# Searching for a photocycle of the cryptochrome photoreceptors

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## Abstract

The initial photochemistry of plant cryptochromes has been extensively investigated in recent years. It is hypothesized that cryptochrome photoexcitation involves a Trp-triad-dependent photoreduction. According to this hypothesis, cryptochromes in the resting state contain oxidized FAD; light triggers a sequential electron transfer from three tryptophan residues to reduce FAD to a neutral semiquinone (FADH$^\bullet$); FADH$^\bullet$ is the presumed signaling state and it is re-oxidized to complete the photocycle. However, this photoreduction hypothesis is currently under debate. An alternative model argues that the initial photochemistry of cryptochromes involves a photolyase-like cyclic electron shuttle without a *bona fide* redox reaction mediated by the Trp-triad residues, leading to conformational changes, signal propagation, and physiological responses.

## Introduction

Cryptochromes are photolyase-like proteins that regulate photomorphogenic development in plants and the circadian clock in plants and animals [1–3]. The photolyase/cryptochrome superfamily is classified into five families: CPD (cyclobutane pyrimidine photolyase) photolyase, 6–4 photolyase, CRY-DASH (probably a single-stranded DNA repairing enzyme), plant cryptochromes, and animal cryptochromes [4]. The animal cryptochromes are further divided into two types. Animal type 1 cryptochromes, such as *Drosophila* dCRY and monarch butterfly CRY1, act as photoreceptors, whereas animal type 2 cryptochromes, such as mouse cryptochromes, human cryptochromes, and monarch butterfly CRY2, act as light-independent transcription repressors [5,6]. All plant cryptochromes examined so far act as photoreceptors and they are best studied in Arabidopsis. The Arabidopsis genome encodes three cryptochromes, CRY1, CRY2, and CRY3. CRY1 and CRY2 primarily mediate light stimulation of de-etiolation and photoperiodic control of floral initiation, respectively, whereas CRY3 may act as a single-stranded DNA photolyases in mitochondria or plastids [7–11]. Cryptochromes are composed of two domains, the N-terminal PHR (Photolyase-Homologous Region) domain of about 500 residues, and the C-terminal extension CCE (Cryptochrome C-Terminal Extension) of various lengths. The PHR domain of cryptochromes bind non-covalently to the chromophore flavin adenine dinucleotide (FAD) and possibly a second chromophore, 5,10-methenyltetrahydrofolate (MTHF) [12•– 14•]. The CCE domains of Arabidopsis CRY1 and CRY2 are approximately 180 and 110

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residues in length, respectively, and they appear to be intrinsically unstructured, but to act as an effector domain [15–19].

Although the light-dependent catalytic mechanism of DNA photolyase, the presumed ancestor of cryptochromes, is well understood [2], the signaling process of cryptochromes, especially their photoexcitation mechanism, remains not fully understood. A cryptochrome that absorbs a photon is expected to be "photoexcited", by which an electron of the chromophore molecule is promoted from the ground state orbital to a higher energy orbital. It is generally agreed that photoexcited cryptochromes undergo a series of biophysical and biochemical changes, including structural rearrangement, protein modifications, protein-protein interactions, and a chain of signal transduction events that leads to changes in gene expression and physiological responses of plants. However, it is not clear whether and how a light-dependent redox reaction or the photoreduction cycle is involved in the cryptochrome photoexcitation *in vivo*. This article attempts to discuss recent studies concerning photoexcitation mechanism of cryptochromes and different opinions about our current view of the initial phase of cryptochrome signal transduction.

## The cryptochrome photoreduction

Under physiological conditions, flavins are usually synthesized in the oxidized state. But flavin bound to proteins may exist in any one of the three redox states in five different forms: oxidized (e.g. FAD), semireduced semiquinones (e.g. neutral radical $FADH^{\bullet}$ or anion radical $FAD^{\bullet-}$), or fully reduced hydroquinones (e.g. $FADH^-$ or $FADH_2$) [20•] (Fig. 1A). It is often presumed that a light-dependent flavoprotein undergoes a photocycle or photon-driven redox exchanges between the flavin chromophore and its protein environment. A possible photocycle of flavin can be analyzed *in vitro* by monitoring changes of absorption spectra of a purified flavoprotein, because flavoproteins of different redox states have different absorption spectra. For example, cryptochromes/photolyases containing oxidized FAD or anion radical semiquinones ($FAD^{\bullet-}$) absorb primarily UV-A and blue light; that containing a neutral radical semiquinone ($FADH^{\bullet}$) has a broad absorption spectrum extending from blue to green and red light, whereas that containing fully reduced flavin ($FADH^-$ or $FADH_2$) absorbs little visible light (Fig. 1B). The fact that Arabidopsis cryptochromes mediate blue light responses in plants argues that oxidized flavin, which has the strongest absorption in blue regions of light, may be the resting state chromophore of these photoreceptors.

