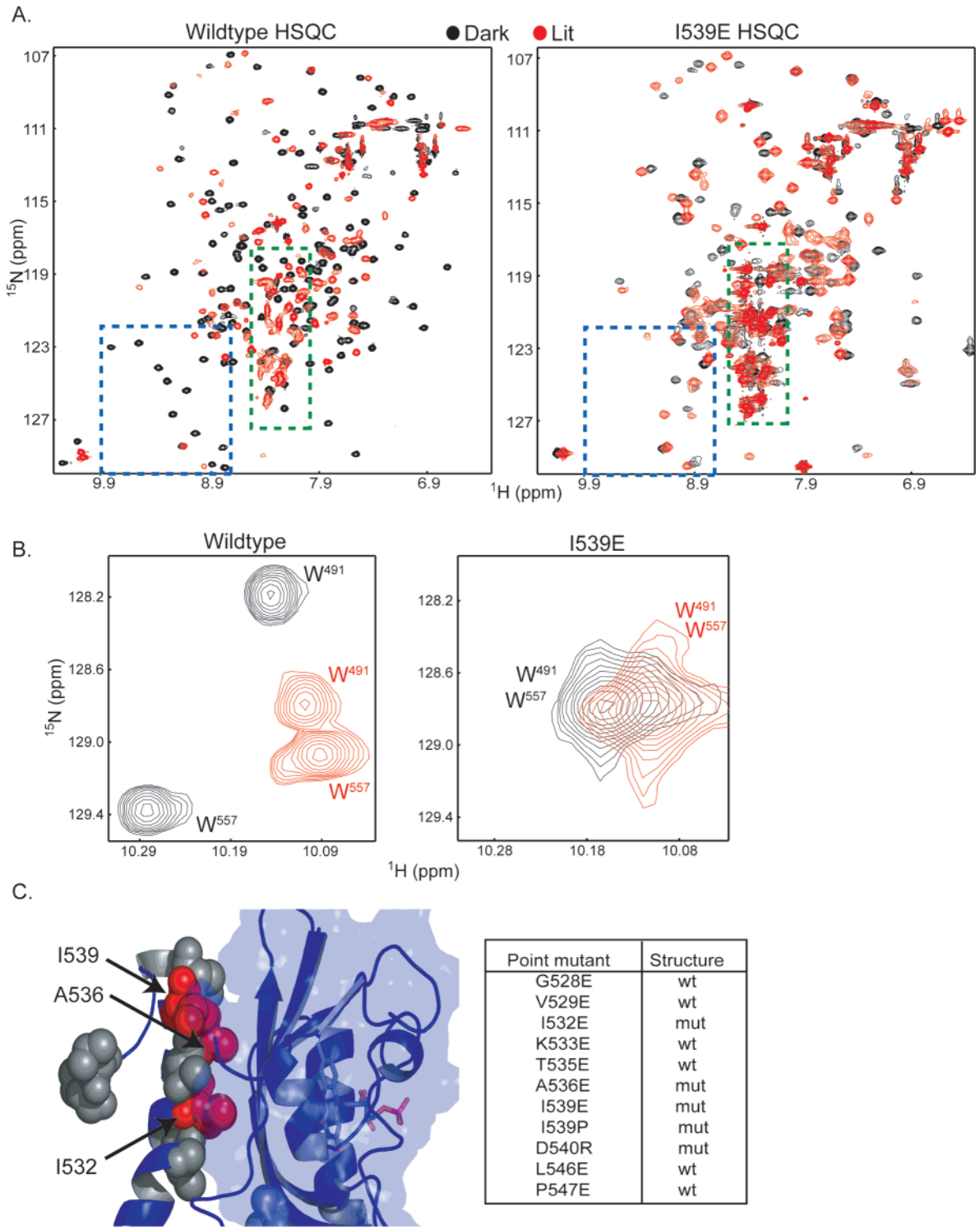

FIGURE 2: Structural effects of point mutations in AsLOV2 Jα helix. (A) ^{15}N−^{1}H HSQC spectra of wild-type and mutant proteins taken under dark (black peaks) and lit state (red peaks) conditions. Highlighted in the blue box is a region particularly susceptible to peak broadening in the wild-type lit-state and both states of the I539E mutant. In the green box is the unfolded region of the spectrum. (B) Expansion of the Trp indole region of ^{15}N−^{1}H HSQC spectra acquired on wild type and the I539E mutant of AsLOV2. (C) Locations and summary of the structural effects of Jα point mutations, mapped onto the AsLOV2 structure in the orientation shown in the top of Figure 1C. AsLOV2 carrying point mutations at residues colored in gray showed minimal spectral changes from wild type, while those in red exhibited significant changes, suggesting a conversion to a pseudo-lit-state structure as described in the text.

mutations that constitutively dissociate this helix should have stimulated autophosphorylation in the absence of any light stimulus. To test this, the autophosphorylation activity of the full-length form of phototropin 1 was assayed both with and without these point mutations in the LOV2 domain. For these studies, we used the *A. thaliana* phototropin 1 protein expressed in insect cells by use of recombinant baculovirus. In addition to wild-type phot1, we generated recombinant forms of phot1 that contained Jα point mutations analogous to those produced for the AsLOV2 (oat I532 = *Arabidopsis* V601, oat A536 = *Arabidopsis* A605, and oat I539 = *Arabidopsis* I608). The wild-type and mutant phot1 proteins

