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Conformational Changes in a Photosensory LOV Domain Monitored by Time-Resolved NMR Spectroscopy

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Plants control a large number of biological functions with protein-based photosensors. One class of these are phototropins, a group of blue light-activated kinases that regulate processes including phototropism, stomatal opening, and chloroplast photoavoidance.[1,2] In addition to a serine/threonine kinase, phototropins contain two light−oxygen−voltage (LOV) domains, members of the PAS family of protein interaction modules that have been optimized for light detection through the use of flavin chromophores.[3,4] In the dark, a single FMN cofactor is noncovalently bound within each LOV domain in a complex known as LOV^{D}_{447}.[5,6] Blue light generates the suggested signaling state, LOV^{S}_{390}, wherein a covalent adduct is formed between the thiol of a conserved cysteine residue and the C4a position of the FMN isoalloxazine ring.[6,7] Formation of this bond results in a conformational change that destabilizes the surrounding protein and unfolds an α-helix over 15 Å away, providing a possible signaling pathway connecting the cofactor and kinase.[8] While this shows that the chromophore and protein conformations are coupled under steady-state conditions, we sought to explore if these are also linked during the kinetic processes that regenerate LOV^{D}_{447} after excitation.

To study this in a site-specific manner we used time-resolved NMR spectroscopy to record a series of two-dimensional (2D) spectra after laser excitation, thereby observing the kinetics of protein conformational changes during the return to the dark-state structure. NMR spectroscopy has successfully measured the folding of several proteins in real time, typically with one-dimensional (1D) experiments.[9,10] This takes advantage of the inherent sensitivity of 1D methods, facilitating collection of high signal-to-noise spectra despite the rapid time scale of many protein-folding processes ($\tau \leq 10$ s). However, crowding in such spectra typically limits the number of unambiguously assignable sites that can be monitored. This problem can be addressed with multidimensional versions of these time-resolved experiments, although their complexity has limited their use to studying very slow conformational changes ($\tau \approx 5-20$ h).[11,12] Proteins involved in faster processes can be studied with this approach, provided these changes can be repeatedly and synchronously triggered. These criteria are met by the spontaneous regeneration of LOV^{D}_{447} in the absence of illumination, which occurs with $\tau \approx 10-100$ s.

Using this approach, we acquired a series of time-resolved $^{15}N/^{1}H$ HSQC spectra on a ^{15}N-labeled sample of the *Avena sativa* phototropin 1 LOV2 domain (AsLOV2). By using an interleaved acquisition mode, we obtained a total of 91 2D spectra that sampled the dark-state regeneration process every 1.1 s. Spectra 2−11 were preceded by a 125-ms pulse of 488-nm light to generate the LOV^{S}_{390} state, leaving the remaining 80 spectra (12−91) to monitor the return to the dark state over a period of 88 s (Figure 1A; Supporting Information). Rates were measured from the time-dependent

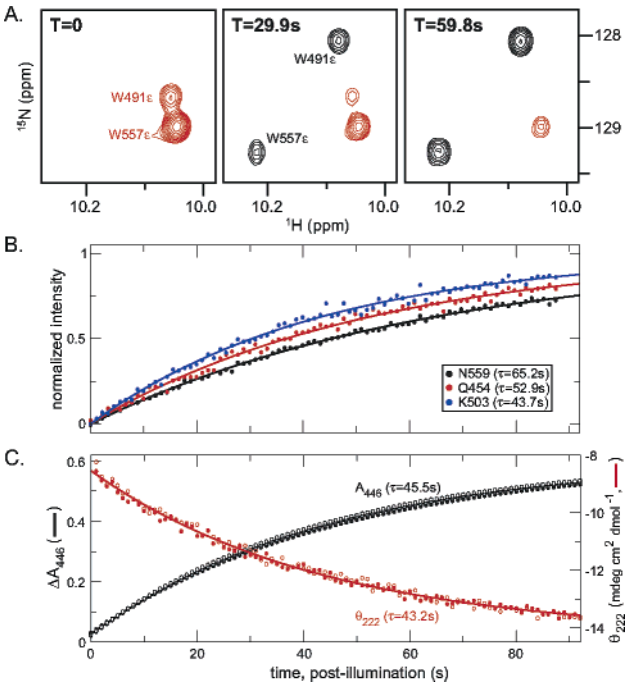

Figure 1. Monitoring the dark-state recovery of AsLOV2 using different spectroscopic probes. (A) Time-resolved $^{15}N/^{1}H$ HSQC spectra acquired after laser illumination (lit state of two tryptophan indole peaks colored red; dark-state peaks colored black). (B,C) Kinetics of LOV^{D}_{447} regeneration as monitored by the return of dark-state NMR signals from three backbone amides (B) and changes in visible absorbance (black) and circular dichroism (red) (C).

recovery of intensity of the dark-state peaks of 107 backbone amides and tryptophan side-chain sites throughout the protein (Figure 1B). These data revealed a range of LOV^{S}_{390} lifetimes ($\tau = 43.7-70.5$ s, each determined with an error of ∼15%; $\tau_{mean} = 54.4 \pm 5.1$ s) among these sites. Analyses of lit-state peaks yielded comparable statistics, with individual residues correlating well with their dark-state counterparts. This 1.6-fold range in lifetimes corresponds to an energetic difference of only 0.3 kcal mol^{-1} in transition-state energies ($\Delta\Delta G^{\ddagger}$), which is significantly less than thermal energy at 303 K, indicating that the protein tertiary structure returns to the dark state in a concerted manner. Consistent with this, mapping these lifetimes onto the AsLOV2 dark-state structure[8] revealed no obvious correlations with proximity to the FMN chromophore or among different protein structural elements.

