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Photochemistry of Flavoprotein Light Sensors

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Abstract

Three major classes of flavin photosensors, LOV domains, BLUF proteins and cryptochromes regulate diverse biological activities in response to blue-light. Recent studies of structure, spectroscopy and chemical mechanism have provided unprecedented insight into how each family operates at the molecular level. In general, the photoexcitation of the flavin cofactor leads to changes in redox and protonation states that ultimately remodel protein conformation and molecular interactions. For LOV domains, issues remain regarding early photochemical events, but common themes in conformational propagation have emerged across a diverse family of proteins. For BLUF proteins, photoinduced electron transfer reactions critical to light conversion are defined, but the subsequent rearrangement of hydrogen bonding networks key for signaling remain highly controversial. For cryptochromes, the relevant photocycles are actively debated, but mechanistic and functional studies are converging. Despite these challenges, our current understanding has enabled the engineering of flavoprotein photosensors for control of signaling processes within cells.

Three major classes of flavoprotein light sensors: Light Oxygen Voltage (LOV) domains, Blue Light sensor Using FAD (BLUF) proteins and Cryptochromes (CRYs) mediate a vast range of biological responses to light that include phototropism, cell and organelle motility, regulation of photosynthesis, stress responses, organismal development and entrainment of circadian rhythms[1–8]. Extensive structural, spectroscopic, biochemical, and computational studies have been brought to bear on the associated light-sensing mechanisms but many of the resolved features and their implications remain contested. Central to these debates is the fact that chromophore excitation generates meta-stable protein conformations capable of propagating signals; however, due to their short lifetimes and modest structural differences, intermediates in the conversion processes are especially difficult to characterize. Moreover, the flavin redox status for both the beginning (dark) and ending (light-adapted or signaling) states *in vivo* can be difficult to define. Compounding these problems is the question of what is a physiologically relevant photocycle; in some cases the “outputs” are behavioral changes that are not easily assayed. Finally it is often assumed, but not always known, that the various homologs, domain constructs and residue variants studied actually display the same mechanistic details. Nevertheless, these proteins have received considerable attention due to their relevance for energy conversion and information processing at the molecular level.

Competing Financial Interests

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Indeed, flavoprotein light-sensors have been co-opted to control cellular processes with that most rapid, specific, and easily delivered substrate: light.

Flavins have long been known to possess rich chemistry, which stems from their access to multiple redox states under physiological conditions and their ability to connect these states to the efficient absorption of UVA, blue, green, and in some cases red light (Fig.1)[2,4,7]. Flavoprotein light sensors have taken advantage of this broad reactivity to drive protein conformational processes that lead to new interactions among protein domains, partners and targets, with the newly forged associations having lifetimes much longer than the initial photophysical events. How these states of varying stability are coupled to allow the polypeptide to read-out flavin photochemistry has not been fully resolved. In this review we summarize the key features of LOV, BLUF and CRY proteins, whose details have been well discussed elsewhere[1–8] and then focus on recent work aimed at resolving mechanism and applying this understanding.

I. LOV Domains

Originally discovered in plant phototropins[2,8], LOV domains are photosensor proteins found in plants, fungi, archaea, and bacteria. LOV domains are a subset of the PER-ARNT-SIM (PAS) domain superfamily that contain a non-covalently bound flavin cofactor (FMN or FAD) that absorbs blue and UVA light[2,6,8]. Upon light exposure the flavin forms a flavin-C4(a)-cysteinyl adduct with a conserved cysteine residue in the LOV domain active site (Fig. 2a,d). Due to the ubiquity of LOV as a modular unit and the diversity of its linked output domains a variety of physiological functions have evolved under control of this photochemistry.

The core LOV domain structure is composed of a β-scaffold with 5 antiparallel β-strands (Aβ, Bβ, Gβ, Hβ, Iβ) and 4 α-helices that connect these elements (Cα, Dα, Eα and Fα). Eα and Fα pack against the β-sheet to form a pocket to bind the flavin isoalloxazine ring (FAD or FMN)[6] (Fig. 2a,b). The Cys residue that forms the flavin-cysteinyl adduct resides on Eα within the conserved sequence GXNCRFL(Q). Variability in the LOV structure is found in core flanking helices at N- and/or C-terminal ends that usually assemble against the β-sheet on the side that opposes the flavin binding pocket (Ncap or Ccap). These helices serve as critical connective elements to (output) domains and are also found in LOV domain proteins that lack an effector unit; in both cases it appears their role is to propagate light signals from the chromophore pocket (Fig. 2c).

The photochemistry of the LOV flavin is relatively well defined[2–4], but several key issues remain unresolved. These include the identity of intermediates preceding adduct formation, proton transfer during the reaction, and factors influencing the highly variable dark-state recovery rates. In the ground (dark) state, the flavin is fully oxidized and noncovalently bound, (visible absorption at λ = 447 nm). Formation of the flavin-(C4a)-cysteinyl covalent bond upon blue-light exposure blue shifts the absorbance (λ = 390 nm). The cycle progresses through an initial excited singlet state (S_1) that intersystem crosses within 0.1-10 ns to a triplet state (λ = 650/715 nm), that then decays on the order of 100 ns to 5 ms[910] (Fig. 2d). Adduct formation could potentially proceed through an ionic intermediate where a

proton is transferred to flavin N5 prior to covalent bond formation[2–4,8]; however, ultrafast spectroscopy reveals that the FMN of phototropin LOV2 remains unprotonated in the productive triplet state immediately prior to reacting with the active site Cys[9,10] (Fig. 2d). In contrast, there is some recent evidence for a neutral semiquinone intermediate, (presumably flavinH$^{\cdot}$-$H_2CS^{\cdot}$) between the triplet and adduct states[11]. Although the proton source for N5 protonation has not been definitively identified, the conserved Cys is a likely candidate[2–49,10] and in the fungal light-adaptation protein Vivid (VVD), adduct formation shows a solvent isotope effect that depends upon a slowly exchanging protonation site close to the active center, presumably the adduct forming Cys[12]. Hence, a proton-coupled electron transfer (PCET) between Cys and the flavin triplet state (E° = ~1.7 V) may generate the adduct[9–11]. Given spatial constraints usually required for PCET, it is remarkable that, the reactive Cys can be repositioned in the active center and still complete the photocycle[13]. The question of whether a biradical intermediate participates in the reaction speaks to the general importance of flavin excited-state quenching by electron transfer (ET) in other photosensing mechanisms (e.g. BLUF and CRY) and sets requirements for creating functionality in designed systems.

