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Dynamics of Light-Induced Activation in the PAS Domain Proteins LOV2 and PYP Probed by Time-Resolved Tryptophan Fluorescence†

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ABSTRACT: Light-induced activation of the LOV2-Jα domain of the photoreceptor phototropin from oat is believed to involve the detachment of the Jα helix from the central β-sheet and its subsequent unfolding. The dynamics of these conformational changes were monitored by time-resolved emission spectroscopy with 100 ns time resolution. Three transitions were detected during the LOV2-Jα photocycle with time constants of 3.4 μs, 500 μs, and 4.3 ms. The fastest transition is due to the decay of the flavin phosphorescence in the transition of the triplet LOV^{L}_{660} state to the singlet LOV^{S}_{390} signaling state. The 500 μs and 4.3 ms transitions are due to changes in tryptophan fluorescence and may be associated with the dissociation and unfolding of the Jα helix, respectively. They are absent in the transient absorption signal of the flavin chromophore. The tryptophan fluorescence signal monitors structural changes outside the chromophore binding pocket and indicates that there are at least three LOV^{S}_{390} intermediates. Since the 500 μs and 4.3 ms components are absent in a construct without the Jα helix and in the mutant W557S, the fluorescence signal is mainly due to tryptophan 557. The kinetics of the main 500 μs component is strongly temperature dependent with activation energy of 18.2 kcal/mol suggesting its association with a major structural change. In the structurally related PAS domain protein PYP the N-terminal cap dissociates from the central β-sheet and unfolds upon signaling state formation with a similar time constant of ∼1 ms. Using transient fluorescence we obtained a nearly identical activation energy of 18.5 kcal/mol for this transition.

LOV1[1] and LOV2 are the light sensing domains of phototropins, a class of blue light photoreceptors which control phototropism, light-induced stomatal opening, and chloroplast movement in response to changes in the light intensity in plants (*1*−*5*). The LOV domains which are members of the PAS-domain superfamily are activated by blue-light absorption of their flavin mononucleotide (FMN) cofactors (*6*, *7*). This triggers the autophosphorylation of a C-terminal kinase domain which is presumed to be essential for the downstream signal transduction (*3*, *8*). The dark state of the LOV domain, LOV^{D}_{450}, has its absorption maximum at 450 nm. Upon absorbing blue light, the FMN chromophore forms within 5 ns a triplet state called LOV^{L}_{660} with absorption maximum at 660 nm via intersystem crossing from the excited singlet state (*9*). The triplet state then decays with a time constant of 4 μs to the long-living intermediate LOV^{S}_{390} which absorbs at 390 nm (*10*). In this transition a covalent adduct is formed between the C(4a) of FMN and the sulfhydryl group of a conserved cysteine (*6*, *10*−*13*). The LOV^{S}_{390} intermediate is the signaling state of the LOV domains, and its formation is supposed to trigger conformational changes of the protein (*13*−*18*) . It recovers to the ground state with a time constant of seconds to minutes. During its long lifetime conformational changes must occur but were not detected by transient absorption spectroscopy.

In LOV2-Jα from *Avena sativa* photoactivation leads to the dissociation and partial unfolding of an amphipathic α-helix (Jα) which follows the C-terminus of the LOV2 domain and which interacts and docks onto the central β-sheet of the LOV2 core in the dark (*14*) (see Figure 1). The timing and interpretation of these events remain unclear, however. Significant light-induced structural changes of the tryptophans W491 and W557 in LOV2-Jα were detected by $^{1}H/^{15}N$ NMR spectroscopy (*14*, *15*, *19*). In the dark the two tryptophans are in different environments; in the photoactivated state their environments change and become more similar (*15*, *19*). The indole NMR cross-peaks return to their dark positions with the same kinetics as the photocycle recovery (*15*). The indole side chain of tryptophan is also highly fluorescent and provides a natural site-specific fluorescent marker. Moreover, since the wavelength maximum of the tryptophan fluorescence is quite sensitive to the polarity of its environment (*20*), it provides a suitable signal to monitor conformational changes. NMR spectroscopy already showed that W491 and W557 undergo conformational changes but could not resolve the kinetics of their formation. It is thus obviously worthwhile to try to monitor the kinetics of the signaling state formation using transient tryptophan fluorescence. Time-resolved fluorescence has been used in chemical relaxation kinetics. Tryptophan fluorescence was used for example to follow protein folding on the nanosecond to microsecond time scale after a laser-induced temperature jump (*21*). It was recently shown that transient fluorescence spectroscopy provides a sensitive signal to monitor the kinetics of the structural changes of photoreceptors that can be triggered by a short laser flash (*22*, *23*). With a time resolution of 100 ns this method is appropriate to resolve the conformational transitions that occur during the life span of the LOV^{S}_{390} intermediate. Two mutants

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[1]Abbreviations: PAS, Per-ARNT-Sim domain; LOV, light−oxygen−voltage sensing domain; phot1, phototropin 1; LED, light emitting diode; PYP, photoactive yellow protein; FMN, flavin mononucleotide; ORD, optical rotatory dispersion; FRET, fluorescence resonance energy transfer; TG, transient grating; TrL, transient lens.

