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Article

Blue Light Induces Global and Localized Conformational Changes in the Kinase Domain of Full-Length Phototropin[†]

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ABSTRACT: The blue-light photoreceptor phototropin plays a crucial role in optimizing photosynthesis in plants. In the two light-, oxygen-, or voltage-sensitive (LOV) domains of phototropin, the light stimulus is absorbed by the flavin chromophores. The signal is assumed to be transferred via dissociation and unfolding of a conserved Jα helix element to the serine/threonine kinase domain. We investigated full-length phototropin from the green alga *Chlamydomonas reinhardtii* by Fourier transform infrared spectroscopy to shed light on the signal transfer within the protein and on the structural response of the kinase. Light-induced structural changes were assigned by comparing signals of the full-length protein with those of the truncated LOV1-LOV2-Jα and LOV1-LOV2 and with those of deletion mutants. A loss of helicity originating from the Jα linker helix was observed in LOV1-LOV2-Jα in agreement with previous studies of LOV2-Jα. Full-length phototropin showed reversible global conformational changes via several turn elements. These changes were suppressed in a deletion mutant lacking the Jα linker and are attributed to the kinase domain. The loss of turn structure is interpreted as a light-induced opening of the kinase tertiary structure upon release of the LOV2 domain. Concomitant protonation changes of Asp or Glu residues in the kinase domain were not observed. A light-induced loss in helicity was observed only in the presence of a phototropin-characteristic 54-amino acid extension of the kinase activation loop, which is predicted to be located apart from the catalytic cleft. This response of the extension might play a significant role in the phototropin signaling process.

Blue light has a strong effect on plant development and on photomovement responses for optimization of photosynthesis. The blue-light photoreceptor phototropin (phot)[1] regulates these responses in plants such as phototropism (*1*), chloroplast relocation (*2*−*4*), stomatal opening (*5*), rapid inhibition of hypocotyl elongation (*6*), and promotion of cotyledon expansion (*7*). In the unicellular green alga *Chlamydomonas reinhardtii*, a single phototropin homologue was proposed to regulate sexual differentiation (*8*) and to modulate chemotactic activity (*9*). Expression of the *Chlamydomonas* phototropin homologue in a phot-deficient *Arabidopsis* mutant successfully restored functionality despite their different functions in vivo (*10*).

Phototropin is a blue-light-dependent kinase classified into subfamily VIIIb of the large group of AGC kinases (protein kinases A, G, and C) (*11*, *12*). The serine/threonine kinase domain of phototropin is regulated by two light-, oxygen-, or voltage-sensitive (LOV) domains at the N-terminus (Figure 1A). Each LOV domain carries a noncovalently bound flavin mononucleotide (FMN) as a chromophore. The kinase domain is highly homologous to the mouse cyclic adenosine monophosphate-dependent protein kinase A (PKA). A major difference between the light-activated phototropin kinase and PKA is an extension of 30−50 amino acids inserted into the 14-amino acid activation loop (A-loop) of the catalytic subdomain (*11*, *13*) (Figure 1B). An extension of this size is absent in the majority of AGC kinases (*14*) and has so far been found only in subfamily VIIIa (*12*), and in NDR (nuclear Dbf2-related) kinases of subfamily VII (*15*). There, the extension has been shown to play important roles either as a localization sequence or in carrying out an autoinhibitory function (*12*, *15*).

Blue light induces formation of adducts between the sulfur atom of conserved cysteines of the LOV1 and LOV2 domains and the flavin C_{4a} atoms (*16*−*18*) (Figure 1C). These adducts are stable on the order of seconds to minutes and finally revert to the dark form. Adduct formation in LOV2 induces detachment of a conserved Jα helix in the linker region between the LOV2 domain and the kinase domain, which has been shown by nuclear magnetic resonance (NMR) spectroscopy on a 40-amino acid extended LOV2 domain construct (*19*) (Figure 1D). It has been proposed that this detachment is followed by unfolding of the helix due to observation of light-induced changes in the diffusion constant (*20*). This proposal has gained support in the recent results from Fourier transform infrared (FT-IR) spectroscopy (*21*, *22*), with the reservation that unfolding was not detectable in LOV2-Jα from the phytochrome−phototropin chimera neochrome 1 (*23*). Blue light finally activates the C-terminal kinase domain, which autophosphorylates phototropin (*24*). Inactivation of the LOV2 domain by point mutation

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[1]Abbreviations: A-loop, activation loop; FMN, flavin mononucleotide; FT-IR, Fourier transform infrared; LOV, light-, oxygen-, or voltage-sensitive; NMR, nuclear magnetic resonance; phot, phototropin; PKA, protein kinase A; AGC kinases, protein kinases A, G, and C.

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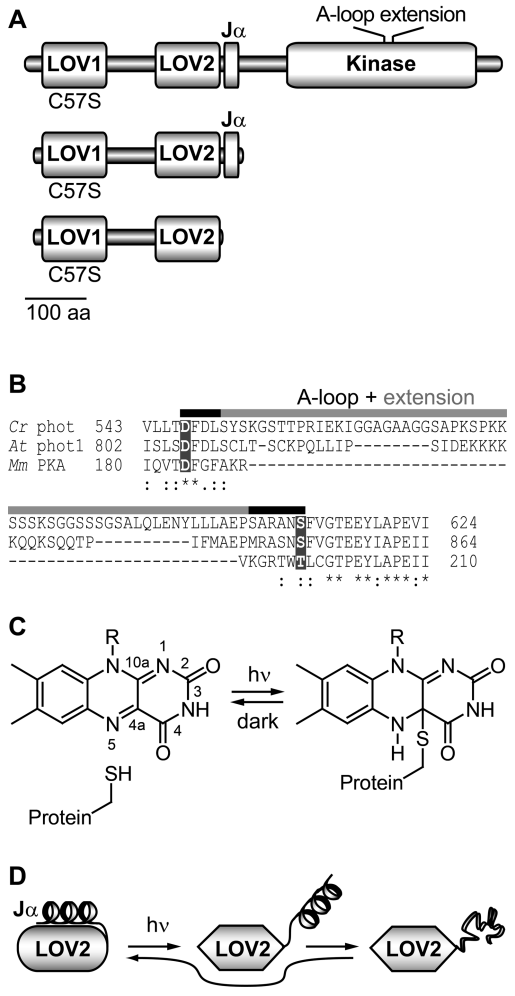

FIGURE 1: (A) Domain organization of phototropin. Schematic representation of the constructs investigated in this study from *Chlamydomonas* phot. Furthermore, deletion mutants were constructed that lack the Jα helix and the A-loop extension. The LOV1 domains were inactivated by mutation of the reactive cysteine 57 to serine to prevent overcrowding of the spectra. (B) Sequence alignment of the activation loop in the catalytic subdomain of kinases from *Chlamydomonas* phot, *Arabidopsis* phot1, and *Mus musculus* PKA. Phototropins contain an extension of the loop of ~30−54 residues as compared to PKA and many other homologous kinases. Highlighted residues show the conservation of the Mg^{2+}-binding aspartate and the serine/threonine phosphorylation site. (C) Chemical structure of the flavin chromophore in the regulatory LOV domains in the dark and after light-induced formation of the adduct. The adduct is characterized by a covalent bond between flavin C_{4a} and the cysteine thiol. (D) Current model of light-induced conformational changes of the LOV2 domain and the adjacent Jα helix. Dissociation of the helix is followed by partial unfolding and results in activation of the kinase.

abolished autophosphorylation in vitro and phototropic responses in transgenic *Arabidopsis*, whereas inactivation of LOV1 led to a wild-type phenotype (*25*). Autophosphorylation became light-independent in vitro in variants with a modified Jα helix that show permanent helix displacement (*26*). This prominent role of the Jα helix for kinase activation was challenged by the in vitro observation that the isolated LOV2 domain without Jα was able to act as light-dependent inhibitor of the kinase (*27*). In the absence of the inhibiting LOV2 domain, the kinase domain triggers constitutive physiological responses, when fused to green fluorescent protein (*28*).