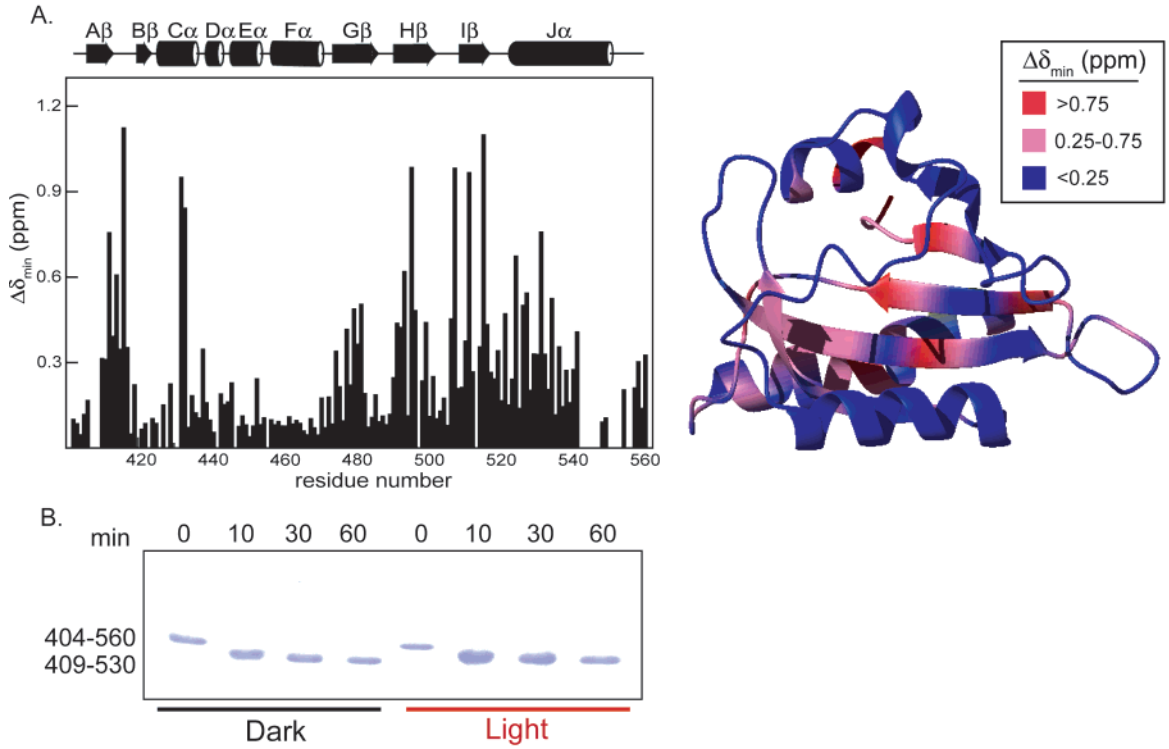

FIGURE 3: Structural analyses of I539E point mutant of AsLOV2. (A) Minimum chemical shift difference from 3D HNCO spectra obtained in the dark for both the wild type and I539E point mutant of AsLOV2. Changes in peak locations between these two spectra were mapped onto the AsLOV2 wild-type structure, with a color gradient encoding the magnitude of the shift change as shown in the legend. (B) Limited proteolysis of I539E in dark and lit states with chymotrypsin. Proteolysis reactions were stopped after 10, 30, and 60 min. Mass spectrometric analysis indicated that chymotrypsin cleaved the full-length domain (residues 404−560) to a protease-resistant fragment (residues 409−530).

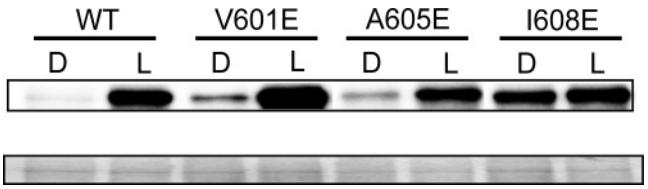

FIGURE 4: Functional effects of V601E, A605E, and I608E mutations on *A. thaliana* phototropin 1 autophosphorylation activity. Top row, lysates of Sf9 insect cells expressing full-length wild-type *A. thaliana* phot1 or mutated forms of phot1 harboring Jα mutations (V601E, A605E, and I608E), assayed for autophosphorylation activity under dark (D) or light (L) conditions as described under Materials and Methods. Bottom row, a Coomassie-stained SDS−PAGE gel of the samples used for autophosphorylation testing to confirm equal loading of phototropin 1 proteins in each lane.

were expressed and assayed for kinase activity in both the presence and absence of light (Figure 4). As found previously, phot1 becomes heavily phosphorylated following a brief irradiation with high-intensity white light. Similarly, each of the Jα point mutations displayed autophosphorylation activity in response to illumination. However, in contrast to wild-type phot1, recombinant forms of phot1 carrying each of the Jα point mutations generated showed autophosphorylation activity in the dark, particularly the