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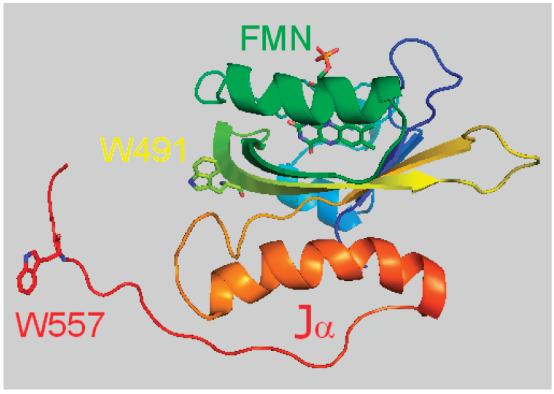

FIGURE 1: Structure of phot1 LOV2-Jα based on NMR data provided by Dr. K. H. Gardner. The FMN chromophore and tryptophans W491 and W557 are shown as sticks. The Jα helix (orange) is on the opposite side of the β-sheet as the flavin chromophore.

were prepared to determine which tryptophan residues contribute to the fluorescence signal. In the PAS-core mutant the Jα helix is absent, and this mutant only contains W491 (see Figure 1). The mutant W557S includes the Jα helix and only W491.

PYP is a related PAS domain protein with similar structure (*24*). In fact, PYP is the structural prototype of the PAS domain superfamily (*24*). Upon formation of the signaling state a global conformational change occurs which has been very well characterized by structural and dynamical methods. This structural change occurs about 1 ms after flash excitation and resembles in many respects what happens in LOV2-Jα: the N-terminal cap which contains two short α-helices detaches from the central β-sheet and unfolds. PYP contains a single tryptophan residue W119 which is bound to the central β-sheet. Its fluorescence differs significantly in the various intermediates of the photocycle (*22*, *25*). For comparison with LOV2-Jα we therefore also measured the transient tryptophan fluorescence of PYP and its temperature dependence. This allowed us to determine a benchmark value for the activation energy of the signaling state formation in PAS domain proteins.

MATERIALS AND METHODS

Protein Production and Purification. LOV2-Jα samples were prepared as previously described (*6*). The LOV2-Jα domains were expressed in *Escherichia coli*, purified by calmodulin affinity chromatography (Stratagene), and lyophilized. Prior to use the dried protein was resuspended in buffer (10 mM Tris, 30 mM KCl, pH 7.5). The complete sequence of the LOV2-Jα fusion protein is shown in ref *13*. It contains three tryptophans, W491, W557, and a third one in the calmodulin binding domain which is attached at the N-terminus of the LOV2 domain and enables the purification of the protein. Experiments were also conducted with constructs which contain a His tag instead of the calmodulin tag. These constructs have only the two tryptophans, W491 and W557. The mutant W557S (amino acids 404−567) and a core mutant (amino acids 404−521) in which the Jα helix was deleted were prepared with His tags as follows. Full-length constructs of LOV2-Jα and LOV2 core were cloned in the modified pET-15b directional vector that carries an N-terminal His tag sequence followed by a TEV site and three cloning sites (MCLAB, South San Francisco). Mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions and confirmed by DNA sequencing. PYP from *Halorhodospira halophila* was prepared as described (*26*).

Transient Fluorescence Spectroscopy. The transient fluorescence measurements were performed with a modification of a setup for time-resolved absorption spectroscopy described in more detail in ref *22*. The tryptophan fluorescence is excited by an LED emitting at 280 nm with a full width at half-maximum of 10 nm. The emission is detected by a photomultiplier tube (PM), and the time course of the signal is recorded from 100 ns to seconds after exciting the sample with a 20 ns flash at 460 nm from a dye laser (Coumarin 102 dissolved in ethanol) pumped by a XeCl-excimer laser (EMG50; Lambda Physics). To protect the PM from stray light from the LED and the excitation flash, the filters WG305 and UG11 (both Schott) were placed in front of it. In this way the time course of the steady-state fluorescence of the tryptophans during the photoactivation is recorded. The cuvette was placed in a thermostated sample holder. The absorption spectrum of the UG11 filter used in our experiment is shown in the inset of Figure 3B. In the UV−vis wavelength region it is transparent from 245 to 395 nm and from 680 to 775 nm. The lower wavelength bandpass matches the emission spectrum of tryptophan, the upper one will pass the red edge of the phosphorescence spectrum of LOV^{L}_{660} which is excited by the intense excitation flash (*27*). To separate the contributions of the emission of both chromophores to our signal, we performed a control experiment measuring the transient fluorescence of LOV2-Jα with an additional color filter (OG590) in front of the photomultiplier tube. With this setup only light in the wavelength region from 680 to 775 nm contributes to the signal (see inset of Figure 3B).

The transient fluorescence signal is defined as the difference of the fluorescence intensity $F(t)$ at time t after the laser flash at $t = 0$ and the initial fluorescence F_0 before the flash divided by F_0: $\Delta F/F_0 = (F(t) - F_0)/F_0 = (I(t) - I_0)/I_0$. The photocycle is triggered at $t = 0$ by the laser flash. It is calculated from the corresponding photomultiplier currents ($I(t)$, I_0).

To calculate the transient signal in the control experiment, we used for F_0 the fluorescence intensity of the sample before placing the color filter OG590 in the beam. The signal therefore shows in a first approximation the FMN phosphorescence excited by the flash normalized by the tryptophan fluorescence emission of the ground state which is excited by the UV LED.

Transient Absorption Spectroscopy. Flash spectroscopy experiments with 50 ns time resolution in the visible and UV were performed as described (*28*).

RESULTS

Steady-State Fluorescence Measurements. The effect of background illumination on the steady-state tryptophan fluorescence of LOV2-Jα is shown in Figure 2. The background illumination was provided by an LED emitting at 470 nm and leads to a photostationary equilibrium between the longest living intermediate LOV^{S}_{390} (85%) and the dark state LOV^{D}_{450} (15%). The values of the percentages were derived from an analysis of the absorption spectra of the dark and illuminated samples (data not shown). The data of Figure 2 show that activation of LOV2 with blue light leads to an increase in the tryptophan fluorescence of about 16%. There is moreover a slight red shift from 340 to 342 nm. Together, these effects suggest that one or more tryptophan residues enter a different environment or change their conformation in the long-living photointermediate LOV^{S}_{390}.