Several signaling components and downstream events of phototropin signaling have been identified in higher plants (as reviewed in ref *13*) and in *Chlamydomonas* (*29*), but a natural phosphorylation substrate has not been found. On the other hand, it has been shown that autophosphorylation of a conserved serine residue at the end of the A-loop is a prerequisite for signal mediation of *Arabidopsis* phototropin (*30*). In *Chlamydomonas*, a light-induced, proteolytic degradation of phototropin has been observed after exposure to blue light (Y. Lu and P. Hegemann, unpublished results).

Kinase activation of phototropin has been studied in vitro by autophosphorylation assays, which, however, did not provide any information about the signal transfer to the kinase after activation of LOV2-Jα. Biophysical studies of phototropin were restricted to shorter fragments such as the LOV domains, the LOV2-Jα construct, and the LOV1-LOV2 tandem protein without the Jα linker. Purification of full-length phototropin from heterologous expression systems was unsuccessful.

In this study, we obtained full-length *Chlamydomonas* phototropin from *Escherichia coli* in sufficient yield and purity to apply FT-IR spectroscopy. The aim of this first biophysical investigation of the full-length protein was to resolve secondary structural responses to light. FT-IR spectroscopy is an established technique for studying secondary structure of proteins via the coupled C=O stretching modes of the backbone. Different elements can be distinguished by this amide I band with β-sheets absorbing at 1620−1640 cm^{-1} with a weak contribution at ~1680 cm^{-1}, helices and loops at 1640−1660 cm^{-1}, and turn elements at 1660−1690 cm^{-1} (*31*, *32*). The method has already been applied to LOV domains for the assignment of light-induced structural changes in static (*33*−*35*), time-resolved (*36*), and temperature-dependent (*37*) approaches. Furthermore, an FT-IR study on the LOV-containing, bacterial protein YtvA (*38*) revealed light-induced conformational changes in the STAS (sulfate transporter anti-sigma-factor antagonists) domain by comparison of spectra of the full-length protein with those of its single LOV domain (*39*).

Here, we analyzed the transfer of the signal from the LOV2 domain to the kinase domain in phototropin by comparing light-induced responses from full-length protein with those from the shorter LOV1-LOV2-Jα and LOV1-LOV2 constructs (Figure 1A). Additionally, we employed deletion mutants lacking the Jα helix and the A-loop extension. The comparison reveals changes in secondary structure upon light activation of LOV2, which are assigned to a global response of the kinase domain and a specific change in its A-loop extension.

MATERIALS AND METHODS

Expression Constructs. cDNA of full-length phototropin from *C. reinhardtii* (accession number CAC94941) was amplified by PCR and inserted in frame with an N-terminal Strep-Tag-II into pASK-IBA-7plus using *Eco*RI and *Hin*dIII restriction sites. Additionally, a six-His tag was fused in frame to the C-terminus by insertion of a double-stranded oligonucleotide using *Pfl*23I and *Hin*dIII sites. Cysteine at position 57 was replaced with serine through site-directed mutagenesis (Quikchange, Stratagene) with oligonucleotides previously described (*40*). In the resulting pASK-IBA-7plus-*phot*_C57S_HTc plasmid, bases encoding amino acids 330−352 were replaced with those for four glycine residues by overlapping PCR to yield pASK-IBA-7plus-*phot*_C57SΔJα_HTc. Deletion of the A-loop was accomplished by subcloning from pKS-*phot*-Δext using *Eco*72I and *Pfl*23I sites to yield pASK-IBA-7plus-*phot*_C57SΔext_HTc. In the resulting

gene product, four glycine residues replace residues 551−605. For expression of the tandem LOV1-C57S-LOV2 (amino acids 16−329) and LOV1-C57S-LOV2-Jα (amino acids 16−363) constructs, the corresponding DNA sequence was amplified by PCR from pASK-IBA-7plus-*phot*_C57S_HTc and inserted in frame with a His tag into Hisp2x using *Eco*RI and *Hin*dIII sites as previously described (*41*).

Protein Purification. Tandem constructs were expressed and purified as previously described (*41*). Full-length constructs were purified from BL21(DE3) cells after overnight expression at 18 °C in LB containing 0.2 μg/mL anhydrotetracycline for induction. Cells were collected and resuspended in 50 mM Tris (pH 8.0), 200 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM PMSF. After disruption using a microfluidizer (Avestin EF-C3) at 1300 bar, insoluble material was removed by centrifugation at 16000*g* (Avanti, Beckmann). The cytosolic fraction prepared by ultracentrifugation at 150000*g* was applied to a Ni-NTA column (Qiagen, Hilden, Germany) and washed extensively with buffer containing 25 mM imidazole. The full-length protein was eluted from the column with 500 mM imidazole and dialyzed twice against 1000 volumes of 50 mM Tris (pH 8.0), 200 mM NaCl, 10 mM $MgCl_2$, and 10% glycerol. The identity of the full-length phototropin was confirmed by mass spectrometry (data not shown).

Sample Preparation. Proteins were transferred into 10 mM Tris buffer (pH 7.5) containing 200 mM NaCl and 5% glycerol by repeated ultrafiltration using a Vivaspin 500 filter device with a 10 kDa cutoff (Sartorius). The final concentration of the sample was adjusted to 800 μM with respect to chromophore content. The concentration was determined by the absorbance at 445 nm using an extinction coefficient of 12500 L mol^{-1} cm^{-1} for free FMN (*42*). For FT-IR spectroscopy, 2 μL of the protein solution was applied on a BaF_2 window. All experiments were performed in solution without any drying except for those on full-length phot, where a suitable concentration could not be achieved by ultrafiltration. Instead, the water content was slowly reduced at a pressure of 300 mbar. This procedure is gentler than the established procedure of drying and rehydration, which might interfere with the functionality of the protein. The sample was sealed by a second window.

