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Estimation of Available Free Energy in a LOV2-J α Photoswitch

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Abstract

Protein photosensors provide versatile tools to study ligand-regulated allostery and signaling. Fundamental to these processes is the amount of energy that can be provided by a photosensor to control downstream signaling events. Such regulation is exemplified by the phototropins, plant serine/threonine kinases that are activated by blue light via conserved LOV (Light, Oxygen and Voltage) domains. The core photosensor of oat phototropin 1 is a LOV domain that interacts in light-dependent fashion with an adjacent α -helix (J α) to control kinase activity. We used solution NMR measurements to quantify the free energy of the LOV domain:J α helix binding equilibrium in the dark and lit states. These data indicate that light shifts this equilibrium by ~ 3.8 kcal mol⁻¹, quantifying the energy available through LOV-J α for light-driven allosteric regulation. This study provides insight into the energetics of light sensing by phototropins and benchmark values for engineering photoswitchable systems based on the LOV-J α interaction.

Keywords

phototropin; free energy; conformational dynamics; NMR; relaxation dispersion

Information transfer in biological signaling pathways often takes the form of stimulus-induced changes in protein structure that consequently alter a functional output. Many signaling proteins are composed of linked arrays of modular domains, which cooperatively function to control activity^{1,2}. In such proteins, sensor domains receive inputs related to changes in the local environment, *e.g.* the binding of a metabolite or other protein, or the alteration of a bound cofactor by a change in redox state. These events change the interactions of the sensors with other domains, which subsequently transmit the signal downstream through changes in their activities. A critical parameter in the construction of such sensors is the magnitude of input-induced changes in the energetics of the sensor domain and its interactions with downstream effectors. It is this change in energy, and the corresponding change in interaction equilibria, that determines the dynamic range of the sensor along with several other fundamental signaling properties.

Several examples of this principle are provided by photosensory proteins, which have evolved to sense and respond to light across the UV/visible spectrum at a wide range of intensities³. These sensory processes are typically achieved through protein domains that bind light-absorbing chromophores and convert photon energy into structural, dynamic and functional changes. One class of these proteins are phototropins, a group of blue light-activated serine/

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threonine kinases that control a range of biological responses in algae and plants, including phototropism, chloroplast migration and stomatal opening⁴. Phototropins sense light through two LOV (Light-Oxygen-Voltage) domains, LOV1 and LOV2, which are located on the N-terminal side of the conserved kinase domain (Fig. 1a)⁵. LOV domains are a subgroup of the larger PAS (Per-ARNT-Sim) domain family, whose members function as sensors of a wide variety of environmental processes throughout biology^{6,7}. To sensitize phototropin to blue light, both LOV1 and LOV2 contain non-covalently bound FMN chromophores (Fig. 1a)⁸. Blue light absorption induces formation of a covalent adduct between a conserved cysteine residue in the LOV domain and the C4a carbon of the isoalloxazine ring of FMN⁹. While the functional significance of light absorption by LOV1 is still unclear, adduct formation in LOV2 is crucial for light-dependent enhancement of phototropin kinase activity as assessed *in vitro* by autophosphorylation and *in planta* by phototropism and other responses¹⁰. Crystal structures and high-resolution solution NMR data of several LOV domains^{11–16} show they all adopt typical PAS domain folds, consisting of a mixed α/β fold of approximately 110 amino acid residues surrounding the flavin chromophore. Solution NMR studies¹³ and a subsequent crystal structure¹⁵ of a fragment of the *Avena sativa* phototropin 1 (AsPhot1) containing the LOV2 domain plus a 40 residue C terminal extension have revealed an additional 20 residue α -helix, J α , that packs onto the PAS core in the dark state. As demonstrated by NMR and other biophysical data, light induces conformational changes in the LOV2 domain that lead to J α unfolding and dissociation from the LOV domain after the Cys-FMN adduct is formed^{13,17}. Complementary biochemical studies have shown that destabilization of the LOV2-J α interaction by mutagenesis activates full-length *Arabidopsis thaliana* phototropin 1 independent of light¹⁸. Together, these data argue that dissociation of J α – or more precisely, a shift in the LOV2-J α binding equilibrium to favor the helix-dissociated state – upon photon absorption is the principal mechanism by which phototropin is controlled by light (Fig. 1b).

