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Mechanism Based Tuning of a LOV Domain Photoreceptor

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Abstract

Phototropin-like LOV domains form a cysteinyl-flavin adduct in response to blue light, but show dramatic variation in output signal and the lifetime of the photo-adduct signaling state. Mechanistic studies of the slow-cycling fungal LOV-photoreceptor Vivid reveal the importance of reactive cysteine conformation, flavin electronic environment and solvent accessibility for adduct scission and thermal reversion. Proton inventory, pH-effects, base catalysis and structural studies implicate flavin N₅ deprotonation as rate-determining for recovery. Substitutions of active site residues Ile74, Ile85, Met135 and Met165 alter photoadduct lifetimes by over four orders of magnitude in VVD and similar changes in other LOV proteins show analogous effects. Adduct state decay rates also correlate with changes in conformational and oligomeric properties of the protein necessary for signaling. These findings link natural sequence variation of LOV domains to function and provide a means to design broadly reactive light-sensitive probes.

Light Oxygen Voltage (LOV) domains sense and respond to environmental stimuli by converting changes in cofactor chemical state to alterations in protein:protein interactions¹. A subset of LOV domains bind flavin cofactors and function as blue-light photoreceptors in plants², bacteria and fungi^{3–8}, albeit with considerable differences in photocycle kinetics and output mechanisms among family members.

These flavin-binding LOV domains act as reversible photo-switches to regulate a diverse array of blue-light responses, including phototropism², chloroplast movement², resetting of circadian clocks⁹, and cell-cell attachment in bacteria⁴. These proteins can bind either FMN ('1') or FAD ('2'), but all form a cysteinyl flavin C4a adduct following excitation with blue light (Fig. 1). Despite undergoing essentially the same photochemical reaction, the phototropin-like LOV domains can be broken down into two groups based on their light-adapted or "on" state lifetimes: (1) fast cyclers with short-lived adduct states, exemplified by the LOV2 domains of phototropins, and (2) the slow cyclers with long-lived (stable) photo-adducts, exemplified by some phototropin LOV1 domains and the bacterial and fungal LOV photoreceptors.

Whereas phototropin LOV domains have adduct state lifetimes on the order of seconds, the bacterial and fungal LOV domain photoreceptors have dramatically extended photocycles that range from hours to days. In this class, studies thus far have been confined to the photoreceptors YtvA^{3,10}, LOVK⁴, a series of LOV histidine kinases^{7,11}, and the circadian clock proteins FKF-1¹², WC-1⁹ and VVD^{6,8}. These proteins have shown a tendency to form LOV:LOV dimers, which are either constitutive (YtvA¹⁰) or undergo rapid interconversion following photoexcitation (VVD¹³). Isolated domains for the fast cycling phototropins also dimerize but how dimerization relates to function is not yet clear^{14,15}. Currently, it is not well understood

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how cysteinyl adduct stability varies so much within the LOV family and importantly, how it relates to the conformational dynamics of the light-adapted state that ultimately produces output signals. Phototropin solvent isotope effects (SIEs) suggest that the rate-limiting step in thermal reversion (adduct decay) involves deprotonation of the N5 position of the flavin ring^{16–18}. Notably, the dependence of adduct decay on pH varies considerably among many LOV proteins. For instance, phot1 AsLOV2 demonstrates an SIE of adduct decay equal to 3 (and a SIE of adduct formation equal to 5) when reconstituted in pure H₂O or D₂O ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$)¹⁶. The decay rate in AsLOV2 is not appreciably affected by the bulk pH and shows only a 2-fold increase in rate from pH 5–10 with an apparent pK_a of 6.8¹⁶. On the other hand, the decay rates of *C. reinhardtii* LOV1 domains are strongly affected by bulk pH, and show an order of magnitude increase in rate with increasing pH (pK_a~5.3)¹⁹. Notably, the decay rates of phototropin-like LOV domains are accelerated several orders of magnitude by relatively small bases such as imidazole²⁰. Details of how bases catalyze thermal reversion in LOV domains are lacking, although a number of possible mechanisms include: a direct or general base effect on N5²⁰, steric stabilization of the ground state²¹, interruption of a hydrogen bonding network that stabilizes N5 protonation²⁰, or changes in solvation of the active site. The biological role for the diverse range of adduct-state lifetimes has remained elusive. However, recent characterization of a fungal LOV domain photoreceptor (VVD) whose function is well defined, promises to provide a link between flavin reactivity and signaling mechanism^{8,13}.