Time-Resolved Fluorescence Measurements. Figure 3A shows the transient tryptophan fluorescence of the isolated LOV2-Jα domain of oat phototropin1. The transient fluorescence signal $\Delta F/F_0$ is defined as the difference of the fluorescence intensity

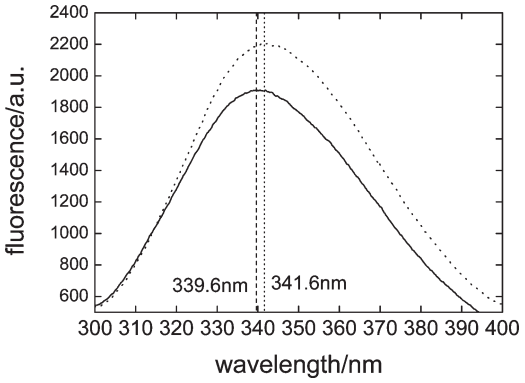

FIGURE 2: Fluorescence emission spectra of the tryptophans of the LOV2-Jα construct in the dark (solid line) and under background illumination from an LED emitting at 470 nm (dotted line). The emission maxima are indicated by vertical dotted lines. Conditions: 10 mM Tris and 30 mM KCl, pH 7.7.

after ($F(t)$) and before (F_0) a laser flash at 460 nm which triggers the photoactivation at $t = 0$, normalized by F_0. Over the entire time course the fluorescence change is positive. In the millisecond time range when the LOV^S_{390} intermediate dominates the signal, the positive fluorescence change is in agreement with the steady-state results. The signal starts at 100 ns with a large positive value and then decreases in several microseconds to a minimum around 20 μs, followed by a slower increase. For times beyond 200 ms the signal rises further, probably due to photoactivation of LOV2 by the excitation light at 280 nm. The time trace was fitted up to 200 ms by a sum of three exponentials with time constants of 3.4 μs, 500 μs, and 4.3 ms. The corresponding fit curves are superimposed on the data.

The time constant of 3.4 μs of the initial decrease strongly suggests that it corresponds to the transition from the triplet LOV^L_{660} to the singlet LOV^S_{390} state as detected by transient absorption spectroscopy (*10*) (for comparison we show the transient absorption time trace at 390 nm in panel C). We suggest that the observed emission signal is due to the phosphorescence of the flavin triplet state LOV^L_{660} which decays with this time constant. The transmission properties of the filter in front of the PM for the experiment of panel A are shown in the inset of Figure 3B. As discussed in Materials and Methods, the flavin phosphorescence will pass through this band-pass filter for wavelengths longer than 680 nm. We therefore measured the transient signal of LOV2-Jα with an additional cutoff filter OG590 in front of the photomultiplier which completely blocks off the tryptophan fluorescence but passes the phosphorescence (see vertical dotted line in inset of Figure 3B). The corresponding data (Figure 3B) show that under these conditions only the 3.4 μs component remains. This proves that the 3.4 μs component is not due to tryptophan emission but most likely to the decay of the triplet phosphorescence. This control experiment moreover shows that the two transitions at 500 μs and 4.3 ms are caused by tryptophan emission in the wavelength range from 305 to 395 nm.

The transitions at 500 μs and 4.3 ms which lead to an increase in tryptophan fluorescence have no counterparts in the UV−vis photocycle of the LOV2-Jα domain (*10*). We verified this for our samples by measuring the transient absorption at 390, 470, and 600 nm from 100 ns to 1 s. As shown in Figure 3C the transient absorption trace at 390 nm which monitors changes sensed by the flavin cofactor is flat from 10 μs to 1 s, indicating that the changes detected by the tryptophans are not sensed by the FMN chromophore in this time range.

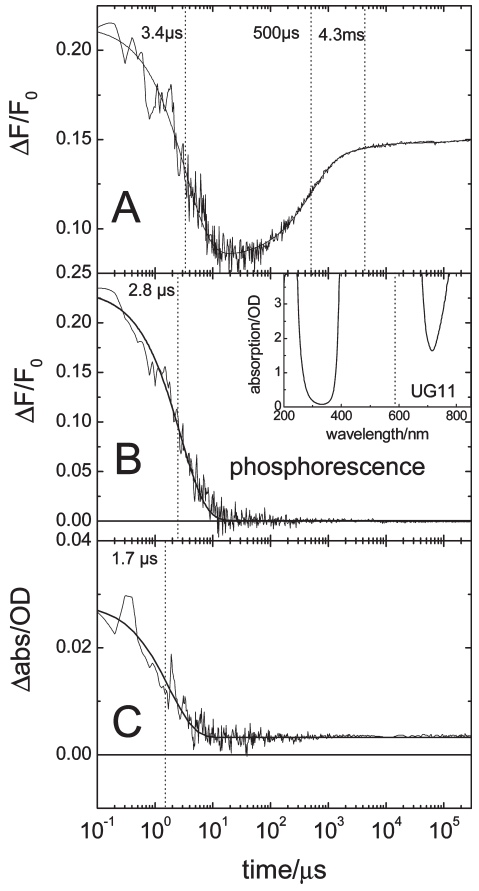

FIGURE 3: (A) Transient tryptophan fluorescence of the LOV2-Jα domain. The signal is the average of 100 scans with waiting times of 80 s between the laser flashes to allow for complete recovery of the cycle. Note the logarithmic time scale. A fit curve corresponding to a sum of three exponentials is superimposed on the data. The corresponding time constants are indicated by the vertical dotted lines. Conditions: 10 mM Tris and 30 mM KCl, pH 7.5, 20 °C. (B) Transient phosphorescence of the LOV2-Jα construct. The signal is an average of 30 scans with waiting times of 80 s between the laser flashes. A fit curve with one exponential is superimposed on the data. The corresponding time constant is indicated by the vertical dotted line. Conditions as in (A). The inset shows the absorption spectrum of the band-pass filter UG11 used in the transient fluorescence experiment. The vertical dotted line indicates the cutoff wavelength of the color filter OG590. (C) Transient absorption signal at 390 nm. Conditions: 10 mM Tris and 30 mM KCl, pH 7.7, 20 °C.

