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Structure and function of the ZTL/FKF1/LKP2 group proteins in *Arabidopsis*

Brian D. Zoltowski[*,1] and **Takato Imaizumi**[†,1]

[*]Department of Chemistry, Southern Methodist University, Dallas, TX, USA

[†]Department of Biology, University of Washington, Seattle, WA, USA

Abstract

The ZTL/FKF1/LKP2 group proteins are LOV domain based blue-light photoreceptors that control protein degradation by ubiquitination. These proteins were identified relatively recently and are known to be involved in the regulation of the circadian clock and photoperiodic flowering in *Arabidopsis*. In this review, we focus on two topics. First, we summarize the molecular mechanisms by which ZTL and FKF1 regulate these biological phenomena based on genetic and biochemical analyses. Next, we discuss the chemical properties of the ZTL family LOV domains obtained from structural, biophysical and photochemical characterizations of the LOV domains. These two different levels of approach unveiled the molecular mechanisms by which plants utilize ZTL and FKF1 proteins to monitor light for daily and seasonal adaptation.

Keywords

ZTL; FKF1; LKP2; Blue-light photoreceptor; LOV domain; Flavin; Photocycle; Circadian clock; Photoperiodism; F-box; Protein-protein interaction; Protein degradation; *Arabidopsis*

1. INTRODUCTION

Plants utilize light not only as an energy source for photosynthesis but also as an information source to enable assessment of environmental conditions. They sense changes in the ambient light and adjust their development to optimize their fitness to the local environment. Plant cells carry several different classes of photoreceptors that can absorb different spectra of light. These photoreceptors include red/far-red light receptor phytochromes, blue-light receptors, cryptochromes and phototropins [1]. These photoreceptors, which are well characterized at the molecular level, regulate many aspects of physiology in plants. In the year 2000, a likely new family of blue-light photoreceptor with three members was identified through *Arabidopsis* genetics. These are referred to as ZEITLUPE (ZTL), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), and LOV KELCH PROTEIN 2 (LKP2) proteins, all of which are involved in the regulation of the circadian clock and flowering time [2–4]. All three proteins were deduced to have a potential light-absorbing domain called the LOV domain. Since the identification of the *ZTL*/*FKF1*/*LKP2* genes, research into signal transduction within this family has focused on elucidating the answer to

[1]Corresponding authors: bzoltowski@mail.smu.edu; takato@u.washington.edu.

three fundamental questions. 1) How are these proteins integrated on an organism wide scale to affect diverse aspects of circadian function and flowering? 2) How do environmental factors regulate the function of these proteins through activation of the light-absorbing LOV domain? 3) How does LOV chemistry regulate protein structure to induce formation of protein-protein complexes and activate the F-Box and Kelch repeat domains? In this review, we will outline the current state of the functional studies of ZTL group proteins and how these proteins relate to blue-light photoreception in *Arabidopsis thaliana*.

2. THE CIRCADIAN CLOCK REGULATION BY ZTL

Various physiological and developmental events, such as stem elongation and leaf expansion, gas exchange, flower opening and so on, occur at specific times of the day, and the timing of these events are regulated by the circadian clock [5,6]. To understand how the circadian clock regulates these events at the molecular level, elucidating how the molecular circadian clock is constituted is necessary. The molecular genetic approaches performed using *Arabidopsis* have facilitated analysis of the architecture of the plant clock.

Among the known plant circadian clock genes, *ZTL* was the first one cloned from mutants that possess altered circadian clocks. Kay and colleagues succeeded in isolating the first set of clock mutants using luciferase-based live imaging in 1995 [7]. To monitor the status of the endogenous molecular clock, they generated transgenic *Arabidopsis* plants possessing a firefly luciferase (*luc*) reporter gene [8]. The expression of *luc* gene was controlled under the 320-bp fragment of the *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2*, also known as *LHCB*) promoter [8]. The *CAB2* gene was the first circadian-regulated gene described in plants [8], and the small 320 bp of the *CAB2* promoter was sufficient to show robust circadian oscillation of luciferase activity. The resulting transgenic *Arabidopsis* plants showed diurnal oscillation of reporter activity with a morning peak, similar to the peak of endogenous *CAB2* mRNA expression [8]. This transgenic line was treated with EMS to screen the clock mutants with aberrant oscillations. A mutant that displayed a longer-period oscillation with 27 hours duration under free-run conditions was named as *zeitlupe* (*ztl*), which means “slow motion” in German [2]. The *ZTL* gene encodes a pioneer protein that possess three unique protein-protein interaction domains; LOV (Light, Oxygen, or Voltage), F-box, and Kelch-repeat domains. The LOV domain is a specialized PAS domain with potential blue-light absorbing capacity, as the blue-light photoreceptor phototropins use this domain to capture ambient light information [9,10] (we will discuss the structure, photochemistry, and functions of the LOV domains in detail later). This prompted Kay and colleagues to speculate that ZTL may be able to absorb blue light and may even be a novel photoreceptor. However, the *ztl-1* mutant showed a red-light (but not blue-light) specific shorter hypocotyl phenotype, so there was not an obvious phenotype that indicates a potential role of blue-light. The *ztl-1* allele also showed a late flowering phenotype under long-day conditions. However, subsequent work indicated that this late flowering phenotype is either an allele specific or ecotype specific phenotype (*ztl-1* is in C24 background), since the *ztl* mutants in Col-0 background do not show any late flowering phenotypes under long day conditions [11].

The *ztl* clock phenotypes seemed to be conserved in both the C24 and Col-0 accessions, as the *ztl* mutants in both backgrounds showed similar longer period phenotypes [2,11]. Conversely, transgenic plants overexpressing *ZTL* showed dosage-dependent shorter period phenotypes [11]. In addition, in the line where *ZTL* expression level is immensely elevated, the oscillation of the circadian clock ceased to become arrhythmic [11]. These results informed us that the amount of *ZTL* mRNA regulates the pace of the clock in a dosage-dependent manner. These transgenic lines with increased *ZTL* mRNA levels also showed longer hypocotyl phenotypes particularly under red-light conditions, and delayed flowering in long day conditions [11]. However, these hypocotyl growth and flowering phenotypes might not be directly caused by the altered pace of the circadian clock. In other words, ZTL protein might be involved in the regulation of hypocotyl growth and flowering time in addition to its role in the clock.