FT-IR Spectroscopy. FT-IR spectra were recorded on a Bruker IFS 66v spectrometer with a resolution of 2 cm^{-1}. A long-wave pass filter with a cuton of 5.35 μm (OCLI) was placed in front of the detector to block stray light and to improve the signal-to-noise ratio. The filter restricted the spectral range to 1900−900 cm^{-1}; 1024 or 2048 scans before and after illumination of the sample for 2 s were recorded for the difference spectra. Blue light was provided by a light-emitting diode (Luxeon Star, Lumileds) with an emission maximum at 455 nm and a power of 20 mW/cm^2. The temperature of the sample was adjusted to 20 °C by a circulating water bath. The experiment was repeated after 60 min, when the protein had completely returned to the dark state. Several illuminations of independent preparations were performed and representative spectra averaged to a total number of 4096 scans for LOV1-C57S-LOV2 and LOV1-C57S-LOV2−Jα constructs, 26624 scans for phot-C57S, 10240 scans for phot-C57S-ΔJα, and 16384 scans for phot-C57S-Δext. The sample-to-sample variation is exemplarily shown for LOV1-C57S-LOV2-Jα and phot-C57S in the Supporting Information (Figures S2 and S3). The spectra were scaled to the isolated C_4=O difference band of LOV2 at 1717 (−)/1730 (+) cm^{-1}. In this spectral region, the apoprotein does not contribute to the signal of LOV2.

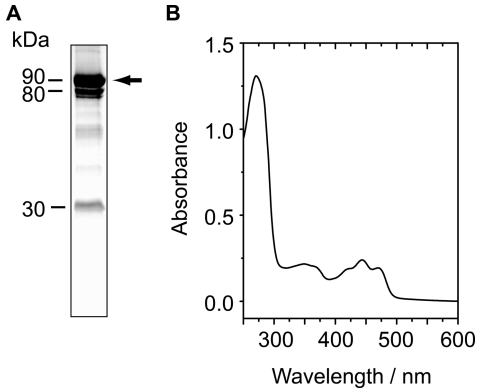

FIGURE 2: (A) SDS−PAGE of full-length phototropin carrying a C-terminal His tag after purification by affinity chromatography. The major band marked with an arrow corresponds to the apoprotein with a theoretical mass of 85.7 kDa. (B) UV−vis spectrum of the holoprotein showing the typical absorption bands of protein-bound flavin.

RESULTS

Full-length phototropin from *C. reinhardtii* was obtained from *E. coli* as a yellow-colored, soluble protein. SDS−PAGE after purification showed a band at ∼90 kDa with a purity of > 70% (Figure 2A). The molecular mass corresponds to the theoretical mass of the apoprotein of 85.7 kDa. Contributions by impurities are visible at ∼80 and ∼30 kDa. Impurities with high molecular weights are likely to consist of N-terminally degraded phototropin molecules that still contain the C-terminal His tag. The 30 kDa impurity was identified as the cAMP receptor protein of *E. coli*, which was copurified using this procedure. The UV−vis spectrum of the protein exhibits the typical fine-structured absorption of protein-bound FMN with absorption maxima at 444, 368, and 349 nm (Figure 2B). The purified protein is not phosphorylated according to phosphoprotein staining and mass spectrometry (C. Gasser and T. Mathes, unpublished results).

Light-induced FT-IR difference spectroscopy was applied to the algal phototropin. To assign signals of the full-length protein to different structural subunits, the LOV1-LOV2 domain tandem (amino acids 16−329) and the tandem additionally containing the conserved Jα helix (LOV1-LOV2-Jα, amino acids 16−363) were investigated (Figure 1A). In all three constructs, the LOV1 domain was inactivated by replacement of the reactive cysteine 57 with a serine (*40*) to prevent crowding of the spectra by simultaneous contributions of both LOV domains. It has been shown that activation of the kinase is dominated by the LOV2 domain (*25*, *27*).

Great care was taken to maintain a high water content in all experiments (Figure S1 of the Supporting Information), which is paramount for resolving structural changes in phototropin (data not shown). The water content by far exceeded that of previous studies where a strong effect of hydration has been shown by using glycerol/water mixtures (*43*). Any artificial effects by dehydration can therefore be ruled out.

LOV1-LOV2 Tandem Protein with and without the Jα Helix. The light-induced FT-IR difference spectrum of LOV1-LOV2-Jα shows a typical pattern of vibrational changes caused by adduct formation (Figure 3a). Negative bands originate from the dark form of LOV2, and positive bands characterize the light state. Only bands of those vibrations are visible that are affected by the reaction. Most of the vibrational bands in the mid-infrared spectral range originate from changes in structure of

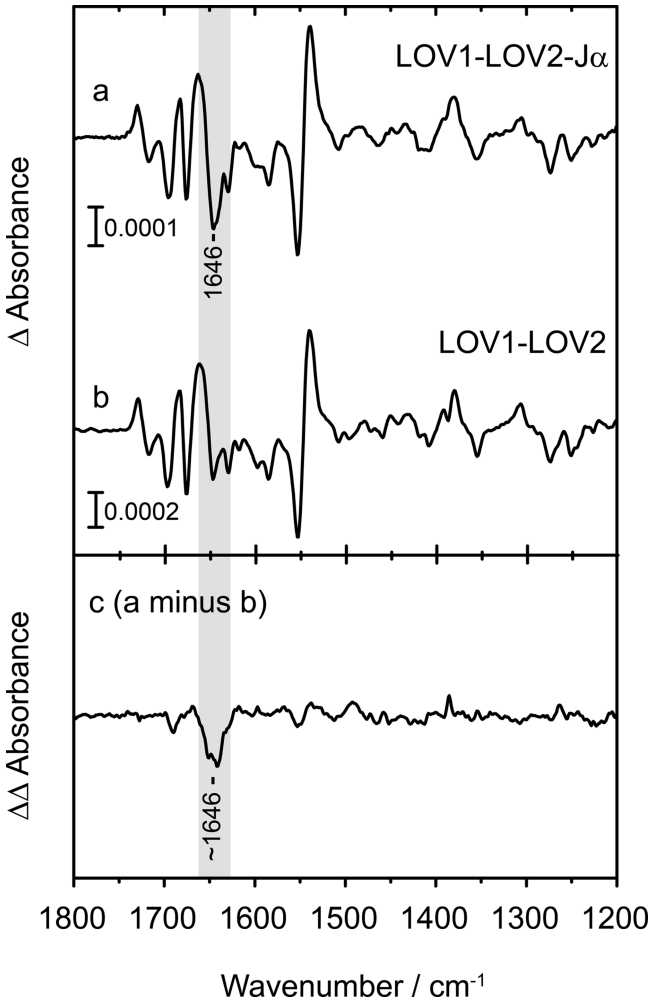

FIGURE 3: (Top) Comparison of the light-minus-dark FT-IR difference spectra of LOV1-LOV2-Jα (a) and LOV1-LOV2 (b). The contribution of the Jα helix is evident at 1646 cm^{-1}. The position and negative sign of the band point to a light-induced loss of helicity. (Bottom) The calculated double difference spectrum (c) supports the assignment of the band to an isolated structural change of the helix, because other signals are not evident within the experimental accuracy.