I608E variant. These data indicate that single point mutations, located outside the LOV core, are sufficient to decouple kinase activation from light sensing by LOV2. While each of the Jα point mutations generated appear to result in varying degrees of kinase activation in the dark, these findings nevertheless demonstrate that unfolding of the Jα helix from the rest of AsLOV2 is sufficient to induce phototropin activity in the absence of light.

DISCUSSION

While the connection between light-induced covalent adduct formation and protein conformational changes had been previously demonstrated in several phototropin LOV domains (*11*−*13*, *20*), the details of how this event upregulates kinase activity were unknown. It was previously shown that the overall conformational change within the LOV2 domain resulting from light activation is crucial for the biological activity of the phototropins, as elimination of its activity or the domain itself abolishes phot1 autophosphorylation and phototropin-activated responses, including phototropic curvature and chloroplast movement (*6*, *13*, *21*). In contrast, photoactivation of LOV1 alone is not sufficient to induce phototropism or chloroplast avoidance movement, demonstrating that the function of LOV1 is distinct from that of LOV2. Indeed, collective alignment of LOV1 or LOV2 domains from all phototropins identified to date reveals that protein sequences that can form the Jα helix are only found associated with LOV2 and not LOV1. Thus, the structural consequences accompanying LOV1 photoexcitation are likely to differ from those following photoactivation of LOV2, consistent with the apparent distinct functionality observed for these two LOV domains. Although the functional role of LOV1 is currently unclear, recent findings indicate that LOV1 may be involved in photoreceptor dimerization (*22*).

