Factors That Control the Chemistry of the LOV Domain Photocycle

Josiah P. Zayner1, Tobin R. Sosnick1,2*

1 Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois, United States of America, 2 Institute for Biophysical Dynamics, The University of Chicago, Chicago, Illinois, United States of America

Abstract

Algae, plants, bacteria and fungi contain Light-Oxygen-Voltage (LOV) domains that function as blue light sensors to control cellular responses to light. All LOV domains contain a bound flavin chromophore that is reduced upon photon absorption and forms a reversible, metastable covalent bond with a nearby cysteine residue. In Avena sativa LOV2 (AsLOV2), the photocycle is accompanied by an allosteric conformational change that activates the attached phototropin kinase in the full-length protein. Both the conformational change and formation of the cysteinyl-flavin adduct are stabilized by the reduction of the N5 atom in the flavin’s isalloxazine ring. In this study, we perform a mutational analysis to investigate the requirements for LOV2 to photocycle. We mutated all the residues that interact with the chromophore isalloxazine ring to inert functional groups but none could fully inhibit the photocycle except those to the active-site cysteine. However, electronegative side chains in the vicinity of the chromophore accelerate the N5 deprotonation and the return to the dark state. Mutations to the N414 and Q513 residues identify a potential water gate and H2O coordination sites. These residues affect the electronic nature of the chromophore and photocycle time by helping catalyze the N5 reduction leading to the completion of the photocycle. In addition, we demonstrate that dehydration leads to drastically slower photocycle times. Finally, we investigate the requirements of an active-site cysteine for photocycling, we moved the nearby cysteine to alternative locations and found that some variants can still photocycle. We propose a new model of the LOV domain photocycle that involves all of these components.

Citation: Zayner JP, Sosnick TR (2014) Factors That Control the Chemistry of the LOV Domain Photocycle. PLoS ONE 9(1): e87074. doi:10.1371/journal.pone.0087074

Received October 15, 2013; Accepted December 20, 2013; Published January 27, 2014

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: trsosnic@uchicago.edu

Introduction

In response to blue light, algae and plants use LOV domain-containing phototropins to activate signaling cascades that end in phototropism or chloroplast rearrangement [1–3]. LOV domains contain 100-150 amino acids and are members of the PAS domain superfamily [4]. Many LOV domains are found as a part of larger proteins [5] whereas others can function as single domains [6].

Light activation occurs when the non-covalently bound flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) chromophore absorbs a photon [7] that excites the chromophore into a singlet state. This state converts with high probability through an intersystem crossing into a triplet excited state [8]. In the triplet state, the flavin forms a metastable covalent bond between its C4a atom and the sulfur of the neighboring cysteine [8]. The N5 atom of the flavin is reduced, binding a hydrogen atom that is thought to come from the active site cysteine [8–10]. The resulting cysteine adduct spontaneously decays in seconds to hours depending on the local side chain environment [11–13]. The decay rate is controlled by the deprotonation of the N5 atom, returning the chromophore to its non-covalently bound dark state [14].

The photocycle of the flavin in LOV domains typically elicits a reversible conformational change [8,15–18]. In Avena sativa LOV2 (AsLOV2), the change involves the unfolding of the A’α and Jα helices [19,20]. This conformational change has been shown to activate kinase activity in full-length phototropins [21–23].

The photocycle of LOV domains is highly tunable by a large number of factors [11,13]. Mutations to residues near the chromophore can shorten the photocycle of AsLOV2 from 80 s to 6 s or lengthen it to days [13,19,24,25]. The known mechanisms that shorten the lifetime of the photocycle include increased solvent accessibility and modification of the electrostatic environment [11,19,24]. The photocycle can be lengthened by altering the packing around the active-site cysteine or through the removal of electrostatic interactions [13,26].