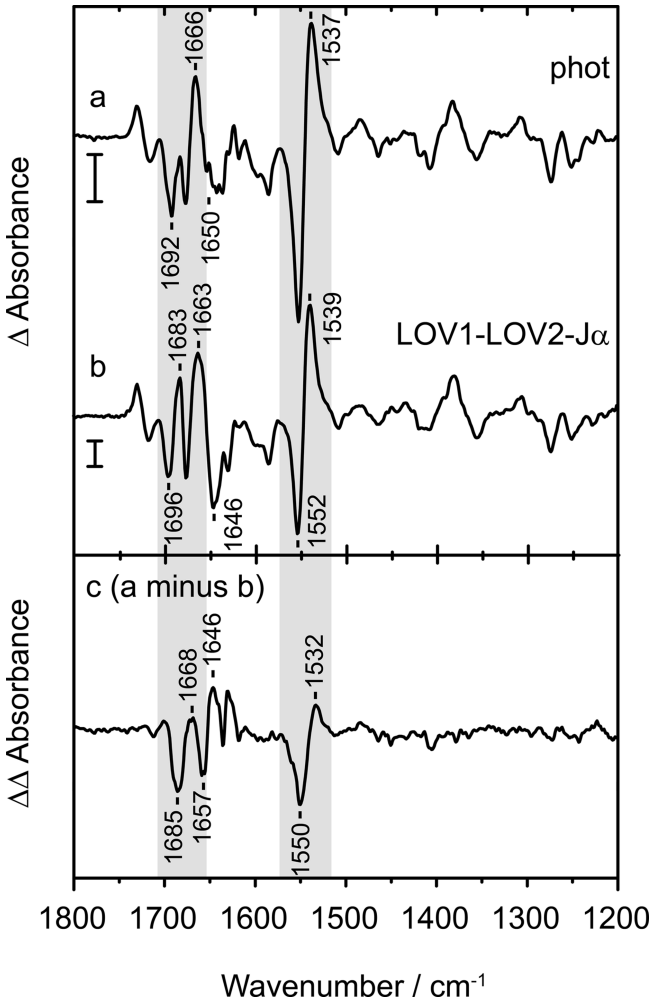

FIGURE 4: (Top) Light-minus-dark FT-IR difference spectra of full-length phototropin (a) and LOV1-LOV2-Jα (b). The scale bar represents a difference absorbance of 0.0001. (Bottom) The double difference spectrum (c) illustrates the differences between the two spectra in the frequency and amplitude of the bands. Prominent signals located in the amide I and amide II regions point to secondary structural changes attributed to the kinase domain. The negative band at 1685 cm^{-1} is assigned to loss of turn structural elements. The gray bar highlights those signals that are above the limit of sample-to-sample variation in the experiment.

the FMN chromophore by adduct formation (*33*, *34*). Further contributions are due to changes in the secondary structure of the LOV2 domain (*35*). The latter are spectrally confined to the amide I region between 1615 and 1695 cm^{-1} and the amide II region between 1520 and 1570 cm^{-1}.

The difference spectrum of LOV1-LOV2-Jα closely resembles that of the LOV1-LOV2 tandem protein (Figure 3b) except for the pronounced negative band at 1646 cm^{-1}. Since all other vibrational contributions of LOV1-LOV2 remain unperturbed, this band is assigned to a change in the vibrations of the ~20-amino acid Jα helix upon illumination. The extent of change is illustrated by calculation of a double difference of the spectrum with Jα minus that without the Jα helix (Figure 3c). The double difference spectrum shows a broad and prominent band in the amide I region at ~1646 cm^{-1}, which indicates a secondary structural change as a response of the Jα helix to light. This band was detected only in experiments with protein in solution, as employed in this study. The loss of the Jα contribution upon concentration of the protein to a film (data not shown) might be interpreted as an aggregation effect that restricts the changes in the secondary structure of the Jα helix.

Full-Length Phototropin. Vibrational bands in the difference spectrum of full-length phot are again dominated by changes in the chromophore structure of the LOV2 domain (Figure 4a). The photoreaction of the chromophore is not influenced by the presence of the kinase domain, as is evidenced by the identical band pattern in the range of 1200−1500 and 1700−1800 cm^{-1} as compared to LOV1-LOV2-Jα (Figure 4b). The comparison reveals, however, alterations in band positions and intensities in the amide I and amide II regions by the presence of the kinase domain. It should be noted that changes in band position cannot be explained by variations in sample preparation but are a clear indication of additional vibrational contributions in the full-length protein (Figure S3 of the Supporting Information). The most prominent change is a strong increase in intensity of the difference band at 1552 (−)/1539 (+) cm^{-1} with a concomitant shift to 1537 cm^{-1}. This band has been assigned to comprise contributions of secondary structural changes (*35*) together with the $C_{10a}=N_1$ vibration of flavin (*33*, *34*). Further pronounced differences are evident in the amide I region.