Despite substantial progress in understanding the structural mechanisms of phototropin signaling, the energetics of this core LOV2-J α switch have not yet been explored. Here we use NMR spectroscopy to quantify the equilibria between the helix-bound and helix-dissociated conformations of LOV2-J α in the dark and lit states, yielding an estimate of the energy that is potentially available in this photoswitch to achieve this change in functional states. Using NMR ¹⁵N and ¹³C relaxation dispersion analyses, we found that the J α helix and its binding surface on LOV2 undergo substantial fluctuations on micro- to millisecond timescales in the dark. Fitting these dispersion data to a model for two-site exchange, we determined that the populations of the low- and high-energy conformations are 98.4% and 1.6%, respectively, with a corresponding free energy difference of 2.4 kcal mol⁻¹ across this equilibrium. By comparing the fitted chemical shifts of the high energy state to those directly measured in an isolated J α peptide, we suggest that J α is unfolded and dissociated in the high energy conformation despite the lack of a Cys-FMN adduct. Systematic differences between the chemical shifts of the J α peptide and those of a photoactivated sample suggest that the LOV2-J α system samples an analogous bound-dissociated equilibrium for J α after blue-light absorption. A quantitative analysis of these differences indicates that the population of the helix-dissociated conformation in the lit state is ~91%, corresponding to approximately -1.4 kcal mol⁻¹ of free energy across the equilibrium. Thus, we determined that the primary effect of light absorption and the corresponding Cys-FMN adduct formation is to shift the LOV2-J α binding equilibrium by ~3.8 kcal mol⁻¹. This provides the first estimate of the energy potentially available through the LOV2-J α photoswitch to activate the kinase domain in AsPhot1, and also yields a critical value for the engineering of artificial photoswitches where the LOV2-J α interaction is coupled to regulation of the activity of other proteins.

Results

Relaxation dispersion measurements indicate μ s-ms timescale dynamics in LOV2-J α

Conformational exchange processes occurring on micro- to millisecond timescales contribute to the transverse relaxation rate (R_2) of nuclear spins in NMR spectroscopy. This additional contribution to R_2 is termed R_{ex} and can be suppressed by a Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse train sequence, provided that the exchange process has a similar frequency as the applied 180-degree pulses in the CPMG train (ν_{CPMG} ; effectively 100–1000 s^{-1}). By examining the effective relaxation rate (R_2^{eff}) as a function of ν_{CPMG} , one obtains a relaxation dispersion curve that contains thermodynamic, kinetic and structural information on the underlying exchange process^{19,20}. For two-site exchange between a low energy conformation A and high energy conformation B, we define k_{ex} as the sum of the forward and reverse reaction rates, p_A and p_B as population of the two states and $\Delta\omega$ as the chemical shift difference between them. Under favorable conditions, these parameters can be extracted from the relaxation dispersion curve.

We used CPMG-based relaxation dispersion experiments on ^{15}N -labeled backbone amides as well as ^{13}C -labeled sidechain methyl groups to probe μ s-ms conformational dynamics in the LOV2-J α protein. Representative dispersion curves of K534 ^{15}N backbone amide (Fig. 2a) and L514 $^{13}C\delta 1$ methyl (Fig. 2b) show significant variation in R_2^{eff} as ν_{CPMG} was increased from approximately 100 to 900 Hz, providing clear evidence for motions on the μ s-ms timescale at these two sites. Complete analysis of these relaxation dispersion data revealed that 25 sites exhibited substantial amide ^{15}N and methyl ^{13}C R_{ex} ($> 4.0 s^{-1}$ and $> 2.0 s^{-1}$, respectively). Mapping these residues onto the LOV2-J α structure (Fig. 2c for ^{15}N ; Fig. 2d for ^{13}C), we observed that residues with high R_{ex} contributions were clustered on the J α helix, the IJ loop that connects it back to LOV2 and the β -sheet of LOV2 that faces J α . Global fits of all ^{15}N and ^{13}C dispersion curves to a model for two-site exchange converged to yield $k_{ex} = 1320 \pm 36 s^{-1}$ and $p_B = 1.6 \pm 0.03\%$. The convergence of the fitting indicated that more complex exchange models were not needed to describe the data, and that the system is reasonably represented as a rapid two-state equilibrium between low and high energy conformations.