VVD is a blue-light photoreceptor containing a single LOV domain that regulates carotenoid production as well as attenuation of circadian clock responses under changing levels of blue light^{22–24} (Fig. 1). Photo-adduct formation induces conformational changes within an N-terminal element external to the PAS core (Ncap). These conformational changes lead to formation of a rapidly exchanging VVD homodimer. The adduct-state in VVD is unusually stable ($t_{1/2}$ ~18000 sec), placing this protein in the slow-cycling class of flavin-binding LOV domains such as YtvA (3000 sec²⁵) and LOVK (~6000 sec⁴)^{6,8}. In the present study, we characterize VVD thermal reversion in terms of pH dependency, kinetic isotope effects, and base catalysis, for both the wild-type protein and rate affecting variants. We identify two regions within the active site where residue substitutions affect the rate of adduct decay in VVD by four orders of magnitude and similarly change adduct decay in other LOV proteins. Moreover, we provide strong evidence that the N5 deprotonation is indeed the rate-limiting step in thermal reversion and that small molecule bases accelerate decay by acting in a solvent channel conserved in this family of LOV proteins.

Results

Sequence variations of active site residues across the LOV family suggest that subtle changes in the active site environment may be responsible for tuning thermal reversion rates (Fig. 2 and 3). Crystal structures of phototropin-like LOV domains indicate two variable regions where steric and electronic interactions could regulate photocycle kinetics⁸. Site 1 residues (74, 76, 83, 85; VVD numbering) would be expected to affect conformational stability of Cys108 as well as solvent accessibility to the flavin active site (Fig. 2b, 3). Site 2 residues contact the *re*-face of the flavin ring (Fig. 2b, 3). In VVD and other phototropin-like LOV domains, three positions that contact the isoalloxazine ring (135, 163, and 165; VVD numbering) can hold either diffuse electron containing Met/Phe residues or β -branched, aliphatic residues such as Ile/Val/Leu (Fig. 2b, 3). Indeed, substitutions of Site 1 or Site 2 residues have dramatic effects on VVD recovery kinetics and thereby impart control over the photocycle kinetics (Table 1).

In addition to thermal recovery, UV light cleaves the flavin-cysteinyl bond of LOV domains²⁶. However, these reactions are even faster (<100 ps) than the already fast adduct formation step (μ secs) and therefore generate a photostationary equilibrium during steady state

illumination over seconds or longer. With ambient light sources the yield of adduct in the steady state is still > 90% (see Supplementary Table 1 online). However, increased UV intensity in deuterium monitoring light sources will reduce the steady state yield of the adduct (to ~ 70%) and increase the apparent recovery rates if exposures are excessive (see Supplementary Table 1 online). Importantly, the active site variants studied here have small, if any effects of the UV cleavage efficiency (see Supplementary Table 1 online).

Fast Cycling Variants

Residue substitutions in Site 1 have large effects on adduct state stability that depend on their proximity to Cys108 (Table 1). Substitutions at Ile74 and Ile85 greatly accelerated the rate of adduct decay in a 36 residue N-terminal truncation of VVD necessary for solution stability (VVD-36^{8,13}). (Recovery times of VVD-36 are the same as the longer VVD-22¹⁴ and at least similar to the full-length protein, which precludes an accurate kinetic analysis due to its instability.) Ile85 is nearly invariant across the LOV family; its substitution has been shown to affect thermal reversion in AsLOV2²¹. Similarly, in VVD, adduct decay in the I85V adduct state proceeds ~25 fold faster than in VVD-36 (Fig. 2c). In contrast to I85V, Ile74 varies in residue type across the LOV domain family. A I74V substitution in VVD-36 also results in a 25-fold increase in the rate of adduct decay (Fig. 2c). This substitution alone is sufficient to increase the conversion rate of the photo-adduct to values comparable with fast cycling phototropins²⁷. Moreover, the double variant I74V:I85V, increases recovery by 600-fold relative to VVD-36 (Fig. 2c), which is comparable with those rates observed for the fastest cycling phototropins. Both substitutions remove a methyl group that projects towards Cys108. In particular, Ile74 positions a methyl group in van der Waals contact with both of two structurally resolved conformations of Cys108 (Fig. 2b). Thus, the rate increase in I74V may reflect steric destabilization of the adduct state. However, additional factors, such as an increase in solvent accessibility to the flavin and/or Cys108 may also play a role.

A channel lined by the phosphodiester side chain of FAD, Cys76 and Thr83 provides solvent access to the isoalloxazine ring (Fig. 2b). Substitution at positions 76 and 83 had differential effects on the rate of adduct decay that were less drastic than those observed for the position 74 and 85 variants (Table 1). Changes at Cys76 both stabilize and destabilize the adduct-state. C76S maintains a photocycle similar to native VVD-36, whereas a C76A substitution, which was designed to increase accessibility to the active site and decrease steric crowding of the Cys108 adduct (Fig. 2b), only slightly accelerated adduct decay (1.6 fold, Table 1). Similarly, introduction of a more bulky hydrophobic residue at position 76 (C76V) only mildly increased the lifetime of the adduct state (by 1.2 fold). Overall, relatively conservative substitutions at Ile74 and Ile85 generate the most substantial increases in thermal reversion rate (Table 1).