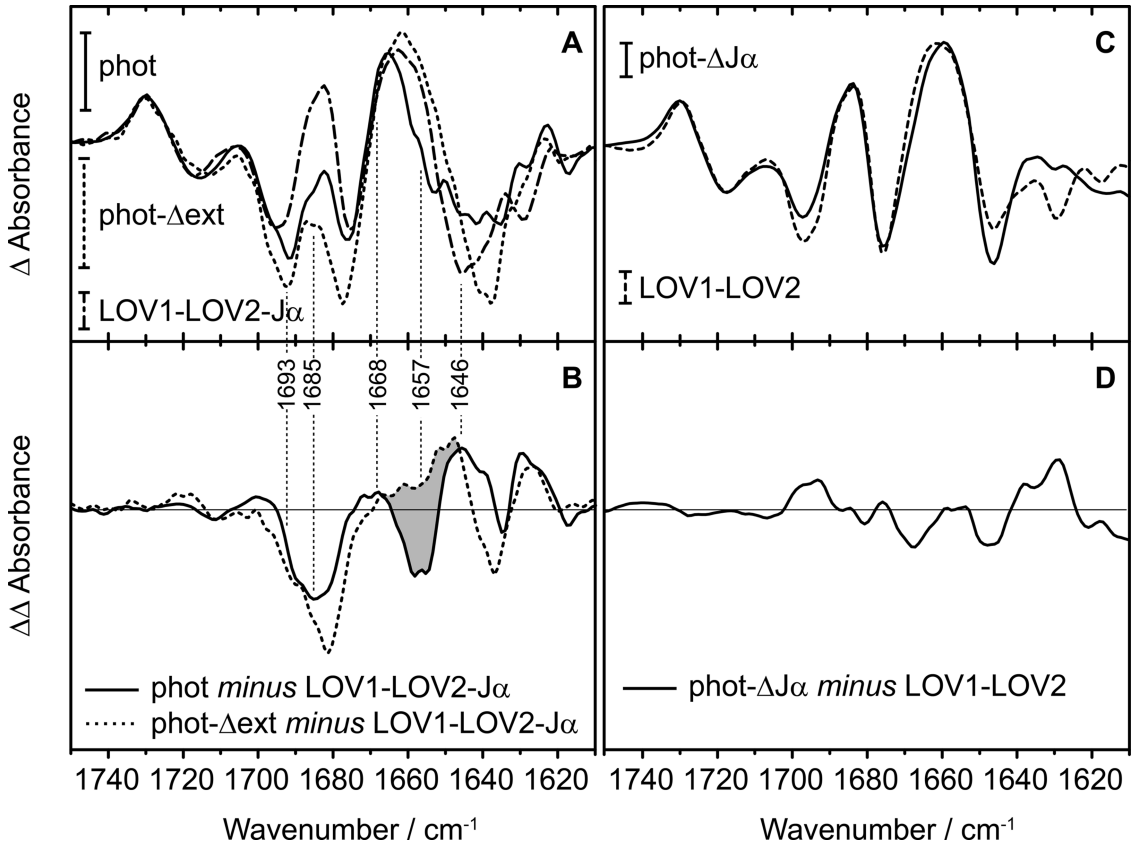

FIGURE 5: Detail of the amide I region of the light-minus-dark FT-IR spectra. Scale bars represent a difference absorbance of 0.0001. (A) Difference spectra of full-length phototropin (—), a deletion mutant without the A-loop extension (phot-Δext) (···), and LOV1-LOV2-Jα (– – –). (B) Corresponding double difference spectra of full-length phototropin minus LOV1-LOV2-Jα (—) and phot-Δext minus LOV1-LOV2-Jα (···) illustrate secondary structural changes attributed to the kinase domain. In the deletion mutant without the extension, the negative band at 1657 cm^{-1} is absent. This finding is interpreted as light-induced structural changes in the A-loop extension of full-length phototropin. (C) Difference spectrum of phot-ΔJα, a full-length phototropin without the Jα linker helix, in comparison to that of LOV1-LOV2. The spectrum of phot-ΔJα resembles more that of LOV1-LOV2 than that of full-length phototropin. (D) The double difference spectrum shows only minor changes in the amide I region despite an increase in size of the protein by a factor of 2. Prominent signals that were assigned to the response of the kinase domain of full-length phot are absent.

The positive band at 1683 cm^{-1} of LOV1-LOV2-Jα is missing in the spectrum of full-length phot. This band has been assigned to the C_2=O vibration of flavin in the adduct (*35*), a contribution that cannot be lost. Instead, it is (over)compensated by a strong negative contribution. At 1692 cm^{-1}, a negative band is detected in the presence of the kinase, whereas the band of LOV1-LOV2-Jα at 1696 cm^{-1} remains in the form of a shoulder. The positive band of LOV1-LOV2-Jα at 1663 cm^{-1} is shifted to 1666 cm^{-1}. The band assigned to the Jα helix at 1646 cm^{-1} in the spectrum of LOV1-LOV2-Jα is less pronounced in the difference spectrum of full-length phot. However, infrared light at ~1650 cm^{-1} is strongly absorbed by water vibrations (Figure S1 of the Supporting Information). This absorbance results in an increase in the noise of the spectrum and of the sample-to-sample variation (Figure S3 of the Supporting Information). Because of the extremely small difference signal of the full-length protein, the presence or absence of a Jα contribution to the spectrum cannot be decided on the basis of these data. All differences are illustrated by a double difference spectrum (Figure 4c). Prominent bands are detected at 1685 and 1657 cm^{-1} in the amide I region and at 1550/1532 cm^{-1} in the amide II region, which are attributed to a response of the kinase domain to illumination of the LOV2 domain. This response provides evidence that the signal relay was not impaired by the overexpression of full-length phot in a heterologous system. Outside of the spectral range of amide vibrations, signals are absent. The lack of signals above 1712 cm^{-1} implies that the response of the kinase to illumination does not involve changes in protonation state or hydrogen bonding of glutamic or aspartic acids (*31*).

Full-Length Phototropin without the A-Loop Extension. To further localize the light-induced structural changes occurring in the kinase domain, the 54-amino acid extension in the A-loop that is characteristic for phototropins (amino acids 551−605) was replaced with four glycines (Figure 1B). The overall difference spectrum of this extension deletion mutant (phot-Δext) is similar to that of full-length phot (data not shown). The difference bands in the amide I and amide II regions are even more pronounced than those of full-length phot as the protein exhibited a weaker tendency to aggregate during preparation. A more detailed analysis of the amide I region reveals similarities and differences in the band pattern of phot-Δext to full-length phot and LOV-LOV2-Jα (Figure 5A). The positive band at 1683 cm^{-1} is absent from and the negative band at 1693 cm^{-1} present in the spectra of both full-length phot and phot-Δext. The latter band therefore serves as a marker band for kinase response. In the spectral region at around 1657 cm^{-1}, a similar trend would be expected with a strongly reduced positive band intensity for phot-Δext as compared to LOV-LOV2-Jα. On the contrary, the band intensity is found to be similar to that of the construct without kinase,

taking some experimental variability into account. For illustration, the corresponding double difference spectra were calculated (Figure 5B). The double difference bands of full-length phot minus LOV1-LOV2-Jα represent the pattern of response induced by the kinase. When this pattern is compared to the double difference spectrum of phot-Δext minus LOV1-LOV2-Jα, it is evident that the negative double difference band at 1657 cm^{-1} is completely absent in the latter. From theoretical considerations, this double difference pattern might be caused either by a structural change in the light state of LOV1-LOV2-Jα that is suppressed in full-length phot but not in phot-Δext or by a loss of secondary structure in the extension that is accordingly only observed in full-length phot. As the latter case is considered much more likely, the double difference band at 1657 cm^{-1} is tentatively assigned to conformational changes in the A-loop extension. The light-induced movements are not confined to the highly conserved parts of the kinase domain but propagate to the extension with its unknown function.

Full-Length Phototropin without the Jα Helix. A negative control was performed to support these assignments. Transfer of the signal to the kinase was stopped by inactivation of the Jα helix by mutation. A suitable candidate would be the I608E point mutation in this helix, which has been shown to induce permanent kinase activity in *Arabidopsis* phot1 (*26*). However, this mutation could not be introduced into *Chlamydomonas* phototropin due to limited conservation of the linker sequence. Furthermore, autophosphorylation of *Chlamydomonas* phototropin in vitro is weaker than that of *Arabidopsis* phot1 (*10*), which makes a screening of the effect of mutations difficult. Therefore, a Jα deletion mutant was constructed in which the 23 amino acids of the (conserved) helix were replaced with four glycines. The FT-IR difference spectrum of phot-ΔJα resembles much more those of LOV1-LOV2 and LOV1-LOV2-Jα than that of the active full-length phot (Figure 5C). Prominent spectral changes detected in the presence of the linker in full-length phot are absent in the deletion mutant. In comparison to that of LOV1-LOV2, the spectrum shows minor changes in the amide I and amide II region. The overall double difference between the two protein spectra is small considering that the constructs differ in size by a factor of more than 2 (Figure 5D). The Jα-deficient phototropin shows a much weaker structural response to blue light. These findings confirm the prominent role of the Jα helix in signal transfer. They further support the conclusion that conformational changes detected in the spectrum of full-length phototropin are induced by the presence of the kinase domain.