Considering that ZTL possesses a LOV domain, does ZTL have a (blue) light specific function? The answer is yes. Although the characterizations of *ztl* mutants and *ZTL* overexpressors did not reveal many light specific phenotypes, the stability of the ZTL protein was later found to be regulated by light quantity. The expression levels of *ZTL* transcripts are constitutive throughout the day and aren't regulated by the circadian clock [12]. However, its protein abundance shows daily oscillation with a peak at the end of the day, and this is due to the presence of time-specific changes in the degradation rate of ZTL [12]. It turned out that this diurnal oscillation of ZTL protein abundance was regulated by GI [13]. ZTL protein is unstable in the *gi* mutants, while the constitutive overexpression of GI protein stabilizes ZTL protein throughout the day. Therefore, the presence of GI stabilizes ZTL protein (Figure. 1). GI protein directly binds to ZTL through the LOV domain, and this interaction is enhanced by blue light [13]. Since GI protein is highly abundant in the afternoon [14], together with the presence of light, the ZTL-GI complex is formed in the afternoon [13]. Although how GI stabilizes ZTL remains unclear, this work also uncovered the role of the LOV domain as a light-dependent protein-protein interaction domain. In other words, the function of ZTL is regulated by light.

In addition to GI protein, a molecular chaperon, HSP90, is also involved in stabilizing ZTL protein [15]. When HSP90 activity was reduced by either the RNAi technique or the application of specific HSP90 inhibitor geldanamycin (GDA), the circadian rhythm of the plants lengthened. The GDA treatment reduced the stability of ZTL, and HSP90 also interacts with ZTL *in vivo* [15], showing that ZTL protein is a HSP90 client. Since the GDA treatment in *GI*-ox reduced the amount of stabilized ZTL, HSP90 and GI additively stabilize ZTL protein (Figure 1A). Furthermore, the ZTL-GI interaction mutually stabilizes both proteins [16] (Figure 1). To stabilize GI, the presence of the entire portion of ZTL protein is not necessary. The partial ZTL peptide, which contains the LOV domain, was sufficient to stabilize GI protein and also the resulting ZTL LOV-GI complex captured GI protein in the cytosol [16].

ZTL also contains F-box and Kelch repeat domains, and this domain combination is typical for the F-box proteins that comprise the SCF complex [17]. The SCF complex functions as an E3 ubiquitin ligase, and is composed of Skip, Cullin, F-box, and Rbx1 proteins. ZTL directly interacts with at least five ARABIDOPSIS SKP1-LIKE (ASK) proteins in yeast

[18] and form the SCF complex with ASK1 *in vivo* [19]. In addition, both the *Rbx1* RNAi lines and the temperature sensitive *cul1* mutant showed longer period phenotypes similar to the *ztl* mutant phenotype, indicating that proteasome-dependent protein degradation is important for ZTL clock function [19,20]. The next obvious question was “what does ZTL protein degrade to regulate the clock?”.

The core clock component, TIMING OF CAB EXPRESSION 1 (TOC1), was found as a first SCF^{ZTL} substrate for degradation [21] (Figure. 1A). In contrast to the *ztl* clock phenotype, the *toc1* mutants showed a short period phenotype with ca. 19-hour periods under continuous light conditions [7,22,23]. When the *TOC1* mRNA level was reduced by RNAi, the plants with lower *TOC1* levels showed shorter period phenotypes, while when *TOC1* mRNA was overexpressed, the *TOC1* overexpression plants displayed longer period phenotypes. Additionally, excess amount of *TOC1* led the circadian clock into arrhythmia. Similar to the function of ZTL, the amount of *TOC1* regulates period length, although ZTL and TOC1 had the opposite effect on period-length determination. Interestingly, the period phenotype of the *ztl toc1* double mutant was similar to that of the *toc1* mutant [21], indicating that TOC1 is a downstream component of ZTL. Supposing that ZTL is involved in protein degradation, Mas and colleagues investigated the possibility that ZTL regulates TOC1 protein turnover. They found that TOC1 interacted with the ZTL LOV domain in yeast. TOC1 protein was degraded in the dark in wild type plants, and was more stable in the *ztl* mutant regardless of the light conditions [21]. In addition, the proteasome inhibitor treatment stabilized TOC1. These results indicate that SCF^{ZTL} interacts with TOC1 and regulates TOC1 turnover during the night. The TOC1 homolog, PSEUDO-RESPONSE REGULATOR5 (PRR5), was also found as a substrate of SCF^{ZTL} [24] (Figure 1A). PRR5 protein peaked at a time similar to the TOC1 peak [25,26], but PRR5 does not have a strong influence on period determination. *PRR5* overexpressors showed reduced amplitudes of *LHY*, *CCA1*, and *GI* expression without changing the period length [27]. In the *ztl* mutant, both *LHY* and *CCA1* mRNA levels were also reduced [26]. Similar to TOC1 protein, PRR5 protein also interacts with the ZTL LOV domain [26] and PRR5 protein was more stable in the *ztl* mutant, while the PRR5 level was reduced in the *ZTL* overexpressors [24]. In addition, similarly to TOC1, PRR5 protein was degraded by ZTL during the night. Based on these observations, PRR5 stability is controlled by ZTL in a similar manner, and the lower amplitudes of *LHY* and *CCA1* expression in *ztl* mutants might be caused by the accumulated amount of PRR5 during the night.

Are TOC1 and PRR5 proteins regulated by ZTL independently? A recent report showed that PRR5 also directly regulates TOC1 activity. Both TOC1 and PRR5 proteins are phosphorylated at a specific time of day [25]. TOC1 and PRR5 form a dimer through their N-terminal domains, and this interaction stabilizes TOC1 protein and also recruits TOC1 into the nucleus [28] (Figure 1A). ZTL is localized in the cytosol inside the cell [13], while PRR5 mainly exists in the nucleus [28]. The amount of TOC1 in the cytosol increased in the *prr5* mutant, while more TOC1 is localized in the nucleus in *PRR5* overexpressors [28]. Wang and colleagues proposed that the competition of TOC1 between ZTL and PRR5 regulate temporal and spatial distributions of TOC1 protein inside the cell. This also indicates that PRR5 and TOC1 might be degraded by ZTL separately.