J α mutations shift the conformational exchange equilibrium

Since the majority of resonances with large R_{ex} values were located at the LOV2- J α interface, we speculated that the dynamics we observed by NMR might involve fluctuations between helix-bound and helix-dissociated conformations. To test this hypothesis, we examined the relaxation characteristics of three different LOV2 variants, each of which contained a single point mutation that changed a hydrophobic J α residue (V529) to Ala, Glu or Asn. This residue lies on the hydrophobic surface of the J α helix proximal to LOV2^{13,15}, and we anticipated that each of these changes would perturb the binding equilibrium and thus the relaxation dispersion behavior of the protein. Overall, the ^{15}N - 1H HSQC spectra of the mutants were very similar to those of the wild type, suggesting that the mutations had little effect on the overall structure of the protein (data not shown). In addition, backbone ^{15}N CPMG experiments showed that the mutants are dynamic in similar regions as in the wild type protein. However, the mutations have clearly altered the dynamic properties of the system, since the magnitude of R_{ex} for individual resonances varies substantially among them (Fig. 3a). For all resonances, R_{ex} values increased from wild type to V529A to V529E to V529N. Consistent with the data collected for the wild type protein, we were able to globally fit all of the dispersion data for the three mutants to a model for two-site exchange. Figure 3b shows the fitted absolute values of ^{15}N $\Delta\omega$ for all dynamic residues in all constructs. In the majority of cases, we obtained very similar $\Delta\omega$ values for a given residue across all four constructs, suggesting that the wild type and mutated proteins all share the same low and high-energy conformations in the dark. Consistent with the rank of R_{ex} magnitudes, the proteins showed increasing populations of the

high-energy conformations, from $1.7 \pm 0.02\%$ in WT to $2.0 \pm 0.03\%$ in V529A to $2.5 \pm 0.03\%$ in V529E and $7.6 \pm 0.1\%$ in V529N. These observations are consistent with the model that the observed conformational exchange dynamics reflects a binding equilibrium between LOV2 and J α . Mutations that destabilized the LOV-J α interaction more significantly than the V529N mutant were difficult to analyze with CPMG-type experiments due to significant line broadening that resulted from more abundant high-energy conformation such that the dynamic residues are no longer observable in the NMR spectrum. For example, only four dynamic residues are observable in CPMG spectra obtained of the Q479T mutant and fits of the corresponding relaxation dispersion curves yield a p_B of 22.2%, consistent with the expected increase in the high-energy state population. Analyses of these proteins are also complicated by generally poorer solution behavior than the corresponding wildtype LOV2-J α construct.

J α is unfolded in the high-energy conformation in the dark state

To gain insight into the structure of the high-energy conformation, we used solution NMR spectroscopy to examine the structural properties of a peptide corresponding to an isolated J α helix and its surrounding elements (AsPhot1 residues 511–545). In the full LOV2-J α helix construct (AsPhot1 residues 404–560) protein, $^{13}\text{C}\alpha$ chemical shift values for residues in the J α helix region are all positive (Fig. 4a), consistent with the α -helical structure indicated by NOEs and backbone $^3J_{\text{HN-H}\alpha}$ coupling constants¹³. In contrast, the same $^{13}\text{C}\alpha$ CSI values for the peptide are very close to zero (Fig. 4a), suggesting that J α exists as a random coil in the absence of stabilizing interactions with the LOV2. To facilitate comparisons of structure of the J α helix in the LOV2-J α context and in isolation, we calculated the difference in ^{15}N chemical shifts between the assigned values for J α peptide and LOV2-J α protein. This parameter, which we refer to as $\Delta\omega_{\text{unfolding}}$, represents the chemical shift change upon unfolding of J α and dissociation from the LOV2 surface. A comparison of $\Delta\omega_{\text{unfolding}}$ with $\Delta\omega_{\text{Rex}}$, the chemical shift difference between the low and high-energy conformations of the LOV2-J α protein obtained from the relaxation dispersion measurements above, shows a very high degree of correlation (Fig. 4b). A linear fit of the data gives a slope of 0.57, an intercept of 1.0 and an R-value of 0.8. One possible reason for the fair quality of this fit is that the free J α peptide and the dark high-energy conformation has different sequence context. In the dark high-energy conformation, the J α residues are still connected to the LOV2 domain and the extra 20 residues in the C terminus. To address the latter of these concerns, we generated a longer J α peptide (residue 517–560 vs. 511–545 as used above) that extended to the same C-terminus as the LOV2-J α construct. Using ^{15}N chemical shift values from this peptide to calculate $\Delta\omega_{\text{unfolding}}$ of J α residues, we obtained a linear fit with the same parameters as the shorter peptide.