DISCUSSION

Previous spectroscopic studies on the photoactivation of phototropin were limited to LOV domains or LOV1-LOV2 tandem constructs. Further progress was impeded by the low solubility of the full-length blue-light receptor from higher plants. We have overcome this limitation by heterologous expression and purification of the 86 kDa phototropin homologue from the unicellular alga *Chlamydomonas*. FT-IR spectroscopy was used in a systematic study of the responses of the Jα linker and the kinase domain.

Light-Induced Unfolding of the Jα Linker Helix. The position of the negative difference band assigned to the Jα linker at 1646 cm^{-1} points to a long, rigid helical structure in the dark (Figure 3a) (*31*). This helical fold in LOV1-LOV2-Jα is lost upon illumination, which corroborates previous results on the shorter LOV2-Jα construct from transient grating spectroscopy (*20*) and FT-IR spectroscopy (*21*, *22*). The absence of a corresponding positive band might be explained by a strong broadening of the signal by formation of fluctuating random coil elements. In full-length phot and the deletion mutants, the lower signal-to-noise ratio in the water region around 1650 cm^{-1} impairs a final evaluation of the Jα contribution. The question of whether light-induced Jα unfolding takes place in the presence of the kinase will have to be resolved by other approaches.

Light-Induced Conformational Changes in the Kinase Domain. The assignment of signals in the double difference spectrum of full-length phototropin minus LOV1-LOV2-Jα (Figure 4c) is not as straightforward as in the case of the Jα helix. First, the spectral range points either to alterations by secondary structural changes or to changes in amino acid side chains. The cooperative appearance of signals in both amide I and amide II regions argues in favor of secondary structural changes. Second, signals exclusively observed in the longer construct can originate either directly from the additional kinase domain or indirectly from strong changes in the structure of LOV1-LOV-Jα induced by binding to the kinase domain. We cannot exclude the latter case but will provide in the following a conclusive interpretation of the signals in the framework of the current understanding of kinase responses.

The negative band at 1685 cm^{-1} in the kinase difference spectrum (Figure 5B) can be assigned to turn structural elements due to its frequency (*31*, *32*). The negative sign of the band points to a loss of the hydrogen bonding within the turns. The extent of this change can be estimated by comparison to the signal generated by Jα helix unfolding (Figure 3). Both bands are comparable in amplitude and integral band intensity. Both bands have a similar origin, a coupled carbonyl vibration of the backbone. The molar extinction coefficient of helices is considered to be similar or even higher than that of turns (*44*, *45*). Since Jα unfolding comprises the loss of several helical bonds, it is correspondingly concluded that several turn structural elements are lost upon illumination of full-length phototropin.

Turn elements can be visualized like joints that hold structural elements together. An unfolding of several of these joints upon illumination might be interpreted as a reorganization of whole subdomains of the kinase. Structural changes in kinases have been demonstrated before in crystal structures of open and closed conformations of the homologous mouse PKA (*46*, *47*) and by subsequent NMR analysis (*48*). A similar transition from a closed to open state could be discussed for the light-regulated kinase of phototropin. However, the closed state of PKA is formed by binding of ATP and substrate, whereas in this study, phototropin kinase can bind only the regulatory LOV domain. Such a binding has been shown by NMR to take place for the LOV-homologous PAS domain to the human PAS kinase without providing insight into the kinase interaction site or response (*49*). For phototropin, it has been shown that LOV2 acts as an inhibitor of kinase action (*27*). From docking studies, the LOV2 domain has been proposed to inhibit the kinase by binding to the catalytic cleft between the C- and N-terminal lobes of the kinase (*11*). The inhibition can proceed either intra- or intermolecularly, because phototropin has been suggested to form a dimer (*50*, *51*) similar to the homologous PKA in the inactive state (*52*). It has been proposed that light initiates a dissociation of LOV2 from the kinase (*27*). The changes in turn structural elements identified in this study may reflect the light-induced opening of the kinase by release of the inhibitory LOV domain.

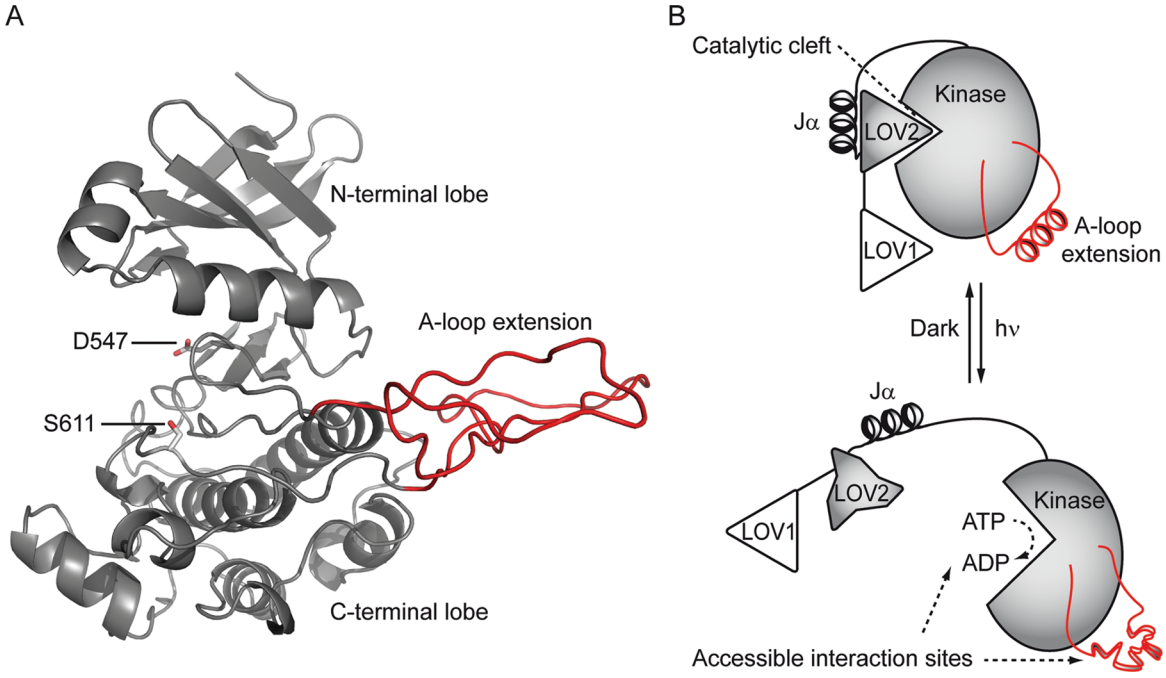

FIGURE 6: (A) Three-dimensional structural model of the kinase domain of *Chlamydomonas* phototropin. Templates for modeling were determined by homology detection using the HHpred server (http://toolkit.tuebingen.mpg.de/hhpred) (*56*). The model was created with MODELER (*57*) using 30 templates of crystal structures of homologous kinases. The A-loop comprises amino acids D547−S611. The A-loop extension is colored red. Secondary structure prediction identifies helical content in the extension. (B) Model of phototropin activation. In the dark state, the catalytic cleft of the kinase is covered by the LOV2 domain. After illumination with blue light, the secondary structure of the LOV2 domain changes, the Jα helix dissociates, and the interaction with the kinase domain is disrupted. The lobes of the kinase domain respond and move to form an open state. These responses gate (auto)phosphorylation. Some helical structure of the A-loop extension is lost by illumination. The light state of phototropin exposes interaction sites where a substrate or regulation partner might bind.