We compared these results to the time dependence of chromophore and global protein conformational changes as monitored by visible and CD spectroscopy (Figure 1C). Increases in the absorbance at 446 nm (A_{446}) report on the decay of the covalent protein/FMN adduct,[13] while CD measurements at 222 nm (θ_{222}) provide complementary information about changes in global protein

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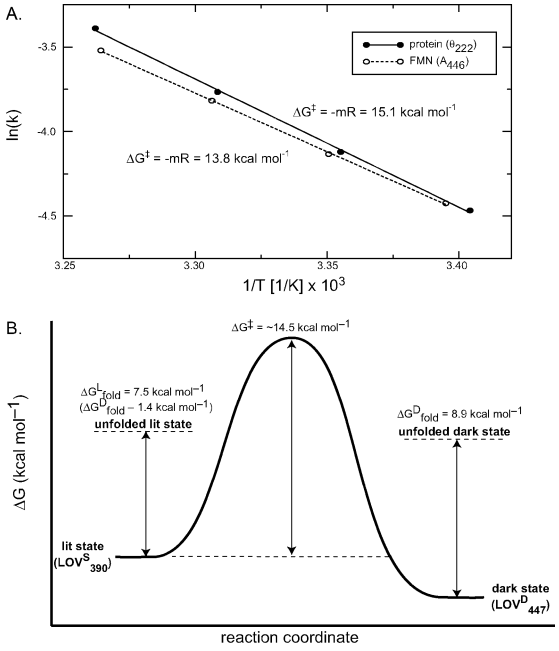

Figure 2. Energetic analysis of the dark-state regeneration of AsLOV2. (A) Arrhenius plot of rates of chromophore and protein conformational changes as monitored by visible and CD spectroscopy; (B) Summary of AsLOV2 thermodynamic parameters.

secondary structure. Both methods monitor processes with equivalent lifetimes ($\tau[A_{446}] = 45.5 \pm 2.0$ s; $\tau[\theta_{222}] = 43.2 \pm 0.4$ s), synchronizing these events as previously reported.[14] These agree with the shortest lifetimes measured by NMR (τ_{min}[NMR] = 43.7 ± 3.3 s), establishing that the tertiary-structure changes monitored by NMR do not precede either cleavage of the FMN adduct or changes in secondary structure. However, we note that the mean lifetime of the NMR signals is longer (τ_{mean}[NMR] = 54.4 ± 5.1 s), suggesting that the regeneration of the dark-state protein fold is a more complex process.

An interesting comparison is provided by another PAS-based blue-light photoreceptor, photoactive yellow protein (PYP).[3] Although structurally homologous with AsLOV2, PYP uses a different chromophore, a single covalently bound 4-hydroxycinnamic acid (4HCA). 4HCA has markedly different photochemistry than FMN, undergoing *trans/cis* isomerization to generate protein conformational changes. Time-resolved NMR data from PYP establish that the photoexcited protein also cooperatively returns to the dark state, exhibiting a 1.6-fold range of lifetimes ($\tau = 0.41-0.67$ s) similar to that of AsLOV2.[15] Intriguingly, optical spectroscopic measurements have established that 4HCA returns to the dark state with the same kinetics as the slowest measured protein rates, contrasting with the AsLOV2 where the chromophore resets to the dark state as quickly as the fastest of the protein NMR signals.

Complementing the kinetic parameters described above, thermodynamic information about the regeneration of the AsLOV2 LOV$^{D}_{447}$ state can be obtained by assuming that chemical transition-state theory applies to the complex rearrangements occurring during this process. In this context, an Arrhenius analysis of the temperature dependence of the lit-state lifetime provides the activation energy ($\Delta G^{\ddagger}$). Examination of the recovery of A_{446} and θ_{222} signals between 293 and 308 K indicates that $\Delta G^{\ddagger}$ is approximately 14.5 kcal mol^{-1} (Figure 2A). Further information obtained from NMR-derived ^{2}H-exchange protection factors provides an estimate of the free energy of global protein folding/unfolding when done under EX2 conditions.[16] Such measurements indicate protection factors of approximately 3×10^6, corresponding to $\Delta G^{D}_{fold} \approx 8.9$ kcal mol^{-1}.[8] By comparison, our observation of a light-induced 10-fold decrease in protection factors indicates that the free energy of unfolding the lit state is approximately 1.4 kcal mol^{-1} less than that that of the dark state ($\Delta G^{L}_{fold} \approx \Delta G^{D}_{fold} - 1.4$ kcal mol^{-1} = 7.5 kcal mol^{-1}; Figure 2B).[8] Given that our prior ^{2}H-exchange measurements of the lit state showed significant protection in secondary-structure elements despite going through numerous photocycles, we suggest that the transition state is not globally unfolded even though it is likely to be of higher energy than the unfolded form of either the dark or lit state. This implies that the high energy of the transition state is partitioned in a very different manner than in the equilibrium unfolded state and may reflect a general property of photoreceptors.

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Supporting Information Available: Details of the protein sample preparation, spectroscopic data collection and analysis (PDF) and a movie of the time-resolved 2D ^{15}N/^{1}H HSQC spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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