Although ZTL is a major protein within the group for circadian clock regulation, the ZTL homologs, LKP2 and FKF1, also contribute to control the pace and robustness of the circadian clock through the regulation of TOC1 and PRR5 protein stability. Both LKP2 and FKF1 directly bind to TOC1 and PRR5 through their LOV domains *in vitro* [26]. LKP2, which is closer to ZTL than FKF1 in amino acid sequence levels [2], shows a similar arrhythmic circadian clock phenotype when it is overexpressed [4]. The circadian clock phenotype of the *lkp2* single mutant is very similar to that of wild-type plants; this is due to the very low expression of *LKP2* mRNA compared with the amount of *ZTL* mRNA [26]. Both TOC1 and PRR5 proteins became more stable in the *ztl lkp2* double mutant than in the *ztl* single mutant [26,28]. The *fkf1* mutant also showed a very minor clock phenotype, but when this mutation was combined with the *ztl* mutation, the *ztl fkf1* double mutants showed about a 2-hour longer period-length phenotype (about 29 hours) than the *ztl* mutant. In the *ztl fkf1* mutant, the trough levels of both TOC1 and PRR5 proteins were further increased than those in the *ztl* single mutant [26]. Importantly, the clock phenotypes of either the *lkp2* or *fkf1* mutants are only visible when the *ztl* mutation is integrated, clearly indicating that ZTL is a major component within the ZTL group family in terms of regulation of the circadian clock.

3. PHOTOPERIODIC FLOWERING REGULATION BY FKF1

The circadian clock regulates the timing of events that occur not only throughout the day, but also throughout the year [29,30]. Many plants flower at a specific time of year to maximize reproductive success. Plants measure changes in day length (=photoperiod) to regulate the timing of flowering, and this phenomenon is called photoperiodic flowering. A functional circadian clock is necessary to accurately measure photoperiod changes [31]. *Arabidopsis* wild-type plants flower early when days are getting longer. The ZTL family proteins, especially FKF1, play important roles in the photoperiodic flowering pathway [30].

The *fkf1* mutant was isolated as a late flowering mutant under long-day conditions, and the causal gene of the *fkf1* phenotype turned out to be a homolog of *ZTL* [3]. Unlike *ZTL* and *LKP2* mRNA expression patterns, the abundance of *FKF1* transcript clearly oscillates throughout the day with an afternoon peak [3,32]. The resulting FKF1 protein profiles also showed a robust daily oscillation with an early evening peak [32].

In the photoperiodic flowering pathway in *Arabidopsis*, the time-dependent regulation of CONSTANS (CO) protein activity is critical [29,30]. FKF1 functions as a positive regulator for flowering by inducing *CO* transcription specifically in the afternoon. This induction of *CO* expression is light dependent, indicating that FKF1 function is regulated by light [32]. To induce *CO* transcription under light, as FKF1 binds to ASKs in yeast [18], FKF1 likely functions as an E3 ubiquitin ligase to regulate turnover of its substrate proteins, which regulate *CO* transcription. To identify targets for degradation, the interacting proteins to the FKF1 Kelch repeat were identified. These interactors belong to the Dof-type transcription factor family, and were named CYCLING DOF FACTORs (CDFs), as they all showed daily oscillation patterns for mRNA expression [33]. Currently, there are five *CDF* genes (*CDF1* to *CDF5*), all of which function as repressors of flowering [34]. Among them, CDF1 is the most characterized. CDF1 protein peaks in the morning and directly binds to both *CO* and

FT promoters to repress their transcription [33,35]. FKF1 degrades CDF1 at the end of the day to release the repression of *CO* and *FT* expression. This facilitates the induction of *CO* and *FT* under long-day conditions. To induce *FT* expression at the end of the day, FKF1 protein also directly binds to CO protein to stabilize it. This interaction is mediated by the FKF1 LOV domain, and it is regulated by blue light. Thus, FKF1 regulates *FT* expression by inducing *CO* transcription and concomitantly stabilizing CO protein [33,35]. FKF1 has light-dependent and independent functions, and both are important for the proper regulation of photoperiodic flowering in *Arabidopsis*.

Do ZTL and LKP2 also regulate flowering time in a similar fashion? Overexpression of *ZTL* and *LKP2* also caused late flowering phenotypes in long days [4,11,36]. In the *ZTL* overexpressors, both *CO* and *FT* expression levels are diminished throughout the day in long days [11,36]. There are at least three possible explanations for the mechanisms underlying the late flowering phenotypes of *ZTL*/*LKP2* overexpressors. One of the likely mechanisms that regulate flowering phenotypes in the *ZTL* and *LKP2* overexpressors is that ZTL and LKP2 directly capture FKF1 protein in the cytosol. FKF1 LOV domain homodimerizes when it is expressed in *E. coli* [37], and FKF1 heterodimerizes with either ZTL or LKP2 in yeast and *Arabidopsis* protoplasts [18,38]. Interestingly, FKF1 can bind to both the LOV domain and Kelch repeat domain of ZTL and LKP2, independently [18,38]. It remains uncertain whether FKF1 always interacts with ZTL/LKP2 through two protein-protein interaction domains or if FKF1 interacts with each domain under different circumstances. FKF1 protein has a mono-particle nuclear localization signal at the N-terminus [39] and YFP-FKF1 is strongly localized in the nucleus [38], while both CFP-ZTL and CFP-LKP2 are evenly distributed throughout entire cells in protoplasts [38]. We should be cautious when we interpret the data using GFP (and its derivative) fusion proteins, since GFP-LKP2 in *Arabidopsis* localized mainly in the nucleus [18,40] and GFP-ZTL is also localized in the nucleus at night [41], while endogenous ZTL and GFP-ZTL in tobacco exists in the cytosol [13,16]. ZTL and LKP2 Kelch repeat domains interact with FKF1 and FKF1 protein abundance in the *ztl lkp2* mutant background is higher than that in the wild-type plants in the morning [38]. This indicates another possible explanation of late flowering caused by *ZTL*/*LKP2* overexpression, of which ZTL and LKP2 may degrade FKF1. The other potential mechanism is that overexpression of *ZTL* captures GI in the cytosol, with the result that GI is sequestered from the FKF1-GI complex in the nucleus [16]. *CO* and *FT* expression profiles in the *ZTL* and *LKP2* overexpressors resemble those in the *gi* mutant [11,38,42], providing support for this explanation. At least, these three mechanisms may contribute to repress flowering in *ZTL*/*LKP2* overexpressors.

ZTL and LKP2 also may control the stability of CDFs. In the *ztl lkp2 fkf1* mutant, CDF2 protein is even more stable than that in the *fkf1* single mutant [34], and the *ztl lkp2 fkf1* mutant showed a late flowering phenotype [34,38], similar to the *fkf1* mutant as well as the *ZTL*/*LKP2* overexpressors. CDF1 is mainly localized in the nucleus [33], while ZTL is mainly localized in the cytosol [13]. Whether ZTL can directly degrade CDFs remains unclear. As we discussed above, even though both the *ztl lkp2 fkf1* mutant and the *ZTL*/*LKP2* overexpressors exhibited a similar late flowering phenotype, the mechanisms causing these phenotypes might be different.

