Light-Oxygen-Voltage (LOV)-sensing Domains: Activation Mechanism and Optogenetic Stimulation

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Abstract

The light-oxygen-voltage (LOV) domains of phototropins emerged as essential constituents of light-sensitive proteins, helping initiate blue light-triggered responses. Moreover, these domains have been identified across all kingdoms of life. LOV domains utilize flavin nucleotides as co-factors and undergo structural rearrangements upon exposure to blue light, which activates an effector domain that executes the final output of the photoreaction. LOV domains are versatile photoreceptors that play critical roles in cellular signaling and environmental adaptation; additionally, they can noninvasively sense and control intracellular processes with high spatiotemporal precision, making them ideal candidates for use in optogenetics, where a light signal is linked to a cellular process through a photoreceptor. The ongoing development of LOV-based optogenetic tools, driven by advances in structural biology, spectroscopy, computational methods, and synthetic biology, has the potential to revolutionize the study of biological systems and enable the development of novel therapeutic strategies.

Introduction

This introductory review aims to provide the readers with a fundamental understanding of light-oxygen-voltage (LOV) domains while highlighting the vast and multifaceted nature of these domains. This review covers various aspects related to LOV domains, with a particular focus on LOV2 domains. The readers are encouraged to use this text as a starting point for exploring the subject further and delving into specialized reviews and research articles for a deeper understanding of LOV domains, their complex roles in cellular processes, and the latest advancements in such a fascinating field of study. This is exemplified in publications of LOV domains, ranging from mechanistic elucidation[1], design of protein light switches[2], reviews focused on cell control with light-induced LOV fusion proteins[3], and specifically in optogenetics applications[4] and the special issue published in this journal[5].

Phototropins were first described as crucial light receptors in plants because they contain LOV domains, which respond to light stimulation and changes in the redox environment. The study of these domains began in the late 1990s with the identification of the *NPH1* gene, which encodes *Arabidopsis thaliana* blue light-activated

phototropin NPH1 (*At*NPH1), a critical component of the initial steps of phototropism signal transduction[6]. In higher plants, the effects of light on development are controlled by several classes of photoreceptors, including red-light-activated phytochromes, blue/UV receptors UV Resistance Locus 8 (UVR8), and blue-light-activated cryptochromes and phototropins[7–10].

Phototropins (Phot1/2) are plasma membrane-associated proteins possessing an effector protein in the C-terminus and hydrophilic chromophore domains (*i.e.*, LOV domains) in the N-terminus[11]. Specifically, *At*NPH1 is a 120-kDa soluble protein containing two flavin mononucleotide (FMN)-binding domains[12,13] and an autophosphorylating serine/threonine kinase output domain[6,14]. The flavin-binding domains of *At*NPH1 share similarities with other blue light proteins discovered in the decade prior to the identification of *At*NPH1, such as *Halobacterium salinarum* (*Hs*)Bat[15], *Neurospora crassa* White Collar-1 (*Nc*WC-1)[16], and *Bacillus subtilis* (*Bs*)YtvA[17]. These domains can be identified by a conserved Cys residue that binds to FMN and is situated within the highly conserved sequence GXNCRFLQ[18]. Table 1 summarizes the LOV domains of several species, their C-terminal effector domains, and the conserved active-site residue sequences.

LOV domains have been classified as a subfamily of the Per-ARNT-Sim (PAS) domains, and are described as signaling modules that monitor changes in light, redox potential, and effectors, and participate in protein–protein interactions[19,20]. LOV domains generally bind to FMN, or less commonly flavin adenine dinucleotide (FAD), as their chromophore. The binding of FMN to the LOV domain induces structural changes that propagate to the domain surface, altering its intra- and inter-protein interactions. Once the function has been exerted, the LOV domains return to a resting state, deemed the dark state, which denotes the end of the LOV photocycle[21–24]. Subsequent studies have investigated the signaling pathways triggered by LOV domains and their involvement in inducing structural alterations in effector domains[25–29], as described in the following sections.

Effectors identified as binding partners of LOV domains include proteins involved in phototropism (Phot1/2)[14,30–32], seasonal gene transcription[33], bacterial stress responses[34–37], bacterial virulence[38], and circadian clock photosensors[21,39–41], among others. In association with LOV domains, these molecules control the activation of a diverse range of proteins, including kinases[11,42,43], sulfate transporter/anti-sigma factor antagonists[44], phosphodiesterases[29] and phosphatases[45], and DNA-binding domains[29,46]. The latter includes the algal basic leucine zipper, Aureochrome[22,47,48], and the zinc finger-containing *Nc*WC-1[49,50].

Notably, not all LOV domains require an effector domain to exert these functions; such domains are known as "short" LOV domains[41]. Examples include VIVID from *Neurospora crassa* (*Nc*VVD), which is involved in photoadaptation[21,51] and binds a protein partner in the White Collar Complex[16,52,53], the LOV domains from *Pseudomonas putida* (*Pp*SB1-LOV[54] and *Pp*SB2[55]), and the LOV domain from *Rhodobacter sphaeroides*, which has an unusually extended C-terminal α-helix (∼50 aa) and shows very slow dark-state recovery[56]. Short LOV proteins lack a covalently attached effector and presumably transduce signals in *trans* to a distinct protein[57].

The output of the LOV domains photocycle affects protein signaling pathways in cells. For example, when sensing redox changes, LOV domains respond by acting on different proteins or target genes; *Erythrobacter litoralis* LOV (EL222) acts on, among others, NAD synthetase[58], *Pp*-SB1-LOV acts on photosynthetic genes[59], and *Bs*YtvA acts on the general stress transcription factor *sigB*[29,60,61].

Moreover, many LOV domains exist as monomers or dimers in the resting state or dimerize following blue light absorption. Because of this modularity and their participation in sensing and signaling pathways, LOV domains have attracted considerable interest, and engineered LOV fusions have been developed for use in optogenetics, which denotes the generation of genetically encoded light-gated molecules capable of modulating biochemical pathways and organelle compartmentalization in living cells and organisms[62–65]. This interesting-but hitherto incompletely understood-activation mechanism is, to our consideration, a major bottleneck in the study of LOV domains. Elucidating this process is essential for advancing our knowledge of both LOV domains and their associated signaling pathways. Owing to the experimental obstacles associated with studying the rapid dynamics of light-responsive proteins and the slower interdomain signal transduction, this area of research presents unique challenges[4,26,28,62].

As LOV domains perform diverse functions and are present in numerous species, understanding their structure, mechanism of action, and functionality in nature requires cooperation from multiple scientific disciplines. This review outlines recent experiments as well as computational studies that elucidate the complex molecular mechanisms of LOV-FMN-effector systems, their three-dimensional structures, and photocycle dynamics. Because the overarching goal of studying natural phenomena is to replicate and use these processes for practical applications, and thus contribute to advancements in health and environmental sustainability, a section of this review is dedicated to discussing the application of LOV domains in optogenetics.

Table 1 Light-oxygen-voltage (LOV) domains present highly conserved residues surrounding the flavin mononucleotide (FMN)-binding site.

