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Tripping the Light Fantastic: Blue-Light Photoreceptors as Examples of Environmentally Modulated Protein−Protein Interactions[†]

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ABSTRACT: Blue-light photoreceptors play a pivotal role in detecting the quality and quantity of light in the environment, controlling a wide range of biological responses. Several families of blue-light photoreceptors have been characterized in detail using biophysics and biochemistry, beginning with photon absorption, through intervening signal transduction, to regulation of biological activities. Here we review the light oxygen voltage, cryptochrome, and sensors of blue light using FAD families, three different groups of proteins that offer distinctly different modes of photochemical activation and signal transduction yet play similar roles in a vast array of biological responses. We cover mechanisms of light activation and propagation of conformational responses that modulate protein−protein interactions involved in biological signaling. Discovery and characterization of these processes in natural proteins are now allowing the design of photoregulatable engineered proteins, facilitating the generation of novel reagents for biochemical and cell biological research.

Organisms have evolved elegant mechanisms devoted to adapting to environmental changes. Alterations in an organism's exposure to sunlight provide cues about the time of day, further coupled with information about positioning in aquatic or forest habitats (*1*−*15*). Found in all kingdoms of life, these pathways rely on nonvisual photoreceptors that involve discrete families of proteins that paradoxically share photochemical mechanisms and architectures but regulate a wide array of signal transduction responses.

To biochemists and biophysicists, the ability to synchronously excite photoreceptors gives them a unique advantage for mechanistic studies of conformational changes and their effects with respect to signal transduction. By simply applying photons to these proteins in solution, crystals, or cellular environments, scientists have a facile way to control them with exquisite temporal and spatial resolution. Subsequent analyses can then be conducted at precise delays from the initial point of excitation to create "snapshots" of photoexcitation and subsequent activation processes that often alter protein−protein interactions. Such studies are difficult, if not impossible, to conduct in other sensory systems.

While nature has evolved numerous photoreceptors sensitive to all wavelengths of light (*16*), we focus here on a specific subset, those sensitive to wavelengths between roughly 400 and 480 nm, in the visible blue region of the electromagnetic spectrum, and discuss how they harness photochemical changes to regulate protein−protein interactions that are critical for signal transduction. After presenting a brief overview, we comment in depth on the photochemistry and signal transduction mechanisms of four families of blue-light photoreceptors (summarized for the flavin-binding photoreceptors in Table 1). For those interested in additional commentaries on these topics, we note that several other recent reviews of blue-light photoreceptors offer complementary viewpoints on the photochemistry, structure, and biological function of blue-light photoreceptors (*4*, *16*−*21*).

OVERVIEW

The primary families of blue-light photoreceptors fall into two categories: (1) domain modules that serve as sensory elements in large multidomain signaling proteins and (2) self-contained photoreceptors that harbor both the sensory and signaling mechanisms in a single sequence-conserved module (Figure 1). The first class primarily consists of small domains (∼110−120 amino acid residues) that constitute the light oxygen voltage (LOV)[1] (*5*), blue light using FAD (BLUF) (*14*), and photoactive yellow protein (PYP) (*22*, *23*) families. Notably, domains within each of these families share a single conserved photochemical mechanism but control diverse biochemical responses, including kinase activity, phosphodiesterase activity, DNA binding, and protein degradation (*5*, *14*). The second class is comprised of the cryptochrome (CRY) family (*24*, *25*), although some members of the LOV, BLUF, and PYP families effectively fit this description.

Regardless of whether a given photoreceptor stands alone or is part of a larger protein complex, it couples local conformational changes in the cofactor binding pocket to rearrangements of intraprotein contacts that modulate downstream protein−protein interactions. These conformational responses in photoreceptors can be classified in accordance with their proximity to the initial photochemical event, which we will divide into three different categories for the purposes of this review. First, we consider

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[1]Abbreviations: LOV, light oxygen voltage; BLUF, sensors of blue light using FAD; PYP, photoactive yellow protein; CRY, cryptochrome; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; PHR, photolyase homology region; CCT, CRY C-termini; PER, Period; PAS, Per-ARNT-Sim; Ncap, N-terminal cap; Ccap, C-terminal cap; AsLOV2, *Avena sativa* phototropin 1 LOV2; WC-1, White Collar 1; PDB, Protein Data Bank.

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Table 1: Overview of Flavin-Binding Blue-Light Photoreceptors

representative protein	chromophore	interaction partners	function	refs
		Cryptochromes (*3*, *24*, *37*−*39*, *41*, *43*, *47*, *51*)		
Drosophila melanogaster (dCRY)	FAD, MTHF	Timeless (TIM), Period (PER), Jetlag	circadian photoreception	*9*, *34*, *44*−*46*, *48*, *50*, *52*−*55*, *59*, *67*, *68*
Arabidopsis thaliana (AtCRY)	FAD, MTHF	COP-1	hypocotal elongation	*1*, *42*, *49*, *57*, *58*, *60*, *61*, *63*−*65*
Homo sapiens (hCRY)	FAD, MTHF	Period (PER), Clock, BMAL	circadian regulator, crosstalk with diverse pathways	*11*, *35*, *53*, *55*, *62*
		LOV Domains (*5*, *92*, *94*)		
phototropins from *Avena sativa* (AsLOV)*A. thaliana* (AtLOV)	FMN	unknown	Ser/Thr kinase, phototropism, chloroplast movement	*4*, *74*−*77*, *86*−*91*, *93*, *95*, *96*, *98*−*103*, *152*
Vivid (VVD)	FAD	WC-1	carotenoid expression	*7*, *33*, *73*, *78*, *79*, *104*−*106*
White Collar 1 (WC-1)	FAD	VVD, WC-2, Frequency (FRQ)	circadian photoreceptor, transcription factor	*2*, *6*, *106*
YtvA	FMN	unknown	stress response	*70*, *80*, *81*, *107*
LOVK	FMN	unknown	His kinase	*10*, *97*
		BLUF Domains (*14*, *128*−*130*, *136*)		
BlrP1	FAD	unknown	phosphodiesterase	*112*, *114*
BlrB	FAD	unknown	unknown	*113*, *126*, *137*
AppA	FAD	PpsR	regulation of photosynthesis	*13*, *109*−*111*, *119*−*124*, *127*, *130*, *133*, *135*, *138*−*140*, *142*
YcgF	FAD	unknown	phosphodiesterase	*117*
PixD	FAD	PixE	phototaxis	*118*

"proximal" conformational responses to be those within the chromophore and any covalently attached protein elements directly coupled to the photocycle. The exact nature of these changes depends on the chromophore and surrounding protein environment, as photoexcitation of these systems can alter redox chemistry through electron transfer reactions, form new chemical bonds, or isomerize existing bonds within a conjugated network (*16*). These local chemical changes, which are chiefly configurational in nature, trigger "intermediate" structural responses, which alter the immediately surrounding protein structure though rearrangements of noncovalent chromophore−protein interactions. In turn, changes in local structure often generate a "distal" response by altering protein−protein interaction surfaces that either facilitate downstream signaling processes or release inhibition of signaling domains. Below we will characterize these structural changes and their effects on protein−protein interactions for blue-light photoreceptors.