Given this linear correlation between $\Delta\omega_{\text{Rex}}$ and $\Delta\omega_{\text{unfolding}}$ for residues in the J α helix, our data suggest that the high-energy conformation of LOV2-J α resembles that of the isolated and unstructured J α peptide. That is, since,

$$\Delta\omega_{\text{Rex}} \approx \Delta\omega_{\text{unfolding}} \quad (1)$$

then

$$\omega_{\text{highenergy}} - \omega_{\text{lowenergy}} \approx \omega_{\text{peptide}} - \omega_{\text{LOV-J}\alpha} \quad (2)$$

and since

$$\omega_{\text{lowenergy}} \approx \omega_{\text{LOV-J}\alpha} \quad (3)$$

then,

$$\omega_{\text{highenergy}} \approx \omega_{\text{peptide}} \quad (4)$$

Since the ^{15}N chemical shifts of the high-energy conformation approximate those of the J α peptide, we concluded that the excited state being sampled in the dark has the J α helix being

dissociated from the LOV2 domain and largely unfolded. Thus, the dynamic equilibrium in the dark state involves transient excursions to a higher energy conformation (or conformational ensemble) that closely resembles the photoactivated state of the system.

Our data also indicate that the correlation between $\Delta\omega_{\text{Rex}}$ and $\Delta\omega_{\text{unfolding}}$ is not ideal, suggesting that a few J α residues might retain some low degree of residual structure in the high-energy dark state that is not present in the unfolded peptides, perhaps by maintaining some loose contacts with the LOV core. This would give rise to deviations between $\Delta\omega_{\text{Rex}}$ and $\Delta\omega_{\text{unfolding}}$, as we observed for several residues located at either end of the J α helix (D522, A523; A542) or in a cluster near its middle (M530, K533, K534) (Fig. 4b). Notably, most of these sites show $\Delta\omega_{\text{Rex}} < \Delta\omega_{\text{unfolding}}$, as is expected if a small degree of residual structure is present in the high-energy dark state. We emphasize that these deviations from linearity are small (<1 ppm ^{15}N) and are not inconsistent with our overall finding that the high-energy dark state has a chiefly-unfolded J α helix.

An analogous equilibrium in the lit state

Previous analyses of NMR chemical shift dispersion, proteolytic sensitivity and other solution biophysical parameters have suggested that J α is dissociated from the LOV domain and melted in the lit state^{13,17,21}. Consistent with these observations, ^{15}N - ^1H HSQC spectra of the isolated J α peptide and LOV2-J α protein in the lit state are very similar (Fig. 5a, b). However, the resonance overlap is not exact, and most of the lit state J α resonances are displaced from their peptide equivalents. A detailed comparison of the dark state, lit state and free J α peptide spectra reveals that the displacement is not random. Rather, for 13 of the 15 J α peaks that can be assigned in the lit state spectrum, the lit state position is systematically displaced approximately along a line that connects the corresponding peptide and dark state peaks (Fig. 5c and Fig. 5d; Supplemental Fig. S1). This behavior suggests that in the lit state J α may be sampling a rapid equilibrium between two conformations, one that resembles the peptide, unfolded and dissociated from the LOV domain, and a second that resembles the predominant dark state structure, folded into an α -helix and bound to LOV2. Such an equilibrium in the lit state would be analogous to that sampled in the dark state, but should be oppositely biased to favor the helix-dissociated conformation. To estimate the populations of the two conformations in the lit state, we assumed that the peptide chemical shifts represent the fully dissociated conformation, and that the dark state chemical shifts of the J α resonances are identical to those of the helix-bound lit state. While the latter assumption is obviously not exactly correct, the similar behavior of the large majority of J α resonances indicates it is reasonable. Based on the chemical shifts of the endpoint conformations, the positions of the J α resonances yield an approximate population of the helix-dissociated conformation of $91 \pm 5.7\%$ in the lit state.