SPECIES	LOV DOMAIN	EFFECTOR DOMAINS	CONSERVED RESIDUES[42,*]
Higher plants[11, 31, 43]			
Arabidopsis thaliana	*At*LOV1/2	Serine/Threonine kinase	GRNCRFLQ
Oryza sativa	*Os*Phot2-LOV		GRNCRFLQ
Avena sativa	*As*LOV1/2		GRNCRFLQ
Green algae[32]			
Chlamydomonas reinhardtii	*Cr*LOV1	Serine/Threonine kinase	GHNCRFLQ
Chlamydomonas reinhardtii	*Cr*LOV2		GRNCRFLQ
Fungi[20, 40]			
Neurospora crassa	*Nc*WC-1	Unknown	GRNCRFLQ
Eubacteria[34, 38, 44, 64]			
Xanthomonas campestris	*Xc*LOV	Histidine kinase	GNNCRFLQ
Xanthomonas axonopodis	*Xa*LOV		GNNCRFLQ
Erythrobacter litoralis	*El*LOV (EL222)		GRNCRFLQ
Brucella abortus	BaLOV	Histidine kinase (virulence)	GRNCRFLQ
Listeria monocytogenes	*Lm*LOV	Sulfate transporter/anti-sigma factor antagonist (STAS)	GKNCRFLQ
Bacillus subtilis	*Bs*LOV (YtvA)		GRNCRFLQ

*In the column *Conserved residues*, the variable amino acid is marked in green and the conserved photoactive cysteine in orange.

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LOV Domain Structure and Dynamics

Many photoreceptors detect light by absorbing photons via their chromophore co-factor, although there are exceptions such as UVR8, that does not have any external chromophores and uses a natural amino-acid tryptophan for light sensing[10,66]. LOV domains use a co-factor that, upon photon absorption, initiates the photocycle of the photoreceptor, which comprises a series of

A. Flores-Ibarra, Raiza N.A. Maia, B. Olasz, et al.

events involving conformational and dynamic changes in both the chromophore and the protein domain[3,11,23,32,41,67–69].

The chromophore co-factor in the LOV domain is a flavin nucleotide (FMN or FAD) that is responsible for the light-sensing properties of LOV. Studies on *Avena sativa* LOV1 and LOV2 domains (*As*LOV1/2) have shown that both domains, when expressed alone or in tandem, undergo a self-contained photocycle involving the formation of a covalent adduct between the FMN chromophore and a conserved active-site Cys residue[70]. In another study, NMR spectroscopic analysis of a recombinant *Avena sativa* (*As*)LOV2 domain with a $^{13}C/^{15}N$-labeled FMN under light and dark conditions revealed that blue light irradiation resulted in the addition of a thiol group from *As*LOV2-Cys450 to the C4a position of the FMN chromophore, which reversed spontaneously in the dark[71]. The Cys residue (Cys450 in *A. sativa*) is conserved among many LOV domains (see Table 1) and is the most common residue involved in the initiation of the photocycle due to its capacity to covalently bind the FMN molecule. This process is conductive to a series of conformational changes in the adjacent residues and eventually in the entire protein, allowing the signal to reach its effector domain(s)[68,72,73].

The LOV photocycle comprises distinct dark (resting) and light (signaling) states, as well as several intermediate states. Flash photolysis studies have demonstrated that the reaction proceeds through an excited singlet state of flavin to a triplet[23] state that decays monotonically to form a thioadduct[74,75]. Typically, the photocycle is fully reversible, as the photoreceptor thermally reverts to its ground state after light absorption[74–76]. X-ray crystallography studies on various LOV domains have confirmed the described reactions[68,77–79], and spectroscopic studies have helped create a more complete understanding of the LOV photocycle.

X-ray crystallography structures of LOV domains

Studying LOV domains through crystallography can be challenging, as X-ray exposure in the active state can radiolyse the Cys–C(4a) bond and revert the protein to its dark state[79]. Nevertheless, the first X-ray structure of *Adiantum capilllus-veneris* Phot-LOV2 domain (at a resolution of 2.7 \AA) revealed a structure comprising five β-sheets connected by α-helices that formed the framework where the FMN chromophore bound to the photoreceptor[77]. The photoreactive cysteine was located 4.2 Å from the FMN-C(4a)[77] carbon and, after light induction, moved approximately 2 Å closer to form the adduct[78].

The conformational changes in the LOV domain after light activation are facilitated by several of these structural elements (Figure 1 shows a graphical depiction), specifically the core β-sheet scaffold, the N-terminal cap with an A'α-helix, and

a Jα-helix[78,79]. The core β-sheet scaffold, comprising five antiparallel β-strands (Aβ, Bβ, Gβ, Hβ, and Iβ) forms the chromophore binding pocket and hosts residues that directly interact with the FMN, participating in signal transmission to the flanking helical regions or even farther to the effector domains, stabilized by hydrophobic interactions and a conserved hydrogen-bond network (Figure 2A)[77,78,80,81]. The A'α- and Jα-helices play key roles in signal propagation and protein–protein interactions, and thus regulate the functionality of the light response of the LOV domain[25,29,43,82]. In *As*Phot-LOV2, the Jα-helix is located in the C-terminus and has been shown to unfold away from the active core[25,39,79].

Several more structures of LOV domains have been described, which further contribute to our understanding of the three-dimensional structures and mechanisms of these proteins. Crystallographic data from the *Cr*LOV1 domain (at resolutions of 1.9 and 2.8 \AA in the dark and light states, respectively) revealed two conformations of the binding-site cysteine (Cys57) in the resting state and confirmed that blue-light absorption readily causes covalent bond formation between the FMN-C(4a) and the thiol of Cys57[79]. The crystal structure of the *Bs*YtvA-LOV domain (at 1.45 \AA-res dark-state, and 1.95 \AA-res light-state) showed that this domain, dimerizes via a hydrophobic interface[68]. Structural analysis of *Nc*VVD and *Nc*WC-1 proteins showed that the N-terminal α-helix-β-strand extension of the LOV domain of *Nc*VVD reorients upon illumination, promoting its interaction with *Nc*WC-1, which in turn regulates photoadaptation. In both structures, the external helices interact with the β-sheet surface of the LOV domain, and both the dark and light states display an N-terminal helix-turn-helix (HTH) motif that is adjacent to the core domain and in close proximity (or even interacting) to the C-terminal Jα helix[21,52].