Most blue-light photoreceptors, including those in the BLUF, LOV, and CRY classes, utilize flavin chromophores in the form of either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). The biochemistry of flavin binding proteins has been a rich subject for both photoreceptors and nonphotoactive enzymes because of their role in one- and two-electron transfer reactions in biochemistry (*26*). Changes in the electronic properties of the flavin isoalloxazine ring induce local chemical reactions as well as rearrangements of hydrogen bonding networks involved in protein signaling cascades. Photoexcitation of flavins rapidly induces the formation of singlet and triplet excited states, which can induce direct electron transfer from nearby tryptophans or tyrosines (*27*). Flavins exist in five distinct redox states: fully oxidized (FAD), one-electron-reduced anionic (FAD^{-}), neutral (FADH), and fully reduced ($FADH_2$ or $FADH^{-}$) (*28*, *29*) (Figure 2). Shuttling between these redox states can be achieved either chemically or through direct photoreduction (*26*, *28*, *30*). Each redox state has distinct chemical and electronic properties that dictate signaling mechanisms (*29*). For instance, the FAD^{-} and FADH states differ by protonation at the N5 position, which can distort the electron density at N5 and the adjacent C4a atom (*29*). Moreover, the chemical identity of the redox species dictates reactivity in electron transfer reactions (electron donor vs acceptor), covalent attachment of small molecules or side chains, and stability in oxygen environments (*26*, *28*, *31*). Taken together, these changes in the electron density and planarity of the flavin isoalloxazine ring alter steric and electronic interactions with surrounding protein residues (*29*, *32*, *33*). These changes can provide the impetus for signal propagation by inducing local conformational changes within the protein.

Below we will outline the role of flavin chemistry in specific blue-light photoreceptors and detail mechanisms for alterations in intraprotein interactions that affect downstream signaling partners. Coupling of initial photochemical events to networks of protein−protein interactions is an area rich in information; however, less is currently known at the atomic level about how these changes control protein signaling networks. Finally, we will review how the recent advances in understanding these signaling processes at a basic level have led to efforts to manipulate photoreceptor signaling mechanisms for biotechnology.

CRYPTOCHROMES

Cryptochromes were initially postulated to exist as unknown blue-light photoreceptors responsible for growth and development in plants (*1*). They were later found in a wide array of organisms, including insects and mammals, where they function as both light-regulated or light-independent regulators of circadian function (*11*, *34*−*36*) (Table 1). The initial discovery of CRYs in *Arabidopsis thaliana* uncovered distinct homology between these proteins and photolyase DNA repair enzymes, although the former did not demonstrate any ability to repair DNA lesions (*1*). CRYs can be divided into three families on the basis of their phylogeny: (1) plant CRYs, (2) animal CRYs, and (3) CRY-DASH proteins (*37*, *38*). The latter group has recently been

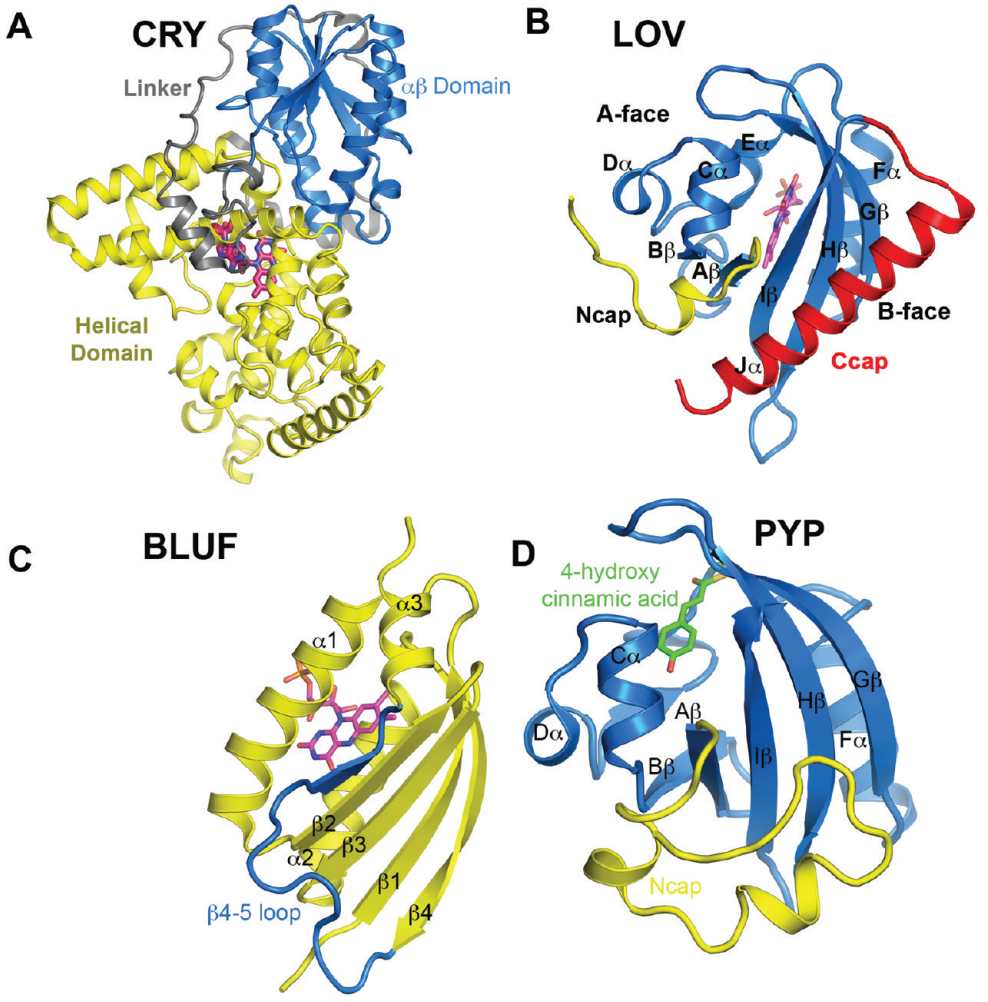

FIGURE 1: Representative structures of the four families of blue-light photoreceptors. (A) *Arabidopsis* CRY1 (PDB entry 1U3C) (*42*), with the two domains (helical in yellow, $\alpha\beta$ in blue) linked by an extended linker. The FAD chromophore is colored magenta. (B) AsLOV2 (PDB entry 2VOU) (*76*) contains a typical PAS domain fold with the PAS core (blue) β-sheet flanked by Ncap (yellow) and Ccap (red) elements. (C) Structure of AppA 1−125 (PDB entry 2IYJ) (*109*). The dynamic $\beta4-\beta5$ loop is colored blue. (D) PYP (PDB entry 2PHY) (*22*) is also comprised of a typical PAS fold with a covalently attached 4-hydroxycinnamic acid chromophore (green). As in AsLOV2, an Ncap is present (yellow).