The fluorescence increases beyond 10 μs, when the phosphorescence has completely decayed; 86% of this increase is due to the 500 μs component, and the remaining amplitude stems from the minor 4.3 ms component. A fit of the fluorescence rise with only one exponential was unsatisfactory (data not shown). Presuming a sequential photocycle, this suggests that there are at least three LOV^S_{390} intermediates with identical UV−vis absorption spectrum but which differ in tryptophan fluorescence.

The 500 μs and 4 ms components were absent in the transient fluorescence of the mutant W557S and of the core construct in which the Jα helix is missing. These data are shown in Figure S1 of Supporting Information. Both constructs only contain tryptophan 491. These results suggest that the tryptophan signal is due to W557. Deletion of the Jα helix did not affect the photocycle as measured by transient absorption (data not shown). In agreement with this, the transient fluorescence time traces of Figure S1 in Supporting Information still contain the 3.4 μs component which is due to the flavin phosphorescence and is associated with the formation of the adduct.

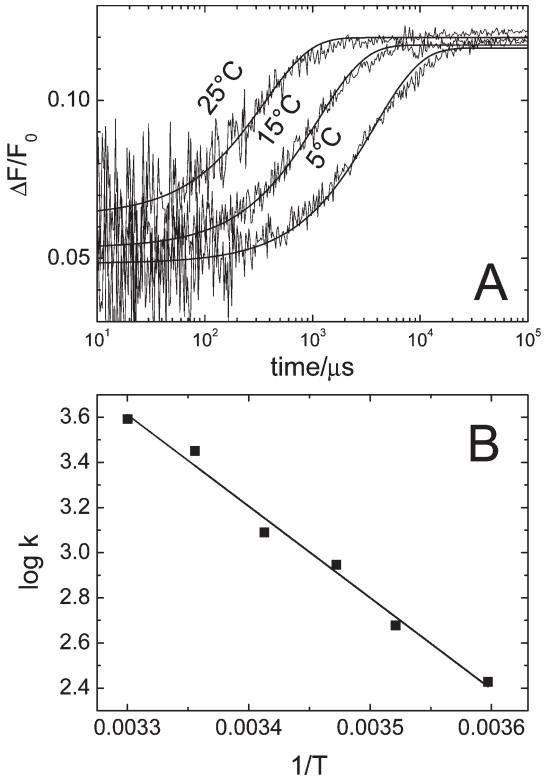

FIGURE 4: (A) Temperature dependence of the transient tryptophan fluorescence of the LOV2-Jα domain. The signals at 5, 15, and 25 °C are an average of 10 scans and are scaled to the same end value at 100 ms. Curves for a single exponential fit of the transient tryptophan fluorescence are superimposed on the data. (B) Arrhenius plot. The rate constants (in units of s^{-1}) were derived by fitting the average of 10 transient fluorescence scans measured at each temperature with a single exponential. The activation energy of 18.2 kcal/mol was calculated from the slope of the linear fit of the logarithm of the rate constants plotted versus $1/T$ (in units of K^{-1}).

Temperature Dependence. To further characterize the photocycle of LOV2-Jα, we calculated the activation energy from the temperature dependence of the rate constant. For this purpose the transient fluorescence was measured at six temperatures between 5 and 30 °C and fitted with a single exponential. This procedure is justified since the data of Figure 4A were acquired with a considerably lower signal-to-noise ratio than those of Figure 3A (average of 10 scans, versus 100 scans for Figure 3A). The activation energy thus refers to the major 500 μs component. The data at 5, 15, and 25 °C are shown in Figure 4A and indicate that the temperature dependence is quite strong. Figure 4B is a plot of the logarithm of the rate constant versus $1/T$. The Arrhenius plot is to a good approximation linear with activation energy of 18.2 kcal/mol, as calculated from the slope of the fit curve. This value is similar to the activation energy of 14.5 kcal/mol reported for the recovery to LOV^{D}_{450} dark state (*15*), in which the structure is reset to its initial conformation.

For comparison we determined the activation energy of the conformational change in the related photoreceptor photoactive yellow protein (PYP) from *H. halophila*. The rationale for doing so is that both LOV2 and PYP are PAS domain proteins with similar structures. PYP is a 125 amino acid long photoreceptor which binds *p*-coumaric acid as the chromophore. Light-induced isomerization initiates a photocycle with several thermal relaxations, which ends in the recovery of the dark state in less than 1 s (*24*). PYP is a PAS-domain protein with an ~30 amino acid long N-terminal extension. This part called the N-terminal cap resembles in many respects the C-terminal Jα helix in the LOV2-Jα domain. Like Jα it packs in the dark against the central β-sheet of the PAS core and contains two short α-helices (see Figure 5C). The N-terminal cap dissociates from the β-sheet and unfolds upon photoactivation (*29*, *30*). The step in the photocycle of PYP associated with this global conformational change is the transition from the I_2 to I_2' intermediate as shown by a number of time-resolved biophysical methods (*31*−*36*). At room temperature and neutral pH this transition occurs around 1 ms and can be monitored very well by transient tryptophan fluorescence spectroscopy, since the fluorescence lifetime of tryptophan W119 in the I_2 and I_2' intermediates differs by a factor of about 20 (*22*, *25*). To determine the temperature dependence of the I_2/I_2' transition, we measured the transient tryptophan fluorescence signal of PYP at pH 7.9 at five temperatures between 5 and 25 °C. The corresponding signals at 5, 15, and 25 °C are shown in Figure 5A. Fit curves with a sum of three exponentials (corresponding to the three sequential transitions from I_1 to I_2, from I_2 to I_2', and the recovery to the dark state) are superimposed on the data. An Arrhenius plot of the rate constant for the I_2/I_2' transition, the second transition in Figure 5A with negative amplitude, is shown in Figure 5B. The plot is to a good approximation linear with activation energy of 18.5 kcal/mol. Remarkably, the rates of the conformational changes in PYP and LOV2-Jα differ only by a factor of about 2 under the investigated conditions. Moreover, the activation energies of the reactions, which in both cases involve the dissociation of a domain from the central β-sheet and its partial unfolding, are nearly identical. We note that an activation energy of 15.5 kcal/mol was obtained for the I_1 to I_2 transition that precedes the I_2 to I_2' transition and has a time constant of about 100 μs (*37*).