Experiments performed in Phot-LOVs, and in the prokaryotic LOV domains *Bs*YtvA and *Nc*VVD, demonstrated the presence of a LOV dimer, wherein upon light absorption, each monomer tilts ~5.0° away from each other, the active Cys rotates around the dark-state Cα-Cβ, and the covalent adduct is formed (Figure 2B). The FMN isoalloxazine ring, originally planar, tilts by ~7.0° and the orbital of the C4a atom hybridizes from sp^2 to sp^3 in the adduct, thus protonating the adjacent N5 atom (transforming it from a H-bond acceptor in the dark state into a donor in the light state)[43,68,83]. In these LOV domains, the N5 protonation causes two conserved Asn residues to change their orientation and a Gln on strand Iβ to "swing and flip," *i.e.*, this residue turns to stand in juxtaposition of its original orientation (Figure 2C), forming a new H-bond network among the three residues that bridges the Gln and Jα-helix in the LOV domain core (Figure 2C), which is considered the first step of signal propagation to the effector
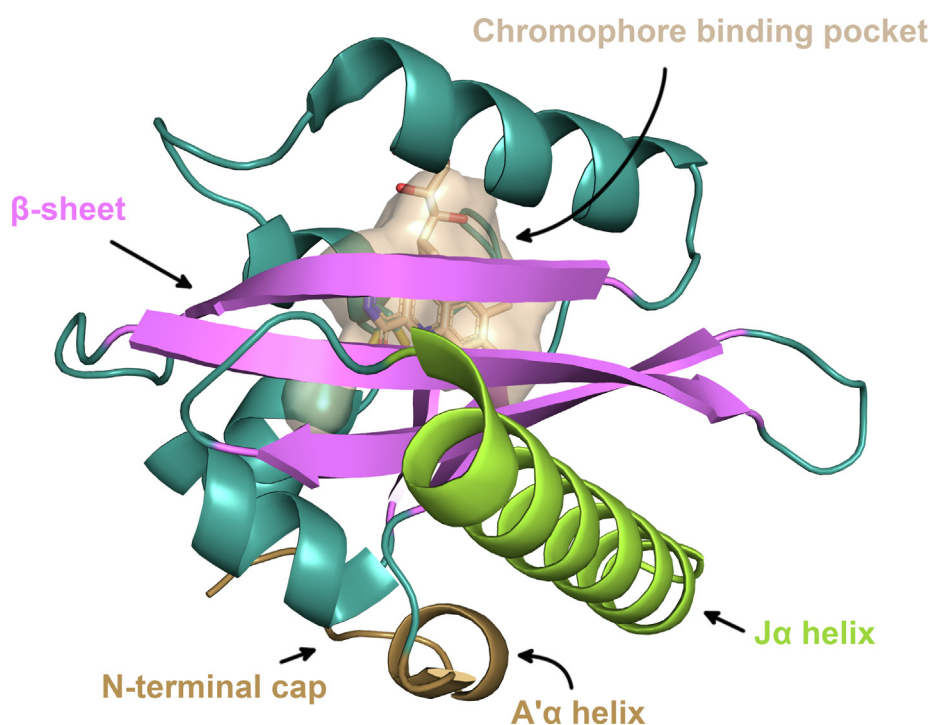
Fig. 1. Structural elements forming the light-oxygen-voltage (LOV) domain. LOV domains consist of a core scaffold comprising five antiparallel β-sheets (purple structure) where the chromophore binding pocket is located (yellow sticks and surface), an N-terminal cap (Ncap) with an A'α-helix (ochre structure), and the C-terminal Jα-helix that interacts with effector proteins (green structure) [PDBID: 2V1A].

domains[39,43,73,84]. Although this is not always the case, studies performed on an LOV domain from *Ochromonas danica* showed that signal propagation is associated with LOV dimerization, as the disruption of dimer formation using a 5-deazaflavin mononucleotide effectively prevents all subsequent reactions of the photocycle[73].

The crystal structure of an LOV domain isolated from the 222-amino acid *Erythrobacter litoralis* HTCC2594 ocean proteobacteria (EL222, 2.1 \AA-res) exhibited direct interactions between the surface of the β-sheet of EL222-LOV and the 4α-helix and 1α-2α loop of the HTH domains in its dark state, inhibiting DNA binding in EL222-LOV. The plasticity of this interface enables smooth conformational transition through allosteric changes in the structure of the LOV domain. Upon light exposure, the interaction between the LOV and HTH domains is disrupted, allowing EL222-LOV to bind to DNA[80].

The cumulative insights from crystallographic structures demonstrate the versatility of LOV domains, and indicate that beyond their shared core, these domains can be differentiated based on the structural variations present in their N- and C-terminal extensions (Figure 3). This is reflected in characteristics such as the variety of light-dependent molecular mechanisms, different tendencies to dimerize, and distinct activation of effector domains that each LOV domain exhibits[63,85–88].

Dynamics of the photocycle of LOV domains

In addition to structural biology approaches, several steady-state and transient spectroscopic studies have been widely conducted on LOV domains to address questions regarding their activation, adduct formation, bond breaking, and relaxation. Moreover, these studies have characterized the physical and chemical changes in the protein and chromophore during the primary reactions, namely the transition from the electronic singlet to triplet state.[75,83,89,90].