Although these effects focus on the stabilization and removal of the covalent adduct, reversion to the dark state is likely limited by a base catalyzed deprotonation of the flavin. This step has been suggested to involve a highly conserved glutamine residue [14,26]. However, mutations to this residue in Arabidopsis thaliana LOV2 (Q1029L [27]) and AsLOV2 (Q513L [26] and Q513A [19]) slow but do not abolish the photocycle, with the Q513A photocycle time remaining within a factor of three of the wild-type value. The ability of the photocycle to remain intact with mutations to Q513 is perplexing because this is the only obvious side chain within the vicinity of the chromophore with the proper functional groups to catalyze the deprotonation process.
Recent studies have suggested that water molecules can enter the chromophore binding pocket [28] and directly participate in the photocycle chemistry [28–30]. This possibility would rationalize the maintenance of the photocycle upon mutation to residues such as Q513, by allowing a side chain independent mechanism to facilitate the proton transfer.

Here we investigate the deprotonation step and other chemical events of the AsLOV2 photocycle using a battery of mutations designed to alter side chain interactions with the FMN and the surrounding water molecules. We found mutations near the chromophore that can alter photocycle times from 2 s to over 2000 s. The N414, F494 and Q513 triad are nonessential for...

Figure 1. Effects on AsLOV2 side chains during the photocycle. (A) Side chain positions in the chromophore binding pocket in the dark and light state crystal structures [32] (B) Chemical processes that occur to the FMN chromophore during the photocycle. doi:10.1371/journal.pone.0087074.g001
photocycling but are necessary for the minute lifetime likely needed for biological function. The data can be explained with a model where the N414 position, located ~10 Å away from the chromophore, controls water access and H2O coordination to alter the photocycle lifetime. Experiments on dehydrated protein further establish the role of H2O molecules in the photoadduct decay. In an effort to understand the requirements to build and use photoactive PAS domains, we also created new active-site cysteine residues that photocycle on similar timescales as the wild-type protein. From these results we develop a new model of photocycle function and provide new directions that can be followed for the tuning and creation of optogenetic tools.

**Materials and Methods**

**Cloning, Expression and Purification**

A clone of *A. sativa* phot1 LOV2 (404-560) with a His6-Gβ1 fusion was used. Mutations were made using the quickchange site directed mutagenesis strategy. All proteins were expressed in *Escherichia coli* BL21 (DE3) grown in M9 minimal medium supplemented with 15NH4Cl (1 g/L) at 37°C to an OD 600 nm of 0.6 and induced with 1 mM IPTG. Cultures were then incubated for ~18 hours at 18°C and pelleted and frozen. Frozen pellets were resuspended in 50 mM Tris 100 mM NaCl and 0.01% SDS and cells were lysed using sonication and clarified with centrifugation at 10000g for 45 min. The proteins were then purified using metal affinity chromatography and exchanged into 50 mM Tris, 1 mM EDTA, 5 mM DTT, pH 8. The His6-Gβ1 tag was removed by incubating overnight at 20°C with His6-TEV protease. Metal affinity chromatography was then used to remove His6-Gβ1 and His6-TEV protease from the solution. The final protein contains residues GEF on the amino terminus and G on the carboxy terminus as cloning artifacts. Proteins were run on a Sephadex S100 size exclusion column (GE Healthcare) and if the protein contains residues GEF on the amino terminus and G on the carboxy terminus as cloning artifacts. Proteins were run on a Polyacrylamide Gel Electrophoresis (PAGE) with a 2 nm bandwidth and a 1 cm pathlength cuvette at 22°C. Kinetic traces were acquired using Origin software (OriginLab). The percent of a population excited was calculated by linearly extrapolating a bleached minimum at 448 nm and comparing it to the dark maximum at 448. Salt was removed from protein solutions before drying. Approximately 100μL of 10μM protein in solution was placed on the outside of a cuvette and air dried in the dark.

**UV-Vis**

UV-Vis spectra were acquired using an Olis HP 8452 Diode Array (Bogart, GA) with a 2 nm bandwidth and a 1 cm pathlength cuvette at 22°C. Kinetic traces were acquired after photosaturation. Samples were illuminated using a 40 W white LED (Model no: BT DWNL4T A, TheLEDlight.Com) for 30 seconds and the absorbance at the λmax, usually 448 nm, was measured every 1-30 seconds depending on the photocycle rate. The data were fit to a single exponential decay using Origin software (OriginLab). The percent of a population excited was calculated by linearly extrapolating a bleached minimum at 448 nm and comparing it to the dark maximum at 448. Salt was removed from protein solutions before drying. Approximately 100μL of 10μM protein in solution was placed on the outside of a cuvette and air dried in the dark.