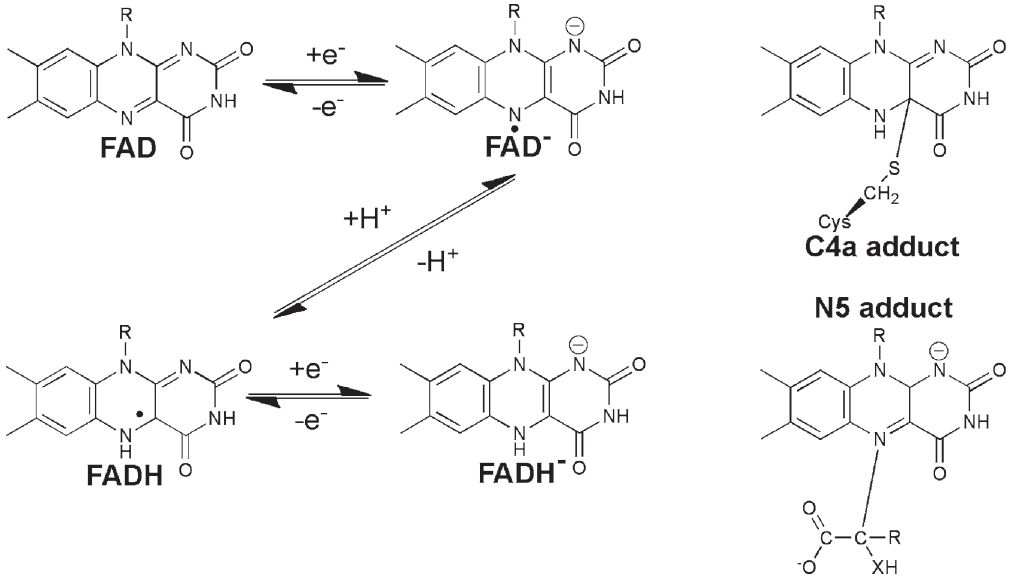

FIGURE 2: Flavin chemistry. FAD or (FMN) can undergo a series of electron and proton transfers to form FAD (oxidized), FAD^{-} (anionic semiquinone), FADH (neutral semiquinone), and $FADH^{-}$ (hydroquinone). Flavin species often perform chemistry at the C4a and N5 positions, including the generation of covalent adducts to both locations.

shown to have single-strand DNA-specific photolyase activity and will not be discussed here (*39*). Also, animal CRYs have been divided into various subtypes depending on sequence homology and their respective roles in cellular signaling (*40*, *41*). For the

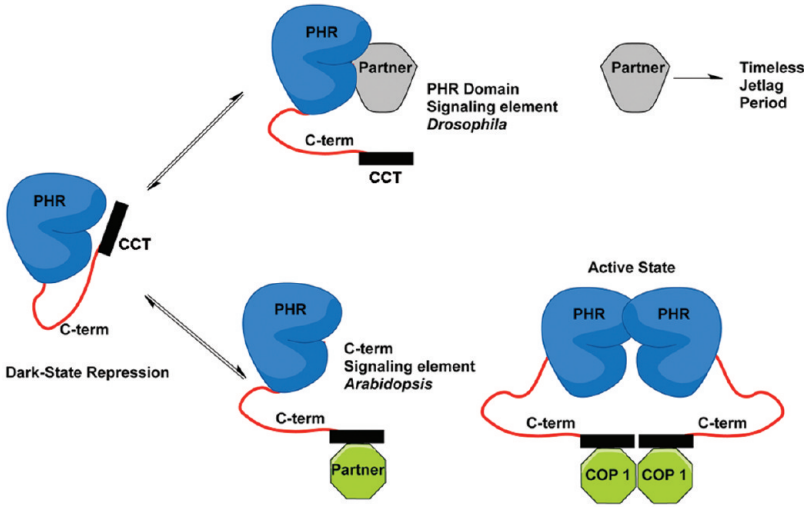

FIGURE 3: Schematic of cryptochrome activation and protein−protein interactions. The CRY CCT can act either as a directly repressive element inhibiting interactions at the PHR domain (top *Drosophila* model) or as a recognition element (bottom *Arabidopsis* model). In *Drosophila*, dCRY has multiple recognition partners, while the active state of *Arabidopsis* CRY is a dimer that interacts with COP1.

scope of this review, we will focus on the plant CRYs and type I and type II animal CRYs.

Cryptochromes are composed of two structural domains linked by a long disordered segment, bearing overall structural homology to photolyases (Figure 1). The N-terminal $\alpha\beta$ domain and the C-terminal helical domain comprise the photolyase homology region (PHR) and harbor the photoactive cofactors (*25*, *42*, *43*). The primary pigment, FAD, is sequestered in the C-terminal domain in a U-shaped conformation that places the adenine group directly beneath the isoalloxazine ring (*25*, *42*, *43*). On the other side of the isoalloxazine ring is a conserved salt bridge that is required for stability and function in animal CRYs (*44*). While CRY photochemistry is confined to the PHR, protein−protein interactions and signaling are dictated by variable C-terminal extensions (*45*−*49*). Current structural data on CRY C-termini (CCTs) are confined to limited proteolysis and qualitatively interpreted solution NMR spectra (*50*, *51*), confirming predictions that CCTs are largely disordered but contain regions of residual structure with conserved signaling motifs. At present, the relevant photocycle and the mechanism of transducing a signal from the PHR to the CCT are largely unknown; however, some insight can be garnered from biochemical assays and comparisons to photolyases.

While the photolyase photocycle has been well characterized, the corresponding CRY photocycle is less understood. This has been complicated by many factors, including differential outputs, alternative cofactors, various functional redox states, and debate over the ground state of the catalytic FAD (*24*, *52*−*55*). In photolyases, two photoactivation mechanisms exist. Excitation from UV-A light promotes the transfer of energy from the antennae pigment (MTHF, HDF, and flavins) to $FADH^-$, with subsequent transfer of an electron from $FADH^-$ to DNA lesions resulting in DNA repair (*25*). A second electron transport pathway involving a conserved sequence of three Trp residues (Trp triad) exists as a reactivation mechanism for maintaining the population of the $FADH^-$ ground state in photolyases (*25*, *53*, *56*, *57*). Importantly, in CRYs the Trp triad may play a direct role in photic signaling via the direct transfer of an electron to the active site flavin (*53*, *57*).

Many CRYs do not purify with an antennae pigment, and studies differ with regard to the wavelength specificity in action spectra and the role of the Trp triad in CRY function (*53*, *54*, *58*, *59*). Purified CRYs have been shown to be incapable of forming the $FADH^-$ functional state of photolyases (*52*). Moreover, studies of *Arabidopsis* CRY1 and CRY2 and *Drosophila* CRY (dCRY) indicate that the Trp triad is required for function, and the signaling state is likely the semiquinone (*53*, *55*, *57*, *58*, *60*). In both cases, treatment with green light populates the $FADH^-$ state and inhibits protein function (*55*, *60*). Notably, type II animal CRYs may not function directly as photoreceptors but rather function as light-independent circadian regulators. Strong evidence of this is provided by the fact that mutation of the Trp triad does not affect function (*35*, *53*).

Importantly, unlike BLUF and LOV domains discussed below, photolyases do not undergo significant photoinduced conformational changes. Instead, photolyases bind DNA in a light-independent manner and act as a scaffold to orient and sequester damaged DNA for reactivation (*25*). CRYs presumably differ with regard to blue-light-induced conformational dynamics as they function in light-dependent protein−protein interactions that require the CCT for fidelity of the light−dark response (*45*−*47*, *49*, *61*).