Upon blue light exposure, the LOV domain is bleached, losing the complex spectral structure of FMN between 400 and 500 nm and instead forming a product with a single absorption maximum near 385 nm and three isosbestic points between 300 and 410 nm, corresponding to the formation of the Cys–C(4a) adduct[70]. Nanosecond laser flash spectroscopy facilitated the identification of triplet-state photoproducts in the photocycle, revealing transient species with broad absorption peaks at 510 and 660 nm, characteristic of triplet-state flavin[74]. Once in darkness, the original flavin spectrum is restored, and the domains can be repeatedly reactivated by light to form the Cys–C(4a) adduct[70]. Notably, different decay times for the triplet state have been observed when using different buffers for *As*LOV2, such as 2–4 μs (sodium phosphate buffer)[74,91], 6 μs (tris buffer)[91,92], and 9.5 μs (deuterated tris buffer)[93]. Furthermore,
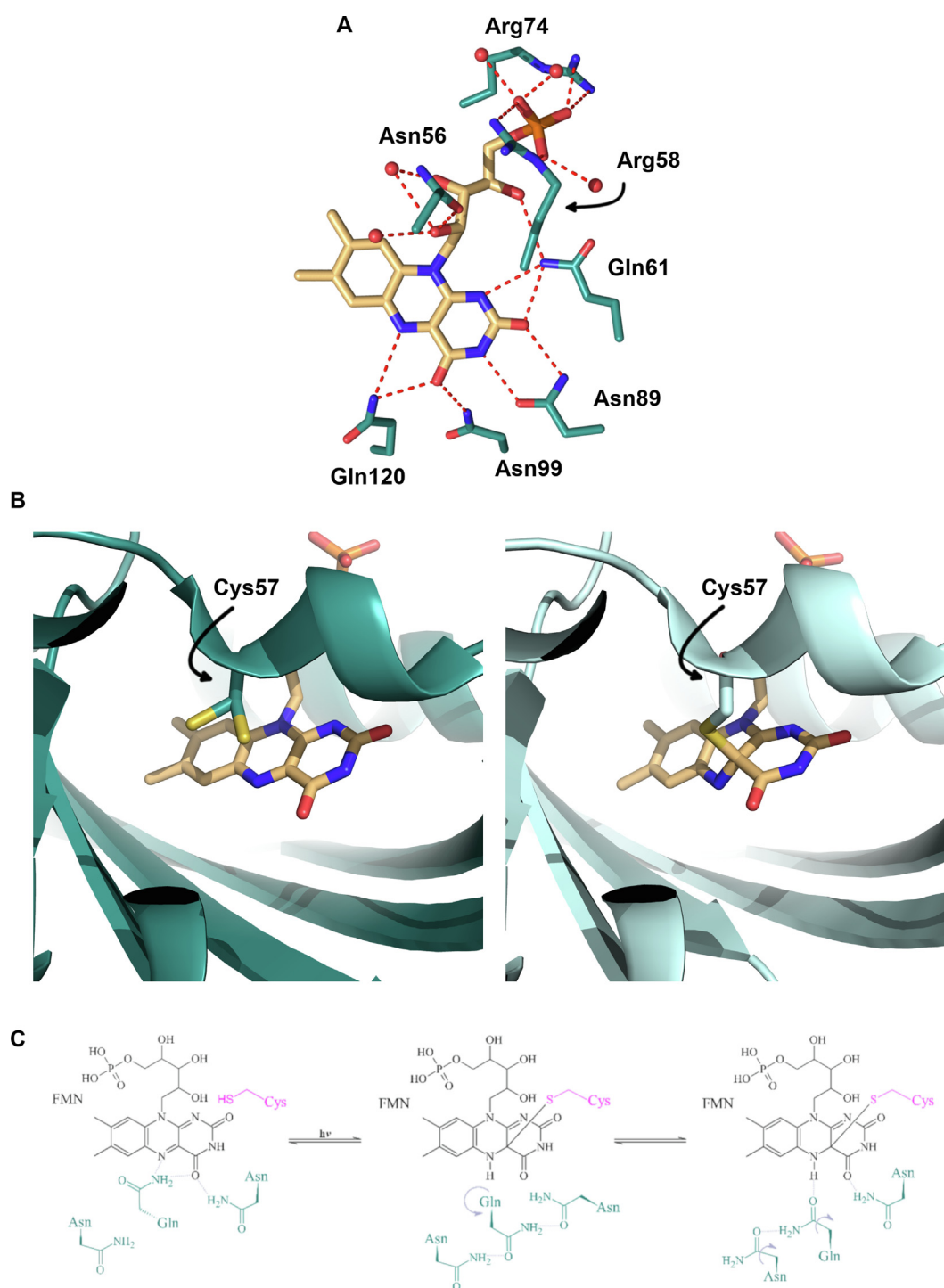
Fig. 2. Photocycle and binding mode of the flavin mononucleotide (FMN) chromophore to the Phot1-LOV1 domain of Chlamydomonas reinhardtii (PDBIDs: 1N9L and 1N9O). (A) The hydrogen bond network securing the FMN molecule in the chromophore binding site (FMN is shown as orange sticks, the residues of LOV1 as dark teal sticks, water molecules as red spheres, and H-bonds as red dashed lines). (B) The photoactive Cys exhibits two conformations in the crystallographic structure of the dark state (left, dark teal structure, PDBID: 1N9L), and it is the rotation of this Cys that enables photoadduct formation with the FMN (orange sticks) after blue-light exposure (left, light teal structure, PDBID: 1N9O). (C) Chemical schematic representation of the photocycle of a light-oxygen-voltage (LOV) domain and its "switch and flip" effect on the neighboring Gln.
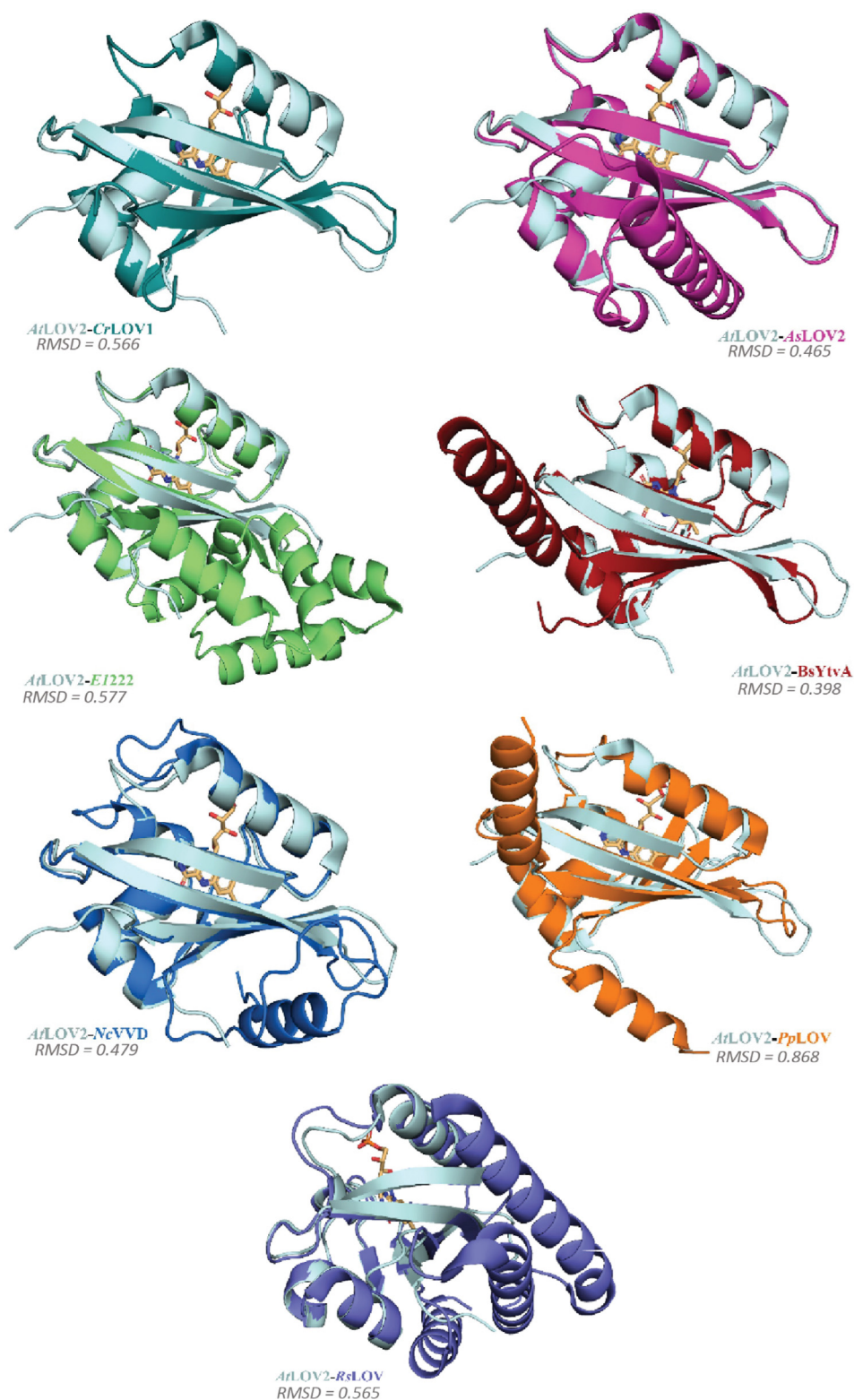
Fig. 3. Comparison of monomers of light-oxygen-voltage (LOV) domains with the canonical ***At*Phot1-LOV2.** While the flavin mononucleotide (FMN)-binding N-terminal domains almost fully align with each other in seven out of eight species compared, the C-terminal of each protein is very variable, pointing at the distinctive functionalities of LOV domains in nature [PDBIDs: *At*LOV2 (4EEP), *Cr*LOV1 (1N9L), *As*LOV2 (2V1A), *El*222 (3P7N), *Bs*YtvA (2PR5), *Nc*VVD (2PD7), and *Pp*LOV (3SW1) [RMSDs are given in Å].