Another compelling feature of LOV proteins is the large range of recovery to dark state timescales (seconds to hours), albeit one whose physiological consequences are still uncertain. Recovery back to the ground state requires flavin N5 deprotonation, covalent bond breakage between Cys S and flavin C4a, and Cys S protonation[8]. The LOV_{390} state recovers through a thermally driven process that can be base catalyzed through the deprotonation of N5[4,12,14] (Fig. 2d). Rates of adduct decay generally depend on solvent access to the active site, the hydrogen bonding network to the flavin cofactor, and the electronic environment surrounding the flavin[4,8,12,14–16]. Dark state recovery occurs in > 500 ms, but can range up to many hours. Recent evidence supports involvement of an organized water cluster as the N5 proton acceptor in the native adduct decay reaction[17]. Residue substitutions far from the active site have striking effects on the recovery reaction and often impact the signaling activity of the entire domain[13,15,16].

A central question surrounding LOV domain signaling concerns the generality of conformational responses triggered from the conserved photochemistry of the LOV core. These signaling mechanisms depend on oligomeric state, flanking helical content, and effector domain character[5,6] (Fig. 2c). In these processes, the blue-light signal induces unfolding of flanking helices, dimerization, and rotation of the LOV modules. A highly conserved Gln residue that hydrogen bonds to N5 and O4 in the dark state, responds to flavin N5 protonation of the adduct and plays a key role in transducing the light signal, although the precise nature of this process, and its generality across the LOV family is not fully resolved[2,1819] (Fig. 2b). In the case of the well-studied LOV2 domains of phototropins, adduct strain in the active center spreads through the β-sheet to induce dissociation and unwinding of the Jα helix preceding the C-terminal kinase effector [2,20]. However, in addition to the C-terminal Jα, adjacent Ncap helices also appear to have importance in the signaling mechanism. For example, an N-terminal helix acts a control element in light-activated conformational change of phototropin LOV2[16]. This is supported by MD simulations that show how dynamics of a conserved glutamine residue in the flavin binding pocket and the J_α helix are coupled through a shift in the main β-sheet and a secondary

process involving the N-terminal A′α helix[18]. Nevertheless, despite their close proximity, the A′α and Jα may undergo independent conformational changes when the adduct forms[21]. Studies of an aureochrome LOV domain demonstrate that the Jα helix maintains an important role in signaling despite the atypical fusion of the effector domain to the LOV N-terminus[22]. The Jα of the LOV2 preceding the sensor kinase domain in phototropins couples molecular interactions to the LOV2 photocycle, but other structural elements may also be involved[23]. SAXS data on full-length phototropins containing LOV1, LOV2, and the kinase domain indicates that light does not cause a large scale change to the overall shape of the protein[24]. In contrast, the LOV-HTH prokaryotic transcription factor EL222 demonstrates how alternations in the LOV flavin center influence domain juxtaposition. Changes induced by the adduct state propagate across the β-sheet to destabilize the packing of associated helices, in this case the 4α-helix of the HTH domain[25], which releases to participate in HTH homodimerization and DNA binding.

In the fungal light sensor VVD, light induces dimerization via the Ncap and an extended segment called the N-terminal latch, which releases from each subunit.[26] VVD was crystallized in the light and dark states, providing a clear view of the structural changes induced by light (Fig.3a). Protonation of flavin N5 flips the Gln182 amide to rearrange hydrogen bonding in the connection between the Iβ and the Ncap. This releases the latch to bind into the restructured Iβ-Ncap linkage of the opposing subunit. Importantly, residue substitutions at key positions in the hydrogen bonding network from the flavin ring to the Ncap and at the light-state dimer interface abrogate transcriptional repression by VVD *in vivo*[26]. Hydrogen-deuterium solvent exchange (HDX) experiments[27] indicate that the subunits of the VVD dimer exchange quickly, perhaps facilitating its interaction with other clock components. MD simulations of VVD are consistent with changes in the hydrogen bonding of the FAD-cysteinyl adduct to Gln182, but predict larger amplitude rearrangements of this residue than observed crystallographically and additionally implicate Asn161 in restructuring of the β-sheet to alter the Aβ-Bβ loop[19]. Another small LOV domain from *Rhodobacter* (RsLOV) also undergoes light-driven changes in oligomerization, but opposite to those of VVD, a dark-state dimer of RsLOV dissociates in light[28]. The RsLOV C-terminal helices pack especially tightly in the dark state due to a conserved interface of Gly and Ala residues, but this interaction becomes perturbed in the light-adapted state.

The signaling mechanism of YtvA, a LOV protein from *B. subtilis* involved in stress responses, also involves changes in subunit contacts. Dimeric YtvA has subunits associated through hydrophobic contacts on the LOV β-sheet, but instead of the Jα helix flanking the LOV β–scaffold, it extends from the LOV core to form a pseudo-coiled coil arrangement with the opposing subunit[29,30]. In full length YtvA, interactions in the coupled STAS domain also stabilize dimerization[31,32]. Structural studies on the dark and light-adapted states of the YtvA LOV domain suggest a rotation of the two monomers by 4–5° relative to one another[5,6], consistent with little conformational change in the full-length protein observed by dipolar ESR studies[39]. Additionally, the YtvA LOV domain has been engineered to regulate the histidine kinase (HK) domain of FixL[29]. The structure of the full-length fusion protein [33], referred to as YF1, indicates that signals indeed propagate through the coiled-coil linkage to the HK. Comparison of this structure with the adduct state of YtvA

reveals a change in the LOV-LOV Ncap-mediated interface that could propagate a potential rotation of 40-60° through the Jα coiled-coil linker to the HK domains (Fig.3b) [33]. Thus, in most LOV domains adduct formation initiates structural changes through the β-strands to the juxtaposed Ncaps, Ccaps or both, which then alter their association with the core LOV and/or partners. Determining the generality of the currently defined mechanisms across the LOV superfamily will require detailed study of more full-length proteins, but promises to provide great insight into how a diverse group of effectors are regulated by a relatively conserved input module.

II. BLUF Domains

Discovered through their roles in photosynthetic gene expression of purple bacteria, and photo-avoidance response of flagellates[1], BLUF (blue light sensor using FAD) domains are known to function in a variety of cellular processes and can be found coupled to several different types of output domains[1]. Well characterized BLUF domains are AppA (Activation of photopigment and *puc* expression A), PixD (Slr1694), PAC-α/PAC-β, BlsA, BlrB and BlrP (YcgF), with AppA as the most extensively studied[1,2]. All BLUF domains consist of a ferredoxin-like module with two helices aligned parallel to a 5-stranded mixed β-sheet (Fig. 4a)[1,2]. A more variable helical Ccap often packs against the β-sheet on the side opposing the N-terminal helices. The isoalloxazine ring inserts between the parallel α-helices of the ferredoxin fold with the N5-C4a-O4 edge directed toward the β-strands and the ribityl side chain directed outward to expose the adenosine moiety on the protein surface. The photoresponse depends on interactions of the isoalloxazine ring with several conserved residues projecting from the β-sheet. Taking AppA numbering, Tyr21, Gln63, Met106 and Trp104 (the latter of which can also be a Thr) form a tetrad of residues that undergo photoinduced rearrangements in electronic state, hydrogen bonding and conformation. Mutagenesis studies have established that these residues are critical to the signaling mechanism in all BLUF proteins (see review [1]). In some cases, residue substitution will even lock the protein in a signal-on state without need for irradiation[34].