Structural dynamics and light-induced signal propagation by CCTs have been validated in *Arabidopsis*, *Drosophila*, and mammalian CRYs (*45*, *49*, *62*). Notably, CCT functions are species-specific, although cross activation by *Arabidopsis* CRY1 and CRY2 via CCT swapping has been observed (*63*). In *Arabidopsis*, CRY1 and CRY2 form constitutive dimers that activate COP1-mediated, light-dependent degradation of HY5 (*1*, *51*, *61*, *63*, *64*) (Figure 3). CCT fusion proteins indicate the CCT induces a constitutive response when presented as a dimeric species, indicating dark-state CRY1 and CRY2 act by suppression of a COP1−WD40 interaction element (*49*, *64*−*66*). Similarly, light activation of dCRY induces formation of a dCRY−Timeless heterodimer that leads to Jetlag-dependent proteasomally mediated degradation (*45*, *67*, *68*). Degradation of Timeless is followed by light-dependent degradation of dCRY that is facilitated by CRY−Jetlag interactions (*45*, *68*). Notably, in dCRY, truncation of the CCT results in constitutive activity (*45*, *46*), indicating that the dCRY CCT inhibits a protein-docking interface, in contrast to *Arabidopsis* CRYs (Figure 3). In dCRY, additional CCT-dependent interactions with the clock protein Period have been observed in yeast two-hybrid experiments (*48*); the functional role of similar interactions within the *Drosophila* clock has not been validated.

Like dCRY, mammalian CRYs are involved in circadian regulation and protein−protein interactions. In mammals, CRYs may not directly sense blue light but rather function in a light-independent mechanism. hCRY regulates the circadian clock through a CRY−PER heterodimer that interacts with BMAL−CLOCK transcription factors, resulting in repression of BMAL activation (*11*, *62*). Notably, repression of BMAL requires elements within both the PHR and the CCT for functional activity. These elements include a coiled-coil-like helix at the end of the PHR domain that appears to be required for interaction with Period and proper circadian output (*62*).

To better elucidate mechanisms of CRY function and signal propagation, a clearer understanding of the CRY photocycle is required. Moreover, structures of CRY proteins incorporating C-terminal elements or in complex with physiological partners would aid in the development of a model of conformational changes in CRYs. Regardless, distinct differences in *Arabidopsis* and dCRY signaling indicate that activation can proceed by either release of the signaling element (*Arabidopsis* CCT) or release of a repressive element (dCRY CCT) (Figure 3). This theme of multiple activation pathways for a given class of homologous photoreceptors will be echoed in other groups of receptors, as seen below.

LOV DOMAINS

LOV domains are present in prokaryotic and eukaryotic organisms, where they function as blue-light-sensing modules regulating enzymatic activity and signaling cascades (*5*). LOV domains are found in multidomain proteins, including histidine kinases (*4*, *10*, *12*), DNA binding domains (i.e., leucine zipper, bHLH, and zinc finger) (*2*, *69*), STAS domains (*70*), and myriad others. Recently, "short" LOV proteins containing only the LOV core and N- or C-terminal extensions have been identified in bacteria and fungi (*71*−*73*). Most knowledge of LOV domain structure and signal propagation stems from biophysical studies of LOV domains from phototropins (*74*−*77*), the fungal photoreceptor Vivid (VVD) (*78*, *79*), and the isolated LOV domain from *Bacillus subtilis* YtvA (*80*, *81*) (Table 1).

LOV domains are members of the Per-ARNT-Sim (PAS) domain family of environmental sensors and have conserved PAS fold and signaling elements. Primary surfaces involve an antiparallel 2−1−5−4−3 β-sheet flanked on one side (A-face) by a series of short α-helices. The A-face comprises the photoreceptor core by binding FAD or FMN within a cavity formed between the α-helices and the β-scaffold. The other face (B-face) often mediates intraprotein and interprotein interactions in PAS and LOV domains (*77*, *79*, *82*), as observed in homo- and heterodimer interactions among PAS domains in the hypoxia response pathway (*83*, *84*) and Period proteins (*85*). Analogously, in LOV domains, the β-scaffold forms dimeric contacts in structures of YtvA (*80*) and is implicated in dimerization of phototropin LOV1 and truncated LOV2 constructs (*86*−*89*). Alternatively, regions located to the N- or C-terminal side of the canonical LOV domain (called Ncap or Ccap elements) are bound to this surface in many LOV proteins (*33*, *76*, *77*). For instance, in *Avena sativa* phototropin 1 LOV2 (AsLOV2), an extended C-terminal helix (Jα) docks to the β-sheet. Similarly, the fungal photoreceptor VVD contains an N-terminal β-strand and helix that dock to the β-scaffold (*33*). In both cases, photoexcitation alters interactions between the core domain and additional structures, leading to functional implications for downstream signaling (*33*, *90*) (Figures 4 and 5).

Significant research has characterized the modes of coupling photon input to downstream signaling cascades. On the nanosecond time scale, blue light rapidly induces the formation of the flavin singlet state that undergoes intersystem crossing to form a flavin triplet within picoseconds. Transfer of an electron from a cysteine within a conserved GRNCRFLQ motif followed by radical recombination forms a long-lived cysteinyl−C4a adduct (*91*−*94*) (Figure 2). The resulting C4a adduct disrupts the planarity of the flavin isoalloxazine ring, tilts the ring slightly within the frame of the surrounding protein, and leads to protonation of the adjacent N5 position (*75*, *79*, *80*). The N5 position is the focal point of the protein conformational response, where a conserved glutamine in the Iβ strand (Q513 in AsLOV2) rotates to form a hydrogen bond with the newly protonated N5 (*75*, *79*, *80*). Importantly, mutations of the glutamine residue impair light−dark responses in LOV proteins (*70*, *79*, *95*).

In addition to rotation of the conserved glutamine, intermediate elements within the adjacent Aβ and Iβ strands have been implicated in signal transduction. The conserved glutamine side chain H-bonds with elements within Aβ in structures of VVD and AsLOV2 (*76*, *79*) (Figure 4), allowing the rotation of Gln to propagate changes at long range to Ncap or Ccap elements. Additionally, other residues in Iβ are also in prime positions to respond to changes in planarity or electron density in the isoalloxazine ring itself (*33*). An Hβ strand phenylalanine unique to phototropin LOV2 domains (F494 in AsLOV2, often a leucine in other LOV domains) regulates conformational dynamics within the β-scaffold (*96*). This region is directly adjacent to Ncap or Ccap signaling elements, which are required for the fidelity of light−dark responses in phototropins and VVD (*79*). Indeed, FTIR studies of LOV domains from phototropin, *Caulobacter crescentus* LovK, and YtvA indicate the initial conformational response involves the β-sheet near the conserved Phe as well as neighboring loop regions (*97*−*101*). Importantly, the degree to which β-sheet elements undergo conformational changes in LOV1, LOV2, and bacterial LOV proteins differs according to LOV subtype (*97*, *99*, *101*). Recent studies of the histidine kinase LOVK indicate that the bacterial LOV domain undergoes modest conformational responses that correlate with changes in full-length proteins (*97*). Similarly, LOV1 and YtvA undergo modest rearrangements in β-sheet structure (*99*, *101*). Consistent with these studies, LOV1, YtvA, and LOVK all contain a Leu at the equivalent Phe position. Moreover, Phe → Leu LOV2 variants confer a LOV1-type response to LOV2 domains (*96*). Similar to the phototropins, substitution of these residues along the β-sheet in VVD can lead to stabilization of the C4a adduct, increased propensity for dimerization, and residual dark-state activity (*33*). These systems present a model for signal propagation in LOV domains that involves both H-bonding networks and conformational responses within the central β-sheet that can propagate to a conserved protein−protein interaction surface (Figure 4).