In principle, we should be able to also apply relaxation dispersion analyses to the lit state protein as well to obtain conformational exchange information. Unfortunately, the narrow linewidths of J α peaks from lit state spectra suggest that these residues are in fast exchange conditions where CPMG methods cannot provide accurate population measurements. Further, lit state samples are only moderately well-behaved over time at high concentration, making it difficult to extract quantitative information from CPMG experiments which require > 48 h data acquisition. Given these issues, we have opted to use the simpler, but more robust, method for measuring population changes by chemical shift changes to estimate the relative p_A and p_B populations in the lit state.

Discussion

Free energy of the photoswitch

We have shown that in both the dark and lit states, the LOV2-J α core of AsPhot1 exists in equilibrium between two major conformations (or more precisely, conformational ensembles): one with J α bound to LOV2, the other with J α dissociated away from the rest of the domain. Both conformations are sampled in dark state and illumination state, but to different degrees. Thus, the essential nature of the photoswitch is to change the relative stabilities of these two conformations. In the dark, the helix-bound form is more stable, while this reverses in the light as the helix-dissociated form becomes the more stable form. Relaxation dispersion analyses indicate that the dissociated conformation is populated to only 1.6% in the dark state. Chemical shift analyses of the lit state indicate that FMN adduct formation produces a 91% population of this conformation. Thus, the population of the active state increases ~57-fold upon photo-excitation. Viewed in terms of equilibrium constants (and thus free energy), photo-excitation changes the bound-dissociated J α equilibrium from 98.4:1.6 in the dark state to 9:91 in the lit state. This corresponds to an overall shift of 620-fold in the bound: dissociated ratio, representing a 3.8 kcal mol⁻¹ change in free energy. We note that this value is more than fifteen-fold smaller than the energy of the blue light photons being absorbed by the internally-bound flavin chromophore, which is ~64 kcal mol⁻¹. Thus, only a small fraction of the total energy of excitation is transmitted to conformational changes in the protein in the form of useful work, while the majority must be dissipated in formation of the flavin-cysteine adduct and as heat.

To confirm these results, we carried out similar measurements on LOV2-J α protein at 20°C with independently-generated samples. In the dark state, the J α unbound conformation decreased from 1.6% at 25°C to 0.97% at 20°C, corresponding to 2.7 kcal mol⁻¹ of free energy. In the lit state, the J α unbound conformation is ~92%, equivalent to ~-1.4 kcal mol⁻¹ free energy. Therefore there are about 4.1 kcal mol⁻¹ of free energy available at 20°C, quite similar to the ~3.8 kcal mol⁻¹ value measured at 25°C. An additional line of independent validation of this value is provided by potential of mean force (PMF) calculations on LOV2-J α , which indicate a displacement of the J α helix and a ~3.0 kcal mol⁻¹ change in free energy upon illumination (P. Freddolino, S.M. Harper, K.H.G., K. Schulten; manuscript in preparation).

Use of the photoswitch energy to activate the phototropin I kinase domain

Previous biochemical and NMR data indicate that dissociation of J α from LOV2 is sufficient for activation of the AsPhot1 kinase domain¹⁸. These observations suggest that in the full length protein the conformational equilibrium in the LOV2-J α core is coupled to a regulatory equilibrium in the kinase domain. This model suggests that the light-induced shift in the former equilibrium drives activation of the kinase, although the physical mechanisms of this coupling are not currently known. It is difficult in the absence of experimental data to predict how thermodynamic coupling to the rest of the phototropin protein will affect the equilibrium in the LOV2-J α core, since the covalent connection and interdomain contacts can perturb this equilibrium in unknown ways.