To investigate the role of unfolding the Jα helix from the LOV2 core independently of any light stimulus, we created point mutations that disrupted the Jα helix in the absence of light. The combination of biophysical and biochemical data presented here demonstrate that such a disruption between

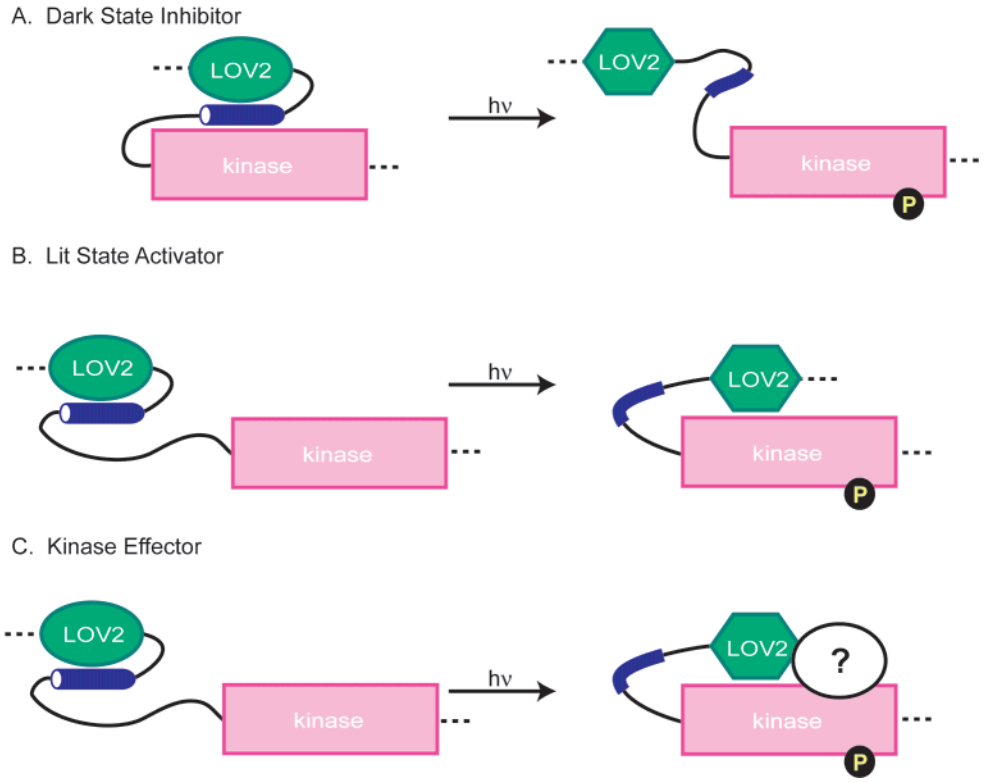

FIGURE 5: Models for LOV2 kinase regulation. (A) LOV2 domain acting as a dark-state inhibitor for kinase phosphorylation. In this model, the Jα helix interacts with the kinase domain to lock it into an inactive conformation, and upon illumination, Jα becomes unfolded and releases the kinase domain. (B) Alternatively, the LOV2 domain could act as a lit-state activator. Here, the kinase normally resides in an inactive conformation. Upon illumination, the β-sheet surface is exposed and can bind the kinase domain to promote it into an active conformation. (C) The LOV2 domain could also act as an effector, where upon binding the kinase domain, no conformational change occurs, but other essential proteins are recruited to activate catalytic activity.

the interaction of the Jα helix and the rest of AsLOV2 leads to activation of the kinase activity in the absence of light. We therefore conclude that either the unfolding Jα helix or the newly revealed β-sheet surface of the LOV2 core is important for coupling light sensing to kinase activation. Our initial biochemical analysis on the effect of Jα point mutations on phot1 kinase activity in vitro shows that both V601E and A605E substitutions are less effective than I608E at inducing phot1 kinase activity in the absence of light (Figure 4). Further structural analysis of V601E and A605E mutations will determine whether the ability of these mutations to trigger displacement and unfolding of the Jα helix is somehow compromised relative to that found for I608E, which may account for the differential effects observed for these mutations on phot1 kinase activity.