Similar to the β-scaffold dynamics, the distal conformational responses involved in regulation of kinase activity in phototropins are also divergent in LOV1 and LOV2 domains. Whereas LOV1 domains seem to be primarily involved in dimer formation that is suspected to occur in the full-length protein (*87*, *102*), LOV2 domains regulate kinase activity through blue-light-induced displacement of the Jα helix (*4*, *90*). Displacement of the Jα helix requires disruption of the β-scaffold, and the magnitude of Jα movement is variable among phototropin species (*98*, *103*). In general, the phototropin model implies that a combination of

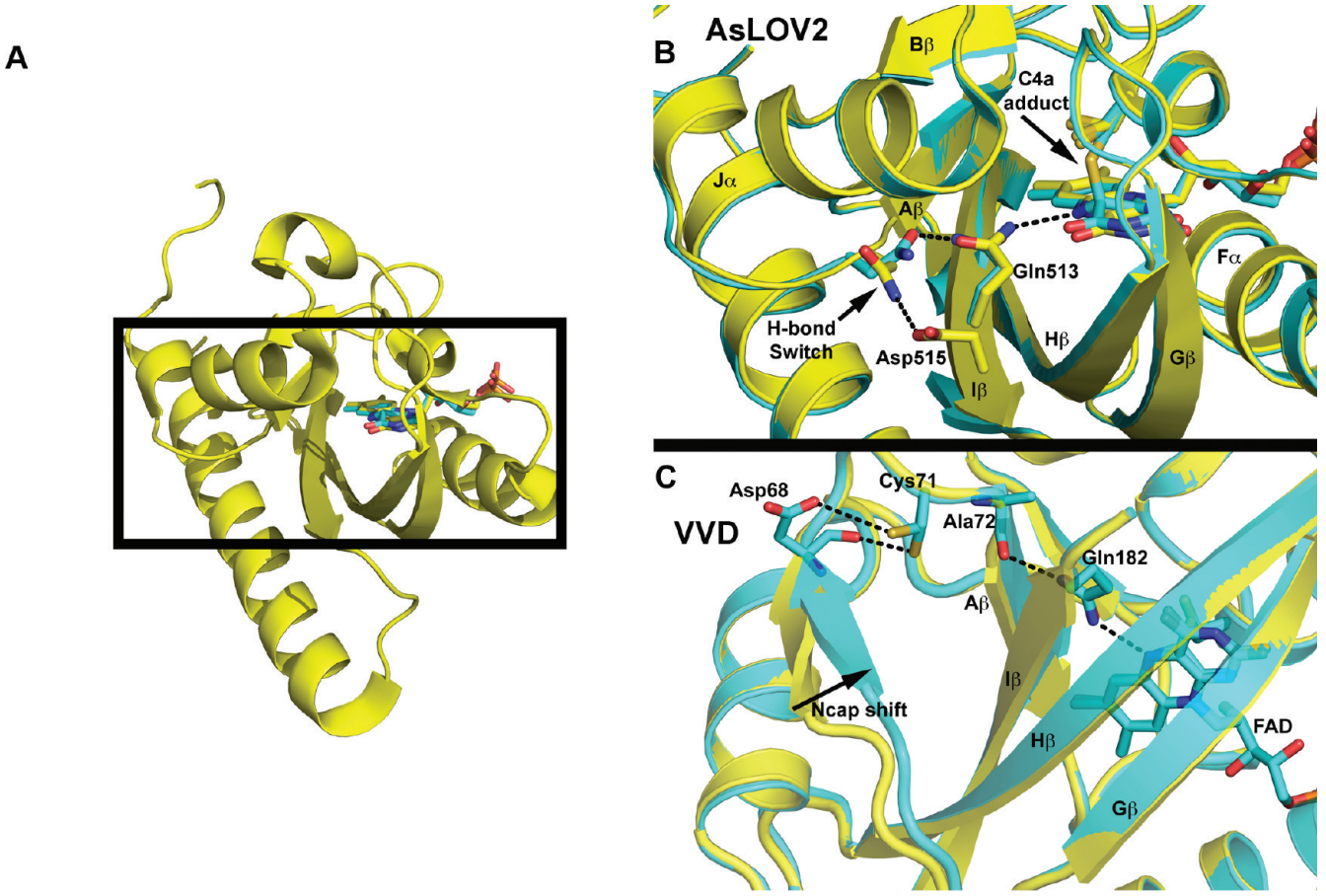

FIGURE 4: Models of signal propagation in LOV domains. Both AsLOV2 (A and B) and VVD (C) initiate signal propagation from rotation of a conserved Gln in the Iβ strand (Q513 in AsLOV2 and Q182 in VVD). Rotation induces interactions with residues at the beginning of the adjacent Aβ strand, which induces alterations of H-bonds between Asp414 (AsLOV2) or induces rotation of Cys71 (VVD). Direct observation of movement of the Ncap was seen in VVD light-state crystals.

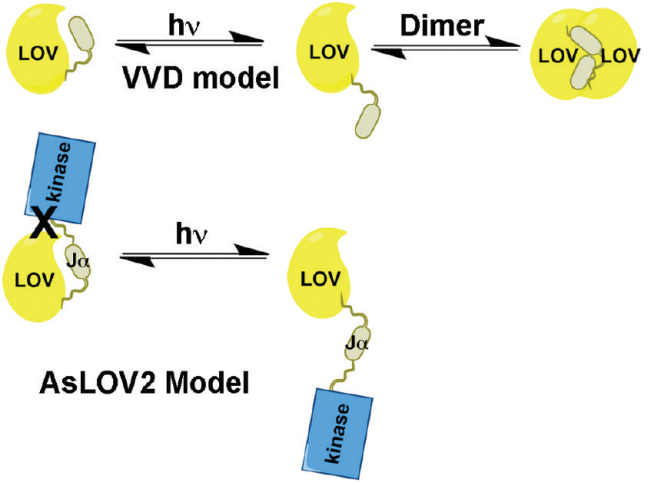

FIGURE 5: Schematic of LOV domain signaling. LOV domains sequester signaling elements via Ccap or Ncap elements. In VVD, conformational changes at the Ncap induce dimerization involving Ncap elements. In AsLOV2, Ccap elements lock the signaling domain (kinase blue) in an inactive conformation. Changes in Jα docking relieve inhibition.

Gln flipping and alterations in β-scaffold conformation disrupt interactions at the B-face of the β-sheet. Subsequent displacement of Ccap elements leads to rearrangements of the phototropin domain architecture, relieving repression of kinase activity (Figure 5).

A modified excitation pathway involving the β-scaffold was observed in the fungal photoreceptor VVD. Examination of light- and dark-treated crystals reveals alterations in H-bond distances stemming from the conserved Gln182 in the Iβ strand, through the β-scaffold to the Ncap (Figure 4). The alteration in H-bonding interactions induces a distal conformational response in the orientation of an Ncap Cys71 residue and displacement of N-terminal structural elements (*78*, *79*). Cys71 variants have been shown to abrogate function in vivo (*79*) or in the case of an activating Cys71Val variant to result in hyperactivity as demonstrated by an extended N-terminus via SAXS (*104*, *105*). Notably, these responses are required for in vitro light-induced dimer formation and in vivo formation of a VVD−White Collar 1 interaction that attenuates gene expression (*78*, *79*, *106*).