To this point, we have summarized how ZTL family proteins, especially ZTL and FKF1, were identified and how they regulate the circadian clock and flowering time. Molecular genetic and biochemical analyses for the functions of the ZTL group proteins reveals that these proteins are LOV-domain based blue-light photoreceptors, and the role of the LOV domain is important for the regulation of the protein functions. To decipher the initial signaling process of the ZTL group photoreceptor proteins, investigating how light signals are perceived by the ZTL group LOV domains is imperative. In the next section, we introduce our current knowledge of how LOV photochemistry regulates protein structure to induce formation of protein-protein complexes and activate the F-Box and Kelch repeat domains to regulate circadian-associated physiology.

4. GENERAL LOV CHEMISTRY

LOV domains constitute a subclass of the Period-ARNT-Single-minded (PAS) domain superfamily that is sensitive to environmental conditions [9]. Originally identified by Winslow Briggs and coworkers as a photosensory domain that imparts regulation of phototropic responses in plants [43]; LOV domains were later found to regulate a diverse array of blue-light signal transduction pathways in plants [2,32], fungal [44,45] and bacterial species [46,47]. Key to their diverse functions is a modular design, where the ~100 amino acid LOV domain attenuates the activity of downstream effector elements through blue-light induced chemistry within a bound flavin cofactor (FMN or FAD) [48,49].

Structurally LOV and PAS domains conserve a mixed α/β fold that is characterized by an anti-parallel central β-scaffold with 2-1-5-4-3 topology (Figure 2A). Two distinct differences impart blue-light sensitivity to the LOV subclass. First, the βscaffold is flanked on one side by a series of α-helices that recognize an oxidized FMN or FAD cofactor required for photoreception [49]. Second, they contain a conserved GXNCRFLQ motif, where the central cysteine residue forms a covalent adduct to the C4a position of the flavin cofactor following blue-light exposure [43]. Upon return to a dark-state environment the Cysteinyl-flavin adduct spontaneously breaks on a time scale from seconds to days.

The ZTL/FKF1/LKP2 family was first identified as functional LOV photoreceptors by Imaizumi et al. in 2003 when they recombinantly expressed the N-terminal LOV domains of all three members of the ZTL family and demonstrated spectra consistent with LOV photochemistry (Figure 2B). The ZTL family copurified with oxidized FMN and blue-light exposure resulted in formation of a 390 nm absorbing light-state species (LOV_{390}) consistent with a C4a adduct. Mutation of the central cysteine residue (C91) to alanine abolished photochemical conversion to LOV_{390} confirming LOV-type photochemistry [32]. Intriguingly, when returned to darkness, FKF1, LKP2 and ZTL did not reconvert to the dark-state oxidized flavin (LOV_{450}), in contrast to other LOV proteins [32]. However, kinetic analysis of the LOV constructs were complicated by instability of protein samples.

5. LOV DOMAIN PHOTOCYCLE

The LOV domain photocycle has been studied in detail [50–55], however some debates as to the identity of reaction intermediates still remains (Figure 3A). The current consensus indicates that the ground state exists as an oxidized flavin with the active site Cys residue

directly above the *si*-face of the isoalloxazine ring [55–57]. In crystal structures of LOV proteins, typically two orientations of the active site Cys residue are observed with only the conformation placing the cysteine thiol moiety above the C4a position competent for photoactivation [55,57]. Following excitation by blue-light, the flavin is promoted to an excited singlet state that rapidly converts to a triplet species [53,54,58,59]. The flavin triplet then causes an electron transfer event between the flavin and active site Cys to generate a flavin-semiquinone and a Cys-radical [37,58]. Rapid recombination of the radicals leads to C4a adduct formation with the neighboring N5 position now protonated [53,54,58]. The adduct state spontaneously decays under dark-state conditions with time constants ranging from seconds to days [55,60,61]. Currently, the longest known photocycles exist for a bacterial LOV protein (BmLOV, $t_{1/2}$ unknown) [62] and those of LKP2 and FKF1 ($t_{1/2}$>100 hours) [32,37,63]. The biological function of the long lifetimes is still debated, however several factors have been elucidated that affect LOV photocycle lifetime and may influence sensitivity of LOV proteins to environmental light levels.

Detailed studies of the bacterial LOV protein YtvA and the fungal circadian clock protein Vivid (VVD) revealed several distinct factors attenuating the lifetime of LOV photocycles [55,61]. First, formation of the C4a adduct generates an electron-rich flavin species that resembles reduced flavins [55]. Thus, factors that tune flavin reduction potentials directly affect photocycle lifetimes [55]. Indeed, variants of VVD with lifetimes approaching those found in FKF1 and LKP2 purify from *E. coli* in the dark as a neutral semiquinone that retains some light-state functionality in the dark [55,64]. Second, solvent isotope effects, proton inventory methods and spectral properties reveal that deprotonation of the N5 position is rate-limiting in adduct decay [55]. Small molecule bases or solvent access to the active site can then promote N5 deprotonation and accelerate adduct scission [51,55,63]. Combined these effect have proven to tune the photocycle lifetime across the entire range observed in nature. Despite detailed LOV photocycle studies in other systems, analysis of the ZTL family has been more limited and largely focused on determining the origin of the unusually long lifetimes and potential biological functions of the photocycle kinetics.

Although initial studies were not able to observe dark-state reversion in the ZTL/FKF1/LKP2 family, subsequent studies of FKF1 on long-length scales did observe adduct scission with a time constant of 62.5 hours [37]. These studies also revealed an alternative activation mechanism not observed in other LOV proteins. Specifically low temperature spectroscopy revealed that light promotes formation of electron transfer to form an anionic semiquinone as an off pathway intermediate [37]. Equivalent studies of other LOV proteins either could not verify the intermediate [58] or observed neutral semiquinones [65], where the proton stems from either the Cysteine thiol or active site solvent. The presence of an altered pathway indicates that H-bonding interactions in the protein active site or lack of solvent access may attenuate both adduct formation and adduct scission in ZTL group proteins. Additional attempts to identify a potential source of the unusually long lifetime identified a 9-residue loop insertion between the E-F helices that typically accommodates FAD binding instead of FMN [66]. Deletion of the loop insert increased the rate of adduct decay 3-fold, indicating coupling between the E-F loop and active site chemistry that may be important for signal transduction [66]. Initially, the long lifetimes of ZTL group proteins seemed

incongruous with their role in measuring day length, as the proteins would be constitutively activated following even brief exposures to light. Thus, it was presumed that dark-state reversion was not required, but rather protein levels were regulated at the level of protein degradation [32,67]. Several factors complicated such a scenario and suggested that at least some members of the family must have divergent photocycle kinetics. First, FKF1 and ZTL are stabilized in the light and protein turnover only occurs following return to a dark-state environment [13,24,67,68]. Second, ZTL functionality required dark-state reversion to allow dissociation from GI to target TOC1 and PRR5 [11,38]. Thus, either an alternative photoreceptor targets them for degradation under dark-state conditions or at least one member of the ZTL family must have divergent photocycle kinetics.