With these results demonstrating the importance of dissociating the Jα helix from the LOV2 core in upregulating phototropin kinase activity, two key mechanistic questions remain open. The first of these concerns the manner by which the Jα helix is dissociated in response to light stimuli, a process that we have artificially circumvented here with our use of point mutations at the Jα helix−LOV core interface. We have previously demonstrated that the light-driven formation of the FMN−protein adduct in AsLOV2 leads to structural rearrangements and an accompanying destabilization of the LOV core (*13*). As shown by deuterium exchange measurements, these changes occur without disrupting the native LOV core secondary structure, suggesting that a tertiary structure change propagates the initiating event of adduct formation to dissociate the Jα helix. A critical residue in this relay has recently been suggested by Fourier transform infrared studies of a Q1029L point mutant of the *A. capillus-veneris* phy3 LOV2 domain (*23*). This mutation changes a highly conserved glutamine that participates in hydrogen-bonding interactions with the FMN C4(O) and N5 positions (*7*, *9*, *10*). Elimination of these interactions significantly reduces the amplitude of light-induced protein conformational changes observed in this domain, indicating that the link between FMN and protein conformational changes has been disrupted. Notably, the AsLOV2 equivalent of Q1029 (AsLOV2 Q513) is located on the Iβ strand, on the opposite face from where residue I532 from the Jα helix docks onto the β-sheet face of the LOV core. Given the ability of the I532E (and adjacent A536E and I539E) point mutations to disrupt the Jα helix−LOV2 core interaction and activate the kinase, these data further implicate this Q513 as being a possible link between the conformational changes in the LOV core and Jα helix dissociation.

A second class of mechanistic questions concern the role that the disrupted Jα−LOV core plays in activating kinase catalytic activity. One model for kinase activation would be for the LOV complex to act as a dark-state inhibitor (Figure 5A). This model is reminiscent of that for type 1 transforming growth factor β receptor (TβR-1) kinase (*24*). TβR-1 contains an N-terminal segment, called the GS region, that binds to and distorts the N-terminal lobe of its kinase domain so that integrity of the site that binds ATP and magnesium is disrupted. This inhibitory interaction is maintained by binding of FKBP12 to the GS region. Phosphorylation of the GS region relieves these interactions, preventing associa-

tion of GS with the N-terminal lobe and allowing the kinase to adopt an active conformation. While neither the LOV core nor the Jα helix is phosphorylated in phototropin (*25*), light activation could provide the stimulus to disrupt interactions in the LOV complex, preventing association with the catalytic domain and relieving the autoinhibitory interactions.

Alternatively, the LOV complex could act as a lit-state activator (Figure 5B), with the light-induced unfolding of Jα providing a crucial element of kinase activation in a manner similar to protein kinase B (PKB) (*26*). Activation of PKB occurs through phosphorylation of a C-terminal hydrophobic motif (HM) that allows its association with the N-terminal lobe of the kinase domain. This interaction orders the Cα helix within the lobe, which in turn reorganizes the kinase activation loop to allow proper substrate positioning. This disorder-to-order transition induced by phosphorylated HM binding could be mimicked in phototropins by light-induced release of Jα from the LOV core, allowing it to interact with the kinase domain and rearranging segments to force an active conformation.