DISCUSSION

Three transitions were detected in the transient emission signal in the course of the LOV2-Jα photocycle. Based on the equality of the first time constant with that obtained from transient absorption spectroscopy for the decay of the LOV^{L}_{660} triplet state (Figure 3C) and on its emission spectrum, the 3.4 μs component was assigned to the decay of the LOV^{L}_{660} phosphorescence (Figure 3B). Protein phosphorescence is rarely observed at room temperature (*38*). In the case of LOV2 the FMN chromophore is rigidly held in the binding pocket, as reflected in the vibrational fine structure of its absorption spectrum. The highly protected environment prevents rapid collisional quenching by solvent molecules such as oxygen. The relative amplitudes of the FMN phosphorescence and the tryptophan fluorescence in Figure 3A cannot be compared directly, since the tryptophan emission is excited by the steady-state UV LED, whereas the phosphorescence is excited by the very intense nanosecond laser flash at 460 nm, which leads to a high fraction of molecules cycling of about 30%.

The second time constant of 500 μs was assigned to the dissociation of the Jα helix from the LOV2 core. A binding equilibrium between the LOV core and the Jα helix already exists in the dark (*39*). From the NMR exchange dynamics a value of 1320 s^{-1} was determined for the relaxation rate for fluctuations around this equilibrium (*39*). It is intriguing that the corresponding relaxation time of 750 μs is in the same ball park as the 500 μs time constant for the light-induced dissociation of the Jα helix.

Kinetic evidence for light-induced conformational changes in LOV2 after formation of the FMN adduct (formation of LOV^{S}_{390}, 3.4 μs) was previously obtained from several time-resolved methods.

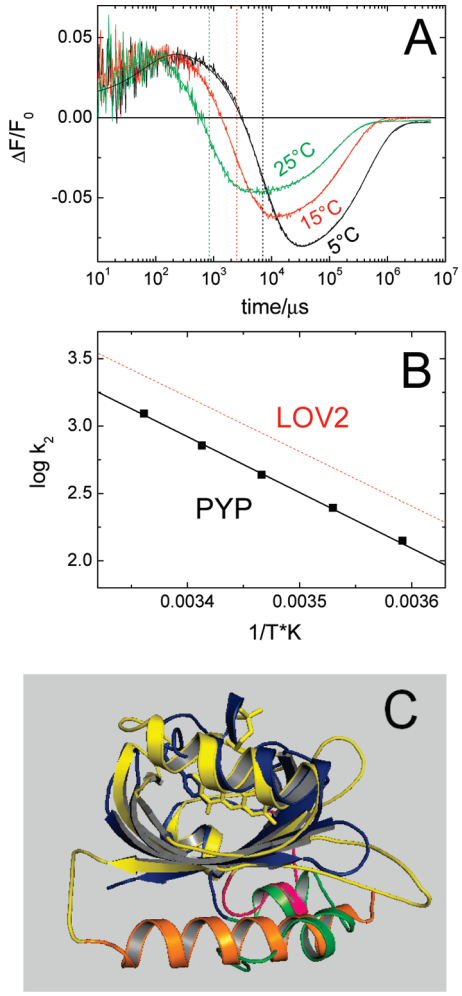

FIGURE 5: (A) Temperature dependence of the transient tryptophan fluorescence of photoactive yellow protein. The signals at 5.4 (black), 15.5 (red), and 24.5 °C (green) are the average of 20 shots. Curves for a three-exponential fit of the transient absorption data are superimposed on the data. The time constants of the I_2/I_2' transition which is associated with a global structural change of the protein are indicated by vertical dotted lines. Conditions: pH 7.9, 30 mM KCl and 10 mM Tris. (B) Arrhenius plot of k_2, the rate constant of the I_2/I_2' transition associated with global conformational changes in PYP. The activation energy of 18.5 kcal/mol was calculated from the slope of the linear fit of the logarithm of the rate constants. The dotted line is the fit curve of the LOV2 data in Figure 4B. (C) Structural alignment of the crystal structures of *H. halophila* PYP (PDB code 2PHY (*44*) and oat LOV2-Jα (2V0U (*41*)) performed with tools available from the RCSB web site (http://www.rcsb.org) using the jCE algorithm (*45*). The domains are colored as follows: PYP, PAS domain (blue) and N-terminal cap (green); LOV2, PAS domain (yellow), Jα helix (orange), and N-terminal turn−helix−turn motif (magenta).