It has been shown in *Arabidopsis* that autophosphorylation of phototropin kinase in the A-loop is a key event in signaling (*30*). The FT-IR experiments on full-length phototropin from *Chlamydomonas* were performed on an unphosphorylated protein and in the absence of ATP. Therefore, ATP binding is not a prerequisite for light-induced structural changes. Furthermore, the secondary structural changes observed cannot be directly correlated with an autophosphorylation response. Autophosphorylation is weaker in *Chlamydomonas* phototropin than in *Arabidopsis*, and several target residues are not present in the more compact algal phototropin (*10*). On the other hand, *Chlamydomonas* phototropin is functional in *Arabidopsis* (*10*), so that the observed structural changes might directly reflect the gating of autophosphorylation or might modulate the affinity of the kinase with a signaling partner.

Response and Localization of the A-Loop Extension. The double difference band at 1657 cm^{-1} of full-length phot is tentatively assigned to contributions of the A-loop extension, because its contribution is missing in phot-Δext (Figure 5B). The position of this band points to a loss of helical structure. This loss could be differently attributed to a response taking place anywhere in the protein induced by the presence of the extension. In support of its localization to the extension, however, secondary structure prediction using the consensus of seven algorithms (*53*) shows a helical element in the A-loop extension besides random coil elements. More precisely, this helix is predicted to be located within ~20 extra residues that are specific for *Chlamydomonas* phot and not found in other phototropins. Upon activation of the kinase by light, this helix might be disrupted.

The A-loop is part of the catalytic cleft between N- and C-terminal lobes. Its extension, however, is predicted to be located apart from the catalytic cleft, as revealed by tertiary structure modeling to structures of 30 kinases with a level of sequence identity of ≥30% (Figure 6A). The fold of the extension itself could not be modeled accurately because of a lack of templates for this region. Therefore, the localization of the extension is predicted on the basis of the orientation of the A-loop without extension in the template structures. This orientation seems to be a general feature of protein kinases despite a conformational diversity of the A-loop (*54*). Furthermore, positions of starting and end points of the extension were maintained even in the case of a template kinase, where an extension of eight residues was present in the sequence (*55*).

The response of the extension is not a prerequisite for the other light-induced structural changes of the kinase because those are preserved in phot-Δext. The physiological role of the A-loop extension in phototropin is unknown, but recent observations point to a crucial role in blue light-induced proteolytic degradation in vivo (Y. Lu and P. Hegemann, unpublished results).

A Model of Phototropin Activation. In summary, our study provides evidence that full-length phototropin undergoes secondary structural changes upon illumination, which are attributed to the kinase domain in general, and especially to the A-loop extension (Figure 6B). The overall movements can be interpreted as disassembly of the structure of the inactive dark state. All changes are reversible, and the whole process can be initiated again by light several times, as the reproducibility of the spectral changes shows.

On the basis of our findings, we present the following model of phototropin activation, simplified by neglecting contributions of LOV1 (Figure 6B). Upon illumination of LOV2, the Jα helix dissociates as has been shown previously (*19*), which leads to disruption of the inhibitory interaction between kinase and LOV2. The kinase domain in response to LOV2 activation performs a segment movement, which is interpreted as an opening of

the structure. This opening might disrupt inhibition of the kinase and result in an increase in (auto)phosphorylation activity, and/or it might modulate the affinity for a signaling partner. Concomitantly, the phototropin kinase undergoes conformational changes attributed to the A-loop extension. Blue light-induced conformational changes apart from those in the catalytic site of the kinase may serve as a signal mediator for physiological responses. The A-loop extension might represent a second interaction site for outward signaling (protein−protein interaction) or internal allosteric regulation (phosphorylation activity). Out of geometrical considerations (Figure 6A), targets of this structural change might be a substrate or inactive phototropin in a possible dimer. Because of the limited conservation of the A-loop extension, this structural response is not available in most homologous AGC kinases and might therefore be an important and specific component in mediation of the physiological signal in phototropin.

The approach taken in this study has been shown to be capable of differentiating in vitro between a potential functional and a nonfunctional phototropin. A comparable assay has only been available up to now via exploitation of the weak autophosphorylation response. Previous in vitro studies were restricted to mutants in the Mg^{2+} and ATP binding sites of the kinase. Our approach can now be applied to extend the scope to point mutations and deletions in the whole kinase domain. This screening will help in finally resolving the intramolecular signal transduction of phototropin.

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SUPPORTING INFORMATION AVAILABLE