Recent, detailed studies of the ZTL group LOV photocycles identified an alternative model of their photoactivation that employed a neglected light-stimulated adduct decay pathway previously identified in other LOV proteins [54,63]. These studies confirmed unusually long lifetimes for FKF1 and LKP2 (τ>100 hours), but identified a divergent fast photocycle for ZTL (τ~1.6 hours). The divergent ZTL photocycle may resolve some questions regarding dark-state protein turnover of the ZTL/FKF1 proteins under evening conditions. Indeed, biological data suggests that ZTL targets FKF1 for degradation following nightfall [38]. Thus, the divergent kinetics may facilitate proper turnover of ZTL group proteins and facilitate circadian/photoperiodic timing. Variable photocycle kinetics in homologous plant LOV photoreceptors is not unique to the ZTL family. Similar divergent photocycle kinetics have been observed between PHOT1 and PHOT2 and facilitate different fluence sensitivities to maximize the dynamic range for phototropism [69]. An analogous fluence sensitive response in ZTL/FKF1 is complicated by the long lifetimes of both proteins, since environmental light is sufficient to completely populate the light state adduct even under dawn/dusk conditions. Rather, a kinetic model has been proposed by Pudasaini *et al.* that incorporates an additional UV-light activated pathway to stimulate adduct scission [54,63]. Inclusion of a light-stimulated back reaction into kinetic models is sufficient to predict fluence-based responses in circadian timing for ZTL deletion mutants [11,63]. Such fluence sensitive responses may facilitate proper timing of dawn and dusk, where the more rapid photocycle of ZTL allows conversion to the dark-state in the evening to target FKF1 and other proteins for degradation. In contrast, the long-lifetimes of FKF1 facilitate stabilization and accumulation of FKF1 beginning at first dawn. Interestingly, additional photophysical studies of ZTL and FKF1 identify additional factors that indicate spectral properties may be finely tuned to allow differential function of the ZTL group family.

Spectral characterization of ZTL, FKF1 and LKP2 identified altered flavin spectra that may indicate unique flavin environments that optimize LOV function for circadian timing and flowering. Spectra of dark-state LOV species are consistent with oxidized flavins with well-defined vibrational structure centered around the 450 nm peak (Figure 2B). Typically, blue-light absorption leads to bleaching of the 450 nm band with formation of a 390 nm intermediate with three isosbestic points. In contrast, light-state spectra of ZTL are unusually blue-shifted (380 nm) and have a decrease in the extinction coefficient, resulting in a loss of two of the isosbestic points [63] (Figures. 2B, C). Similar spectral changes have been observed in mutants of other LOV proteins, where hydrogen-bonding patterns to the

active site flavin have been altered [61]. Specifically, mutations of conserved residues Gln154, Asn123 and Asn133 (ZTL numbering) that contact the flavin pyrimidine ring result in blue-shifted spectra and altered photocycle kinetics in YtvA [61] (Figure 3B). Given the importance of contacts to the O4 and N5 positions in signal transduction, the altered spectral properties may influence both the kinetics and structural signaling mechanisms and differentiate FKF1 and ZTL in their biological functions. Identification of such mechanisms coupling flavin chemistry to domain reorganization and ZTL group function requires in depth biophysical studies of ZTL group protein structure. Currently little data has been obtained on these systems. Below we outline the current knowledge of ZTL group protein signal transduction and contextualize current photophysical data with known signal transduction pathways in other LOV systems to construct a series of theoretical signal propagation mechanisms.

6. ZTL GROUP PROTEIN STRUCTURE AND FUNCTION

Like most LOV proteins, FKF1/ZTL/LKP2 are composed of a modular domain structure to couple blue-light excitation to regulation of protein degradation and the formation of protein-protein interactions. All three members of the family have an identical domain architecture formed by a N-terminal LOV domain fused to an F-box and series of C-terminal Kelch repeats. These domains are then allosterically regulated by blue-light through LOV mediated regulation of protein complex formation. As all light-induced regulation results from flavin chemistry within the LOV domain [11,32,35,70], currently most biophysical studies of ZTL/FKF1/LKP2 proteins have focused on the initial activation event occurring in the N-terminal LOV domain.

LOV proteins have been well studied in plant, fungal and bacterial species, however a consensus mechanism on how blue-light signals are transduced to regulate effector domains is not known. The lack of a consensus mechanism in part stems from the absence of data on full-length multi-domain containing LOV proteins. Similar complications exist for the ZTL group family proteins and no biophysical studies of the full-length proteins have been conducted. Moreover, due to instabilities and insolubility of ZTL group proteins, no structures of any of the domains of FKF1, ZTL or LKP2 are currently available. Some insight into structural mechanisms of activation, however, has been gleaned from solution studies of FKF1 and comparisons to other LOV proteins.

Sequence analysis of ZTL/FKF1/LKP2 LOV domains reveals several factors important for LOV signal transduction. First, all members of the ZTL family contain a core 5-stranded anti-parallel β–scaffold flanked on one side by a series of α-helices consistent with other LOV/PAS proteins (Figure 4). Second, there is a short insert between the E and F helices that has been termed the FAD-binding loop due to its role in stabilizing interactions with the adenine ring of FAD in fungal circadian clock photoreceptors [71,72]. Third, external to the PAS core, all three members contain N-terminal and C-terminal α-helical elements that vary in length across the ZTL group proteins. In other LOV proteins, these N- and C-terminal elements are required for signal propagation to effector domains and may similarly be involved in signaling and differentiation of function in ZTL/FKF1/LKP2 [72–75]. Finally, sequence alignments of LOV proteins indicate that ZTL-group LOV domains more closely

resemble LOV1 domains of plant phototropins (up to 44% identical) and LOV proteins from fungi and stramenophiles (~40% identical) than they do LOV2 domains of plant phototropins (~35% identical). The former typically form LOV-mediated dimers that are formed by the central β–scaffold with involvement of N-terminal and C-terminal α-helices [76,77].