Time-resolved optical rotatory dispersion (ORD) spectroscopy in the far-UV on the same LOV2-Jα construct as used here detected a transition with a time constant of 90 ± 36 μs at 24 °C which was attributed to secondary structural changes of the protein backbone (*16*). In these measurements ORD spectra were recorded with a multichannel detector. The time resolution was achieved by repeating these measurements at 19 discrete time delays between the actinic flash and the recording of the spectra with a gated detector. As the signal-to-noise ratio of the transient ORD data was very low, we presume that the fluorescence change at 500 μs refers to the same transition.

Application of transient grating and lens methods (*18*) to a LOV2-linker construct from *Arabidopsis* phot1 which had a 110 amino acid C-terminal extension to the LOV2 domain led to the detection of two transitions with time constants of 300 μs and 1 ms at room temperature. These values are close to those from our fluorescence measurements at 20 °C (500 μs and 3.4 ms) which were obtained from a different organism and with a C-terminal extension of only 39 amino acids. The authors interpreted these transitions as the dissociation of the linker domain from the LOV2 core (300 μs) followed by unfolding of α-helices in the linker domain (1 ms), respectively.

In a recent step-scan FTIR experiment (*40*) on a mutant from *Arabidopsis* with fast recovery kinetics a transition was detected with a time constant of 120 μs at 40 °C even in a LOV2 domain without a C-terminal extension. Taking into account the activation energy of 18.2 kcal/mol, the value of this time constant agrees within a factor of 2 with the 500 μs transition we observed at 20 °C. This would imply that this component is due to a conformational change of the LOV2 core itself rather than to the dissociation of the linker domain/Jα helix as suggested from the transient grating and lens experiments. These measurements may be reconciled by assuming that the Jα dissociation follows the structural change of the core in a concerted manner. The 120 μs transition was observed in the FTIR time trace at 1537 cm^{-1} which is diagnostic for the secondary structure (amide II). We note that we did not observe a 500 μs component in the fluorescence signal of the core construct.

The above dynamic methods monitor changes in the overall secondary structure (ORD, FTIR) or in the hydrodynamic properties such as the molecular diffusion constant and molecular volume (transient grating and lens, respectively) but provide no spatial resolution. The transient fluorescence method introduced here is a novel complementary method with the following advantages. It provides high time resolution (100 ns), which can be improved further, and a superior signal-to-noise ratio. Whereas each of the above methods covers only a limited time domain, the transient fluorescence signal covers the entire time range from 100 ns to seconds, seven decades in time. The signal is recorded at 132000 discrete time points per time scan. Moreover, being based on the change in tryptophan fluorescence, this method also has considerable spatial resolution since most proteins contain only a few tryptophan residues, which moreover may be mutated away one at the time.

NMR experiments showed that both W491 and W557 are involved in the conformational changes (*14*, *15*, *19*). For the core construct, in which W557 and the Jα helix are missing, we detected no 500 μs component in the fluorescence. This suggests that the fluorescence change is due to W557 or that the Jα helix is required for the occurrence of this component. In the absence of the Jα helix, W491 is exposed to the solvent in the dark (see Figure 1). If light-induced activation does not change that exposure, no change in fluorescence is expected. This interpretation is in agreement with NMR results on the core construct, which show that in the absence of the Jα helix the chemical shifts of W491 in the dark and the light differ very little (*14*). Moreover, the values of the chemical shifts indicate that this tryptophan is solvent-exposed in both states. It is well-known that changes in the polarity of the environment of tryptophan's indole ring affect the wavelength of its emission maximum and its fluorescence quantum yield (*20*). The emission maximum of 340 nm in LOV2-Jα indicates that the average environment of the tryptophans in the dark is rather polar. The light-induced shift to 342 nm indicates a more polar environment suggesting exposure to the aqueous phase in the activated state. On the basis of the structure

in Figure 1, one would expect that W557 is already exposed in the dark state, whereas W491 may become more exposed when the Jα helix dissociates in the light. For the construct W557S, which includes the Jα helix, we did not observe a 500 μs component in the fluorescence either, again suggesting that the fluorescence change is due to W557. For wild-type LOV2-Jα structural changes were observed in the NMR spectra for both tryptophans. NMR would presumably have detected changes for W491 in the W557S mutant as well. The fact that no 500 μs component was observed from W491 in the W557S mutant suggests that the change in the environment of W491 either occurs on a different time scale (e.g., in parallel with the decay of the triplet state at 3.4 μs) or has only a minor effect on the fluorescence quantum yield. The latter may be due to the fact that in both states the fluorescence of W491 is highly quenched by energy transfer to the FMN chromophore minimizing the effect of other decay channels on the fluorescence quantum yield. For LOV2-Jα in the dark state LOV^{D}_{450} we calculated a Förster radius of 13 Å from the spectral overlap of donor and acceptor making the usual assumption of a κ^2 value of $^2/_3$. Since the Förster radius is comparable to the W491−FMN distance of 14 Å which we obtained from the published oat LOV2-Jα X-ray structure (*41*), considerable energy transfer occurs between this tryptophan and the flavin cofactor in the dark.

Transient absorption measurements indicate that the LOV^{S}_{390} signaling state, absorbing at 390 nm, is formed in about 4 μs and decays in seconds (see also Figure 3C). The fluorescence results show on the other hand that between 4 μs and the end of the cycle two additional transitions occur. There are thus at least three LOV^{S}_{390} intermediates, confirming previous reports (*16*, *18*, *40*, *42*, *43*). The very large activation energy of 18.2 kcal/mol for the 500 μs transition suggests that it is associated with a major conformational change.