Similarly, the amount by which the equilibrium in the core affects the activity of the kinase domain, in both the dark and lit states, will depend on the specifics of the physical connections. In principle, the actual perturbation of the kinase equilibrium by light could be relatively small, since full activation is likely driven by autophosphorylation, as in other kinases²². The Pak kinase, which is regulated by the Rho GTPases Cdc42 and Rac, appears to be regulated in such a fashion²³. GTPase binding to Pak does not substantially disassemble the autoinhibitory apparatus; rather, it simply perturbs the regulatory equilibrium sufficiently to permit autophosphorylation. This covalent modification then fully relieves autoinhibition, raising Pak to a GTPase-independent activation state. On the other hand, there are cases where activity is

dictated by the conformational equilibrium. In nitrogen regulator protein C (NtrC), transcription activity of the protein is turned on by phosphorylation of residue D54, which is located in the N-terminal receiver domain. NMR studies on the NtrC receiver domain suggest the existence of an equilibrium between active and inactive conformations in both the phosphorylated and unphosphorylated form²⁴. Mutations that are partially active in the absence of phosphorylation show a close correlation between their activity and population distribution of the equilibrium. Comparison of the population distribution in the phosphorylated versus unphosphorylated form in NtrC in the context of the full length protein will provide insights into the energetic coupling of those “two-component” signaling systems. Similarly, comparison of the LOV2-J α core and full AsPhot1 in both the dark and lit states will ultimately be necessary to understand how the energy inherent in the photoswitch is used to drive kinase activation.

Engineering light control into other systems

The ability to reversibly control the activity of arbitrary proteins with light would have tremendous utility in basic and applied research and technology. Although reversible regulation has been achieved in several cases through covalent attachment of small molecule photoswitches^{25,26}, and natural light-controlled ion channels and adenylyl cyclases have shown great promise in neuroscience^{27,28}, it would be particularly powerful to generate genetically-encoded photoswitches in a general fashion. Such control might be achieved with chimeras or simple fusions between the LOV2-J α element of AsPhot1 and various functional elements – *e.g.* enzymes or binding domains. The success of such an effort is predicated on efficiently coupling the light-dependent changes in free energy provided by a photoswitchable element with other regulatory equilibria. This is analogous to the LOV2 control of kinase activity in phototropin, but such a coupling would have to be achieved through artificial engineering rather than through the course of natural evolution. Lim and colleagues have used an analogous approach to generate synthetic multi-domain proteins with activities controlled by ligand binding²⁹. By fusing a protein and its peptide ligand to an allosterically regulated functional domain, the domain activity can be controlled by free ligand. However, significant suppression of activity (a necessary prerequisite to ligand-mediated activation) was only observed when the intermolecular K_D between the protein and ligand was $\sim 10 \mu\text{M}$ or less, corresponding to $6.8 \text{ kcal mol}^{-1}$ or higher free energy without any covalent linkage between the ligand and the enzyme²⁹. Given that the dark state suppressive free energy of the LOV2-J α system corresponding to the helix-bound:helix-free conformation is only $2.4 \text{ kcal mol}^{-1}$ with J α tethered to the LOV domain, more efficient coupling, perhaps achieved through a combination of engineering and screening, will likely be needed to generate LOV2-based photoswitches with a large dynamic range.

LOV2-J α versus photoactive yellow protein

Analogous to the LOV2-J α element of phototropins, photoactive yellow protein (PYP), a blue light photoreceptor from halophilic bacteria, also adopts a PAS fold³⁰. PYP does not bind FMN, but rather uses a covalently linked p-coumaric acid as chromophore. Analogous to J α in phototropin, PYP has a N-terminal extension that consists of two short α -helices. Numerous studies have shown that photoexcitation leads to unfolding of this N terminal extension^{31, 32}. This partially unfolded state is presumed to be the signaling state of PYP. Despite these similarities, relaxation dispersion experiments have not shown evidence of significant μs - ms timescale dynamics in PYP under physiologic conditions (data not shown). Thus, if there are analogous fluctuations to an excited state resembling the lit state in PYP, these are either on timescales outside the $0.1\sim 10 \text{ ms}$ range sampled by the CPMG-based technique or the excited state is populated to $< \sim 0.5\%$, based on the detection limit of the method. Although LOV2-J α and PYP may utilize similar structurally similar photoswitch mechanisms, the energetics involved in the two systems appear to be distinct. It remains to be discovered whether the

structural and energetic properties of the photosensory core of AsPhot1 are conserved among other members of the LOV-containing photoswitch family.