The flavin absorption spectra of BLUF domains undergo a rapid ~10 nm redshift on conversion from the dark (signal-off) to light-adapted (signal-on) states that can persist from sec to minutes ; the chemical basis of this change has been the subject of intense study and controversy[1]. This red-shifted spectrum results from modulation of hydrogen bonding among the flavin and the Tyr-Gln-Trp(Met) tetrad. The conserved Gln63 side chain resides between Tyr21 and either Trp104 or Met106, depending on the BLUF state or identity. Despite many crystallographic structures, the orientations of Gln63 and Trp104 in the dark state are an unresolved issue. In the first reported structure of AppA (1YRX), Gln63 was modeled to interact with Tyr21 and Trp104, in the so-called Trp_{in} conformation. However, a subsequent AppA structure (2IYG) has the Gln63 amide flipped so that it interacts with Tyr21 and Met106 in the Trp_{out} conformation (Fig. 4a). Model refinement against Quantum Mechanical (QM) and x-ray data supported the 1YRX Gln orientation[35], but this was also challenged by a subsequent QM assessment[36]. The Trp_{in} conformation is consistent with spectroscopic, NMR and computational studies[37,38] (and see [2] for discussion). However, structures of other BLUF domains (BP-1 (Tll0078); 1XOP and BlrB; 2BYC) and recently that of nearly full-length AppAΔ399[39] have Trp_{out} in the dark state. Highlighting the

sensitivity of the Trp104 conformation to environment, the crystal structure of PixD (Slr1694) contains multiple BLUF domains, with most displaying Trp_{out}, but one harboring Trp_{in}(3MZI). Despite these discrepancies, light-activation of BLUF likely involves conformational change at residue 104 and thus the various structures may all pertain to aspects of the signaling mechanism.

Consensus remains to be established for the photochemical processes that trigger rearrangement in the BLUF active site. Initial spectroscopic studies of several BLUF domains established that excitation of the flavin to the S_1 singlet state is quenched as fast as ~1-10 ps by electron transfer (ET) from neighboring aromatic residues, primarily Tyr21, but to a lesser extent Trp104[3,40,41] (Fig. 4b). The flavin converts to a neutral semiquinone in tens of ps, which then recombines with the Tyr radical in the 100 ps - ns timescale[3,42]. This transient charge-separated state rearranges the hydrogen bonding of the active center to produce a much longer-lived metastable conformation for propagating signals[5,47,48]. However, recent ultrafast vibrational spectroscopy has cast doubt on photoinduced ET as an essential component of the BLUF mechanism. In these studies, evidence for a Tyr radical was found only for PixD, not AppA or BlsA[43]. It is not clear why some BLUF mechanisms would involve charge-separated intermediates to achieve the product state, whereas others would not.

Fast spectroscopies sensitive to protonation state have provided further insight into the mechanism. In the light-adapted state, FTIR spectroscopy shows that Tyr21 becomes a stronger hydrogen bond donor and that flavin C_4=O becomes a stronger acceptor, the latter of which partially explains the red-shifted absorption state[1,44,45]. A revision of hydrogen bond donation to flavin N5 finds increased interaction in the light-adapted state[45]. Moreover, hydrogen bonding between chromophore and protein changes within ~100 ps of light absorption[45–47]. Indeed, the constitutively activated Gln63Glu AppA variant was employed to show that interactions to flavin C_4=O increase so rapidly (< 100 fs) that even side-chain rotations are frozen on this time scale [48]. In the variant, fast vibrational changes in the Glu63 protonated carboxylate indicate increased hydrogen bonding to C_4=O. For the wild-type protein, this result suggests that flavin photoactivation pushes electron density toward C_4=O to encourage hydrogen bond acceptance very early in the photocycle, which may then drive structural rearrangements to supply a proton donor.

Models proposed to explain how hydrogen bonding rearranges in the BLUF active site all invoke combinations of Gln63 rotation and tautomerization (Fig. 4b). The most straightforward mechanism (A) has Gln63 beginning with the amide NH_2 hydrogen bonded to Tyr21 and then photoinduced charge transfer causing a rotation of the Gln side chain so that the carbonyl oxygen accepts a strong hydrogen bond from Tyr21 and the NH_2 group donates a hydrogen bond to C_4=O and possibly N5[3,49]. A competing model (B) involves the dark state having Gln63 oriented with the carbonyl group hydrogen bonding to Tyr21 (as in 2IYG). Photoinduced ET then drives conversion of the Gln amide to the imine tautomer, followed by rotation of the side chain to allow hydrogen bonding between the imine N and Tyr-OH[36,50,51]. Proposed variations on these themes include rotation as in (i), but involve tautomerization without biradical formation as an intermediate step (iii)[48], or initiation from the dark state of (ii) and progression by Gln tautomerization but not subsequent rotation

(iv)[52]. In isolation, the enol tautomer would be less stable than the keto by ~0.5 eV[52], but this is well within the energy available from the photochemical reaction and the protein may stabilize this state: for example, the increased hydrogen bond strength of C_4=O in the light-adapted state could contribute ~0.13 eV of stability alone[48]. Photoexcitation of the light-adapted (product) state also leads to rapid oxidation of the Tyr residue and produces the same FADH• intermediate, but in this case proton transfer is concerted with ET and no FAD•- intermediate appears. This fast proton transfer (< 1 ps) suggests that a donor resides close to both N5 and the Tyr21 proton so that an overall shift of protonation states flows through a Grotthus-type mechanism. Of the light states that satisfy this constraint in Fig. 4b, (i) is favored because it best accounts for the strong hydrogen bond formed by Tyr after photoconversion[53].

It is worth noting that the majority of work on BLUF proteins has been carried out on isolated domains. How active site signals influence interactions with output domains or partners is just being resolved. Many of the active site switching models assume that the hydrogen bonding rearrangements among Tyr21, Gln63 and flavin alter the conformation of the residue juxtaposed to Gln63, whether it be either Trp104 (in the Trp_{in} conformation) or its replacement, Met106. There is good evidence that the 104/106 position is indeed sensitive to the Gln63 hydrogen bonding state[2,51,54]. This implies that conformational changes will be propagated through β5 and surrounding regions (i.e. β4-β5 loop, α1-β2 loop) and out the α-helices of the Ccap. Indeed, solution NMR and other spectroscopy provides evidence for such a path; although structural changes are not large, the properties of the Ccap are clearly affected by light conversion[55–57]. The first structure of a full-length BLUF protein (BlrP1) revealed how the Ccap forms a tight interface with the output di-cyclic GMP photophodiesterase (EAL) dimerization domain [58]. Subsequent HDX studies on BlrP1 in dark and light demonstrated correlation in dynamics between the C-terminal α3-α4 helices and the EAL active sites. Conformational coupling back to the BLUF flavin center implicated the N-terminal α1-β2 loop to a greater extent than the β4-β5 loop, but the two regions are in close proximity[59]. Similar results were found with AppAΔ399[39]. Thus, despite many controversies the picture of BLUF domain signaling is sharpening. Photoinduced changes in flavin electronic state rearranges hydrogen bonding along the N5, C4a, C_4=O edge which propagates through the β-sheet to the Ccap. Modest changes in the Ccap then influence the dynamics and stability of the output domain. Key unresolved issues include establishing the importance of photoinduced ET in the general mechanism, definitive assignment of the Gln63 rotomer and tautomer states and defining how these states influence the conformation of residues at the 104/106 position. Structures of light-adapted BLUF domains bound to their targets would identify conformations more definitively associated with function. Key features could then be tested through the phenotypes of variant proteins designed to perturb these properties.