For the global structural change in PYP which occurs in the transition from the I_2 to the I_2' intermediate we found an almost identical value of 18.5 kcal/mol. This remarkable similarity in both the barrier height and time constant of the light-induced conformational changes in the PAS-domain proteins LOV2-Jα and PYP is presumably due to the structural similarities between the two systems. This result is nevertheless surprising as the chromophore and photochemistry of both photoreceptors are quite different. While illumination of LOV2 leads to a transient covalent bond between the flavin and a conserved cysteine residue, the absorption of light by PYP causes a *trans*−*cis* isomerization of the already covalently bound chromophore *p*-coumaric acid. X-ray diffraction studies of illuminated protein crystals showed that in PYP the isomerized chromophore swings out of the binding pocket, while in LOV2 apart from the covalent bond formation only minor rearrangements in the chromophore binding pocket occur (*12*, *35*). However, in a recent study investigating the light-induced conformational changes of an oat LOV2 construct which included the Jα helix as well as a short conserved N-terminal extension to the PAS-domain that forms a turn−helix−turn motif, the propagation of the structural signal from the FMN to the surface of the protein was found to be similar to that in PYP (*41*). A structural alignment of the crystal structures of this LOV2 construct and PYP is shown as an overlay in Figure 5C. In addition to the structural similarity of the PAS domains of the two proteins (upper part of the figure) we see that the two helices of the N-terminal cap (shown in green) of PYP align very well with the C-terminal end of the Ja helix (orange) as well as with the N-terminal extension of LOV2 (magenta). Remarkably, the light-induced conformational changes of the LOV2 crystal are concentrated in this area (*41*). We therefore suggest that the kinetics of the formation of the signaling state in PYP and LOV2-Jα are determined by the common PAS-domain fold of the two proteins and by the similar interaction of the N-terminal cap and C-terminal Jα linker, respectively, with the central β-sheet of the PAS-domain core and not by the specific interaction between the different chromophores and the protein.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 with the transient fluorescence data for the W557S and PAS-core mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Huala, E., Oeller, P. W., Liscum, E., Han, I. S., Larsen, E., and Briggs, W. R. (1997) *Arabidopsis* NPH1: A protein kinase with a putative redox-sensing domain. *Science 278*, 2120–2123.
2. 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.
3. 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.
4. 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.
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. 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.
7. Christie, J. M., and Briggs, W. R. (2005) Blue light sensing and signaling by the phototropins, in Handbook of Photosensory Receptors (Briggs, W. R., and Spudich, J. L., Eds.) pp 277−303, Wiley-VCH, Weinheim.
8. Salomon, M., Knieb, E., von Zeppelin, T., and Rudiger, W. (2003) Mapping of low- and high-fluence autophosphorylation sites in phototropin 1. *Biochemistry 42*, 4217–4225.
9. Kennis, J. T. M., Crosson, S., Gauden, M., van Stokkum, I. H. M., Moffat, K., and van Grondelle, R. (2003) Primary reactions of the LOV2 domain of phototropin, a plant blue-light photoreceptor. *Biochemistry 42*, 3385–3392.
10. Swartz, T. E., Corchnoy, S. B., Christie, J. M., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2001) The photocycle of a flavin-binding domain of the blue light photoreceptor phototropin. *J. Biol. Chem. 276*, 36493–36500.
11. Salomon, M., Eisenreich, W., Durr, H., Schleicher, E., Knieb, E., Massey, V., Rudiger, W., Muller, F., Bacher, A., and Richter, G. (2001) An optomechanical transducer in the blue light receptor phototropin from Avena sativa. *Proc. Natl. Acad. Sci. U.S.A. 98*, 12357–12361.
12. Crosson, S., and Moffat, K. (2002) Photoexcited structure of a plant photoreceptor domain reveals a light-driven molecular switch. *Plant Cell 14*, 1067–1075.
13. Corchnoy, S. B., Swartz, T. E., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2003) Intramolecular proton transfers and structural changes during the photocycle of the LOV2 domain of phototropin 1. *J. Biol. Chem. 278*, 724–731.
14. Harper, S. M., Neil, L. C., and Gardner, K. H. (2003) Structural basis of a phototropin light switch. *Science 301*, 1541–1544.
15. Harper, S. M., Neil, L. C., Day, I. J., Hore, P. J., and Gardner, K. H. (2004) Conformational changes in a photosensory LOV domain monitored by time-resolved NMR spectroscopy. *J. Am. Chem. Soc. 126*, 3390–3391.