A. Flores-Ibarra, Raiza N.A. Maia, B. Olasz, et al.

amino acid substitutions have been shown to affect overall oxygen accessibility to the FMN binding pocket[91].

Fourier-transform infrared spectroscopy (FTIR) analysis of the infrared absorption spectrum demonstrated that light treatment induced structural changes in both the FMN co-factor and the LOV domain[93]. Notably, the derived *in vivo* reaction kinetics may differ, as demonstrated in the pioneering in-cell FTIR studies[94]. To study the changes in FMN, vibrational spectroscopy was performed to demonstrate that after photoactivation, the dipole moment of FMN underwent substantial modifications as a result of the formation of the cysteinyl-adduct state. Consequently, the dominant changes observed in the infrared spectra of the LOV domains were primarily attributed to variations in the vibrational bands of the FMN chromophore[95]. The $C=O$ region between 1670 and 1750 cm^{-1} is dominated by FMN $C(2)=O$ and $C(4)=O$ vibrational modes. The positive vibrational modes around 1650 and 1620 cm^{-1} have been assigned to carbonyl vibrations in the unprotonated triplet state using transient spectroscopy[89,95]. In addition to studies focusing on FMN, light-induced FTIR spectroscopy has facilitated the identification of S-H stretching modes in the reactive cysteine of *Cr*LOV1[95]. The negative intensity of the band indicates protonation of the reactive cysteine prior to the formation of a covalent bond with C4a of FMN[96]. Subsequent investigations conducted on *Cr*LOV1 and *Cr*LOV2 domains identified S-H vibrations at 2570 and 2573 cm^{-1}, respectively. In the LOV domain of *Bacillus subtilis*, the S-H vibration is observed at a down-shifted frequency of 2569 cm^{-1}, indicative of differences in the hydrogen-bonded environment among the various LOV domains[97].

A strong negative band at 1647–1650 cm^{-1} in *At*Phot1-LOV2 was assigned to the Jα-unfolding event via steady-state light minus dark FTIR[97,98]. Secondary structural changes in the amide I-II ranges were successfully identified for *As*Phot1-LOV2[99] and a variant of AtPhot2LOV2[90] domains. Furthermore, ultra-fast transient spectroscopy yielded valuable insights into a series of significant events in *As*LOV2, including the real-time unfolding of the Jα-helix, conversion of the excited FMN chromophore from singlet to triplet state, and formation of the covalent Cys-FMN adduct[100].

Structural changes resulting in the light state of *At*Phot2LOV1 have been elucidated using time-resolved FTIR spectroscopy and assigned to specific parts of the protein[90]. Recently, time-resolved infrared spectroscopy was used to trace proton transfer from the reactive cysteine to the flavin chromophore in a variant of the short-LOV protein derived from *Dinoroseobacter shibae*[101].

Similarly, independent spectroscopy and time-resolved X-ray solution scattering experiments (TRXSS) conducted on *Bs*YtvA revealed that the FMN-bound LOV domain exhibits spectral changes upon irradiation that are consistent with the formation of the Cys–C(4a) adduct (LOV-383), albeit accompanied by a smaller volume contraction ($\Delta V_T = -1.5$ ml/mol) and a faster adduct decay time (2 μs) than that of the phototropin LOV domains from higher plants[68,72]. Simultaneously, TRXSS provided structural data on *Bs*YtvA, showing that rearrangements occur throughout the LOV domain after adduct formation, in which the two subunits rotate away from each other and the FMN isoalloxazine ring loses its planarity (Figure 4)[102].

However, biophysical and computational studies on LOV domains have also contradicted the previously described steps in the photocycle. For example, the flipping of the β-strand H-bond Gln (see Figure 2C) and the protonation of FMN-N5 to send the activation signal through the LOV domain surface into the effector domain had been considered essential for LOV domain function. More recent research using spectroscopy and molecular dynamics simulations has indicated that several LOV receptors retain signaling responses, albeit attenuated, in the absence of the conserved Gln, which is substituted naturally by Leu and Ile[69,93,103], and experimentally by Trp in *Ostreococcus tauri* LOV1 domain[104]. This, in turn, indicated the presence of a primordial LOV ancestor from which glutamine-deficient LOV domains could stem, but still function as blue-light receptors; LOV signal transduction in this ancestor would have relied on flavin photoreduction and water mediation, employing a less sophisticated action mechanism[1,105].