Infrared absorbance spectra showing the hydration level of protein preparations and sample-to-sample variation in the difference spectra of LOV1-LOV2-Jα and full-length phototropin. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Liscum, E., and Briggs, W. R. (1995) Mutations in the NPH1 locus of *Arabidopsis* disrupt the perception of phototropic stimuli. *Plant Cell 7*, 473–485.
2. Sakai, T., Kagawa, T., Kasahara, M., Swartz, T. E., Christie, J. M., Briggs, W. R., Wada, M., and Okada, K. (2001) *Arabidopsis* nph1 and npl1: Blue light receptors that mediate both phototropism and chloroplast relocation. *Proc. Natl. Acad. Sci. U.S.A. 98*, 6969–6974.
3. Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K., and Wada, M. (2001) *Arabidopsis* NPL1: A phototropin homolog controlling the chloroplast high-light avoidance response. *Science 291*, 2138–2141.
4. Jarillo, J. A., Gabrys, H., Capel, J., Alonso, J. M., Ecker, J. R., and Cashmore, A. R. (2001) Phototropin-related NPL1 controls chloroplast relocation induced by blue light. *Nature 410*, 952–954.
5. Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K. (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature 414*, 656–660.
6. Folta, K. M., and Spalding, E. P. (2001) Unexpected roles for cryptochrome 2 and phototropin revealed by high-resolution analysis of blue light-mediated hypocotyl growth inhibition. *Plant J. 26*, 471–478.
7. Ohgishi, M., Saji, K., Okada, K., and Sakai, T. (2004) Functional analysis of each blue light receptor, cry1, cry2, phot1, and phot2, by using combinatorial multiple mutants in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A. 101*, 2223–2228.
8. Huang, K., and Beck, C. F. (2003) Phototropin is the blue-light receptor that controls multiple steps in the sexual life cycle of the green alga *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A. 100*, 6269–6274.
9. Ermilova, E. V., Zalutskaya, Z. M., Huang, K. Y., and Beck, C. F. (2004) Phototropin plays a crucial role in controlling changes in chemotaxis during the initial phase of the sexual life cycle in *Chlamydomonas*. *Planta 219*, 420–427.
10. Onodera, A., Kong, S. G., Doi, M., Shimazaki, K., Christie, J., Mochizuki, N., and Nagatani, A. (2005) Phototropin from *Chlamydomonas reinhardtii* is functional in *Arabidopsis thaliana*. *Plant Cell Physiol. 46*, 367–374.
11. Tokutomi, S., Matsuoka, D., and Zikihara, K. (2008) Molecular structure and regulation of phototropin kinase by blue light. *Biochim. Biophys. Acta 1784*, 133–142.
12. Zegzouti, H., Li, W., Lorenz, T. C., Xie, M., Payne, C. T., Smith, K., Glenny, S., Payne, G. S., and Christensen, S. K. (2006) Structural and functional insights into the regulation of *Arabidopsis* AGC VIIIa kinases. *J. Biol. Chem. 281*, 35520–35530.
13. Christie, J. M. (2007) Phototropin blue-light receptors. *Annu. Rev. Plant Biol. 58*, 21–45.
14. Hanks, S. K., and Hunter, T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J. 9*, 576–596.
15. Bichsel, S. J., Tamaskovic, R., Stegert, M. R., and Hemmings, B. A. (2004) Mechanism of activation of NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. *J. Biol. Chem. 279*, 35228–35235.
16. 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.
17. 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.
18. 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.
19. Harper, S. M., Neil, L. C., and Gardner, K. H. (2003) Structural basis of a phototropin light switch. *Science 301*, 1541–1544.
20. 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.
21. 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.
22. 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* phototropin1. *Biophys. J. 96*, 2771–2778.
23. 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.
24. 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.
25. 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.
26. 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.
27. Matsuoka, D., and Tokutomi, S. (2005) Blue light-regulated molecular switch of Ser/Thr kinase in phototropin. *Proc. Natl. Acad. Sci. U.S.A. 102*, 13337–13342.
28. Kong, S. G., Kinoshita, T., Shimazaki, K., Mochizuki, N., Suzuki, T., and Nagatani, A. (2007) The C-terminal kinase fragment of *Arabidopsis* phototropin 2 triggers constitutive phototropin responses. *Plant J. 51*, 862–873.
29. Im, C. S., Eberhard, S., Huang, K., Beck, C. F., and Grossman, A. R. (2006) Phototropin involvement in the expression of genes encoding chlorophyll and carotenoid biosynthesis enzymes and LHC apoproteins in *Chlamydomonas reinhardtii*. *Plant J. 48*, 1–16.

30. Inoue, S., Kinoshita, T., Matsumoto, M., Nakayama, K. I., Doi, M., and Shimazaki, K. (2008) Blue light-induced autophosphorylation of phototropin is a primary step for signaling. *Proc. Natl. Acad. Sci. U.S.A. 105*, 5626–5631.
31. Barth, A., and Zscherp, C. (2002) What vibrations tell us about proteins. *Q. Rev. Biophys. 35*, 369–430.
32. Krimm, S., and Bandekar, J. (1986) Vibrational spectroscopy and conformations of peptides, polypeptides, and proteins. *Adv. Protein Chem. 38*, 181–364.
33. 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.
34. 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.
35. 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.
36. 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.
37. 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*, 8183–8191.
38. 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.
39. 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.
40. Kottke, T., Heberle, J., Hehn, D., Dick, B., and Hegemann, P. (2003) Phot-LOV1: Photocycle of a blue-light receptor domain from the green alga *Chlamydomonas reinhardtii*. *Biophys. J. 84*, 1192–1201.
41. Guo, H., Kottke, T., Hegemann, P., and Dick, B. (2005) The phot LOV2 domain and its interaction with LOV1. *Biophys. J. 89*, 402–412.
42. Macheroux, P. (1999) UV-visible spectroscopy as a tool to study flavoproteins. *Methods Mol. Biol. 131*, 1–7.
43. 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.
44. Kalnin, N. N., Baikalov, I. A., and Venyaminov, S. Y. (1990) Quantitative IR spectrophotometry of peptide compounds in water (H_2O) solutions. III. Estimation of the protein secondary structure. *Biopolymers 30*, 1273–1280.
45. de Jongh, H. H., Goormaghtigh, E., and Ruysschaert, J. M. (1996) The different molar absorptivities of the secondary structure types in the amide I region: An attenuated total reflection infrared study on globular proteins. *Anal. Biochem. 242*, 95–103.
46. Akamine, P., Madhusudan, Wu, J., Xuong, N. H., Ten Eyck, L. F., and Taylor, S. S. (2003) Dynamic features of cAMP-dependent protein kinase revealed by apoenzyme crystal structure. *J. Mol. Biol. 327*, 159–171.
47. Zheng, J., Trafny, E. A., Knighton, D. R., Xuong, N. H., Taylor, S. S., Ten Eyck, L. F., and Sowadski, J. M. (1993) 2.2 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MnATP and a peptide inhibitor. *Acta Crystallogr. D49*, 362–365.
48. Masterson, L. R., Mascioni, A., Traaseth, N. J., Taylor, S. S., and Veglia, G. (2008) Allosteric cooperativity in protein kinase A. *Proc. Natl. Acad. Sci. U.S.A. 105*, 506–511.
49. Amezcua, C. A., Harper, S. M., Rutter, J., and Gardner, K. H. (2002) Structure and interactions of PAS kinase N-terminal PAS domain: Model for intramolecular kinase regulation. *Structure 10*, 1349–1361.
50. Nakasako, M., Iwata, T., Matsuoka, D., and Tokutomi, S. (2004) Light-induced structural changes of LOV domain-containing polypeptides from *Arabidopsis* phototropin 1 and 2 studied by small-angle X-ray scattering. *Biochemistry 43*, 14881–14890.
51. Salomon, M., Lempert, U., and Rüdiger, W. (2004) Dimerization of the plant photoreceptor phototropin is probably mediated by the LOV1 domain. *FEBS Lett. 572*, 8–10.
52. Johnson, D. A., Akamine, P., Radzio-Andzelm, E., Madhusudan, M., and Taylor, S. S. (2001) Dynamics of cAMP-dependent protein kinase. *Chem. Rev. 101*, 2243–2270.
53. Subramaniam, S. (1998) The Biology Workbench: A seamless database and analysis environment for the biologist. *Proteins 32*, 1–2.
54. Nolen, B., Taylor, S., and Ghosh, G. (2004) Regulation of protein kinases: Controlling activity through activation segment conformation. *Mol. Cell 15*, 661–675.
55. Jauch, R., Jakel, S., Netter, C., Schreiter, K., Aicher, B., Jackle, H., and Wahl, M. C. (2005) Crystal structures of the Mnk2 kinase domain reveal an inhibitory conformation and a zinc binding site. *Structure 13*, 1559–1568.
56. Söding, J., Biegert, A., and Lupas, A. N. (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res. 33*, W244–W248.
57. Sali, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol. 234*, 779–815.