Slow Cycling Variants

In contrast to most phototropin-like LOV domains, the residues surrounding the VVD flavin ring have diffuse, polarizable electrons. Two of the four Met residues within 10 Å of the flavin ring contact its *re*-face (Fig. 2b). Met-to-aliphatic side chain substitutions at analogous positions in flavodoxins affect the stability of reduced flavin species²⁸. In addition, the presence of sulphur atoms in LOV domain active sites has been shown to impart quinoid character to the flavin^{18,29}. Thus, substitutions of Met135 and Met165 in VVD should alter the electronics of the flavin ring and affect adduct stability.

Ile substitutions at Met135 and Met165 slow adduct decay (Fig. 2d). The M135I variant undergoes thermal reversion approximately 1.4 fold slower than VVD-36, but the M135I:M165I double substitution renders the adduct state nearly irreversible with an at least 10-fold increase in the “on” state lifetime (Recovery of M135I:M165I is so long that it competes protein stability). The rate of decay in M135I:M165I rivals that of FKF1, which until recently

was thought to form an irreversible photoadduct¹². Moreover, spectroscopic analysis of the M135I:M165I variant demonstrates a noticeable perturbation in the electronic environment of the flavin. The vibronic structure around the S0-S2 transition (377 nm) is altered with a more pronounced peak at 377 nm and a distinct shoulder at 361 nm (Fig. 2d). Notably, the M165V and M165L variants are slightly rate accelerating despite removal of the electron rich sulfur. In contrast, the M135L slows adduct state decay by 1.3 fold compared to VVD-36. Moreover, the M135L:M165L substitutions when made together, negate each other's effects, producing a decay rate similar to VVD-36. Thus, the 135 and 165 positions have variable effects on adduct state stability in VVD depending on whether they hold Val, Leu, Met or Ile residues.

Thermal Reversion Mechanism and Solvent Accessibility

To better delineate the factors controlling VVD thermal reversion, site-specific variants were analyzed for base catalysis, solvent isotope effects, and pH sensitivity in adduct decay.

Fast cycling VVD variants maintain similar solvent isotope effects (SIEs) as VVD-36. Indeed I74V, I85V, and I74V:I85V all exhibit SIEs in the range of 1.8–2.2, analogous to those observed in VVD-36. Notably, these relatively small SIEs do not necessarily implicate N5 deprotonation as the rate limiting step in thermal reversion, but may also represent a combination of factors including, additional proton transfers, global stabilization of the protein through osmolytic and viscosity effects, as well as alteration in chemical structure due to H-D exchange. Importantly, the I74V and I85V variants shift the recovery kinetics into a regime where the kinetics can be monitored on a relatively short time scale, and thereby facilitates proton inventory (PI) measurements.

PI measurements, in which the magnitude of an SIE is correlated with solvent deuterium content, provide further insight into the origin of SIEs and the nature of proton transfers involved in the rate-determining step (rds)³⁰. If the rds involves a single proton transfer that exchanges rapidly on the time scale of the experiment, the SIE should vary linearly with D content. However, if the proton in question exchanges slowly on the reaction time scale, the process should be characterized by two kinetically isolated populations and hence two rate constants: one for the process in pure D₂O, and the other in pure H₂O, with their relative contribution weighted by the D₂O/H₂O ratio. This is precisely the behavior observed for VVD I74V (Fig. 4a) and I85V (see Supplementary Fig. 1 online). Moreover, a weighted average of k_{H_2O} and k_{D_2O} is linear with D content (Fig. 4b), thereby indicating similar affinities of H⁺ and D⁺ for the reactive site. Thus, a single proton transfers in the rds, and it does not exchange with bulk solvent during the recovery process. Moreover, SIEs are only observed in samples where H/D exchange occurs in the dark-adapted state and not in samples exchanged into mixed buffer after the light state adduct has been formed. Thus, the proton transfer site that contributes to the rds is either not present or protected in the light-adapted state. Furthermore, for I74V, an SIE in adduct decay is only observed if the protein dark-state is incubated in the counter solvent for >1 min (Fig. 3e), but for I85V, the SIE is realized on a time scale faster than can be measured (see Supplementary Fig. 1 online). Taken together, this data indicates that a proton is transferred from a slowly exchanging staging site ($t_{1/2}$ =2 min), which becomes fast exchanging in I85V, to a virtually non-exchanging position during photo-adduct formation, and the subsequent removal of the proton from this position is rate-limiting for thermal recovery. Very few protonation sites would be expected to give the latter behavior, except for flavin N5.

