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Rationally improving LOV domain–based photoswitches

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Abstract

Genetically-encoded protein photosensors, including the LOV (light, oxygen, voltage) domain, are promising tools for engineering optical control of cellular behavior. We are only beginning to understand how to couple these light detectors to effectors of choice. We report a method that increases the dynamic range of an artificial photoswitch based on the LOV2 domain of *A. sativa* phototropin1 (AsLOV2). This approach can potentially be used to improve many AsLOV2-based photoswitches.

INTRODUCTION

Photoswitchable proteins offer the unique ability to perturb living cells, tissues, and intact organisms with high spatial and temporal precision¹. In particular, genetically encoded photoswitches such as the LOV and phytochrome domains can be conveniently used in many different experimental contexts^{2–7}. The LOV2 domain of *A. sativa* phototropin 1 (AsLOV2) has proven especially useful for controlling functionally diverse effectors including DNA binding proteins, enzymes, and small GTPases^{2,3,5}. To aid the optimization of these artificial LOV-effector fusions, biophysical investigations are needed to understand

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D.S., designed and performed experiments, analyzed data and wrote the paper; X.Y., designed and performed experiments, analyzed data and wrote the paper; G.G., performed experiments and analyzed data; M.K.R., designed experiments, analyzed data and wrote the paper; K.H.G., designed experiments, analyzed data and wrote the paper; T.R.S., designed experiments, analyzed data and wrote the paper.

how these genetically-encoded light detectors are coupled both to their natural effectors and effectors of our choosing⁸.

AsLOV2 and other LOV domains were first identified in phototropins, a class of light-activated serine/threonine kinases that mediate phototropism and other blue light responses in plants and algae^{9,10}. LOV domains share the mixed α/β fold common to the Per-ARNT-Sim class of environmental sensors⁸ and carry a flavin chromophore (either FMN or FAD), that broadly absorbs blue light. While these cofactors are non-covalently associated with the surrounding LOV domain in the dark, photoexcitation generates a covalent adduct between the flavin C4a carbon atom and an invariant cysteine residue^{11,12}. After illumination ceases, this adduct spontaneously decays to the dark state¹¹.

For phototropin LOV domains, the formation of this adduct is associated with kinase activation. In the dark state, the second LOV domain (LOV2) acts as a dark-state repressor of phototropin's kinase activity¹³. Lit-state adduct formation promotes the undocking and unfolding of the carboxy-terminal J α helix^{14,15}, but how structural and dynamic changes within the LOV2 domain couple these events to kinase activation has yet to be fully elucidated^{14,16}.

Our understanding of the light-regulated undocking of the J α helix in AsLOV2 has paved the way for its use as the input component of designed proteins^{2,3,5}. In each of these three engineered proteins, effector activity is suppressed in the dark, concomitant with the J α helix being in the folded and docked conformation. Although the J α is mostly docked in the dark state and mostly undocked in the lit state, both conformations are populated in both the dark and lit states (Fig. 1a). The equilibrium between these two populations dramatically shifts between the dark and lit states, providing a large thermodynamic driving force ($\Delta G \approx 4$ kcal/mol) which can be used to regulate the activity of an effector¹⁷.

Despite this potentially large driving energy, initial attempts at designed AsLOV2-containing photoswitches have generally suffered from a low photoswitching dynamic range^{2,3,5}. This is exemplified by our design of an end-to-end fusion of AsLOV2 and the *E. coli* trp repressor (TrpR) that selectively binds DNA when illuminated². In our initial design, designated "LovTAP," the dynamic range is modest, with only a five-fold change in DNA affinity between the dark and lit states². Here we present an analytical model where mutations that stabilize LOV–J α docking increase the dynamic range of AsLOV2-based photoswitches. We report four such mutations, and demonstrate that these increase the dynamic range of the LovTAP photoswitch to 70-fold.

RESULTS

Analytical model of photoswitching

We sought to identify mutations that improve coupling between the initiating photochemical events at the AsLOV2 chromophore and the resultant activity of an effector domain. Mutations throughout the composite signaling protein (including the FMN binding pocket, the β sheet, the J α linker, and the effector domain) could potentially improve this coupling. However, it is not obvious what type of mutations to make, or in which regions to make

them. Furthermore, because our goal is to increase the ratio of lit- to dark-state activity, mutations that preferentially stabilize either the J α -docked conformation in the dark or the J α -undocked conformation in the lit state would seem to offer the most benefit. Yet, the task of rationally predicting such mutations or screening for them is daunting.