Finally, as LOV domains are known to facilitate protein−protein interactions, the phototropin LOV domain could act as an effector that recruits other proteins required for kinase activation (Figure 5C) (*27*). A similar mechanism is seen in the cyclin-dependent kinase 2 (CDK2) protein in complex with the Cdc28 kinase subunit (Cks1) (*28*). Cks1, which is structurally similar to a LOV domain, utilizes its β-sheet interface to bind the C-terminal lobe of CDK2's kinase domain. As this interaction has no allosteric consequences on the kinase domain, it is believed that Cks1 functions to recruit other proteins to promote CDK2 catalytic activation. For phototropin, light-induced disassociation of Jα exposes the β-sheet face of the LOV domain, which could then, in a manner analogous to Cks1, bind the kinase domain and recruit other protein elements required for kinase activity. Indirect evidence in support of this model is provided by the protein−protein interactions that involve the β-sheet surface of other PAS domains, such as HIF-2α PAS B and *Ec*Dos (*29*, *30*). Given that recombinant phototropins demonstrate light-dependent activation when heterologously expressed in insect cells, it is clear that auxiliary proteins would not be plant-specific in origin.

Phototropins offer the unique opportunity to convert light to trigger kinase activation and in turn initiate signaling, allowing plants to directly respond to changing environmental conditions. The results presented here indicate that the Jα helix, located C-terminal of LOV2, is important for kinase activation. Additional functional analyses of the Jα point mutations described here should provide further insights into the biochemical mechanisms underlying phototropin activation. While further experiments are needed to distinguish among the precise activation mechanism within phototropins and other PAS-regulated kinases, it is interesting to note that other PAS-containing kinases are also directly sensitive to other external stimuli. For example, the bacterial histidine kinase FixL utilizes its PAS domain to bind heme, allowing it to use oxygen as its signaling trigger (*31*). Thus, PAS domains enable kinases to use environmental stimuli for activation, in contrast to the more common mechanism of phosphorylation utilized by other proteins.

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REFERENCES

1. Briggs, W. R., and Huala, E. (1999) Blue-light photoreceptors in higher plants, *Annu. Rev. Cell Dev. Biol. 15*, 33−62.
2. Christie, J. M., Reymond, P., Powell, G. K., Bernasconi, P., Raibekas, A. A., Liscum, E., and Briggs, W. R. (1998) Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism, *Science 282*, 1698−1701.
3. Taylor, B. L., and Zhulin, I. B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light, *Microbiol. Mol. Biol. Rev. 63*, 479−506.
4. 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 photoreceptor, phototropin, *Biochemistry 39*, 9401−9410.
5. Swartz, T. E., Corchnoy, S. B., Christie, J. M., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2001) The photocycle of a flavin-binding domain of the blue light photoreceptor phototropin, *J. Biol. Chem. 276*, 36493−36500.
6. Christie, J. M., Swartz, T. E., Bogomolni, R. A., and Briggs, W. R. (2002) Phototropin LOV domains exhibit distinct roles in regulating photoreceptor function, *Plant J. 32*, 205−219.
7. Crosson, S., and Moffat, K. (2001) Structure of a flavin-binding plant photoreceptor domain: insights into light-mediated signal transduction, *Proc. Natl. Acad. Sci. U.S.A. 98*, 2995−3000.
8. Fedorov, R., Schlichting, I., Hartmann, E., Domratcheva, T., Fuhrmann, M., and Hegemann, P. (2003) Crystal structures and molecular mechanism of a light-induced signaling switch: the Phot-LOV1 domain from *Chlamydomonas reinhardtii*, *Biophys. J. 84*, 2474−2482.
9. Crosson, S., and Moffat, K. (2002) Photoexcited structure of a plant photoreceptor domain reveals a light-driven molecular switch, *Plant Cell 14*, 1067−1075.
10. Crosson, S., Rajagopal, S., and Moffat, K. (2003) The LOV domain family: photoresponsive signaling modules coupled to diverse output domains, *Biochemistry 42*, 2−10.
11. Iwata, T., Nozaki, D., Tokutomi, S., Kagawa, T., Wada, M., and Kandori, H. (2003) Light-induced structural changes in the LOV2 domain of *Adiantum* phytochrome3 studied by low-temperature FTIR and UV−visible spectroscopy, *Biochemistry 42*, 8183−91.
12. Corchnoy, S. B., Swartz, T. E., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2003) Intramolecular proton transfers and structural changes during the photocycle of the LOV2 domain of Phototropin 1, *J. Biol. Chem. 278*, 724−731.
13. Harper, S. M., Neil, L. C., and Gardner, K. H. (2003) Structural basis of a phototropin light switch, *Science 301*, 1541−1544.
14. Harper, S. M., Neil, L. C., Day, I. J., Hore, P. J., and Gardner, K. H. (2004) Conformational changes in a photosensory LOV domain monitored by time-resolved NMR spectroscopy, *J. Am. Chem. Soc. 126*, 3390−3391.
15. Bashford, D. (1997) in *Scientific computing in object-oriented parallel environments* (Ishikawa, Y., Oldehoeft, R. R., Reynders, J. V. W., and Tholburn, M., Eds.) pp 233−240, Springer, Berlin.
16. Lindahl, E., Hess, B., and van der Spoel, D. (2001) GROMACS 3.0: A package for molecular simulation and trajectory analysis, *J. Mol. Mod. 7*, 306−317.
17. DeLano, W. L. (2002) The PyMOL Molecular Graphics System.
18. Farmer, B. T., Constantine, K. L., Goldfarb, V., Friedrichs, M. S., Wittekind, M. G., Yanchunas, J. J., Robertson, J. G., and Mueller, L. (1996) Localizing the $NADP^+$ binding site on the MurB enzyme by NMR, *Nat. Struct. Biol. 3*, 995−997.
19. Clackson, T., and Wells, J. A. (1995) A hot spot of binding energy in a hormone-receptor interface, *Science 267*, 383−6.
20. 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.
21. Kagawa, T., Kasahara, M., Abe, T., Yoshida, S., and Wada, M. (2004) Function analysis of phototropin2 using fern mutants deficient in blue light-induced chloroplast avoidance movement, *Plant Cell Physiol. 45*, 416−426.