Methods

Sample preparation

Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). The LOV2-J α construct consisted of residues 404–560 of *Avena sativa* phototropin 1. Proteins were expressed from plasmid pHis₆-GB1¹³ in *E. coli* strain BL21(DE3) grown in M9 minimal media containing 1 g/L ¹⁵NH₄Cl as the sole nitrogen source for uniform ¹⁵N labeling and 1 g/L 3-¹³C- pyruvate^{33,34} for selective ¹³C labeling of the following methyl groups: Ala β ; Ile γ 2, α 1; Leu δ 1, δ 2; Val γ 1, γ 2. Expression and purification of all constructs were performed as described¹³ except for an additional Source 15Q (GE Healthcare) anion exchange chromatography step after an initial Ni²⁺ affinity chromatography purification. Purified proteins were exchanged into 50 mM phosphate buffer (pH 6), 0.1 M NaCl. All NMR samples contained ~1 mM protein, 10% D₂O, 1 mM DTT, 2 mM FMN and a cocktail of protease inhibitors that included EDTA, benzamidine, leupeptine and antipain.

To generate the J α peptide, AsPhot1 residues G511-E545 were cloned into a modified pET19b vector (Novagen) containing a N-terminal His₁₀-ubiquitin tag and a TEV protease recognition site before the multiple cloning site. The expressed fusion protein was purified using Ni²⁺-NTA (QIAGEN) affinity followed by anion exchange chromatography (Source 15Q, GE Healthcare). The His-ubiquitin tag was cleaved off by TEV protease and separated from the peptide by Ni²⁺-NTA affinity chromatography. A Superdex75 (GE Healthcare) gel filtration column was used to buffer exchange the peptide into the same NMR buffer as used for the LOV2-J α proteins except no FMN was added. Final concentration of the peptide was ~1 mM.

NMR spectroscopy and data analyses

All NMR experiments were performed at 25°C on Varian Inova 600 and 800 MHz spectrometers. Relaxation compensated constant time CPMG pulse sequences were used for both ¹⁵N and ¹³C experiments^{35–37} using a 40 ms constant time period. All NMR data were processed using NMRDraw³⁸ and NMRview³⁹ software. For each CPMG series, repeats of one or two data points were performed and the intensity of the repeats were used to calculate standard deviation of the series. In cases where the standard deviation was less than 2%, 2% was used in data fitting. For data points with repetition, the average of the two repeats was used for data fitting. Data were fit using software kindly provided by Dr. Lewis Kay, using the following equations²⁰

$$R_2(v_{CPMG}) = \frac{1}{2} (R_{2A}^0 + R_{2B}^0 + k_{ex} - v_{CPMG} \cosh^{-1} [D_+ \cosh(\eta_+) - D_- \cosh(\eta_-)]) \quad (5)$$

where,

$$D_{\pm} = \frac{1}{2} \left[\pm 1 + \frac{\psi + 2\Delta\omega^2}{(\psi^2 + \xi^2)^{1/2}} \right] \quad (6)$$

$$\eta_{\pm} = \frac{1}{\sqrt{2}v} \left[\pm \psi + (\psi^2 + \xi^2)^{1/2} \right]^{1/2} \quad (7)$$

$$\psi = (R_{2A}^0 - R_{2B}^0 - p_A k_{ex} + p_B k_{ex})^2 - \Delta\omega^2 + 4p_A p_B k_{ex}^2 \quad (8)$$

$$\xi = 2\Delta\omega (R_{2A}^0 - R_{2B}^0 - p_A k_{ex} + p_B k_{ex}) \quad (9)$$

Where R_{2A}^0 and R_{2B}^0 are the intrinsic R_2 values of A and B states.

Dispersion curves were first individually fit assuming the two-site exchange model. Individual fits give similar exchange rates and excited state populations, justifying the use of a two-state global fit, using the same k_{ex} and p_B for all dynamic residues. Chemical shift values of LOV2-J α were taken from previous assignments¹³. Backbone resonance assignments of the peptide were carried out using HNN, HN(C)N⁴¹, HNCACB^{40,42} and CBCA(CO)NH⁴³ experiments. Lit state assignments were obtained as described previously¹³ using $^{15}\text{N}/^1\text{H}$ SCOTCH experiments⁴⁴ that provide 2D correlations of $^{15}\text{N}_{\text{dark}}-^1\text{H}_{\text{lit}}$ to couple fully assigned $^{15}\text{N}/^1\text{H}$ HSQC spectra in the dark and the corresponding lit state spectra. This approach provided unambiguous assignments for 12 of the 15 residues in the J α helix; the other 3 residues that we could assign had ambiguities in the lit state that were resolved by proximity to crosspeaks from spectra acquired on the free J α peptide.