Direct dimerization of isolated LOV domains of FKF1 has been observed in several solution biophysical studies. Size exclusion chromatography (SEC) and small-angle x-ray scattering (SAXS) of the FKF1 LOV core and adjacent C-terminal helix demonstrate hydrodynamic radii and molecular envelopes consistent with a constitutive dimeric state [78]. Comparisons of FKF1 molecular envelops to those of other LOV proteins indicated that FKF1 likely forms elongated anti-parallel dimers mediated by the core β–scaffold. Such a dimer is in direct analogy to crystallographic dimers of the LOV1 domain of *Arabidopsis* PHOT1 and can allow a predicted structural model of ZTL group proteins [76,78] (Figure 5). Although similar studies have not been conducted on ZTL or LKP2, given the homology across the family and similar patterns of hydrophobic residues in the core β–scaffold (Figure 4), it is currently presumed that other members of the ZTL group family likely from constitutive dimers that may be anti-parallel in orientation.

The presence of dark- and light-state dimers of FKF1 were also confirmed using transient grating spectroscopy, where only subtle alteration in the protein structure was observed following photo-excitation [66]. The small structural changes in ZTL group proteins are consistent with studies of phototropin LOV1 domains, where similar small magnitude conformational changes were observed by the same technique. These small conformational changes contrast from Phototropin LOV2 domains, where light induces large scale changes within the LOV core that induce unfolding of N- and C-terminal helical elements [73,74,79,80]. Recent, studies of *Arabidopsis* PHOT1 LOV2 domain, however reveal the differences in LOV1 and LOV2 type conformational responses may not be distinct, rather the observed differences in magnitude of conformational changes may be dictated by construct length and the presence of N- and C-terminal helical elements [81]. Specifically, AtLOV2 with both N- and C-terminal helical elements crystallizes as a dimeric unit, employing the α-helical elements as dimerization motifs that are coupled in inducing a conformational response [81] (Figure 5). Given, FKF1 has only been studied in constructs containing the C-terminal helix it is unknown if additional elements may facilitate light-induced signaling. At this point, how light-signals propagates to affect the signaling of ZTL/FKF1/LKP2 and light-induced complex formation is unknown, however corollaries with other LOV and PAS proteins provide some guiding principals to signaling of the ZTL group family proteins.

Studies of LOV proteins reveals conformational changes and signal transduction is initiated by C4a adduct formation, protonation of the N5 position, and a tilt in the planarity of the isoalloxazine ring. Light- and dark-state crystal structures of phototropins and *N. crassa* VVD reveal that N5 protonation leads to rotation of a conserved Gln residue in the LOV active site [72,73] (Figure 6A). Gln rotation in turn alters H-bonding contacts to elements within Aβ to affect the conformation of N- and C-terminal helices leading to either protein dimerization or alteration of interactions with C-terminal effector domains [72,73,82,83].

Alternatively, FTIR studies of LOV proteins reveal that alteration in the planarity of the flavin ring system leads to loosening the β–scaffold [79,84] (Figure 6B). Conformational responses in the β–scaffold are dependent on the presence of a Phe residue in Hβ of LOV2 domains [48,85], but VVD, bacterial LOV and ZTL group proteins contain a Leu residue at the equivalent position. A third mechanism of signal propagation has been proposed involving a salt bridge on the helical face of the LOV domain [48]. In the ZTL group proteins, one or more of these signal propagation mechanisms may be employed in blue-light signaling.

Several factors indicate that signal transduction in ZTL group proteins may involve multiple interfaces. 1) LOV domains in ZTL group proteins form specific interactions with multiple protein targets, which implicates multiple light-activated surfaces. 2) Allosteric activation of F-box proteins most likely involves the C-terminal helix that can alter interactions between the LOV and F box domains. 3) The presence of the FAD binding loop between the E-F helices, but absence of FAD in ZTL, FKF1 and LKP2 indicate an alternative function for the 9-residue insert. Biological and biophysical studies confirm the importance of these elements in signal transduction for FKF1, however analogous experiments for ZTL and LKP2 are still lacking. Genetic studies of FKF1 variants indicate that Gln163 (FKF1 numbering) is required for FKF1-CO complex formation [35]. The involvement of Gln163 indicates that N5 protonation propagates light signaling to N- or C-terminal components in FKF1 [35]. Involvement of Gln163 may not be employed by ZTL or LKP2 and differentiate protein function between the two ZTL subgroups. Although, ZTL retains Gln154 at the equivalent position, LKP2 naturally contains the inactivating Leu residue at this site. Thus, if signal propagation from this locus is still employed in LKP2 a unique mechanism of signal transduction must exist. Future, biophysical studies of FKF1, ZTL and LKP2 are required to better understand how signals propagate from this locus.

Transient grating spectroscopy of FKF1 also implicates the E-F loop in mediating light-induced conformational changes. FKF1 constructs lacking the E-F insert do not undergo a conformational response, whereas modest conformational changes were observed in the presence of the E-F loop [66]. Involvement of the E-F loop as a mechanism of regulating LOV/PAS function is not unexpected. Studies of mammalian PAS transcription factors indicate that the helical interface involving the E-F loops can recruit transcriptional regulators to form interprotein helical bundles [86–88] (Fig. 5C). Analogous, protein-protein interactions may be present in ZTL group proteins to allow stabilization of the helical interface following blue-light activation and allow for distinct protein-protein interaction interfaces at the β–scaffold, E-F loop as well as N- and C-terminal helical elements. In this regard, ZTL group proteins may incorporate all the signaling interfaces observed in LOV/PAS proteins to allow diverse signal transduction pathways to integrate circadian and photoperiodic timing.

Taken together, chemical and structural signaling in the ZTL group proteins indicate a complicated intersection of fundamental chemistry and signaling interfaces that allow very specific integration of environmental variables into plant flowering. The lack of biophysical data of these systems limits distinct understanding of signal transduction pathways, but has allowed for several testable hypothesis linking photocycle kinetics and signaling interfaces

in differentiating protein function in the FKF1 and ZTL/LKP2 subclasses. Future studies will hopefully resolve how blue-light induces selective protein complex formation and regulation of the F-box and Kelch repeat domains to allow greater understanding and control of plant flowering.