The I74V, I85V and I74V:I85V substitutions render thermal reversion of the adduct state sensitive to increases in pH and the addition of small organic bases such as imidazole. I74V, unlike VVD-36, but similar to AsLOV2¹⁶ demonstrates a pH dependence for adduct decay with an increase in rate of approximately 40% above pH 9 (Fig. 4c). Likewise, substitution of residues lining the conserved solvent channel to smaller side chains renders VVD more

susceptible to catalysis by small molecule bases such as imidazole (Fig. 4d). Whereas in VVD-36 a maximum rate enhancement of ~100 fold was achieved with 400 mM imidazole (Fig. 4d), I85V achieves similar rates with 6-fold less imidazole. Interestingly, I74V has a low imidazole sensitivity, like VVD-36. This is perhaps because the Ile74 γ -methyl lies interior to Ile85, which in itself blocks access to the active center (Fig. 2b). Removal of this blockage in the I74V:I85V double variant renders VVD-36 2-fold more sensitive to imidazole than the I85V protein and 15-fold more sensitive than VVD-36 (Fig. 2b). (Note that such a pocket is found above the flavin dimethylbenzene moiety in crystal structures of phy3 LOV2²⁰.) The kinetic data also suggests the formation of a binding site for base in I74:I85V, in which catalysis of adduct decay appears to saturate at high imidazole concentrations (Fig. 4d). In fact, the rate dependence on imidazole concentration can be broken into two regimes. Below 80 mM imidazole the reaction shows a strong concentration dependence; whereas above 80 mM the concentration dependence is much more shallow and similar to that of VVD-36 (Fig. 4d). Such behavior is consistent with two sites of action by the base, the first involving a saturatable position close to the flavin ring, only accessible in I74V:I85V, and a second less specific pathway to the active site. Notably, SIE studies of imidazole catalyzed I74V recovery also indicate involvement of a single proton in the rds. The most likely candidate for the protonation site is flavin N5 and thus imidazole acts in the vicinity of the active site and gains access by way of the conserved solvent channel.

Structural Studies

To verify large scale structural rearrangements were not responsible for the observed increase in thermal reversion, I74V and I74V:I85V crystals were soaked with 100 mM imidazole and structures were determined to 2.0 Å and 1.8 Å resolution, respectively. The active site residues were superimposable upon those of native VVD with only the substituted Ile methyl groups absent. Neither structure showed any evidence for an ordered imidazole molecule. However, the variant structures reveal a surprising change in the conformation of Cys108 that correlates with the rate of thermal reversion. Two conformations of the active site cysteine had previously been identified in VVD, as well as CrPhot1 LOV1 crystal structures^{8,31}. Dark state VVD has a ~10:90 ratio between conf1 (sulfhydryl group situated over top of flavin C4a) and conf2 (Cys oriented with the sulfhydryl group directed towards Cys76)⁸. Studies of other LOV domains have demonstrated that the conf1 should be more reactive^{31,32}. Notably, in VVD I74V, the ratio between conf1 and conf 2 is altered (40:60), due to the removal of the methyl group which projects between the two positions of the sulfhydryl group of Cys108 (Fig. 5a). In I74V:I85V the population is biased completely toward conf2, the fastest cycling variant, which shows no evidence of conf1 (Fig. 5b).

Dimerization in VVD Variants

A size exclusion chromatography (SEC) assay was employed to verify that the variants still undergo light induced dimerization^{8,13}. In all cases, variants underwent a light induced shift in elution volume characteristic of dimerization. However, the I74V:I85V double variant, and the site 2 variants have altered behavior on SEC.

Light-adapted VVD undergoes a rapid interconversion between monomer and dimer species that manifests as a single peak on SEC at an elution volume intermediate to that of the monomer and dimer¹³. In VVD I74V:I85V, the lifetime of the light-adapted state is at least an order of magnitude shorter than the required elution time for VVD-36. Moreover, interconversion of the monomer and dimer species in VVD-36 proceeds with a rate constant $> 1 \text{ s}^{-1}$ ¹³. Thus, a shift in v should not be observed under these conditions for I74V:I85V. However, at high concentrations the light-adapted state of I74V:I85V does show a small shift in v , indicative of an expanded hydrodynamic radius (Fig. 5d). Thus, either some dimerization or the

conformational changes coupled to dimerization persist after scission of the photo-adduct in this fast cycling variant.

In addition to lengthening the adduct state lifetime, M135L, M135I, M165I, and M135I:M165I stabilize the VVD light-state dimer. The lack of a concentration dependence in v over a concentration range of 10–200 μM is consistent with a >5-fold increase in dimer affinity for M135I:M165I. Notably even at concentrations below 3 μM , the variant elutes with an apparent MW of 22,000, significantly larger than the dark state of VVD-36 (~16000). Thus, either the M135I:M165I dimer is unusually strong or the monomeric state is expanded relative to that of VVD-36. A related effect is observed for the Cys71Val variant of VVD, which forms an expanded monomer that can partially dimerize, even in the dark³³.

Generality of Site 1 mutations

Site 1 substitutions in other LOV proteins follow the same trends as in VVD. The substitution analogous to I85V in AsLOV2 (I427V) increases recovery rates much like in VVD²¹. The analog of V74I (V416I) in AsLOV2 and YtvA (V28I) decrease recovery rates by factors of 10 and 5, respectively, whereas the YtvA I85V analog (I39V) increases recovery rates by a factor of 5 (Table 1· Supplementary Fig. 2 online). The Phe1010Leu substitution at the *re*-face of the phototropin LOV2 demonstrates a similar effect on recovery rates as the VVD Site 2 mutants in that removal of polarizable electrons from the vicinity of the flavin stabilizes the adduct state³⁴. Notably, YtvA and AsLOV2 do not have Ncaps like VVD, but instead associate C-terminal regions against the PAS core. Also dark state YtvA dimerizes through these C-terminal regions, whereas, AsLOV2 does not. Thus, although the conformational coupling in all these LOV systems must be different, the ability of Site 1 and Site 2 active center residues to modulate recovery rates is qualitatively the same.