Assuming the J α bound state chemical shift values are similar between dark and lit states, the population distribution across the exchange process in the lit state was estimated using backbone ^{15}N and ^1H chemical shifts of J α residues according to the following the equation⁴⁵:

$$\omega = p_A \omega_A + p_B \omega_B \quad (10)$$

where A is the J α bound state in dark, B is the J α dissociated and unfolded state that is equivalent to the J α peptide. The chemical shifts of J α bound state in the dark (state A) is back calculated from the dark state apparent chemical shift values and experimentally determined 98.4% J α -bound state in the dark using equation (10).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

LOV domain, Light-Oxygen-Voltage domain; PAS domain, Period-ARNT-Single-minded (Per-ARNT-Sim) domain; AsPhot1, *Avena sativa* phototropin 1; NOE, Nuclear Overhauser Effect; CSI, Chemical Shift Index.

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Figure 1. Schematic representations of phototropin domain organization and photoswitching mechanism

(a). Domain organization of oat (*A. sativa*) phototropin 1, with the locations of domain boundaries indicated by residue numbers. (b). Phototropin switching mechanism is regulated at the stage of LOV2-J α interactions, which are chiefly intact in the dark. Illumination leads to the formation of a protein-flavin adduct (involving Cys 490 in *A. sativa* phot1 LOV2 and the C4(a) position of the FMN chromophore), distorting the LOV2 structure sufficiently to significantly weaken the LOV2-J α interactions thus freeing the J α helix and allowing it to unfold¹³.

Figure 2. Conformational exchange dynamics at the LOV-J α interface as detected by relaxation dispersion measurements

Representative relaxation dispersion curves of backbone amide groups (a: K534 backbone ^{15}N amide) and sidechain methyl groups (b: L514 $^{13}\text{C}\alpha$ 1 methyl) at 600 (black circles) and 800 MHz (red squares) are shown. Residues with $R_{\text{ex}} > 4 \text{ s}^{-1}$ for ^{15}N (c) and $> 2 \text{ s}^{-1}$ for ^{13}C (d) are colored yellow in the LOV2-J α ribbon diagrams, while the FMN chromophore is shown in green. Figures 2c and d were prepared with PyMOL software⁴⁶.

Figure 3. ¹⁵N relaxation dispersion analyses of LOV2-Ja constructs containing V529A, V529E and V529 point mutations

These mutants give similar $\Delta\omega$ values as the wild type protein but have different high energy state populations. (a) ¹⁵N $|\Delta\omega_{\text{Rex}}|$ of various residues from all constructs. (b) Relaxation dispersion curves of the A524 backbone amide ¹⁵N in WT (black), V529A (green), V529E (blue) and V529N (red) LOV2-Ja proteins at 800 MHz.

Figure 4. The J α helix is unfolded in the high energy conformation of dark state LOV2-J α
 (a) $\text{C}\alpha$ chemical shift index of J α residues (D522 to A542) in the LOV2-J α (open bars) and in the corresponding residues in the peptide (filled bars). (b) ^{15}N $|\Delta\omega_{\text{Rex}}|$ obtained from relaxation dispersion analyses plotted against $|\Delta\omega_{\text{unfolding}}| = |\omega_{\text{peptide}} - \omega_{\text{LOV-J}\alpha}|$. Data are labeled with the number of the corresponding residue. Residues which deviate from the $m=1$ line are indicated in red on LOV2-J α structure.

Figure 5. The J α helix is largely unfolded in the lit state

(a,b). Overlay of methyl region ^{13}C - ^1H (a) and ^{15}N - ^1H (b) HSQC spectra of lit state LOV2-J α (red) protein and the peptide (blue). (c,d). Expansions of ^{15}N - ^1H HSQC spectra of WT LOV2-J α in the dark (black), lit (red) states and the peptide (blue) of the backbone amides for residues R526 (c) and I539 (d).