16. Chen, E. F., Swartz, T. E., Bogomolni, R. A., and Kliger, D. S. (2007) A LOV story: The signaling state of the Phot1 LOV2 photocycle involves chromophore-triggered protein structure relaxation, as probed by far-UV time-resolved optical rotatory dispersion spectroscopy. *Biochemistry 46*, 4619–4624.
17. 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.
18. 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.
19. Harper, S. M., Christie, J. M., and Gardner, K. H. (2004) Disruption of the LOV-J alpha helix interaction activates phototropin kinase activity. *Biochemistry 43*, 16184–16192.
20. Lakowicz, J. R. (2006) Protein fluorescence, in Principles of Fluorescence Spectroscopy, pp 530−577, Springer, New York.
21. Neuweiler, H., Sharpe, T. D., Johnson, C. M., Teufel, D. P., Ferguson, N., and Fersht, A. R. (2009) Downhill versus barrier-limited folding of BBL 2: Mechanistic insights from kinetics of folding monitored by independent tryptophan probes. *J. Mol. Biol. 387*, 975–985.
22. Hoersch, D., Otto, H., Cusanovich, M. A., and Heyn, M. P. (2008) Distinguishing chromophore structures of photocycle intermediates of the photoreceptor PYP by transient fluorescence and energy transfer. *J. Phys. Chem. B 112*, 9118–9125.
23. Hoersch, D., Otto, H., Wallat, I., and Heyn, M. P. (2008) Monitoring the conformational changes of photoactivated rhodopsin from microseconds to seconds by transient fluorescence spectroscopy. *Biochemistry 47*, 11518–11527.
24. Cusanovich, M. A., and Meyer, T. E. (2003) Photoactive yellow protein: A prototypic PAS domain sensory protein and development of a common signaling mechanism. *Biochemistry 42*, 4759–4770.
25. Otto, H., Hoersch, D., Meyer, T. E., Cusanovich, M. A., and Heyn, M. P. (2005) Time-resolved single tryptophan fluorescence in photoactive yellow protein monitors changes in the chromophore structure during the photocycle via energy transfer. *Biochemistry 44*, 16804–16816.
26. Kyndt, J. A., Vanrobaeys, F., Fitch, J. C., Devreese, B. V., Meyer, T. E., Cusanovich, M. A., and Van Beeumen, J. J. (2003) Heterologous production of *Halorhodospira halophila* holo-photoactive yellow protein through tandem expression of the postulated biosynthetic genes. *Biochemistry 42*, 965–970.
27. Gauden, M., Crosson, S., van Stokkum, I. H. M., van Grondelle, R., Moffat, K., and Kennis, J. T. M. (2004) Low-temperature and fine-resolved spectroscopic characterization of the LOV2 domain of *Avena sativa* phototropin 1. *Femtosecond Laser Appl. Biol. 5463*, 97–104.
28. Borucki, B., Otto, H., and Heyn, M. P. (1999) Reorientation of the retinylidene chromophore in the K, L, and M intermediates of bacteriorhodopsin from time-resolved linear dichroism: Resolving kinetically and spectrally overlapping intermediates of chromoproteins. *J. Phys. Chem. B 103*, 6371–6383.
29. Harigai, M., Imamoto, Y., Kamikubo, H., Yamazaki, Y., and Kataoka, M. (2003) Role of an N-terminal loop in the secondary structural change of photoactive yellow protein. *Biochemistry 42*, 13893–13900.
30. Hoersch, D., Otto, H., Joshi, C. P., Borucki, B., Cusanovich, M. A., and Heyn, M. P. (2007) Role of a conserved salt bridge between the PAS core and the N-terminal domain in the activation of the photoreceptor photoactive yellow protein. *Biophys. J. 93*, 1687–1699.
31. Brudler, R., Rammelsberg, R., Woo, T. T., Getzoff, E. D., and Gerwert, K. (2001) Structure of the I-1 early intermediate of photoactive yellow protein by FTIR spectroscopy. *Nat. Struct. Biol. 8*, 265–270.
32. Chen, E. F., Gensch, T., Gross, A. B., Hendriks, J., Hellingwerf, K. J., and Kliger, D. S. (2003) Dynamics of protein and chromophore structural changes in the photocycle of photoactive yellow protein monitored by time-resolved optical rotatory dispersion. *Biochemistry 42*, 2062–2071.
33. Hendriks, J., Gensch, T., Hviid, L., van der Horst, M. A., Hellingwerf, K. J., and van Thor, J. J. (2002) Transient exposure of hydrophobic surface in the photoactive yellow protein monitored with Nile red. *Biophys. J. 82*, 1632–1643.
34. Borucki, B., Devanathan, S., Otto, H., Cusanovich, M. A., Tollin, G., and Heyn, M. P. (2002) Kinetics of proton uptake and dye binding by photoactive yellow protein in wild type and in the E46Q and E46A mutants. *Biochemistry 41*, 10026–10037.
35. Genick, U. K., Borgstahl, G. E. O., Ng, K., Ren, Z., Pradervand, C., Burke, P. M., Srajer, V., Teng, T. Y., Schildkamp, W., McRee, D. E., Moffat, K., and Getzoff, E. D. (1997) Structure of a protein photocycle intermediate by millisecond time-resolved crystallography. *Science 275*, 1471–1475.
36. Hoersch, D., Otto, H., Cusanovich, M. A., and Heyn, M. P. (2009) Time-resolved spectroscopy of dye-labeled photoactive yellow protein suggests a pathway of light-induced structural changes in the N-terminal cap. *Phys. Chem. Chem. Phys. 11*, 5437–5444.
37. Meyer, T. E., Tollin, G., Hazzard, J. H., and Cusanovich, M. A. (1989) Photoactive yellow protein from the purple phototrophic bacterium, *Ectothiorhodospira halophila*. Quantum yield of photobleaching and effects of temperature, alcohols, glycerol, and sucrose on kinetics of photobleaching and recovery. *Biophys. J. 56*, 559–564.
38. Vanderkooi, J. M., Calhoun, D. B., and Englander, S. W. (1987) On the prevalence of room-temperature protein phosphorescence. *Science 236*, 568–569.
39. Yao, X. L., Rosen, M. K., and Gardner, K. H. (2008) Estimation of the available free energy in a LOV2-J alpha photoswitch. *Nat. Chem. Biol. 4*, 491–497.
40. 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.
41. Halavaty, A. S., and Moffat, K. (2007) N- and C-terminal flanking regions modulate light-induced signal transduction in the LOV2 domain of the blue light sensor phototropin 1 from *Avena sativa*. *Biochemistry 46*, 14001–14009.
42. 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.
43. Alexandre, M. T. A., van Grondelle, R., Hellingwerf, K. J., and Kennis, J. T. M. (2009) Conformational heterogeneity and propagation of structural changes in the LOV2/J alpha domain from *Avena sativa* phototropin 1 as recorded by temperature-dependent FTIR spectroscopy. *Biophys. J. 97*, 238–247.
44. Borgstahl, G. E. O., Williams, D. R., and Getzoff, E. D. (1995) 1.4 Å structure of photoactive yellow protein, a cytosolic photoreceptor: Unusual fold, active site, and chromophore. *Biochemistry 34*, 6278–6287.
45. Shindyalov, I. N., and Bourne, P. E. (1998) Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. *Protein Eng. 11*, 739–747.