Discussion

Three general factors contribute to light-state deactivation: 1) acceleration of N_5 deprotonation and/or reduced stability of the flavin $\text{N}_5\text{-H}$ bond; 2) steric destabilization of the Cys108 adduct conformation; and 3) steric and electronic effects that disfavor the “reduced” flavin state. These mechanisms are controlled by residues that modulate solvent/base access through a channel adjacent to the flavin side chain (Site 1) and by steric-electronic effects at the *re*-face of the flavin isoalloxazine ring (Site 2). Importantly, as discussed below the features that stabilize the adduct reveal a conserved mechanism of thermal recovery in LOV domains and identify residues involved in tuning the chemical reactivity of the flavin.

Past studies of phototropin-like LOV domains indicate that proton transfers contribute to the rds for thermal reversion^{16,18–20}. PI studies of VVD indicate that a single labile proton, exchangeable only in the dark state, contributes to the rds of C_{4a} -adduct scission. N_5 is the most obvious protonation site directly connected to the photocycle that would be stable against solvent exchange over the entire course of the adduct lifetime. Crystallographic and spectroscopic data⁸ show that N_5 is unprotonated in the dark state of VVD, becomes protonated in the adduct state and deprotonates on the time scale of adduct cleavage. Further, N_5 protonation is known to stabilize “reduced” flavin rings³⁵ and N_5 undergoes a large change in pK_a when an adduct forms at C_{4a} ^{36,37}. In VVD, the N_5 proton is transferred from a protected site because the protein must be incubated in mixed solvent for minutes to manifest an SIE (Fig. 4e). This protection against solvent exchange is lost in I85V (see Supplemental Fig. 1 online). Crystal structures do not contain ordered solvent within H-bonding distance of N_5 , however Cys108 loses a proton during activation and the thiol group resides within 4 Å of N_5 and I85. FTIR studies and ultrafast spectroscopy have demonstrated that Cys108 is protonated in the dark state and may be the source of the N_5 proton^{17,32,38–41}. QM/MM calculations on LOV domain active site models have suggested that direct hydrogen atom

transfer from Cys108 to N₅ is a plausible pathway for adduct formation, however, the calculated activation energies are higher (11–30 kcal/mol)^{42,43} than would be reflected by the forward rate constants (μsec). Given all of these considerations, coupled with the observation that the donation site exchanges with bulk solvent much faster in I85V, Cys108 is currently the best candidate for proton (or H atom) donation.

The base involved in N₅ abstraction is less obvious and may involve solvent, a conserved Gln (182 VVD numbering), or Cys108. Regardless, the importance of N₅ deprotonation in recovery implies that active site residues that control solvent/base accessibility to this position or affect stability of the N₅-H bond are likely to have a major role in regulating adduct state lifetimes. Phototropin-like LOV domains show varying degrees of base catalysis in their recovery reactions^{16,19,20} but, in general, base sensitivity inversely correlates with adduct lifetime.

Mutational studies of VVD, AsLOV2, and YtvA, (Table 1) show that two residues in particular have a large impact on both solvent access to N₅: 1) Ile85, which resides between the flavin core and the solvent channel and 2) Ile74, which positions its methyl group in steric contact with Cys108, and Gln182 (Fig. 5a,b). The ability of changes at these positions (Site 1) to render VVD susceptible to pH changes and base catalysis is well explained by an increase in solvent access. Consistent with this, substitution at the more peripheral substitution does not have an effect (I85V), unless made in concert with a change at the internal site (I74V). Furthermore, the kinetic data on I74V:I85V indicates that the general base imidazole acts in at least two ways: 1) from a specific site that saturates and may involve the cavity created above the flavin, and 2) from a more distributed set of positions.

It is also important to consider that Val substitutions at positions 74 and 85 not only increases access to N₅ but also alter the equilibrium between the conformation of the Cys108 thiol over flavin C4a (conf1) and its position beside Cys76 (conf2). Stabilization of conf2 in the dark state correlates with faster recovery times. Preference of conf2 over conf1 in the dark state may reflect relative destabilization of Cys108 over C4a. This could manifest as a direct destabilization of the closely related adduct structure, or the creation of a new reaction coordinate for bond scission that is coupled to movement into conf2.