To narrow the search for such mutations, we developed an analytical model of photoswitching. We focused on how fusion with an effector domain might itself restrict the dynamic range of the photoswitch. In isolated AsLOV2, the J α helix is normally docked against the LOV core. When AsLOV2 is part of a composite signaling protein, a competition for J α helix docking is set up between the components¹⁵. To create a molecule that signals effectively, fusion with an effector domain should introduce intra- or intermolecular interactions. These new interactions may favor the undocking of the J α helix from the LOV core, thereby increasing the equilibrium constant of helix undocking by the factor

$$x_{\text{fus}} = \frac{K_{\text{helix}}^{\text{iso}}}{K_{\text{helix}}^{\text{fus}}} \quad \text{Eq. 1}$$

where the equilibrium constant of helix undocking is represented by K_{helix} , while superscripts ^{iso} and ^{fus} refer to isolated and fused AsLOV2, respectively (Fig. 1a–b). The shift in docking equilibrium, x_{fus} , will depend on the strength of the new interactions in the fused state and on the effector's intrinsic conformational equilibrium.

Bringing the conformational equilibrium between active and inactive states into an appropriate regime (Fig. 1b) is a central challenge of engineering allosteric proteins and nucleic acids¹⁸. Photoswitching requires that the effector activity be suppressed in the dark state. However, this suppression is only achieved when the J α helix is docked to the LOV core. The new interactions introduced by fusion likely favor undocking of the helix, and increase unwanted dark state effector activity. Potentially, the active conformation can predominate even in the dark state, such that the molecule is “always on,” and cannot be further activated by photoexcitation (Fig. 1b).

The undesirable effects of competition for the J α helix might be mitigated using mutations that stabilize LOV–J α docking. These mutations would change the equilibrium constant of helix undocking by the factor

$$x_{\text{mut}} = \frac{K_{\text{helix}}^{\text{wt}}}{K_{\text{helix}}^{\text{mut}}} \quad \text{Eq. 2}$$

where the superscripts ^{wt} and ^{mut} refer to wild-type and mutated species, respectively. By definition, for isolated AsLOV2, $x_{\text{mut}} = 1$. For mutations that destabilize the LOV–J α interaction (e.g. I532E¹⁵) $x_{\text{mut}} < 1$. For mutations that stabilize LOV–J α docking, and thereby reduce dark state effector activity, $x_{\text{mut}} > 1$. To be independent of x_{fus} , x_{mut} should not depend on coupling between AsLOV2 and the effector or on the effector's intrinsic conformational equilibrium, but could depend on the photo-state.

To represent the combined effects of fusion and mutation on LOV-J α docking, we used the parameter

$$x_{\text{eff}} = x_{\text{mut}} x_{\text{fus}} \quad \text{Eq. 3}$$

Generally, when $x_{\text{eff}} \ll 1$, the J α helix is undocked from the LOV domain, and the effector domain is active. Conversely, as x_{eff} increases, LOV-J α docking increases and effector activity is suppressed (Fig. 1c).

Intuitively, one expects that mutations which increase LOV-J α docking equally in the lit and dark states would have no net effect on the photoswitching dynamic range (assuming that coupling between LOV-J α and effector is the same in both states, i.e. that x_{fus} is independent of photo-state). We questioned this supposition. In LovTAP, the DNA binding activity obeys a linked equilibrium model in which helix undocking promotes an active, binding-competent conformation (Fig. 1c). The competition for the J α helix reduces the observed DNA binding affinity by the fraction of the population that is in the active conformation:

$$K_{\text{obs}}^{\text{dark}} = K_{\text{DNA}} \frac{K_{\text{helix}}^{\text{dark}}}{K_{\text{helix}}^{\text{dark}} + x_{\text{eff}}^{\text{dark}}} \quad \text{Eq. 4}$$

and

$$K_{\text{obs}}^{\text{lit}} = K_{\text{DNA}} \frac{K_{\text{helix}}^{\text{lit}}}{K_{\text{helix}}^{\text{lit}} + x_{\text{eff}}^{\text{lit}}} \quad \text{Eq. 5}$$

Under the assumption that x_{eff} is the same in the lit and dark states, the photoswitching dynamic range becomes:

$$\beta = \frac{K_{\text{obs}}^{\text{lit}}}{K_{\text{obs}}^{\text{dark}}} = \frac{1 + \frac{x_{\text{eff}}}{K_{\text{helix}}^{\text{dark}}}}{1 + \frac{x_{\text{eff}}}{K_{\text{helix}}^{\text{lit}}}} \quad \text{Eq. 6}$$