Computational studies of the LOV photocycle

The numerous insights that can be obtained from multiscale approaches, molecular dynamics and bioinformatics make computer simulations an essential tool for studying the signaling photocycle mechanisms of LOV domains. The computational toolkit ranges from highly accurate quantum chemical methods for studying the electronic structure and spectroscopic parameters of flavin[106–108] to molecular dynamics simulations using restricted to classical force fields[109]. In molecular dynamics simulations performed with *As*Phot1LOV2[110], different trajectories were computed, some based on the dark- and others based on light-adapted structures. The results showed a correlation between the rotation of the glutamine 513 sidechain and the movement of the Jα-helix. This was established for two different paths; one path involved the shift of the LOV core domain Iβ sheet, while the other path proceeded through coupling with the N-terminal A'α-helix. However, Freddolino et al emphasized that their 200 ns trajectories were insufficient for confirming the unfolding of the Jα-helix, thus necessitating further simulations.
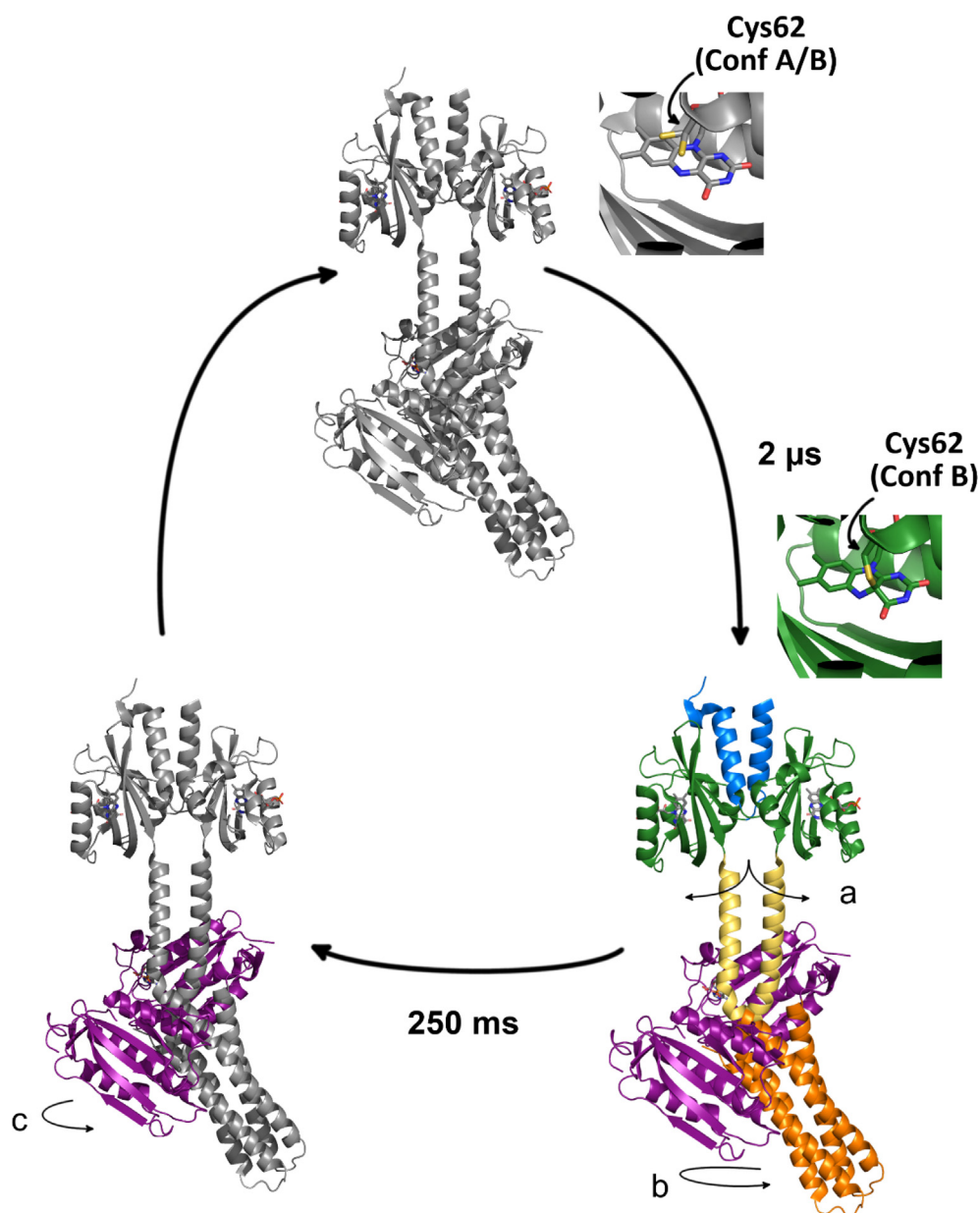
Fig. 4. Structural changes initiated by blue light activation in the YF1 protein. Blue-light irradiation results in sequential structural rearrangement in the protein. After 2 μs, the light-oxygen-voltage (LOV) domains separate by approximately 3 Å from the Jα helices (green, arrow a). This leads to the rotation of the kinase module (purple and orange, arrow b). On a slower timescale, the kinase domain undergoes an internal rearrangement (purple, arrow c). Conformation A of the Cys62 in the inset is 0.7 and Conformation B is 0.3 in the dark state. (Modified from Berntsson et al.[102]).

This shortcoming was addressed by Tao and coworkers, who revisited the transition between the dark- and the light-adapted states in *As*Phot1LOV2 using longer MD simulations (1.5 μs trajectories) with sophisticated analyses, Markov state models, and machine learning techniques, in addition to testing four mutants[111]. The simulations revealed that Thr407 and Arg410, located in the A'α-helix, are responsible for the conformational switch. Moreover, MD simulations at

1.5 μs showed the unfolding of the Jα-helix and linked the conformational change to the rotation of Q513, which is also involved in important interactions with N414[112]. However, the key role of Q513 was recently questioned in an experimental and computational study, where MD simulations showed that the absence of Q513 is compensated for by an influx of water molecules that transiently approach the flavin chromophore. It was hypothesized that these water molecules form a cluster that

subsequently propagates the protonation of the FMN further downstream[1].

Challenges and strategies in the study of LOV domains

In summary, LOV photosensor domains are activated when the dark state co-factor absorbs a photon of blue light (∼450 nm), creating an excited flavin singlet state[75] that decays into a triplet state over several nano-to-microseconds (the duration being species-dependent)[74]. Within microseconds, likely through a radical intermediate[70], a covalent Cys–C(4a) thioadduct is formed between a conserved Cys residue within the LOV domain and C(4a) in the isoalloxazine ring of the flavin co-factor, which is typically FMN[9] but can also be FAD[46,67]. Simultaneously, experiments have shown that mutating Cys to certain amino acids in the binding site maintains light activation of the LOV domain to a certain degree[76,113]. Furthermore, dimer formation following thioadduct formation has been found to occur only in some LOV domains, whereas some LOV domains are already dimeric in the dark and some do not dimerize at all[39,68,73,81,85,114]. Following thioadduct formation, the flavin nucleotide N5 nitrogen is protonated[32,75], which in turn causes nearby conserved residues, glutamine, and sometimes two nearby asparagines, to rearrange in the H-bond network, initiating downstream signaling[21,68,78]. A common motif involves LOV activation modifying a binding site on the surface of a β-sheet adjacent to the chromophore, occupied by either a C-terminal Jα-helix (in short-LOVs) or the N-terminal Ncap A'α-helix[1,67,69,93,103,104,115,116].

However, the light-induced changes in photosensitive proteins may depend heavily on the experimental approach used to measure them. To avoid bias introduced by the choice of technique, the photoactivation mechanism of photosensitive proteins should be validated using complementary techniques to precisely map the steps of both the mechanism and the activation of downstream effector proteins[62,79,95,117,118].

Questions that remain open regarding the study of LOV domains include discrepancies in the initial signal generation and propagation steps, which represent a major bottleneck in LOV research. Furthermore, as it is now possible to engineer other proteins with LOV domains to control them using light signals, understanding the precise activation and deactivation mechanisms of LOV effector proteins could substantially contribute to the development of improved optogenetic tools[62].

In this context, a major focus of LOV domain research is the elucidation of the precise molecular mechanisms governing their activation and deactivation in response to blue light. Research on this subject not only extends our understanding of the dynamic processes within LOV domain photocycles, including the rates at which photoadducts form and decay, but also how these dynamic events impact the propagation of signaling pathways. One particularly significant question pertains to the precise rate and pathway governing the decay of the triplet state into the adduct state, which is pivotal for understanding the kinetics of this process. However, this endeavor presents numerous experimental challenges. Similar to ultrafast timescales, spanning from femtoseconds to microseconds, traditional spectroscopic methods are often too slow to capture these rapid processes. The complexity of the reaction pathways of the LOV domains[74,89], particularly the transition from the triplet state to the adduct state, may involve complex reaction mechanisms[90]. Environmental sensitivity, as previously mentioned, including experimental conditions[74,93,119] such as temperature, pH, and the presence of co-factors or interacting proteins, can significantly influence the rate of decay, making it essential to control and monitor these parameters.