Another factor that may contribute to recovery rate acceleration in I74V is unblocking of Gln182 rotation by removal of the I74 terminal methyl group. Gln182 must flip between the dark and light states in response to changes in N₅ protonation state⁸. As this rotation is restrained in Ile74 in WT (see Supplementary Fig. 3 online), but not in the I74V variants, this substitution may facilitate return to the dark-state, where the Gln182 amide hydrogen bonds to the N₅ position. Such a model is supported by changes in adduct stability seen in the Gln→Leu and Gln→Asn variants of AsLOV2⁴⁴. However, given the very low Gln carbonyl pK_a, this residue probably does not act as an isolated base but rather aids in proton transfer to Cys108 by altering hydrogen bonding to N₅-H. In flavodoxins, removal of a hydrogen bond between an amide carbonyl and the N₅-H bond³⁵ is known to affect the N₅ pK_a and stability of reduced flavin.

The second region where substitutions affect recovery rates (Site 2) resides within Hβ and Iβ on the opposite side of the flavin ring from Cys108. At positions 135 and 165, substitution of electron rich methionine for aliphatic residues decreases the rate of adduct decay in VVD. These changes likely alter both the steric and electronic environment of the flavin cofactor, analogous to those reported in flavodoxins²⁸ and in a Phe→Leu substitution at the flavin *re*-face in phototropins³⁴. However, the consequences of the Met substitutions does not derive solely from removal of the electron-rich sulfur moieties. Ile substitutions at positions 135 and 165 lengthen the VVD adduct-state lifetimes, whereas changes to Leu do not. Met to Ile or Leu substitutions in flavodoxins also differentially affect redox potential²⁸, hence strain in the flavin

ring by altered sterics may modulate the potential and adduct state stability. The maximum lifetime of the AsLOV2 adduct state (~1000 sec) is held by a V416I:L496I variant (equivalent to I74 and Met165 in VVD). The relatively short lifetime in V416I:L496I AsLOV2 highlights the complexity of changes at the *re*-face of the flavin, observed also in the M135I and M165I VVD variants (Table 1). AsLOV2 has Phe at a position equivalent to VVD Leu163, and removal of this polarizable side chain increases AsLOV2 thermal recovery³⁴. Thus, residues at the *re*-face of the flavin (135, 163, and 165) likely work cooperatively in tuning adduct stability.

The perturbations in adduct state decay rate for M135I:M165I (and also I74V:I85V) are so great that coupling between active center photochemistry and the conformational response cycle is altered for these proteins. A related effect was observed for the Phe1010Leu (VVD 163) variants of phototropin LOV2, which has a much smaller, LOV1-type conformational response despite a more stable adduct state³⁴.

Despite conservation of residues that line the flavin binding site, the stability of the photo-adduct and the efficiency of adduct formation vary widely (Fig. 3, Table 1). Through changes in naturally variable residues in VVD, the entire recovery timescale of known phototropin-like LOV domains can be spanned. Whereas, the fast cycling phototropins allow for rapid attenuation and reversal of signaling, long-lived photo-adducts may be employed for a specific one time event such as gating of the circadian clock. In the case of VVD, base catalysis and/or oxidation⁴⁵ may provide additional means to attenuate the response.

Finally, phototropin-like LOV domains have recently been used as *in vivo* photoswitches to regulate cellular function⁴⁶. The current study has outlined methods to manipulate the duration of the photic-reponse by 4-orders of magnitude, in addition to the type of output (VVD for dimerization, AsLOV2 for conformational gating). Increasing the lifetime of the adduct state is important to overcome a primary obstacle in *in vivo* photoswitches. High-intensity light, required to maintain steady state populations of the adduct, induces sample heating. Lifetime extension in existing LOV-based probes will provide high adduct yields under low light intensity. The ability to manipulate LOV domain on-state lifetimes will enhance the design of improved photoswitches for heightened control over cellular signaling.

Methods

Expression and Purification

Point mutations were made in 36 residue N-terminally truncated VVD (VVD-36)^{8,13}, AsLOV2 (*Avena sativa* Phototropin 1 residues 404–560) and YtvA (*Bacillus subtilis* residues 20–147) constructs according to the QuickChange protocol (Stratagene). Mutations were sequenced in their entirety at the Biotechnology Resource Center of Cornell University.

VVD proteins were overexpressed in *E. coli* BL21(DE3) cells for 22 hours at 18° C under constant light. Soluble cell lysate was fractionated by centrifugation and purified with Ni:NTA affinity chromatography. Following thrombin cleavage at 4° C samples were purified in buffer containing 10% glycerol, 150 mM NaCl and 50 mM Hepes pH 8.0 with a Superdex 75 Hi-load 26/60 FPLC column. Fractions containing VVD were concentrated to ~4 mg/ml for all spectroscopic studies.

YtvA and AsLOV2 proteins were overexpressed *E. coli* BL21(DE3) cells for 5 hours at 37° and for 20 hours at 20° C respectively. Soluble cell lysate was fractionated and purified as described above for VVD.