III. Cryptochromes

Cryptochromes (CRYs) are key components of circadian clocks in plants and animals, but serve a variety of roles in all of life's kingdoms[7,8,60]. CRYs are closely related to the photolyase (PL) family of proteins, which use light to repair UV-damaged DNA. CRYs share with PLs a conserved N-terminal Photolyase Homology Domain (PHD) that consists

of an FAD-binding α-helical domain and a nucleotide binding Rossman fold that in PLs recognizes an antenna cofactor (either a pterin or deazaflavin)[7] (Fig. 5a). In PLs, energy transfer from the antenna cofactor excites the flavin hydroquinone (HQ, $FADH^-$), which then repairs a covalent pyrimidine dimer by cyclic electron-transfer[7]. Although PLs require HQ *in vivo*, PLs often purify containing the neutral semiquinone radical (NSQ). Reduction to HQ is photochemically driven through reductive quenching by a triad of Trp residues, highly conserved in both PLs and CRYs that propagates charge to the surface of the protein[7]. In addition to the PHD, CRYs contain a variable C-terminal extension specific to the type of CRY and critical to function. At least some CRYs appear not to bind an antenna cofactor[61,62]. CRYs have been given one of three classifications depending on their ability to serve as a blue light receptor or repair DNA: Type I, or light-sensing CRY, such as Drosophila CRY (dCRY) and CRY1,2 from Arabidopsis (AtCRY); Type 2, or non-light sensing CRY, such as those from mammals (mCRY1,2 and human(h)CRY); and CRY-DASH cryptochromes, which retain some PL DNA repair ability and have roles in signaling[7].

The redox status of the CRY flavin in the cellular ground (dark) and light-adapted signaling states is a matter of controversy. This is particularly relevant for dCRY entrainment of the insect clock. In the insect clock, dCRY triggers the light-dependent degradation of the oscillator protein Timeless (TIM) by facilitating interaction between TIM and the E3-ubiquitin ligase Jetlag (JET)[63]. Two models for light signaling have been proposed (Fig. 5b). In the first, dCRY contains oxidized FAD in the ground state (as purified) and light induces photoreduction to the anionic semiquinone (ASQ), which is sufficient to send conformational signals and engage targets[64,65]. The conserved Trp triad serves as the electron donor *in vitro*; however, an intact Trp triad is not always needed to light activate CRY[66]. Thus, if this model is operative, another electron donor to the flavin may be operative in cells. In the second model, dCRY contains the ASQ in the ground state and light drives conversion to a short-lived excited state, or further reduced state, perhaps analogous to the photochemistry of BLUF proteins[66–68]. Whichever model is correct, light excitation of the flavin is very likely propagated to the C-terminal extension, or C-terminal Tail (CTT) in dCRY. The CTT forms a helix that binds in the active center grove alongside the flavin in analogy to where substrate DNA lesions bind in PLs[61,69,70] (Fig. 5a). Removal of the CTT results in constitutive binding to TIM[63] and there is strong evidence that it releases from the active center in the presence of light[67,70,71].

Lending support to an excited state signaling model are the findings that chemical reduction of the purified dCRY does not cause conformational changes in the CTT, which suggests that a reduced flavin alone is not sufficient to cause conformational signaling[67]. Furthermore, a residue substitution in the Trp triad that prevents photoreduction still allows light-dependent conformational conversion and engagement of JET[66]. However, another study found that reducing agents were sufficient to invoke conformational changes and that these conformations correlated kinetically with flavin reduction and binding to regions of TIM that mimic the CTT[71]. Furthermore, there is evidence that disruption of the Trp triad did in fact prevent dCRY conformational changes in response to light[70]. It also appears that dCRY can be photoreduced to the ASQ when overexpressed inside of insect cells,

independent of the Trp triad[65]. This raises a question as to the source of electrons for CRY reduction. Although the electron source could be exogenous, other internal reductants, such as protein thiols[61,70], are also possible. Moreover, recent data on PLs indicates that internal ET reactions between adenine and isoalloxazine could transiently (< 100 ps) generate changes in the flavin redox state that may contribute to CRY signaling[72]. These rapid perturbations to active center electronic structure could lead to a meta-stable protein conformation, not unlike BLUF.

As with dCRY, there are competing models to explain the mechanism of AtCRY signaling (Fig. 5b) [73]. Both AtCRY1 and AtCRY2 purify from insect cell expression with oxidized FAD, which is then reduced to a NSQ by blue light, and fully reduced to a HQ by green light. Thus, in one model the dark state of AtCRY has oxidized FAD, which light reduces to the NSQ to cause a conformational change in the C-terminal extension. Lifetime of AtCRY signalling state in vivo matches well with the FAD radical state lifetime in insect cells by EPR[74]. The second model holds that light excitation of an AtCRY ASQ causes a rapid photoredox reaction responsible for sending signal. What would AtCRY then reduce? Interestingly, AtCRY1 binds ATP near the FAD cavity, and AtCRY1 catalyzes autophosphorylation in a light-dependent manner[75]. Thus, cyclic electron transfer between FAD and ATP may lead to conformational changes that promote autophosphorylation activity[75]. Substitution of the Trp triad residues in AtCRY modestly affect light-dependent degradation of AtCRY, but do not prevent normal light-dependent association of AtCRY partners[76]. Interestingly, although plant CRYs may use the NSQ as a signaling state, green algae have an animal-like CRY (aCRY) that converts from a ground state NSQ to a HQ in the sensing of blue-to-red light[77].

Although CRYs also play an important role in the mammalian circadian clock, they are likely not the primary light sensors[7]. Instead they appear to be critical components of the central circadian oscillator itself and have a direct role in gene repression[63]. There is some ambiguity as to whether the two mammalian CRYs (mCRY1 and mCRY2) bind flavin *in vivo*, although purified proteins can be reconstituted with FAD, and the FAD binding mode is very similar to that of dCRY[78] (Fig. 5b,c). Furthermore, human CRY, which purifies from recombinant expression with flavin bound, does show light responses in a *cry*-defective fly mutant, although it cannot entrain the circadian clock[79]. The flavin binding pocket of mCRY is targeted by the E3-ligase $SCF^{Flxl3/Skp1}$, which inserts the C-terminus of Fbxl3 into the region normally bound by FAD[78] (Fig. 5a, b). Recent high-throughput screening of small molecules that disrupt circadian rhythms in cultured cells identified a compound that also targets the same FAD binding pocket of mCRY2[80,81] (Fig. 5a). Thus, the CRY FAD pocket is an important recognition motif for flavin cofactors, binding partners and even small molecule mimics (Fig. 5a, b). An important direction to explore is whether flavin competes with protein and small molecule ligands for binding within this pocket and whether such interactions depend on flavin redox state.