Thus, for $x_{\text{eff}} < K_{\text{helix}}^{\text{dark}} < K_{\text{helix}}^{\text{lit}}$, $\beta \approx 1$, i.e. nearly all of the effector is active, even in the dark state. Photoexcitation may further shift the equilibrium to the active state, e.g., from 99% active to 99.9% active, but this has a negligible functional effect. On the other hand, for $x_{\text{eff}} > K_{\text{helix}}^{\text{lit}} > K_{\text{helix}}^{\text{dark}}$, the maximum photoswitching dynamic range is obtained as $\beta \approx K_{\text{helix}}^{\text{lit}} / K_{\text{helix}}^{\text{dark}}$. For intermediate values of x_{eff} , however, β increases rapidly with x_{eff} , even assuming that x_{eff} is the same in the lit and dark states (Fig. 1c and Fig. 2). Because β is small for LovTAP (5.3)², we expect that the protein is near the lower end of this intermediate regime and we should be able to increase β with mutations that stabilize LOV-J α docking.

Mutational stabilization of LOV-J α docking

We used available computational tools to predict mutations that increase LOV-J α docking either by increasing helix propensity, new tertiary interactions or increasing the electrostatic

complementarity (Supplementary Fig. 1 and online methods). We quantified this stabilization (expressed as x_{mut} in our model) for four point mutations and two tandem mutations using nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. The mutations all stabilized helix docking in both the dark and lit states (Fig. 2a, Supplementary Fig. 2 and online methods). Generally the CD-derived $x_{\text{mut}}^{\text{lit}}$ values were similar to the NMR-derived $x_{\text{mut}}^{\text{dark}}$ values (Fig. 2a). However, for the G528A mutant and the G528A-N538E tandem mutant, $x_{\text{mut}}^{\text{dark}} \ll x_{\text{mut}}^{\text{lit}}$. This may reflect experimental error in determining these quantities (online methods), or it may reveal that for some mutations $x_{\text{mut}}^{\text{dark}} \neq x_{\text{mut}}^{\text{lit}}$.

Mutants that increase LOV-J α affinity lead to improvement in photoswitching

To test how mutations that stabilize LOV-J α interaction in the isolated AsLOV2 domain influence the dynamic range of the Lov-TAP photoswitch, we used a restriction enzyme protection assay to measure the DNA binding affinity of LovTAP (Fig. 2b)2. We found that the G528A mutation produced a ~ 2-fold decrease in dark-state DNA binding. Similarly, the I532A and N538E mutations each produced a ~ 4-fold decrease in dark state DNA binding (Table 1). Notably, the N538E mutation decreased lit-state DNA binding ~ 2-fold. This decrease may result from the introduction of a negatively charged residue near the DNA binding site of TrpR and highlights the fact that x_{mut} and x_{fus} may not be fully independent. When introduced together, the G528A and N538E mutations decreased dark-state DNA binding affinity by ~ 9-fold, without changing the lit-state binding, and increase β from ~ 5 to ~ 70 (Table 1).

We compared these DNA binding affinities to the prediction of our model. To determine the relationship between predicted and observed affinities for the dark state, we need a reference value of x_{fus} (Eq. 3). In WT LovTAP2, $\beta = 5.3$ and, by Eq. 6, $x_{\text{fus}} = x_{\text{eff}} = 0.28$. We used this value of x_{fus} as the reference for calculating values of x_{eff} in mutant species. We found that the predicted dark-state DNA binding affinities agreed well with the observed values (Fig. 2c). According to Eq. 6, the lit-state DNA binding affinity becomes independent of x_{eff} when $x_{\text{eff}} < 10$. Consistent with this prediction, all of the mutants had lit-state DNA binding affinities that were approximately equal to the lit-state affinity of the original LovTAP design (Fig. 2c).

Discussion

Fusion of AsLOV2 with an effector introduces new interactions and a competition that depopulates the marginally-stable J α -docked conformation in the dark state. Because suppression of effector activity requires helix docking, the competition leads to undesired activity in the dark. Conversely, mutations that stabilize the J α -docked conformation should shift this docking equilibrium back to a regime where the helix is mostly docked in the dark state. From this condition, light can again be used to drive the protein from a largely docked state to a largely undocked state (Figs. 1c and 2c).

Based on this analysis, we identified helix-stabilizing mutations and demonstrated that the mutations generally stabilized the LOV-J α interaction equally in the dark and lit states. As predicted by our model, these mutations increased the dynamic range of our designed DNA

binding photoswitch by up to ~10-fold, to a final lit-to-dark activity ratio of $\beta \approx 70$. Moreover, our model suggested that greater dynamic range can be achieved by further stabilization of the LOV–J α interaction (Fig. 2c).