To overcome these challenges and improve our understanding of the LOV domain photocycle, we propose employing more sophisticated methods to monitor dynamic occurrences throughout the photocycle at a near-atomic resolution. Among these, ultrafast infrared spectroscopy (UF-IR) has emerged as a useful tool to facilitate the real-time monitoring of ultrafast processes occurring on femtosecond to picosecond timescales[89,93]. UF-IR enables tracking of changes in vibrational modes within specific chemical bonds during the transition from the triplet state to the adduct state. The advent of two-dimensional infrared spectroscopy (2D-IR) further contributes to this, as it offers in-depth insights into the structural and dynamic alterations accompanying the decay of the triplet state. By correlating multiple frequency dimensions, 2D-IR can untangle complex reaction pathways[120,121]. Complementing these methods, time-resolved serial X-ray crystallography enables the observation of localized light-induced changes in crucial amino acid side chains, thus offering high-resolution structural insights[122]. These advanced techniques, with their combined temporal and structural resolution capabilities, offer a means to elucidate the complex photoreaction kinetics, rate decay mechanisms, and dynamic processes inherent to LOV domains[2,5]. These approaches aided by molecular quantum simulations can provide insights into global protein changes, functional mechanisms of LOV domains and effector domains.

Several approaches have already been proposed to resolve this issue. In an experiment monitored every 63 ms, the population of *At*Phot2-LOV2 in a crystal showed that, the crystal volume increased as the LOV domain progressively photoconverted from the dark to the light state. Further experiments showed that after a time period corresponding to twice the relaxation time constant, the space group of the crystals changed

from P4$_3$2$_1$2 to P2$_1$2$_1$2$_1$, suggesting that the *in crystallo* relaxation of the photoadduct population leads to a phase transition and a reordering of the C-terminal region of the protein into two distinct conformations in adjacent monomers in the crystal, in correlation with the flipping of a Trp side chain[123,124].

LOV in Optogenetics: Applications and Advances

As discussed in the previous section, it is essential to expand and improve methods for studying LOV domains and other photoreceptors to facilitate their utilization in controlling physiological responses to light stimuli. In this section, we discuss the topic of optogenetics, noting the recent publication of an entire issue dedicated to it in this journal at the beginning of this decade[5].

Optogenetics refers to the use of light-sensitive domains genetically fused to functional effector domains to create a photosensor protein unit that enables non-invasive control of biological processes in cells and entire organisms with spatiotemporal precision and accuracy. This approach can be used to study complex, highly dynamic biological systems as well as develop novel therapeutic strategies[2,62]. This field originated primarily in neuroscience, with a notable example being the discovery that light-gated channelrhodopsin-2 from *C. reinhardtii*[125] can be expressed in mammalian neurons to modulate neuron depolarization in milliseconds using blue light[126]. Subsequently, using *Saccharomyces cerevisiae* cells mutated to delete their kinase activity kinase activity, a phenotype that exhibits growth defects, it was shown that *Cr*LOV2 can be heterologously expressed in yeast cells to compensate for these defects and recover cell growth in a blue light-dependent manner[127].

The inherent modularity of LOV receptors, along with their ability to undergo structural changes in response to light in a manner that affects the associated domains, make them highly attractive candidates for use in optogenetics[65]. Prokaryotic LOV proteins are notable for their remarkable modularity, with approximately 45% of their effector proteins comprising histidine kinases, phosphodiesterases, and cyclases[128–130]. Table 2 outlines the primary criteria for creating efficient photosensors for optogenetic applications. A comprehensive website cataloging protein systems for optogenetics can be accessed at https://optobase.org.

Engineering LOV-based optogenetic tools

Over the past decade, numerous systems using LOV domains have been established, and research on new methods to engineer light-activated modules with LOV continues (Table 3 summarizes some of the most notable experiments).

To understand the signal reception and processing of the LOV domains, a chimeric LOV domain fused with a light-inert kinase (YF1) was designed to create a blue light-responsive protein, LOV-YF121. In subsequent studies, experiments involving a series of random mutageneses were performed on LOV-YF1, which showed that some of the variants retained their biological activity, while others were abolished or inverted. One variant group carried mutations within the LOV photosensor that disrupted the proper coupling between FMN and the protein, whereas a larger group carried mutations that clustered at the dyad interface and disrupted signal transmission. This information provides a foundation for the customized reprogramming of signal receptors[131].

Experiments on *Nc*VVD-LOV without the Cys-binding site demonstrated light-induced dimerization and signaling due to flavin protonation of the neutral semiquinone, a feature shared by both the cysteinyl adduct and semiquinone. This general mechanism is also conserved in LOV-like regulators lacking conserved Cys, which respond to the chemical or photoreduction of their flavin co-factors. The ability to adjust the LOV reactivity through photoreduction has important implications for studying the mechanism of LOV activation and its applications[132].

The first genetically encodable, light-induced, flavin-binding source of singlet oxygen ($^1O_2$) was developed using *At*LOV2[133]. This was a 106-amino acid engineered LOV domain with six mutations, two of which involved residues surrounding the chromophore that conferred increased rigidity to the FMN environment[134,135]. Owing to its smaller size and the generation of $^1O_2$ upon exposure to blue light, it was considered a miniSOG (singlet oxygen generator). The miniSOG was mainly designed for use in correlative light and electron microscopy[133] although it has now found its way into phototherapeutics as well[136,137]. Notably, $^1O_2$ is highly reactive and photooxidizes substrates, such as proteins, lipids, and nucleic acids, a property that has found application in photodynamic cancer therapy and chromophore-assisted laser inactivation[136,138]. Further research has shown that it is possible to modify the flavin specificity of the miniSOG, thus providing a novel approach for adjusting photosensitization and other physical properties of the protein[135]; for example, electron transfer from the LOV domain to a photosensitizer can be modified by mutating the flavin-binding site[139]; it can be either disfavored by replacing the amino acids around the binding site with amino acids with a comparatively low redox potential[137,140], or enhanced by performing a strategic insertion of a Tyr close to the FMN[141].

Table 2 Components for designing photoactive-effector proteins in optogenetics.

Main features[2,62,128]	
Encoded in a single polypeptide	
Photosensitive domain	Effector domain
e.g., LOV, Rhodopsin	*e.g., histidine kinases, helix-turn-helix*
Co-factor for light-activation	
Cellular constituent	Supplied exogenously or with
e.g., FMN, BV	chromophore-synthesis genes
	i.e., if not widely available or a cellular constituent
Precision for activation/inactivation[76,85]	
Dynamic range	
Leakiness or background activity in light, dark, and/or intermediate states	Energy difference between the light and dark state
i.e., the bigger the energy difference, the higher the background activity	
Safety	
Dark recovery time	Speed of diffusion and spatiotemporal
i.e., how long is the effect going to last and the photoreceptor be unavailable	confinement
	i.e., how accurate is protein location
Complexity[3,27,28]	
Linker between photodomain and effector	
Distribution of conserved domain positions	Linker length
i.e. there is a higher degree of complexity for LOV domains that bind effector domains (HisK, HTH and STAS) than for short-LOVs	*i.e. preservation of heptad repeats in helical linkers*
Type of light-dependent activation	
Association/dissociation:	Induced allostery:
– *reconstitution of function from split fragments*	– *independent of expression levels*
– *recruitment to site of function*	– *protein stays in its natural context*
– *concentration dependency*	
Protein recruitment	Dimerization

Regarding the use of LOV-based optogenetics to drive gene expression, a breakthrough was made when the HTH motif of EL222 was found to have the ability to bind DNA after blue light illumination[142]. Regarding cell compartmentalization, control of protein localization has also been achieved by attaching signaling peptides, such as nuclear import/export or mitochondrial signals, to light-responsive domains. Attaching a nuclear signaling protein to the C-terminus of *As*LOV2 Jα-helix inhibited translocation in the dark[143]. The LOV-TRAP system uses directed evolution to bind a variant protein Zdk (an engineered small protein that binds selectively to the dark state of LOV2) to *As*LOV2, achieving the retention of plasma membrane proteins in the mitochondria in the dark and reversibly releasing them through irradiation[144].