A fascinating function of dCRY currently being explored is its role in light-dependent magnetosensing[82–85]. CRY mutants were found to be defective in the response of Drosophila to magnetic fields and this behavior can be rescued by the introduction of dCRY and mCRYs[85,86]. In birds mCRY activation correlates with behavioral sensitivity to

magnetic fields[83]. The wavelength sensitivity of the behavioral effect suggests involvement of the HQ state[83]. In plants, AtCRY responses are influenced by magnetic fields[84]. Although the underlying mechanism of magnetosensing is largely unknown, the ability of flavin to produce magnetic dipoles in the form of radical pair states may be the key chemical feature[82]. In this mechanism, the conversion between the triplet and singlet states of a correlated spin-pair is influenced by the geomagnetic field and a spin-state selective chemical reaction generates signals. However, the difference in triplet and singlet state energies must be small and the nuclear hyperfine coupling that mediates the spin conversion must be of similar strength as the field effect[82]. This implies separation of the radical pairs within the protein to weaken their coupling as well as anisotropic hyperfine interactions to give directionality [87]. Radical pairs composed of [$FAD^{\cdot -}$ + $W377^{\cdot +}$] or [$FAD^{\cdot -}$ + $W324^{\cdot +}$] satisfy the distance constraint[87] and time-resolved EPR has established such a spin-correlated radical pair between FAD and Trp upon blue-light illumination of frog CRY[88]. Additionally, magnetic fields (albeit considerably stronger than earth's field) affect photoreduced flavin yields in purified CRY[89]. How the protein conformation reads out the spin-pair interaction remains to be determined, but it may involve modulating the rate of return to the oxidized flavin ground state[82].

IV. Engineering of flavoprotein light sensors

The ability of flavoprotein sensors to absorb visible light and undergo well-defined conformational transitions has allowed their usage as both reporters and controllers of molecular interactions within cells[4,8,90,91]. Most relevant to this discussion are cases where non-native domains have been placed under the control of photosensor conformational modulation or oligomerization, such as the previously mentioned YF1[29, 33]. Phototropin LOV2 is probably the mostly highly exploited example. The well-defined displacement of Jα from the β-scaffold has been adopted to release interaction domains, thereby activating them. Examples include the embedding of photoswitchable peptides in Jα that are then irradiated to become free ligands for binding partners,[92] and fusion of LOV2 to the TrpR repressor protein wherein Jα release frees TrpR to bind DNA (LOVTAP)[93]. In a similar strategy, LOV fused to the G-protein regulator of actin cytoskeletal dynamics Rac1 blocks Rac1 from interacting with targets until light caused the unfolding of the intervening helix (PA-Rac1)[94]. A tandem fusion of the light-sensing LOV2 domain and the apoptosis-executing domain from caspase-7 (L57V) rapidly induces apoptosis after light stimulation[95,96]. LOV fused with the Ca^{2+} sensor protein STIM1 gates interaction with Ca^{2+} channels to allow photonic control over Ca^{2+} signals. A more general protein targeting system has been developed in TULIP (tunable light-inducible dimerization tag)[97], which is based on the interaction between LOV2 and a PDZ domain engineered to recognize specific peptide epitopes appended to a modified Jα helix. Light uncovers the epitope and recruits any protein fused to the PDZ domain. Notably, dynamic range of the effectors can be improved by tuning the thermodynamic properties of the LOV-Jα interaction[93]. Dimerization of VVD has been employed to light regulate the association of DNA binding domains and their recognition of target sequences (LightOn)[98,99]. Additionally, the LOV-containing FKF1 and GI (GIGANTEA) interaction domains have been joined to proteins of

interest to allow light-triggered association of the fusions through the native FKF1-GI interaction[100].

BLUF and CRY proteins have been engineered to a lesser extent than LOV, but are currently receiving considerable attention. Light reactions of the BLUF protein PixD have been used to cluster transcription factors[101] , and adenylyl cyclases under BLUF control have been converted to guanylyl cyclases[102]. CRY chimeras have also been applied to control protein interactions with light. By fusing AtCRY and its natural targets (CIBN or CIB1) to proteins of interest[103–108], light dependent heterodimerization has been used to regulate many cellular processes including transcription[104,106] and translation[103,106]. Additionally, AtCRY itself will form photo-aggregates upon illumination that can be exploited to activate a fused regulator by driving oilgomerization[109,110]. The variety of systems that function effectively in these capacities highlight the relative ease in which a given module can be brought under control of a light sensor, providing the key features being regulated are structural juxtaposition and accessibility. Finally, flavoproteins are not the only light sensors being recruited for these so-called “optogenetic” applications[8,90,91]. We are rapidly converging on a future where control of molecular events can be switched on and off, with great temporal resolution and in a wavelength dependent manner. Such tools will help delineate the dependencies in complex processes such as gene transcription, chromatin modification, membrane signaling assemblies and targeted protein degradation.

In closing, despite substantial advances in the study of flavin-based photosensors, there is still much work to be done. At a basic level the relevant chemical states require precise definition and consensus. However, these states are clearly context dependent and care must be taken to prove relevance for the physiological processes regulated. As we attempt to define the sequence of charge transfers and hydrogen bond rearrangements that convert light signals, it is worth considering that there may be multiple paths along which signals can progress, even within the same protein. Moreover, across a photosensor family (e.g. BLUF) there may well be a range of mechanisms and thus, one has to take care in transference of one system onto a related one. The flavin S_1<-S_0 transition provides a considerable amount of energy (2.35 eV), which can be dissipated by ET, bond-forming, and structural rearrangements. The remarkable breadth of possible processes speaks to how effectively the protein environment tunes reactivity of the same chromophore. Nevertheless, the critical output may ultimately be how tightly the core sensor in a given state stabilizes the structure of an appended element (e.g. the LOV2 Jα helix) or output domain (e.g. BlrP1-EAL) through a shared interface. These interactions will be sensitive to stable conformation and dynamics, within the light-sensing module. Specific experimental settings are likely to exaggerate or constrain these effects to some degree. Nonetheless, we should relish the rich information that has been gathered on these many systems and consider it all with an open mind.