22. Salomon, M., Lempert, U., and Rudiger, W. (2004) Dimerization of the plant photoreceptor phototropin is probably mediated by the LOV1 domain, *FEBS Lett. 572*, 8−10.
23. Nozaki, D., Iwata, T., Ishikawa, T., Todo, T., Tokutomi, S., and Kandori, H. (2004) Role of Gln1029 in the photoactivation processes of the LOV2 domain in *Adiantum* phytochrome3, *Biochemistry 43*, 8373−8379.
24. Huse, M., Chen, Y. G., Massague, J., and Kuriyan, J. (1999) Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12, *Cell 96*, 425−36.
25. Salomon, M., Knieb, E., Zeppelin, T., and Rudiger, W. (2003) Mapping of low- and high-fluence autophosphorylation sites in phototropin 1, *Biochemistry 42*, 4217−4225.
26. Yang, J., Cron, P., Thompson, V., Good, V. M., Hess, D., Hemmings, B. A., and Barford, D. (2002) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation, *Mol. Cell 9*, 1227−40.
27. Ballario, P., Talora, C., Galli, D., Linden, H., and Macino, G. (1998) Roles in dimerization and blue light photoresponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins, *Mol. Microbiol. 29*, 719−729.
28. Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I., and Tainer, J. A. (1996) Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1, *Cell 84*, 863−74.
29. Erbel, P. J., Card, P. B., Karakuzu, O., Bruick, R. K., and Gardner, K. H. (2003) Structural basis for PAS domain heterodimerization in the basic helix−loop−helix-PAS transcription factor hypoxia-inducible factor, *Proc. Natl. Acad. Sci. U.S.A. 100*, 15504−9.
30. Kurokawa, H., Lee, D. S., Watanabe, M., Sagami, I., Mikami, B., Raman, C. S., and Shimizu, T. (2004) A redox-controlled molecular switch revealed by the crystal structure of a bacterial heme PAS sensor, *J. Biol. Chem. 279*, 20186−93.
31. Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998) Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction, *Proc. Natl. Acad. Sci. U.S.A. 95*, 15177−82.
32. Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering, *Nucleic Acids Res. 16*, 10881−10890.

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