A third model of LOV domain signal propagation stems from structural studies of the bacterial photoreceptor YtvA. Structures of YtvA are composed of the minimal LOV domain fused to a C-terminal helix that forms an extended coiled-coil-like structure with an adjacent molecule (*80*). As seen in phototropin and VVD, mutagenesis of the active site Gln results in impaired light−dark regulation in YtvA (*107*). Moreover, β-scaffold variants indicate that Hβ residues regulate the light−dark response (*107*). Comparisons of YtvA to PAS-STAS and studies of YtvA−histidine kinase fusion proteins suggest that light generates distortions in the β-scaffold that rotate the Jα helix activating a stress response (*107*, *108*). Thus, similar to VVD and phototropin, the proximal and intermediate conformational responses at the conserved Gln

and β-scaffold initiate chemical signaling; however, the distal structural effects differ depending on Ncap or Ccap elements. These variations according to LOV subtype are consistent with the observed variations in the magnitude of structural changes at the β-scaffold (*97*, *99*). Complex structures of light-activated full-length proteins and protein complexes (e.g., VVD−:WC-1) are required to elucidate a complete model of LOV protein−protein interaction networks.

BLUF DOMAINS

Similar to LOV domains, BLUF domains couple blue-light absorption to effector domains or proteins (Table 1). The minimal BLUF structure consists of an ∼100-amino acid ferrodoxin-like fold with two helices packed to one side of a mixed 4−1−3−2−5 β-sheet (*109*, *110*). These two elements encompass the isoalloxazine ring of a bound FAD molecule, leaving the adenosine side chain protruding to the protein surface. While the minimal BLUF domains of some proteins are sufficiently stable for biophysical study (*111*), this does not hold for all. NMR studies of truncated versions of several BLUF domain-containing proteins required a C-terminal helical extension of both *Klebsiella pneumonia* BlrP1 and *Rhodobacter sphaeroides* BlrB for proper folding (*112*, *113*).

As one might expect from this dependency of certain BLUF domains on auxiliary regions for stability, BLUF domain β-scaffolds often mediate either intraprotein (Ccap) or interprotein interactions (*109*, *110*, *112*, *114*−*116*). For instance, *Escherichia coli* YcgF exists as either a tetramer or a pentamer when purified in the absence of its Ccap (*117*) but is dimeric when expressed with Ccap elements (*112*, *117*). These results are consistent with formation of large oligomeric complexes through the direct interaction of β-sheet regions, which is found in the *Synechocystis* homologue PixD, which lacks Ccap elements (*116*, *118*). In the context of full-length proteins, the β-scaffold likely mediates interdomain interactions. For instance, isolated structures of AppA form dimeric arrangements involving the β-sheet; however, the C-terminal Cys-rich signaling domain is absent in all structures (*109*−*111*). In the dark, the AppA C-terminal domain is bound to the transcriptional repressor PpsR. Following blue-light treatment, PpsR is released and inhibits transcription of target genes (*119*−*121*). Similar mechanisms of activation involving release of C-terminal elements are likely, and experimental evidence of coupling photon input to signal propagation will be discussed below.

The initial photochemical events that comprise BLUF domain signaling have been intensely studied and have spurred much debate. BLUF domains are unique from other flavin-bound photoreceptors in that the ground and signaling state have their bound flavin cofactors in the same redox state (*120*, *122*−*126*). Evidence of this was provided by the early observation that photoexcitation of BLUF domains simply generated a 10 nm red shift in the visible absorbance spectrum (*124*, *127*). Time-resolved spectroscopy, structural data, and QM/MM calculations have elaborated elegant models that depict rearrangements of H-bonds as the sole contributor of BLUF photochemistry (*15*, *122*, *123*, *128*−*132*). Importantly, several models that depict the initial chemical events that induce intermediate and distal structural responses in BLUF domains and their effectors have been proposed.

Briefly, all models incorporate three principal elements into BLUF domain photochemistry: the active site FAD and nearby conserved tyrosine and glutamine residues. The importance of these two residues has been repeatedly demonstrated by the ability of mutations at either site to prevent the formation of the 10 nm red-shifted active state and a corresponding loss of both in vivo and in vitro activity (*124*, *127*, *133*). Blue-light excitation induces a short-lived flavin excited state, which abstracts an electron from the conserved tyrosine forming a transient semiquinone biradical (*122*, *124*, *128*, *129*, *131*). The following chemical events differ between two proposed models involving chemistry at the conserved glutamine: (1) a 180° flip in the side chain (*128*, *134*) and (2) glutamine tautomerization (*129*, *135*, *136*) (Figure 6). Proponents of the latter tautomerization indicate that the short 10 ns photocycle is insufficiently long to allow for reorientation of the glutamine side chain within the constrained protein environment but is consistent with a series of intricate proton transfers (*129*).

The intermediate structural responses, following radical recombination, hinge on chemical changes at the conserved glutamine position. Structural and biophysical studies provide models of conformational responses involving three structural elements conserved in BLUF domains: (1) residues interacting with the adenosine side chain of FAD (*137*), (2) residues lining the central β-sheet and the adjoining C-terminal helices (*112*), and (3) the β4−β5 loop and the β5 strand (*109*, *110*, *112*, *114*, *118*) (Figure 6). While movement of residues interacting with the adenosine side chain has been observed in crystal structures (*137*), they are unlikely to play a substantial role in propagation of chemical signals (*138*). Below we will outline specific demonstrations of light-induced conformational responses with a particular focus on the β4−β5 loop and the β5 strand, as the ground- and excited-state structures of these elements have been the topic of much debate.

A common theme among structural studies of BLUF domain photoregulation is the proposed transmission of configurational changes near the FAD chromophore across the adjacent β-sheet, perturbing interactions with adjacent structural features. For BlrP1 and YcgF, NMR studies show such changes affect the docking of Ccap helices with the BLUF domain β-sheet surface (*112*, *117*). In particular, a three-residue sequence of hydrophobic residues in BlrP1 undergoes significant chemical shifts in response to blue light. These residues directly interact with Ccap residues involved in large-scale conformational responses (*112*). The functional importance of these interactions is validated by the observation of reduced light-dependent conformational changes in proteins containing mutations at these sites (*112*). Similar structural responses were observed in YcgF in the presence of the C-terminal helix and imply a conserved signal propagation path in BLUF-EAL proteins.

Models of PixD−PixE oligomerization also incorporate signal propagation across the β-sheet. The BLUF domain protein PixD is involved in blue-light-induced phototaxis through direct interactions with the two-component signaling protein PixE. The two proteins form a decameric assembly in the dark, which is inhibited by blue light. The decameric arrangement is likely composed of two pentameric rings demonstrating interactions between the β4 and β5 strands of the β-sheet (*116*, *118*). This region has been proposed to undergo a conformational response that disrupts PixD−PixE oligomerization and is implicated in structural responses of most BLUF domains.