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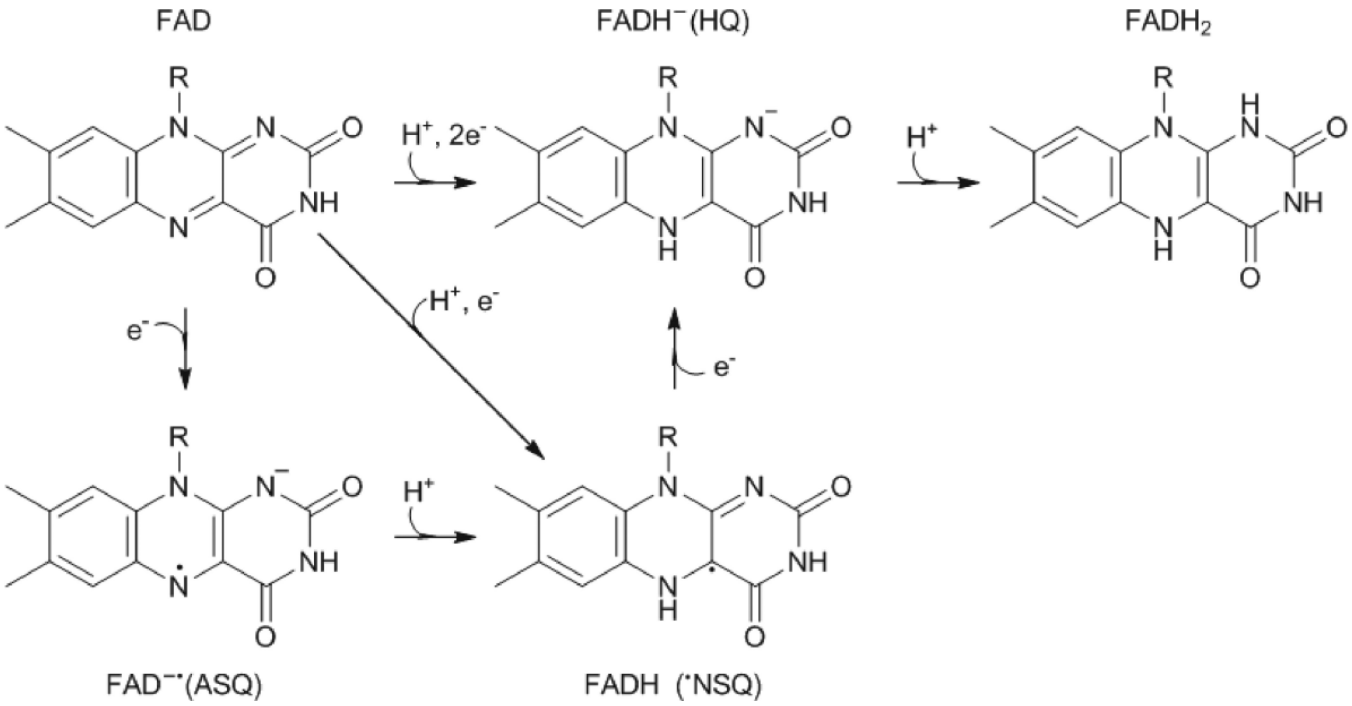

Figure 1.

Redox and protonation states of flavin (FAD or FMN). In flavoprotein light sensors photochemistry drives conversions among these states, which are then coupled to changes in protein conformation.

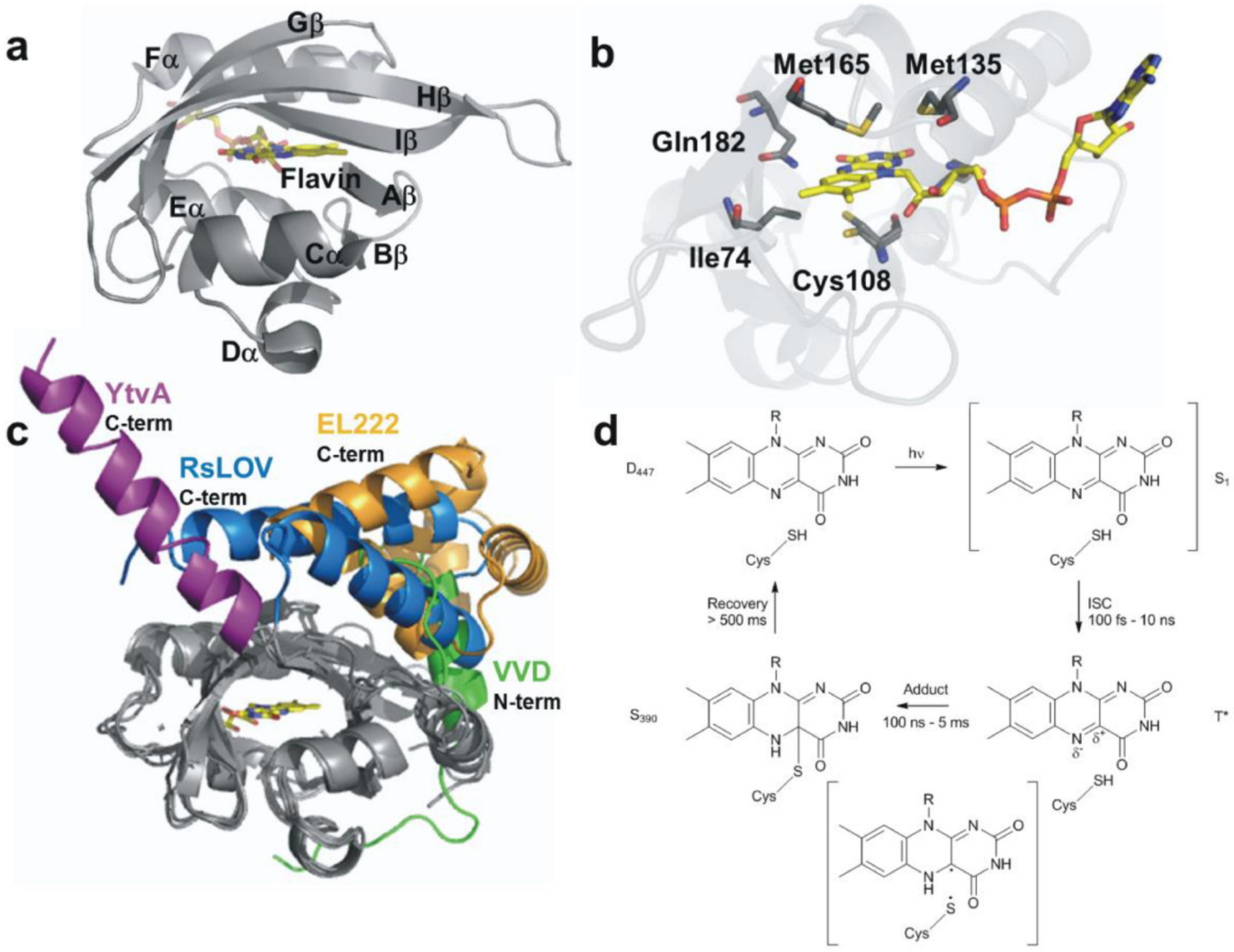

Figure 2.