VVD Spectroscopy and Kinetics

All spectroscopy was conducted on an Agilent 8453 spectrophotometer. Kinetics of adduct recovery was determined by obtaining spectra at 2–3600 second intervals until no further change in absorbance at 450 nm was observed. Absorbance traces at 450 and 478 nm were fit assuming first order kinetics with a single rate constant. Decay curves were fit well with a monoexponential function, $Abs=B+C\cdot\exp(-kx)$, except in the case of proton inventory measurements, where the traces were clearly biphasic. Average rate constants were obtained from measurements at multiple wavelengths.

For the slow cycling M135I:M165I variant the rate of adduct decay was approximated from early time point data and fit as a linear curve of $\text{Log}(A-A_0/A_{asy}-A_0)$ vs. time, where A_{asy} is the absorbance after complete recovery.

Adduct-state lifetimes in VVD are strongly dependent on the frequency of data acquisition. Rapid sampling of VVD spectra with broad spectrum monitoring light causes net photo-induced repopulation of the adduct state. Lifetimes are reported for the longest separation between sampling times after which no change in lifetime is observed (see Supplementary Fig. 4 online).

Base Catalysis

Base catalysis of VVD recovery was tested with imidazole, methyl-imidazole, DTT, ATP and Histidine. Stock solutions were first prepared to 800 mM (imidazole, methylimidazole or DTT) with 13% glycerol, 150 mM NaCl and 50 mM Hepes pH 8.0. Concentrated VVD samples were then diluted with these buffers to appropriate imidazole concentrations prior to analysis via UV-Vis spectroscopy as described above. In the case of I74V:I85V the catalytic rate constant's dependence on imidazole concentration was fit to the following saturation equation including an additional linear component: $\text{Rate}_{cat}=\{V_{max}\cdot[\text{imidazole}]/(k1 + [\text{Imidazole}])\} + A[\text{imidazole}]$.

Proton Inventory

SIE data was obtained by varying the D₂O percentage in buffer containing 10% glycerol, 150 mM NaCl, and 50 mM Hepes pH 8.0. In addition, kinetics of H-D and D-H exchange were determined by allowing for successively longer durations of D₂O exposure prior to illumination with ambient light to initiate the photocycle.

Approximately 100% D₂O content was achieved by three successive 20-fold dilutions in buffer containing 100% D₂O, whereas 100% H₂O was achieved by direct purification via FPLC. Rates of exchange were obtained by monitoring kinetics after dilution (in the dark) of 100% D₂O to a final D₂O concentration of 20%. Exchange was allowed to proceed in the dark for between 0–120 minutes prior to light exposure. In addition, exchange dynamics were assayed by direct dilution of 100% D₂O and H₂O samples to between 20–80%, followed by immediate photobleaching and obtaining kinetic data. To verify that H-D exchange does not occur in the adduct-state, samples were placed in the adduct state and then diluted to 80% D₂O (from 100% H₂O). Samples were then allowed to incubate on ice under constant light prior to kinetic analysis.

Proton inventory measurements were made by allowing for exchange into mixed solvent buffers in the dark for > 10 minutes. After this time, little change in kinetics was observed. Kinetics were then monitored both in the presence and absence of 100 mM imidazole.

pH Dependence of Rates

VVD proteins were purified in a buffer containing 13% glycerol, 150 mM NaCl and 5 mM HEPES pH 8.0. Solutions containing 13% glycerol, 150 mM NaCl, and 100 mM buffers of pH ranging 4.5–10 were used to dilute the protein samples 10-fold. The resulting protein pH was then measured and verified to not deviate from the stock solution by more than 2–5%. Kinetics were monitored spectroscopically as described above.

Crystallization of VVD Variants

Crystals of I74V and I74V:I85V were obtained using equal volumes of protein at ~3.6 mg/ml with 5 mM DTT and reservoir solutions containing 28% PEG 4k, 100 mM ammonium acetate and 100 mM tri-sodium citrate pH 5.6. Large single plates (400 × 400 × 50 μ m) grew after three days and diffracted to 1.6 Å resolution. Incubation with 100 mM imidazole rapidly deteriorated the diffraction quality of the crystals resulting in decreased resolution (2.0, 1.7 Å) for the two variants in the presence of imidazole.

Data Collection and Structure Solution

Data was collected at 100 K with synchrotron radiation on beamline F1 and F2 at the Cornell High Energy Synchrotron Source (CHESS). Data was reduced and scaled with HKL2000⁴⁷ and refinement statistics can be found in Supplementary Table 2 online. Phases were obtained by molecular replacement in AmoRe⁴⁸ using VVD (PDB ID 2PD7) as a search model. Subsequent models were rebuilt using XFIT⁴⁹ followed by refinement in CNS⁵⁰.