**Results and Discussion**

The photocycle of LOV domains can be measured by monitoring A448, the absorbance near the λmax usually at 448 nm. Upon photoexcitation, the N5 atom is reduced and gains a proton, changing the electronic nature of the chromophore and bleaching the spectra, causing A448 to reach a minimal value. Unless otherwise noted, the photo-recovery can be fit with a single exponential having a time constant, τM. At 22°C, τM ≈ 81 ± 2 s in WT ALOV2 (phot1 residues 404-560).

**Side Chain Effects on the Photocycle**

The LOV domain architecture provides an environment suitable for flavin molecules to undergo reversible reduction and oxidation [31]. A multitude of interactions between the flavin molecule and the protein exist inside the flavin binding pocket. In ALOV2, the flavin interacts electrostatically with the residues N462, N492 and Q513 and forms a π-bond or ring stacking interaction with F494 (Fig. 1A). Other residues near the chromophore have been shown to have effects on the photocycle but these generally are through indirect interactions such as

| Construct | τM (s) | λmax (nm) | Source |
|-----------|--------|-----------|--------|
| WT        | 80     | 448       | Zayner et al. 2012 |
| N414A     | 1427   | 448       | This Work |
| N414D     | 69     | 446       | This Work |
| N414G     | 615    | 448       | This Work |
| N414L     | 1847   | 444       | This Work |
| N414Q     | 280    | 448       | This Work |
| N414S     | 685    | 448       | Zayner et al. 2013 |
| N414T     | 892    | 448       | Zayner et al. 2013 |
| C450V     | NM     | 448       | This Work |
| L453V     | 160    | 446       | This Work |
| N492A     | 54     | 448       | Zayner et al. 2012 |
| F494L     | 206    | 448       | Zayner et al. 2012 |
| F494H     | NM     | 444       | This Work |
| Q513A     | 261    | 442       | Zayner et al. 2012 |
| Q513D     | 1793   | 438       | This Work |
| Q513H     | 30     | 446       | This Work |
| Q513L     | 1907   | 444       | This Work |
| Q513A     | 2081   | 442       | This Work |
| Dehydrated WT | 1726 | 448       | This Work |
| Dehydrated Q513A | 864 | 442 | This Work |
| Dehydrated N414L/Q513A | 4076 | 444 | This Work |
| C450V/F494C | 69 | 448 | This Work |
| C450V/Q513C | 44 | 440 | This Work |
| C450V/L453C | NM | 448 | This Work |
| N414T/C450V/Q513C | NM | 440 | This Work |
| N414C/C450V/Q513C | 13 | 440 | This Work |
| WT +1mM Imid. | 12 | 448 | This Work |
| WT +2mM Imid. | 6 | 448 | This Work |
| WT +3mM Imid. | 4 | 448 | This Work |
| C450V/Q513C +1mM Imid. | 9 | 440 | This Work |
| C450V/Q513C +2mM Imid. | 9 | 440 | This Work |
| C450V/Q513C +3mM Imid. | 8 | 440 | This Work |

*Samples were air dried for 48 hours. doi:10.1371/journal.pone.0087074.t001
increasing the solvent accessibility [11,13]. Of these indirectly interacting residues, N414 is of note because of the large effects upon substitution [13], its conformational change between the light and dark crystal structures [4,32] and its interactions with Q513 in simulations [4,13,30].

We used mutations to probe the residues suggested to regulate the photocycle of AsLOV2, N414, N482, N492, F494 and Q513 (Fig. 1A). Except for N414, these residues are highly conserved in LOV domains with F494 occasionally being replaced with a leucine. Using UV-Vis spectroscopy, we measured how these mutations influence the lifetime and changes in $\lambda_{\text{max}}$ (Table 1). Five of the variants (N482A, N492Q, F494N,W or Y) did not express in sufficient quantities to be characterized, as previously observed in other mutants [13], most likely because of compromised FMN binding.