LOV domain structure and reactivity. (a) Conserved LOV domain structure with labeled secondary elements, represented by VVD (PDB: 2PD7). (b) Critical flavin binding site residues, (VVD numbering). (c) Alignments of LOV domain core structures (gray) with helical extensions depicted to show variations in peripheral structure important for signal transduction: VVD (green, 2PD7), EL222 (gold, 3P7N), RsLOV (blue, 4HIA), YtvA (pink, 2PR5). (d) LOV domain photocycle. Photoconversion to the excited triplet state promotes reaction of the C4A position with an active site Cys residue. A neutral bi radical formed by flavin oxidation of the thiol is a likely intermediate. Return to the ground state is relatively slow and rate limited by N5 deprotonation.

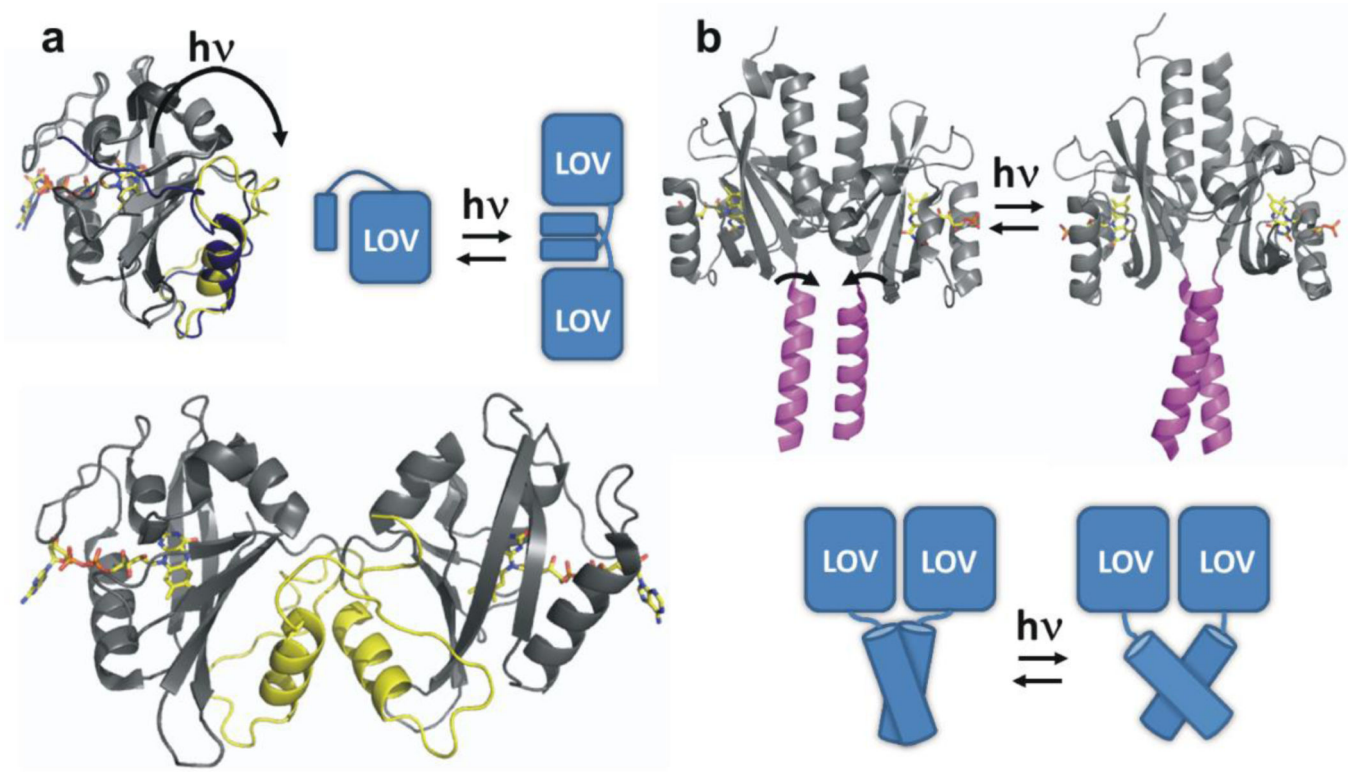

Figure 3.

Protein responses to LOV cysteinyl-adduct formation. (a) Overlay of VVD dark and light state monomer (N-terminal regions in blue and yellow, respectively). Arrow indicates movement of N-terminal latch on conversion to the light state. Inset shows schematic of N/C-terminal movement associated with adduct formation and dimerization. Below: Light state dimer of VVD, (3RH8) N-terminal region in yellow. (b) Proposed model for quarternary structural rearrangements in YtvA/YF1 based on the YF1 dark-state structure (4GCZ) and the light state model on right from alignment of LOV core with Pseudomonas putida LOV (3SW1). Schematic of YtvA/YF1-based rearrangement upon exposure to light with rotation and super-coiling of Jα helix.