Size Exclusion Chromatography (SEC) of VVD Variants

To assay dimer formation in VVD variants SEC was conducted on a Sephadex 75 10/300 analytical column. All samples studied were lysed, digested with thrombin, purified via FPLC and analyzed on SEC within a 36-hour time period to avoid oxidative damage of the proteins. Determination of the dissociation constants of the variants were determined as outlined previously¹³.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Structure of VVD-36 with the PAS core (blue), N-terminal cap (yellow) and FAD binding loop (red). (b) Thermal reversion mechanism for phototropin-like LOV domains. Adduct scission is catalyzed by a base (B), which may include solvent, a conserved glutamine residue that hydrogen bonds to N5, the active site Cys or exogenous compounds such as imidazole.

Figure 2. VVD Kinetics. (a) Thermal reversion of VVD, successive spectra shown at 1.7 hour intervals. Inset demonstrates exponential growth of signal at 450 nm. (b) Active site superposition of VVD and variants: VVD-36 (light blue), I74V (yellow), and I74V:I85V (orange). Residues targeted for mutation near the solvent channel (water-aqua spheres) and the re-face of the flavin are highlighted. (c) Recovery kinetics from the adduct-state for VVD-36 (black), I74V (green) and I74V:I85V (red) and M135I:M165I (blue). (d) Dark state spectra of Native VVD (red) and M135I:M165I (black). M135I:M165I has more pronounced peaks at 364 and 374 nm, which reflect a change in flavin environment. Spectra are offset to aid comparison.

Figure 3.

Sequence alignment of VVD, WC-1, Zeitlupe (ZTL), Flavin-Binding Kelch Repeat F-Box protein (FKF1), *Brucella mellitus* LOV histidine kinase (Bm-LOV), *Arabidopsis thaliana* phototropin 1 LOV1 and LOV2 (Atphot1LOV1, AtphotoLOV2), and *Chlamydomonas reinhardtii* LOV1 and LOV2 (CrLOV1, CrLOV2). Positions that border the VVD solvent channel are highlighted in green, whereas residues at the re-face of the flavin are in red. Additional variant residues that affect recovery rates are shown in blue. Non-variant flavin-interacting residues are shown in grey. The presence of M135 is unique to VVD and WC-1.

Figure 4. Proton Inventory and Base Catalysis. **(a)** Weight of the slow component (equivalent to 100% D₂O, fast component equivalent to 100% H₂O) in biphasic recovery kinetics as a function of the D₂O percentage. **(b)** Overall weight averaged rate constant as a function of the D₂O percentage for imidazole catalyzed (red) and uncatalyzed (black) I74V. **(c)** pH dependence of thermal reversion rate for I74V. **(d)** Comparison of base catalyzed rate constant (see Table 1) by imidazole for VVD-36 (black), I74V (red), I85V (green) and I74V:I85V (blue). In each case concentration dependence is linear with the exception of I74V:I85V, which is best fit as a saturation function with an additional linear term. **(e)** Rate constant as a function of counter solvent (H₂O) exchange time in the dark. Error bars are depicted at ± 1 s.d.

Figure 5.

Crystal Structures of VVD Variants. Electron density for alternate conformations of Cys108 in VVD-36 (a), I74V (b) and I74V:I85V (c). 2Fo-Fc maps are contoured at 1 σ (cyan) and 2 σ (purple); Fo-Fc maps are contoured at 3 σ (green). VVD-36 has mostly conf1; I74V:I85V only conf2, and I74V has both. (d) SEC elution profile of I74V:I85V. I74V:I85V (green) elutes intermediate of the light- (red) and dark-adapted (black) states of VVD-36 on a Superdex 75 26/60 preparative column.

Table 1

Parameters for adduct decay kinetics in VVD Variants

Variant	Lifetime (sec)	rate constant (sec ⁻¹)	k_{cat}^* (s ⁻¹)
VVD-36	18000	5.6E-05	6.1E-04
I74V	730	1.4E-03	6.8E-05
I85V	780	1.3E-03	3.0E-03
C76A	11000	9.1E-05	5.8E-04
C76V	21000	4.8E-05	4.1E-04
T83V	12000	8.3E-05	9.9E-04
I74V:I85V	28	3.6E-02	9.7E-03
M135L	23000	4.3E-05	4.8E-04
M135I	24500	4.1E-05	4.0E-04
M165V	16500	6.1E-05	ND
M165L	12500	8.0E-05	ND
M165I	20000	5.00E-05	ND
M135L:M165L	18000	5.6E-05	ND
M135I:M165I	180000	5.6E-06	ND
WT YtvA	3600	2.8E-04	ND
V74I YtvA (V28I)	16000	6.3E-05	ND
I85V YtvA (I39V)	670	1.5E-03	ND
WT AsLOV2	81	1.2E-02	ND
V74I AsLOV2 (V416I)	821	1.2E-03	ND
V74I:L165I AsLOV2 (V416:L496I)	1009	9.9E-04	ND
Neo1-LOV2	90 ³³	1.1E-02	ND
F163L Neo1-LOV2 (F1010L)	870 ³³	1.1E-03	ND

* k_{cat} is the catalytic rate constant in the presence of 100 mM imidazole, assuming that $k_{\text{observed}} = k_{\text{uncatalyzed}} + k_{\text{cat}}$.