The BLUF domain β4−β5 loop has been thought to play a particularly important role in this signaling process, triggered in part by the discovery of two different conformations in initial

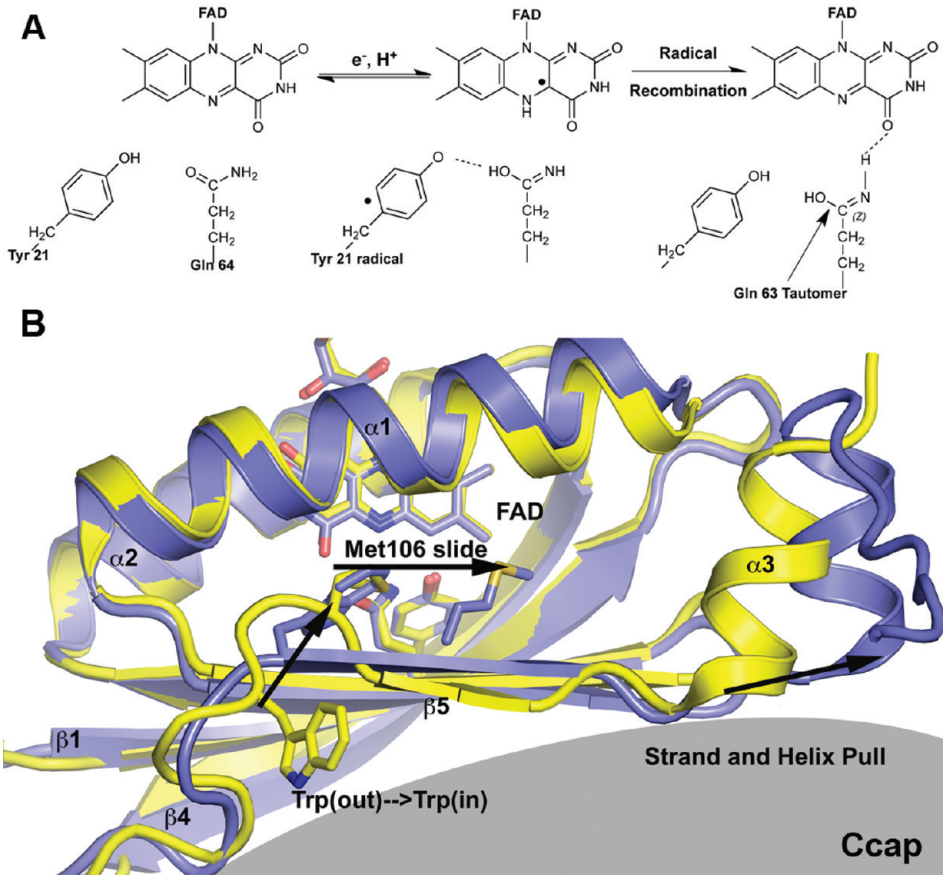

FIGURE 6: BLUF domain activation and signaling. (A) Mechanism of BLUF domain activation. Light induces the transfer of a radical from Tyr21 to FAD, which induces tautomerization of Gln63. (B) Comparison of Trp_{in} (blue) and Trp_{out} (yellow) conformations. Movement of Trp results in alteration of the $\beta5$ loop and adjoining helix. These perturb the conformation of Ccap regions bound on the opposite side of the central β-sheet (gray), altering effector function.

structures of BLUF domains (*109*, *110*). The AppA BLUF domain provided an excellent example of this, where two conformations of Trp104 were observed (Figure 6) in constructs differing in their N-terminal sequences and mutations of Cys residues (Figure 6). The first crystal structure reported of a N-terminally truncated AppA BLUF domain had Trp104 in an internal conformation within the BLUF domain, abutting the conserved active site glutamine residue (*110*) (Trp_{in}). In contrast, in a subsequent structure with an intact N-terminus, AppA Trp104 was solvent-exposed (Trp_{out}) and replaced with Met106 (which formed direct H-bonds with the active site Gln63) (*109*). Despite initial reports that both states represented the dark-state structure, later reports revealed that the Trp_{in} conformation was an artifact of N-terminal truncation (*139*). Notably, some reports still confirm a possible physiological role of the Trp_{in} conformation (*140*).

The two opposing structures revealed a dynamic structural entity in the proximity of both the signaling interface (β-sheet) and the internal photochemistry through interactions with Gln63 (AppA numbering). Fluorescence studies of Trp104 confirm a reduction in the degree of solvent exposure following blue-light treatment (*139*). Similarly, in-depth spectroscopic and theoretical studies of the light−dark response demonstrate that Trp104 undergoes light-dependent alterations in its chemical environment (*129*, *136*, *139*, *141*). Moreover, in vivo studies of Trp104 variants indicate it is required for the fidelity of the light−dark response (*142*). These studies provide compelling evidence of the role of the $\beta4-\beta5$ region in mediating a blue-light response in BLUF domains.

The recent report of a dark-state crystal structure of an intact full-length BLUF-EAL protein, coupled with corresponding NMR studies of the BLUF domain from this protein, solidifies the Trp_{out} ground-state conformation and directly implicates the $\beta4-\beta5$ region in eliciting blue-light-regulated phosphodiesterase activity. Crystal structures of BlrP1 suggest blue-light-dependent conformational changes in three regions (*114*) consistent with the three modes of conformational responses outlined here: (1) residues interacting with the phosphate backbone of the adenosine side chain, (2) the $\beta4-\beta5$ region in the BLUF domain, and (3) residues in the $\alpha3-\alpha4$ loop of the C-terminal cap. Light-induced changes in all of these regions were validated by solution NMR studies of the isolated BlrP1 BLUF domain, which demonstrated that illumination triggered changes in the $\alpha3-\alpha4$ helices of the C-cap, more than 15 Å from the Gln residue (*112*). Importantly, the $\alpha3-\alpha4$ region directly contacts the EAL catalytic domain, providing a mode of propagation of the signal from sensor to effector (*114*).

Currently, BlrP1 is the only BLUF-effector structure and, hence, may not be representative of the modes of excitation of all BLUF domains. In addition, similar to LOV domains, the proximal (Gln tautomerization) and intermediate (disruption of $\beta4-\beta5$) conformational responses are conserved; however, the distal output signal of BLUF domains may be dependent upon their mode of signal propagation and whether they primarily affect intraprotein interactions (inhibitory light−dark state) or whether they function directly as protein−protein interaction domains (PixE−PixD). To further evaluate the role of these sensors and the signal transduction elements, more full-length

structures and structures of BLUF domain−protein interactions are required.

PYP

Research on blue-light photoreceptors was initiated by the discovery of a yellow pigmented photoactive protein (PYP) in 1985, while different cytochromes were being investigated (*143*). The discovery of PYP led to an onslaught of biophysical characterization, in which spectroscopic and structural studies outlined the details of a distinct blue-light-driven photocycle coupled to alterations in protein structure. Unfortunately, the biological role of PYP remains speculative, as it has been assigned only by similarities in the UV−visible absorption profile of this protein and the action spectrum of negative phototaxis by the host bacterium *Halorhodospira halophila* (*144*). This has limited our knowledge of how conformational changes within the isolated domain are propagated in a functionally useful biological environment. Nonetheless, PYP contains the most well characterized photocycle and conformational response within a blue-light photoreceptor.