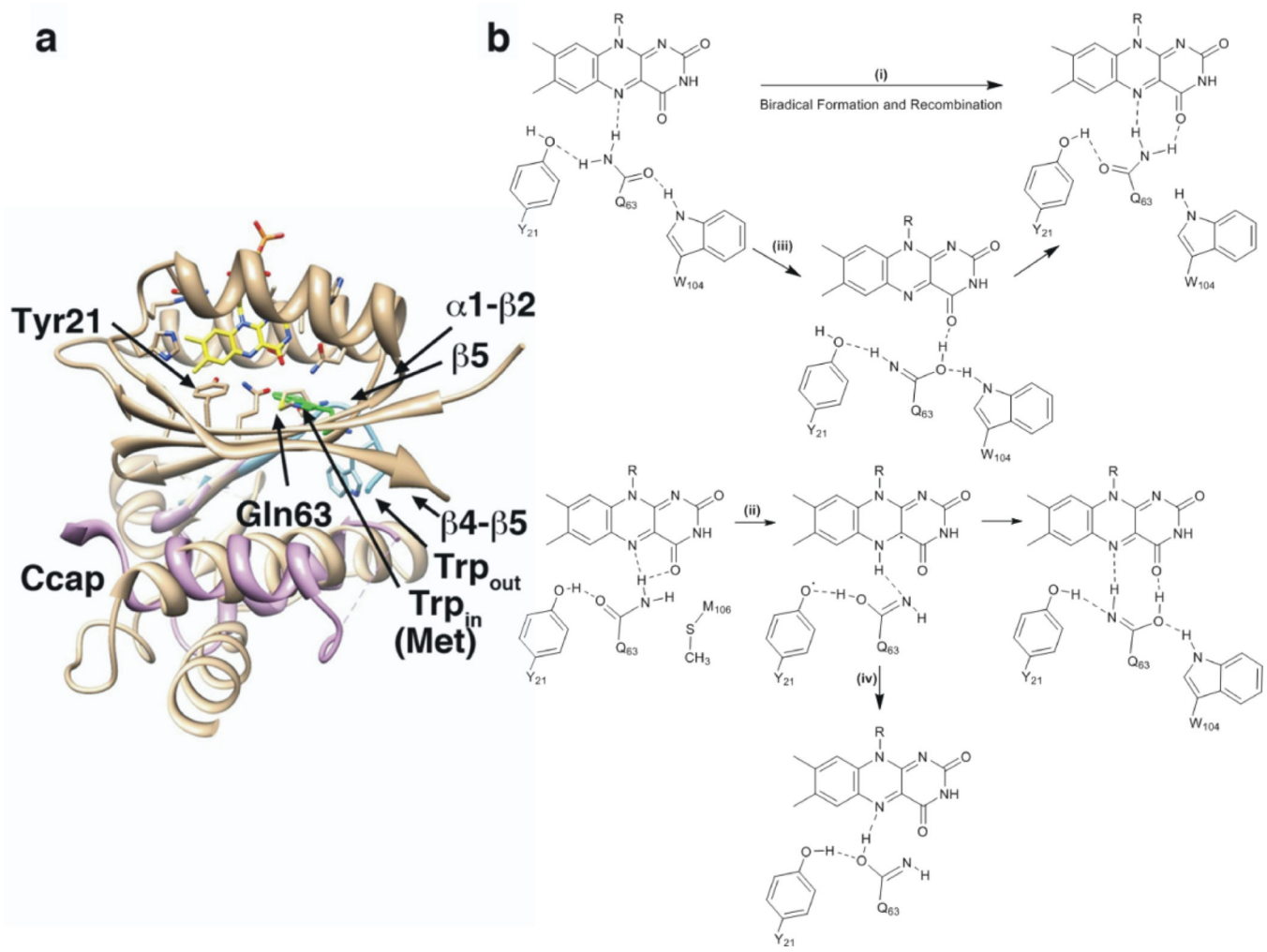

Figure 4.

BLUF domain structure and reactivity. (a) BLUF domain architecture shown as a superposition of AppA with the Trp_{in} (green, 1YRX), full-length AppA with the Trp_{out} configuration (cyan, 4HH1) and the Ccap of BprP (magenta, 3GFX). Tyr21, Gln63 and Trp104/Met106 interact along the edge of the flavin ring (yellow). β5, the β4-β5 loop and the α1-β2 loop propagate conformational changes from the flavin center to the Ccap. The Trp104 residue is found in different conformations (out and in) in various structures (b) Proposed changes in active site hydrogen bonding that accompany photoinduced electron transfer between flavin and Tyr21. Four different proposed reaction pathways are shown (i-iv) that involve variations of Gln63 rotation and/or tautomerization and Tyr21 transient oxidation. Note that pathways (i) and (ii) have different starting (ground) states.

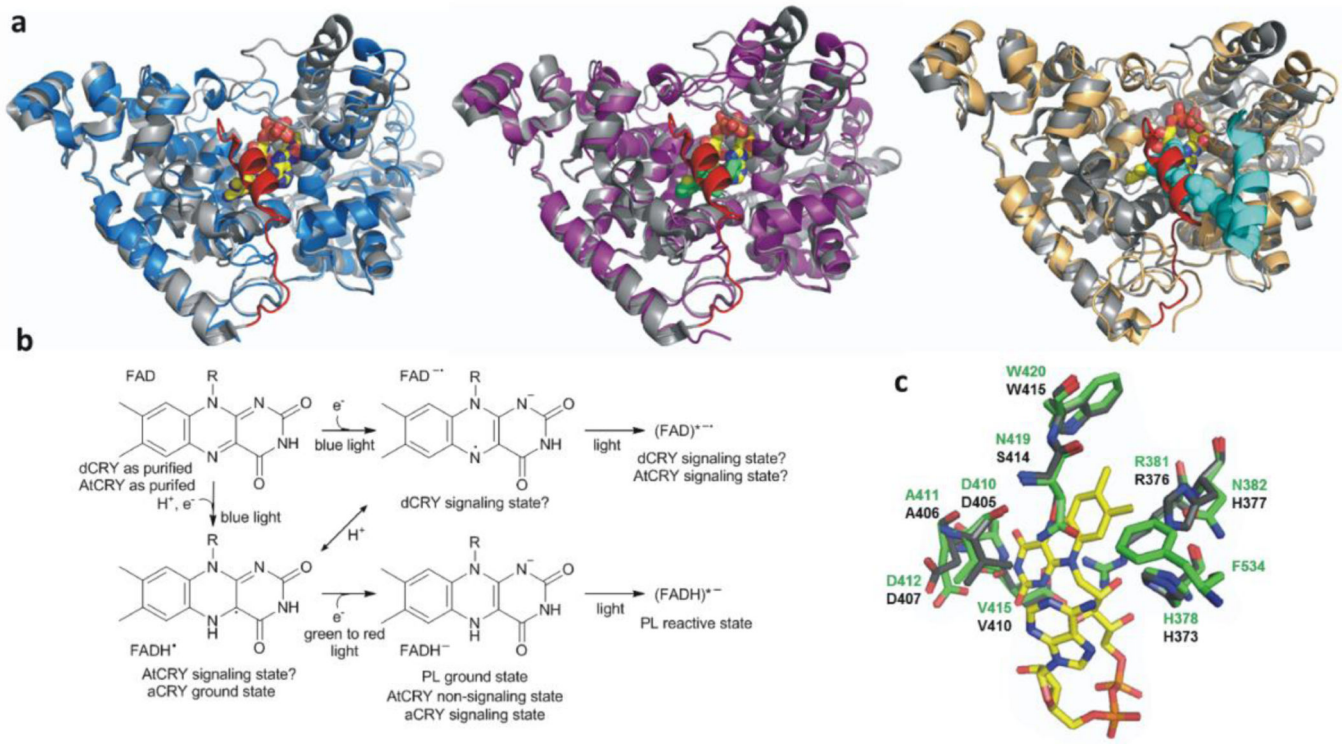

Figure 5.

Cryptochrome structure and reactivity. (a) The flavin pocket in various CRY and PL structures is used to recognize cofactors, substrates, regulatory elements, targets and small molecule inhibitors; thereby providing mechanisms to couple molecular recognition to flavin chemistry. Superposition of dCRY (grey with red CTT and yellow FAD, 4GU5), murine mCRY2 PL domain with FAD (residues 1-512, blue with dark blue FAD, 4I6G), murine mCRY2 PL domain with a small molecule bound (magenta with green small molecule, 4MLP), and murine mCRY2 PL domain with the C-terminus of FBXL3 bound in the FAD pocket (gold with teal FBXL3 residues 400-428, 4I6J). (b) Changes in FAD redox states driven by light in dCRY, AtCRY and aCRY; both the ASQ and a light-excited ASQ may be signaling states of dCRY; similarly the NSQ and light-excited ASQ could be AtCRY signaling states (see text); aCRY signals from a ground NSQ state (c) dCRY and mCRY2 residues that lie near FAD are strongly conserved.