Other variants of N414, N492, F494 and Q513 typically lengthened the photocycle (Table 1). These variants disrupt interactions with the chromophore or change the steric packing

![Image](https://example.com/image1.png)

**Figure 2.** UV-Vis absorbance changes upon photoexcitation and recovery for selected AsLOV2 variants. Single exponential fits to the time dependent data are shown in red. doi:10.1371/journal.pone.0087074.g002

![Image](https://example.com/image2.png)

**Figure 3.** AsLOV2 structure and side chains near the chromophore. (A) Location of side chains mutated in active-site cysteine variants (B) The effects of imidazole on photocycle lifetime in WT and C450V/Q513C. doi:10.1371/journal.pone.0087074.g003
near it, factors that have been previously shown to affect photocycle lifetime [11]. To further understand the influence of N414, F494 and Q513, we mutated them to a number of different functional groups. Based on our prior observation that N414V has a 12+ hour long photocycle [19], we also mutated N414 to A, D, G, L, S, and T substitutions. All except N414D slow the photocycle by at least five-fold (Table 1). Substitutions at the N414 position have the most dramatic effect on the photocycle of any site we have investigated (other than the C450) [13,19].

Based on its location in the structure, the N414 residue may control water entry into the chromophore binding pocket (Fig. 1A). Crystal structures in the lit and dark state indicate rotations of N414 and Q513 during light activation (Fig. 1A) [32]. Likewise, the effects of N414 substitutions on $\tau_{\text{FMN}}$ relate both to residue size and electrostatic properties with larger hydrophobic side chains having the longest times as compared to smaller residues (e.g., N414L: $\tau_{\text{FMN}} = 1847$ s $\approx$ N414A: $\tau_{\text{FMN}} = 1427$ s $\approx$ N414G: $\tau_{\text{FMN}} = 615$ s), polar residues have faster times (N414Q: $\tau_{\text{FMN}} = 280$ s and WT N414: $\tau_{\text{FMN}} = 80$ s) and a negatively charged residue has the shortest photocycle (N414D: $\tau_{\text{FMN}} = 69$ s) (Table 1 and Fig. 2A). Notably, the alanine and glycine side chains still have slower photocycle times than the original asparagine in spite of their smaller size. These results at N414 indicate that sterics are not the overriding factor controlling life-time of the activated state at this position. Therefore, based on polar and charged residues in the N414 position having faster photocycle times and hydrophobic or neutral side chains having slower photocycle times, we suggest that N414 is involved in the coordination of the water molecules to catalyze a faster transfer of the proton.

A number of N414 mutations modify the electronic properties of the chromophore causing blue shifts in the $\lambda_{\text{max}}$. Because the distance of N414 to the chromophore is $\sim 10$ Å, it is unlikely that there is a direct effect. Rather, the shift is likely caused by a neighboring residue or by changes in the FMN’s solvation mediated by N414. Further, the N414’s influence on the FMN allows for a possible signaling mechanism from the chromophore to the A’$\alpha$ helix (located near N414), which has been shown to regulate light activated conformational change [19,30].

Accordingly, we investigated whether the effect N414 has on the chromophore’s $\lambda_{\text{max}}$ and photocycle time occurs independently or through a neighboring residue. The Q513 residue is positioned between N414 and the chromophore. Mutations to Q513 also shift the $\lambda_{\text{max}}$ and change the $\tau_{\text{FMN}}$. We hypothesized that if the effect of N414 on the chromophore is through Q513 then by mutating Q513 to an inert functional group, mutations to N414 should have less or no effect on the chromophore. If N414 is acting directly on the FMN, however, we would expect effects even if the Q513 position is inert. For the N414L/Q513A variant, $\lambda_{\text{max}} = 444$ nm, the same as for the N414L mutant rather than the Q513A variant where $\lambda_{\text{max}} = 442$ nm. Therefore, N414L can act in a dominant fashion. The N414A mutant, however, does not shift $\lambda_{\text{max}}$ but N414A/Q513A mutant shifts $\lambda_{\text{max}}$ to 442 nm, which suggests a Q513 dominant or combinatorial effect for this pair (Table 1).

We observe similar behavior for the photocycle time. The N414L and N414L/Q513A have similar $\tau_{\text{FMN}}$, 1847 and 1900 s,
respectively. However, an additive or synergistic effect is observed for the N414A/Q513A variant; its lifetime, 2048 s, is longer than either of single mutations (N414A: $t_{\text{FMN}} = 1427$ s, Q513A: $t_{\text{FMN}} = 267$ s; Table 1).