Of a broader scope, light-controlled tools have been designed and engineered to tag specific targets in the cell. Notable examples are light-sensitive nanobodies (OptoNBs)[145] and tunable, light-controlled interacting protein tags (TULIPs)[146]. OptoNBs were created as chimeric photoswitchable proteins whose binding to effector proteins could be enhanced or inhibited with blue light by inserting an engineered *Avena sativa* short LOV into the NB. These OptoNBs bind to endogenous intracellular targets and can potentially modulate signaling pathways[145]. TULIPs were built binding an *As*LOV2 domain with an engineered PSD95-DlgA-ZO1 (PDZ) domain (ePDZ). PDZ domains are small globular domains that primarily bind to the C-terminal sequences of target proteins with low affinity[147,148]. TULIP fusion tags are much smaller than other light-dependent engineered proteins, making them preferable for use with proteins that cannot tolerate large fusions[149]. TULIPs can recruit proteins to diverse structures in living yeast and mammalian cells, and can be controlled either through global photoexcitation or with spatial precision using a steerable laser, and have been used to confer light sensitivity to specific pathways in the cell, such as cell polarization, cell compartment recruitment, and activation of the MAPK pathway[146,149]. TULIPs have also been used to achieve optogenetic control of RhoA, a membrane-bound GTPase that primarily controls the cellular basis of cytoskeletal contractility[150,151].

To study the direct electrostatic interactions of LOV receptors with anionic phospholipids, a photosensory signaling mode was engineered using *Botrytis cinerea* LOV4, a regulator of the G-protein signaling (RGS) domain. The light-regulated RGS-LOV shows high-affinity electrostatic interactions between anionic plasma membrane phospholipids and a polybasic amphipathic helix at the LOV interface in a reversible manner in yeast and mammalian cells[152]. Light-dependent regulation at the mRNA level (optoribogenetics) was achieved by heterologously expressing the *Nakamurella multipartita* PAS-ANTAR-LOV protein to specifically bind RNA

Table 3 Experiments performed with light-oxygen-voltage (LOV) domains for use in optogenetics.

Protein (organism)	Type of experiment	Results yielded
LOV (*Bacillus subtilis* YtvA)-**YFI** (*Bradyrhizobium japonicum* FixL)	Random mutagenesis[131]	– disruption of LOV-FMN binding in a variant group – disruption of signal transmission to effectors in another variant group
Phot1-LOV2 (*Avena sativa*)	Attachment of *As*LOV2 Jα-helix to nuclear import/export proteins[143]	– inhibition of translocation in the dark
	LOVTRAP engineering to bind LOV with Zdk[144]	– control of proteins' movement between the membrane and the mitochondria
	Design of light-sensitive nanobodies (OptoNB)[93]	– blue-light enhancement binding of endogenous intracellular targets by the OptoNB
Phot2-LOV2 (*Arabidopsis thaliana*)	Design of mini Singlet-Oxygen-Generator (miniSOG)[133]	– EM applications, photodynamic therapy and chromophore-assisted laser inactivation
LOV2 (*Avena sativa*)-**ePDZ** (engineered small globular domain bound to the C-terminus)	Design of Tunable, Light-controlled Interacting Protein tags (TULIPs)[146]	– light-directed, reversible, local subcellular recruitment – control of signaling molecules activity
LOV4 (*Botrytis cinerea*)-**RGS** (regulator of G-protein signaling)	Design of a photoinducible membrane localization system[152]	– high-affinity electrostatic interactions with plasma membrane phospholipids – control of cell compartment localization
LOV (*Nakamurella multipartita*)-**PAS-ANTAR**	Specific binding to RNA hairpins[153]	– light-dependent regulation at mRNA level

hairpins for blue light absorption. This experiment highlighted the importance of the Jα-helix in LOV domains signal propagation[153].

LOV-based optogenetic input modules are based on the engineering of photoreceptor-protein hybrids to enable light-inducible allostery, with the highly complex task of achieving precise control of the transition between active and inactive protein conformations[3]. To achieve this, a computationally-designed protein was used to design an *As*LOV2 insert to N-acyltransferase to achieve light-inducible allosteric regulation in a manner in which blue-light exposure resulted in the unfolding of the C-terminal Jα-helix of LOV2 to activate the effector transferase domain, but without over-stabilizing the fused protein, thus facilitating its inactivation in a more controlled manner[154].

LOV-based optogenetics perspectives

Despite the progress in the development of LOV-based optogenetic tools, several limitations remain. For instance, there is a critical need for fusion protein libraries, although recent years have witnessed the development of genetic engineering strategies and vector libraries for the rapid generation of protein–protein interactions, many of which have been applied both *ex vivo* and *in vivo*[155–157].

Another major challenge is the limited penetration of visible light, particularly blue light, through biological tissues, whereas the use of energy-rich light of shorter wavelengths (in the UV range) is problematic as it causes phototoxicity and DNA damage[3]. To overcome these issues, researchers have developed strategies for activating optogenetic switches using biological light generated by engineered luciferase proteins (responding to red/ far-red light) fused to the optogenetic switch. This approach enables the activation of LOV-based switches in deeper tissues and in living animals, and aids in mitigating potential phototoxic effects[158,159]. Recently, using an LOV domain from *Chloroflexus aggregans* and performing random mutagenesis of the binding-site Cys and Gln residues, a color palette of 22 finely tuned fluorescent tags was obtained; however, there is still a need to identify mutants with significantly shifted spectra[160].

Finally, when generating tools that depend on protein–protein interactions to control the above-mentioned processes, such as inducible gene expression, protein translocation, homodimerization, heterodimerization, and dissociation, it is necessary to fully understand how to modulate the kinetics and photostationary states of the LOV domains[3,104].

In summary, and as discussed in the previous section, we suggest in this review that the constantly improving knowledge of protein structures through time-resolved crystallography, spectroscopy, and molecular dynamics simulations, combined with more powerful computers and algorithms (such as AlphaFold[161]), is crucial for guiding the elucidation of the entire activation mechanism of LOV domains, as well as the design of optogenetic tools to overcome current obstacles.

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Andrea Flores-Ibarra: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Raiza N.A. Maia:** Writing – original draft, Writing – review & editing. **Bence Olasz:** Writing – original draft, Writing – review & editing, Visualization. **Jonathan R. Church:** Writing – original draft, Writing – review & editing. **Guillaume Gotthard:** Writing – original draft, Writing – review & editing. **Igor Schapiro:** Writing – original draft, Writing – review & editing. **Joachim Heberle:** Writing – original draft, Writing – review & editing. **Przemyslaw Nogly:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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