7. PERSPECTIVES

Since the identification of the *ZTL*/*FKF1*/*LKP2* genes, we have accumulated a significant amount of results for the characterization of mutants and overexpressors. Now we have a better idea about the ways in which these proteins regulate circadian clock progression and flowering time at the molecular levels. The photochemical approach for the LOV domains of the ZTL group family proteins also showed us the likely mechanisms of blue-light perception and photochemical changes induced by the light perception. What is still missing is observations of chemical and structural changes using LOV domain peptides with adjacent domain(s) attached. Analysis from full-length or near full-length proteins will likely bring important insights into the process of converting light information into biochemical signals. Although there might be technical challenges to analyzing the full-length proteins, that information will fill the gaps in our current understanding of the ZTL/FKF1/LKP2 protein-associated signal transduction cascades. An additional challenge will be to understand how ZTL group proteins interact with each other to regulate physiology and developmental processes. This type of work has already been started; however, so far it has often been done using heterologous systems or transient assay systems. Under these conditions, since all fusion proteins were overexpressed, the stoichiometry among ZTL group proteins and also against their interacting partners should be quite different from the situation in wild-type plant cells. To more accurately access the function of the *in vivo* interactions of the members, it would be better to use systems that resemble endogenous conditions. Since ZTL group family-related genes are conserved not only in angiosperms (both monocots and dicots) [89], but also in the lycophyte *Selaginella moellendorffii* [90], it is likely that this protein family plays an important role in all vascular plants. *Selaginella* has only one ZTL homolog [90]. It would be interesting to analyze whether the potential ancestral type of the ZTL homolog has a similar function to ZTL or FKF1. The studies performed in *Arabidopsis* can be a framework for understanding the function of the ZTL/FKF1/LKP2 proteins in each species and how the functions of this protein family may diverge. The studies on the ZTL group proteins also made an impact on animal science. Molecular light switches in mammalian cell culture systems have already been made using the light-dependent FKF1-GI complex [39]. The details of biochemical and biophysical analyses of the LOV domain and interacting domains/proteins will further facilitate our ability to develop and improve new optogenetic tools in cell biology and neurobiology areas.

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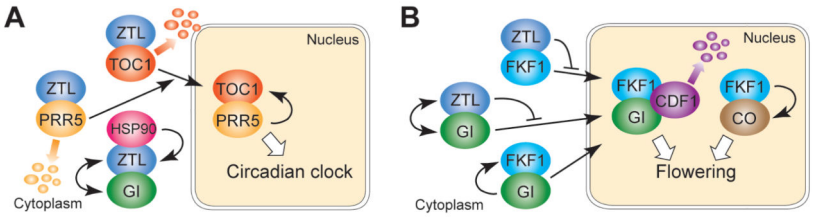

Figure 1.

Intracellular localization of ZTL, FKF1, and interacting partners (A) How ZTL regulates the circadian clock. ZTL is mainly localized in the cytosol and directly controls the protein turnover of TOC1 and PRR5. PRR5 binds to TOC1 to facilitate nuclear transfer of TOC1 and PRR5. Inside the nucleus, PRR5 helps TOC1 to be phosphorylated and stabilized, and both proteins are involved in regulation of the pace and amplitude of the clock gene expression. ZTL is stabilized by GI and HSP90. ZTL binding to GI also stabilizes GI, so they mutually regulate their stabilities. (B) How ZTL and FKF1 regulate flowering time. FKF1 is the main regulator of photoperiodic flowering. The targets of FKF1 E3 ubiquitin ligases are nuclear localized CDFs. FKF1 also binds to CO protein to stabilize it. FKF1 may interact with ZTL in the cytosol, and this interaction may capture FKF1 in the cytosol. FKF1 requires GI to degrade CDFs. ZTL also can capture GI in the cytosol; this likely results in the sequestration of GI from the FKF1-GI complex in the nucleus. GI may also stabilize FKF1 [34], similar to ZTL. Arrows indicate the direction of movement or activation, and repression lines indicate the negative actions of ZTL protein. Small circles drawn from TOC1, PRR5, and CDF1 depict the proteasomal degradation process.

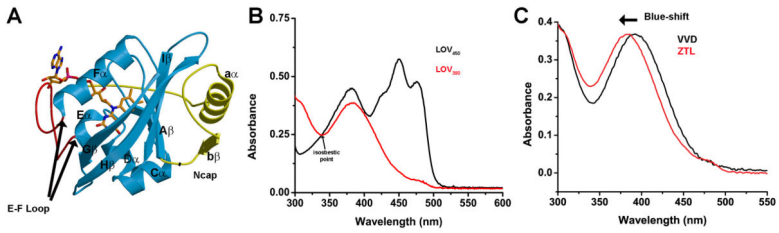

Figure 2.

LOV Structure and Photochemistry. (A) Structure of *N. crassa* VVD. The PAS core (blue) contains a β-scaffold flanked by an Ncap (yellow). Opposite the Ncap lies a helical interface that binds a flavin cofactor. An E-F loop accommodates FAD instead of FMN. (B) Spectra of ZTL photocycle. The dark state (black) resembles oxidized flavin. Blue-light leads to formation of a 380 nm absorbing intermediate (red). (C) Light-state spectra of ZTL (red) are blue shifted relative to other LOV proteins (VVD: Black).

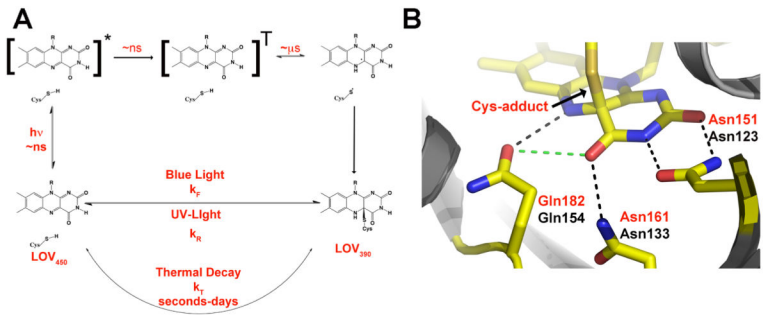

Figure 3.

LOV photocycle and Active-Site H-bonding Network. (A) LOV photocycle proceeds through rapid excitation to a singlet state that rapidly undergoes intersystem crossing to form a triplet intermediate. The flavin triplet induces electron transfer from the active site flavin and rapid radical recombination to form a flavin C4a adduct. The overall kinetic process is characterized by k_F. Reversion to the ground state can either follow a thermal pathway, k_T or can be stimulated by UV-light, k_R. (B) A conserved glutamine and two asparagines (Red VVD numbering; Black ZTL numbering) form H-bonds to the isoalloxazine ring system. A dark-state H-bond (green) is broken following Cys-adduct formation to favor interactions at N5.