The N414 mutations in combination with the Q513 mutations suggest that the N414 affects the chemistry of the photocycle in a manner distinct from Q513. The N414L mutation is functionally dominant over the Q513A mutation in regards to photocycle time and $\lambda_{\text{max}}$, whereas the N414A and Q513A mutations appear additive. We believe that the N414 residue in ALOV2 influences solvent access or $\text{H}_2\text{O}$ coordination with the chromophore thereby regulating the chemistry of the photocycle.

These and other mutations to Q513 confirm that this residue has significant effects on photocycle lifetimes and the electronic nature of the chromophore, as measured by $\lambda_{\text{max}}$ [13,26,27]. With an acidic substitution, Q513D, the protein has a much faster photocycle, 5 s, than most WT phototropin LOV2 domains, which typically range from 56 to 786 s [12] (Table 1). In contrast, hydrophobic residue substitutions slow down the photocycle by 20-fold (Q513L) and 4-fold (Q513A). The mutation of Q513H, intended to place a basic group near the FMN protonation site, has a photocycle time of 27 s, which is one third of the WT lifetime (Table 1). However, Q513H has the peculiar effect of not completely vanishing under our illumination conditions (Fig. 2B). The mechanism of Q513H’s reduced photocycle time, is slowed 2-3 fold and there is a concomitant decrease in the amount of light activated conformational change [19,33]. Many molecular dynamics simulation studies suggest that solvent can enter the FMN binding pocket [28,29] and accessibility increases due to structural changes arising from the multiple mutations. Both experimental and computational studies that have attempted to change the location of this critical residue. We moved the original cysteine (C450V or C450A) to three different locations near the chromophore based on distance and geometry constraints, L453, F494 and Q513 (C450A/L453C, C450V/F494C and C450V/Q513C) (Fig. 3A). The C450A/L453C variant lacked a measurable photocycle. But, the other two mutations C450V/F494C ($t_{\text{FMN}} = 69$ s) and C450V/Q513C ($t_{\text{FMN}} = 44$ s) had photocycle times very similar to WT ($t_{\text{FMN}} = 80$ s) (Table 1). However only a small fraction of the population became photoexcited as indicated by minimal bleaching, <10%, (Fig. 2D and Fig. 3A).

We examined the effect of imidazole on C450V/Q513C variant’s photocycle. In the WT protein, the addition of imidazole hastens the photocycle [11,14], with 1, 2 and 3 mM imidazole reducing $t_{\text{FMN}}$ from 80 s to 12, 6 and 4 s respectively (Table 1 and Fig. 3B). The C450V/Q513C variant also has a noticeable imidazole effect with $t_{\text{FMN}}$ shortened from 44 s to ~9 s in 1-3 mM imidazole, very similar to the changes observed for the WT protein under the same imidazole concentrations (Table 1 and Fig. 3B).

We created a variant with altered solvent accessibility by introducing the N414G and N414T mutations in a C450V/Q513C background. We anticipated observing a change in photocycle times as was found with other variants when these mutations were made. The N414T/C450V/Q513C variant did not have a measurable photocycle, being too fast or non-existent. But the N414G/C450V/Q513C had a decrease in $t_{\text{FMN}}$ to 14 s compared to the 44 s for C450V/Q513C. The decrease may be due to structural changes arising from the multiple mutations causing a higher catalytic rate for water when the N414 group is absent (Table 1). The reversibility of the photocycle was unaffected by the new cysteine residues. However, the ability of the protein to form the covalent adduct or initially reduce the FMN is greatly decreased indicating that geometry plays a role in successful photoactivation.