A systematic mutational screen of the J α helix may uncover other stabilizing mutations. However, for $x_{\text{eff}} \gg 10$, the helix docked, inactive state predominates even in the lit state. In this regime, mutations that equally stabilize LOV–J α interaction in the dark and lit states no longer offer an increase in photoswitching. Additional improvements require the discovery of mutations that act differentially in the dark and lit states, or the exploitation of dimeric or multimeric systems in ways that offer cooperativity (online methods).

Other designed AsLOV2-based photoswitches also rely on helix docking for dark state suppression of activity^{3,5}. In these designs, as in LovTAP, fusion with the effector domain likely introduces competition for helix docking that increases dark state activity and limits the dynamic range of photoswitching. Because the mutations described here are in the LOV domain itself, they should reduce undesirable dark state effector activity and improve these other designs and other future AsLOV2-based photoswitches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Conformational and binding equilibria in AsLOV2 and LovTAP. **(a)** Photoexcitation ($h\nu$) of AsLOV2 (blue) is accompanied by displacement and unfolding of the J α helix (dark blue). **(b)** In AsLOV2 (left), photoactivation shifts the equilibrium from mostly docked to mostly undocked. Black lines, dark-state free energy surfaces; dashed lines, lit-state free energy surfaces. Upon fusion with an effector (center), the equilibrium is shifted due to interactions with the effector and the helix is mostly undocked in the dark and lit states. As a consequence, the effector is mostly active in both states. Helix stabilizing mutations specifically stabilize the docked state ($G = -RT \ln x_{\text{eff}}$), bringing the equilibrium back into a regime where the effector is mostly inactive in the dark, and photoactivation shifts the equilibrium from mostly inactive to mostly active. **(c)** K_{helix} , equilibrium constant of helix undocking for the isolated LOV2 domain. The fusion protein LovTAP is in equilibrium between an inactive conformation (left), in which the shared helix is docked on the LOV domain, and an active conformation (center), in which it is docked on the TrpR domain. Due to competition by TrpR (orange) for the helix, the equilibrium constant of the helix undocking reaction, K_{helix} , is increased by a factor $1/x_{\text{eff}}$. In the active conformation, LovTAP binds DNA with an intrinsic association constant K_{DNA} .

Figure 2.

Mutational stabilization LOV-J α association and its effect on DNA binding activity in LovTAP. **(a)** Comparison of CD-derived x_{mut}^{lit} with NMR-derived x_{mut}^{dark} for AsLOV2 with the indicated mutations. The identity line is shown as a reference. **(b)** Gel image of RsaI protection assay for LovTAP(G528A, N538E). Open and closed triangles denote reactant and product bands, respectively. **(c)** Effects of the helix-stabilizing mutations on LovTAP. In the upper panel, observed affinity constants (K_{obs}) are plotted against x_{eff} . Large data points are average K_{obs} values, and small points show individual measurements. The data are compared to Eqs. 4 and 5 (solid and dashed lines). For wild-type LovTAP, $x_{eff} = x_{fus}$ is calculated using Eq. 3. For mutants, x_{mut} is determined by NMR, and $x_{eff} = x_{mut} x_{fus}$. In the lower panel, $\beta = K_{obs}^{lit}/K_{obs}^{dark}$ is plotted as a function of x_{eff} and compared to Eq. 6. The shaded regions indicate regimes where β is relatively insensitive to changes in x_{eff} .

Table 1

Helix stabilization and DNA binding parameters

	AsLOV2				LovTAP		
	pU ^{dark} (%)	pU ^{lit} (%)	x ^{dark} _{mut}	x ^{lit} _{mut}	K ^{dark} _{obs} (10 ⁵ M ⁻¹)	K ^{lit} _{obs} (10 ⁵ M ⁻¹)	β
WT	5.98 ± 0.08	91 ± 6	1 ^a	1 ^a	14 ± 5	70 ± 20	5 ± 3
G528A	4.5 ± 0.9	70 ± 10	1.3 ± 0.3	6 ± 2	6 ± 3	80 ± 30	13 ± 9
I532A	1.76 ± 0.03	80 ± 10	3.55 ± 0.08	3 ± 2	3 ± 2	110 ± 30	30 ± 20
N538E	0.74 ± 0.02	60 ± 10	8.5 ± 0.3	7 ± 3	3 ± 1	30 ± 20	9 ± 7
G528A–N538E	N.D.	40 ± 10	12 ± 2 ^b	15 ± 6	1.4 ± 0.4	90 ± 20	70 ± 20
D419K–I532A	0.73 ± 0.04	50 ± 10	8.6 ± 0.5	9 ± 3	N.D.	N.D.	N.D.

^aby definition

^bcalculated from G528A and N538E single mutant data