In addition to being the first blue-light photoreceptor discovered, PYP was the first structurally characterized member of the PAS domain family of proteins that includes LOV domains (*22*). Consequently, the structure of PYP is similar to that of LOV domains and contains a central β-scaffold surrounded on one side by the α-helical segments that includes the 4-hydroxycinnamic acid chromophore covalently attached to a conserved cysteine (Figure 1). Time-resolved spectroscopy and X-ray crystallography have mapped reaction pathways and conformational responses in detail, as reviewed elsewhere (*145*, *146*). Briefly, central to conformational responses in PYP is isomerization of a double bond in the 4-hydroxycinnamic acid that leads to global disruption of structure including an Ncap. The mode of signaling from this "molten globule" state has yet to be resolved but likely involves mechanisms similar to those outlined in other PAS and LOV domains.

BIOTECHNOLOGICAL APPLICATIONS

Motivated by the ability to regulate cellular machinery with exquisite spatial and temporal specificity, researchers have modified photoreceptors for use as fluorescence reporters (*147*), transcriptional activation (*148*, *149*), and photoactivation of cellular responses (*150*). A keen understanding of the modes of molecular excitation and the modular nature of LOV domains affords them a unique opportunity to function in biotechnology applications. To date, focus on LOV-based biosensors has been primarily limited to the AsLOV2 and YtvA LOV domains.

Photocoupled unfolding of a Jα helix in AsLOV2 allows for the construction of dark-state-inhibited structures that are activated upon helical displacement (Figure 7). AsLOV2 fusion proteins function as blue-light photoswitches by coupling a target protein to the C-terminal helix of AsLOV2. Such an approach has been effective in the construction of a photoactivated Trp repressor that can achieve an up to 5-fold dynamic switch (*148*). A LOV2−Rac fusion protein uses a similar methodology to allow for photoinduced cell migration through relief of an inhibited Rac surface (*150*). Notably, structures of the LOV2−Rac fusion protein support a model of dark-state inhibition through sequestration of the Rac domain that can be relieved by unfolding of Jα (Figure 7). Importantly, limited amplification of the signal can be achieved by blue light because of the inherent thermodynamic

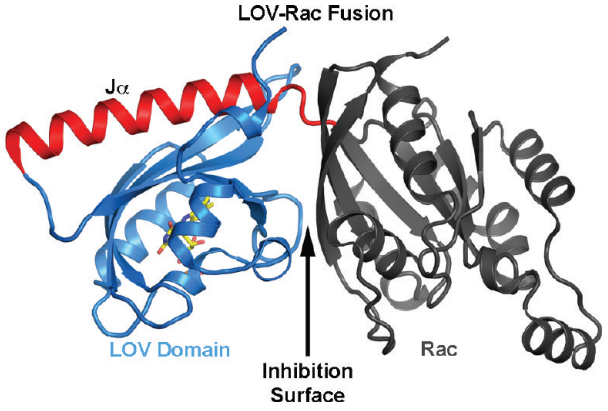

FIGURE 7: Structure of a photoactivatable LOV domain−Rac fusion. The dark state inhibits the activity of Rac (gray) via interactions between AsLOV2 (blue and red) and Rac (PDB entry 2WKP) (*150*). Light-induced unfolding of the Jα helix (red) can relieve inhibition, presumably by allowing freedom between the two domains.

properties of the LOV domain−Jα construct (*151*, *152*). Solution NMR studies have shown that the LOV domain−Jα helix interaction exists in an equilibrium between the bound and free Jα states, with a bias of 98:2 in the dark and 10:90 in the light (*152*). The dynamic range of this conformational switch has been optimized by point mutations within the β-scaffold and Jα helix (*151*).

Alternative photosensor scaffolds have been engineered to confer blue-light sensitivity onto a variety of targets. These scaffolds include other LOV domains that may switch through mechanisms slightly different from that of AsLOV2 and Jα, such as YtvA. Fusion of the YtvA C-terminal helix to signaling helices of a kinase domain of FixL allows for a light-activated kinase (*108*). Analysis of the fusion proteins reveals that effective photoswitches depend upon the maintenance of a proper heptad repeat within the helical connectors (*108*). YtvA has also been employed as a "plug and play" enhancer of enzyme function in *B. subtilis* lipase A (*153*). In these cases, the mechanism of activation is not known, but the ability to enhance function by simply fusing two proteins is intriguing. Finally, non-LOV fusions have recently been reported in the form of PYP fusions to parts of the GCN4 transcription factor, providing blue-light-regulated DNA binding proteins (*154*, *155*).

Notably, the design of fusion proteins provides new tools for biologists but also informs upon the mechanism of signal transduction and limitations of their modular design. Mutation of residues involved in the signaling mechanism allows for amplification of the maximum light−dark response (*151*). Alternatively, knowledge of the mechanism of adduct formation and adduct scission has allowed for modification of the LOV domain so that it acts as a high-quantum efficiency, oxygen insensitive fluorescent probe (*147*). Further, modification of residues important to adduct scission can allow for tunability of the lifetime of photoswitches over many orders of magnitude (*33*). Future tuning of the chemical reactivity of these sensors may allow for greater ranges of wavelengths of excitation and potentially for a photoreversible switch.

FUTURE DIRECTIONS

Since the initial discovery of PYP in 1985, blue-light photoreceptors have been characterized from the point of initial photon absorption to complete changes in organismal behavior. An emphasis on studying the minimal photoreceptor core consistent

with full-length protein photocyles yields constructs amenable to structural studies but may not incorporate all elements required for conformational responses. In fact, in some cases such as BlrP1, what were presumed to be minimal structural units are in fact incapable of photochemistry (*112*). Alternatively, analysis of isolated domains may provide spurious information because of the lack of key regulatory elements. For example, isolated LOV2 domains lacking their Jα helices form constitutive dimers. To completely understand signaling networks, including interdomain and interprotein interactions, complete sensor−effector proteins need to be examined by structural and biochemical methods.

From our perspective, three major questions are at the forefront of the photoreceptor field. (1) What do true atomic-resolution light-state structures, obtained either in solution or through direct crystallization of light-state species, look like? Currently, modes of signal propagation stem from extrapolation of conformational changes observed by illumination of dark-grown crystals, qualitatively analyzing solution NMR data or relying on other spectroscopic data that are difficult to convert into structural restraints. While these methods offer glimpses of conformational changes that lead to activation, each of them has caveats that lead to imprecise characterization of the details of these processes. (2) How do signals structurally and functionally propagate in full-length photoreceptors? Currently, BlrP1 is the only photoreceptor for which there is an atomic-resolution structure of the full-length protein. As such, the generality of this model still remains to be proven for BLUF domains, and atomic-resolution details of CRY- and LOV domain-containing proteins and complexes remain a challenge. These structural studies will provide models of functional regulation that will require experimental validation, often in new pathways that have not yet been characterized. (3) What is the functional cryptochrome photocycle, and how is this coupled to CCT release? The CRY field is rich in biological information; however, little is currently known about the photochemistry or atomic level mechanisms of signal transduction. Notably, an additional relatively unexplored level of regulation in photoreceptors is posttranslational modification, which cannot be fully studied using isolated domains heterologously expressed in *E. coli*. Currently, atomic level details of such levels of control are not entirely tractable. However, as we gain new insight into the nature of molecular excitation of blue-light photoreceptors, new avenues of research and new questions will emerge. Moreover, as we improve our understanding of the molecular underpinnings of photoreceptor function, even more flexible and useful biological tools can be constructed, ultimately affording the control of a wide array of biological responses with precise timing and positioning.

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