### Hydration

In addition to cysteine location, interaction with solvent plays a role in the chemistry of photo-reversibility. Both experimental and molecular dynamics simulation studies suggest that solvent can enter the FMN binding pocket [28,29] and accessibility increases the speed of the photocycle [11,14]. Recently, a cluster of water molecules was implicated in deprotonation [29]. Experimentally, this property has been measured indirectly by inferring solvent accessibility from changes in isotope effects or a proposed base catalyzed imidazole reduction of the chromophore [11,14]. Here we wanted to test directly the effect of water on the speed of the photocycle. To do so, we air dried three different variants (WT, Q513A and N414L/Q513A) of the ALOV2 domain for >48 hours in the dark on the outside of quartz cuvettes at 22°C, and
measured the photocycle. We chose both Q513A and N414L/ 
Q513A variants to remove the possibility that either the N414 or 
Q513 residues were functioning in place of water to facilitate 
reduction of the chromophore. For the dehydrated versions, the 
FMN molecule retains its characteristic spectra and the \( \lambda_{\text{max}} \) does 
not measurably shift from the solvated version (Table 1 and 
Fig. 3C). The photocycle, however, is slowed with \( \tau_{\text{max}} \), decreasing 
by \(~2-20\) fold (Table 1). The dehydrated variants lose their ability 
to undergo conformational change as measured by CD and by 
FTIR [35]. These results suggest that water plays a significant role 
in the decay of the photocycle and conformational change. 

The dehydrated proteins can be rehydrated and they regain the ability 
to undergo conformational change. This recovery is indicative of a 
lack of protein denaturation or disruptive conformational changes 
as we have only observed minimal refolding of \( \alpha \lambda \text{LOV} \) after 
denaturation using guanidine hydrochloride or heat (data not 
shown).

Upon the removal of bulk solvent, we expected, but did not 
observe a change in the electronic environment around the 
chromophore sufficient to measurably shift \( \lambda_{\text{max}} \). Presumably, 
either the protein has tightly bound water molecules or the role of 
solvent in deprotonation is so transient that the effects are not 
easily measurable. The crystal structures of \( \alpha \lambda \text{LOV} \) [32] do not 
contain bound \( \text{H}_2\text{O} \) molecules near the chromophore. However, in 
NiIL, a very similar PAS domain, such molecules are observed 
near the chromophore and are required for oxidation of its bound 
flavin [36]. Together, this suggests that coordination of bound 
water molecules could be a conserved mechanism of PAS domain 
flavin redox reactions.

### Conclusion

Over a hundred mutations have been performed on \( \alpha \lambda \text{LOV} \) by 
us and others [11,13,19,24,26] yet the only residue required for 
photocycling is C450. These experiments suggest that the minimal 
requirements for a reversible photocycle are an active-site cysteine 
residue, a protein cage to protect the chromophore from solvent, 
and water to participate in the oxidation of the FMN [29]. The 
engineered cysteine variants provide new insight into the chemical 
process of how the covalent bond is formed with the flavin upon 
light excitation. Interestingly, the photocycle length in these 
modified cysteine variants is similar to the WT protein indicating 
that this geometry is not a critical factor for the deprotonation 
process.

During the photocycle of \( \alpha \lambda \text{LOV} \), the FMN chromophore is 
reduced to FMNH and is spontaneously oxidized back to its 
ground state at room temperature. At the start of the photocycle, 
the cysteine loses a proton and is the logical donor to the FMN 
based on proximity [9,37]. The other major candidate to be 
involved in deprotonation would be the Q513 residue but a 
number of studies [13,26,27] have shown that even upon mutation 
to inert functional groups LOV domains still photocycle with 
similar time constants. These data suggest that the deprotonation 
process only requires a cysteine and solvent. However, both Q513 
and N414 affect the length of the photocycle and the electronic 
nature of the chromophore as seen in \( \lambda_{\text{max}} \) changes.

Recent molecular dynamics studies suggest that Q513 plays a role 
in conformational change and does not flip to place its oxygen 

### Acknowledgments

The authors thank Keith Moffat, and members of our group for helpful 
discussions and comments on the article. This work was supported by 
research and training grants from the U.S. National Institutes of Health 
(GM088668 to T.R.S. and M. Glotzer, ST32GM07183-34 to B. Glick 
and the Chicago Biomedical Consortium with support from The Searle 
Funds at The Chicago Community Trust (to T.R.S., M. Glotzer, and 
E. Weiss).

### Author Contributions

Conceived and designed the experiments: JPZ TRS. Performed the 
experiments: JPZ. Analyzed the data: JPZ TRS. Wrote the paper: JPZ 
TRS.

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