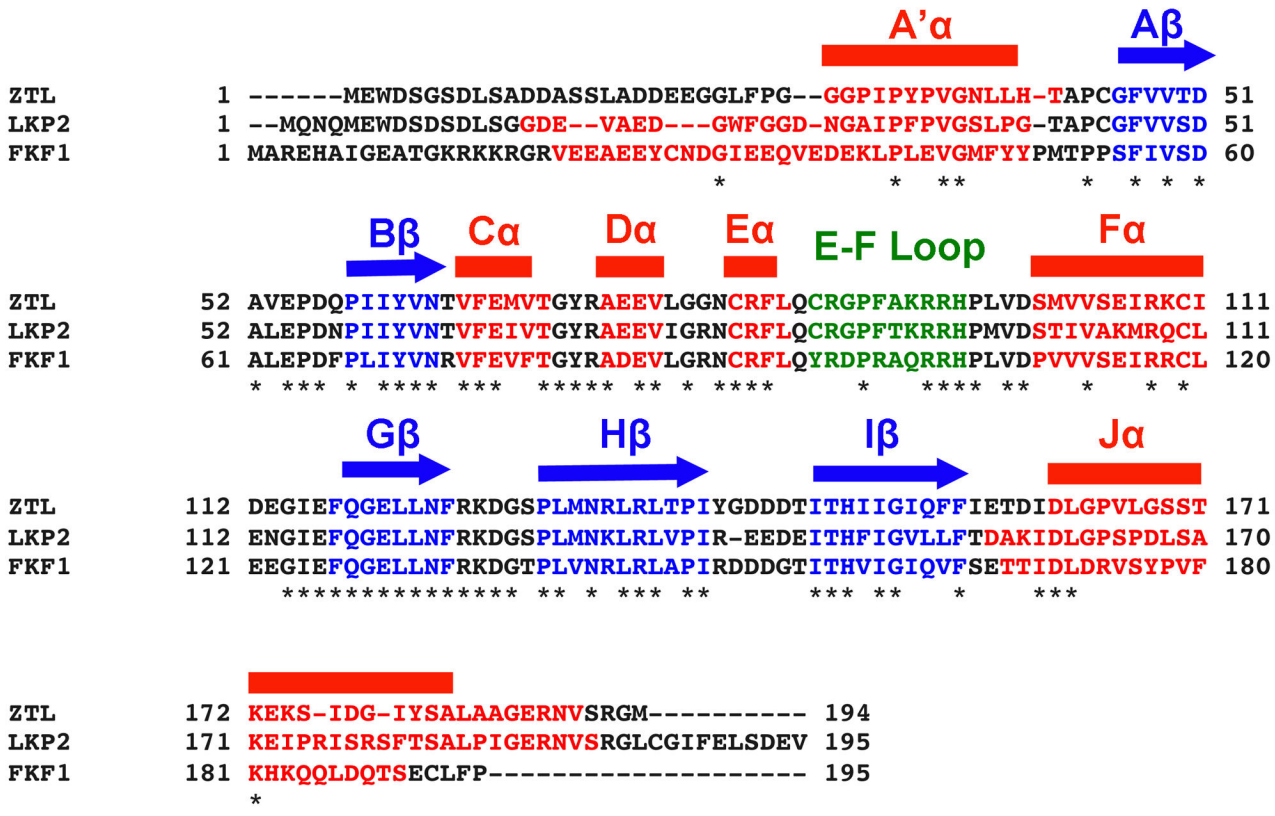

Figure 4.

Sequence Alignment of LKP Proteins LOV domains. Helical segments are shown in red, β-scaffold elements in blue and the E-F loop in green. The core LOV domain is highly conserved (* denotes 100% conserved residues in ZTL group family). Primary differences are in the E-F loop, Jα helix and Ncap (A′α) and likely represent functional areas that distinguish differential function in the family.

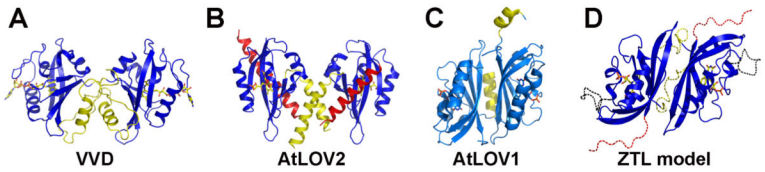

Figure 5.

LOV dimers. (A) Light-state VVD dimer (pdb 3RH8) formation is dictated by an Ncap helical element (yellow). (B) Dimerization of *Arabidopsis* PHOT 1 LOV 2 (pdb 4HHD) is mediated by Ncap helical elements (yellow) that are affected by a C-terminal helix (red). (C) LOV 1 domains of *Arabidopsis* PHOT1 (pdb 2Z6C) involve the core βscaffold. Neighboring Ncap helices (yellow) interact near the dimerization site but adopt different structures in the two monomers. (D) Model of LKP proteins (ZTL) constructed via threading the amino acid sequence on AtLOV1. Dimerization is similarly directed by the core β-scaffold, but N-terminal (yellow) or C-terminal (red) elements may be involved in manners similar to VVD/AtLOV2. Here they are modeled as disordered loops (broken line). The E-F loop (black) is also modeled in as a disordered loop.

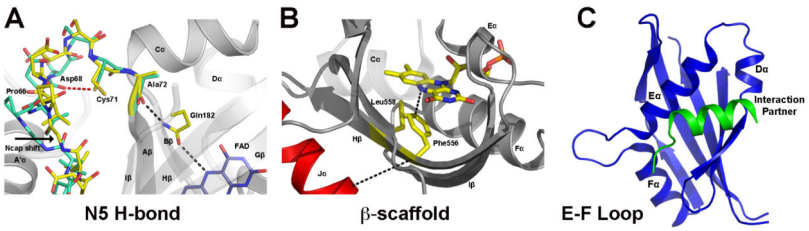

Figure 6.

Putative Signaling Mechanisms in LOV Proteins. (A) N5 protonation dictates a H-bond cascade to affect the position Ncap elements. Conformational changes require rotation of Gln182 to induce a new H-bond to Ala72. Movement of the Ncap causes rotation of Cys71 to break an H-bond to Asp68 leading to a shift in A′α (termed aα in VVD). (B) Adduct formation in LOV2 domains affects interactions with Phe556 that propagate to Jα. Signal transduction pathway is denoted with dashed lines. (C) The helical interface including the E-F loop rearrange to interact with helical effector proteins. Here STAT6 (green) docks to the helical region of the PAS-B domain NcoA-1 (pdb 1OJ5).