Like many other flavoproteins, but in striking difference to most photolyases studied, Arabidopsis CRY1 expressed and purified from insect cells contains oxidized FAD [12•, 13•,21•,22]. Under anaerobic conditions, the FAD of CRY1 can be rapidly (within 2 min) photoreduced *in vitro* to the semireduced flavin ($FADH^{\bullet}$ or $FAD^{\bullet-}$), which absorb blue and/or green lights to become fully reduced ($FADH_2$ or $FADH^-$) [12•,21•]. Photoreduction has also been reported for Arabidopsis CRY2 under an aerobic condition [14•]. Although CRY2 was reported to be photoreduced to only the semireduced form under aerobic condition [14•], it can be reduced, just like CRY1, to the fully reduced form under anaerobic conditions (Zhong and Lin, unpublished data). Because *in vitro* photoreduction is common for flavoproteins regardless of whether their function is dependent on light, it is particularly important to understand whether the photoreduction observed *in vitro* is physiologically relevant to the functions of cryptochrome photoreceptors *in vivo*.

The physiological relevance of the cryptochrome photoreduction observed *in vitro* was initially tested for the hypocotyl inhibition response in seedlings grown under blue, green, or UV-A light, roughly corresponding to the characteristic absorption spectra of oxidized (FAD), semireduced ($FADH^{\bullet}$), and fully reduced ($FADH^-$ or $FADH_2$) form of CRY1

observed *in vitro* [8,12•,23] (Fig. 1B). When grown under continuous broadband fluorescent blue light (~410nm–520nm), green light (~480nm–580nm) or UVA-light (~300–400nm), the wild type Arabidopsis and tobacco seedlings showed fluence rate-dependent inhibition of hypocotyl elongation in all three wavelengths tested, with the apparent effectiveness of blue>UV-A>green light. The transgenic Arabidopsis and tobacco seedlings overexpressing CRY1 showed a hypersensitive growth inhibition under all three wavelengths tested [8,12•, 23]. The light-dependent degradation of Arabidopsis CRY2 was also found to occur not only in blue light, but also in broadband green and UV-A light [24]. Although these earlier experiments failed to demonstrate a different physiological activities of different redox species of Arabidopsis cryptochromes, it was later found that the broadband green light has both stimulatory and inhibitory effect on the cryptochrome-dependent blue light inhibition of hypocotyl elongation, and that cryptochromes may act as sensors for the blue/green ratio in nature [25].

Given that the photoreduction of Arabidopsis CRY1 *in vitro* produces semireduced neutral radical semiquinone (FADH•) that absorbs green light [12•], it is conceivable that a semireduced flavin may represent the signaling state of CRY1. It was reasoned that if the semireduced and green-light-absorbing CRY1 was the physiologically active form, illumination of seedlings with bichromatic green and blue lights might shift the equilibrium toward a decreased level of oxidized and semireduced CRY1 and an increased level of fully reduced CRY1 to undermine the CRY1 function [21•]. Indeed, supplement of green light to blue light partially suppressed blue light inhibition of hypocotyl elongation and blue light stimulation of anthocyanin accumulation in Arabidopsis seedlings, but no such green light effect was observed in the *cry1* mutant [21•]. In addition to the CRY1-mediated responses, the antagonistic effect of green light to blue light was also shown for blue light-induced CRY2 degradation and CRY2-mediated blue light promotion of flowering in SD photoperiods [14•,21•]. In one of these experiments, it was shown that CRY2 was degraded to almost completion after eight cycles of 3-min blue light exposure followed by a 3-min dark period [21•]. In contrast, little CRY2 degradation was detected after eight cycles of 3-min blue light exposure followed by a 3-min green light pulse or eight cycles of green light treatment [21•]. However, in contrast to the far-red light reversion of most phytochrome-mediated red light responses, the antagonistic effect of green light appears relatively weak for the cryptochrome-mediated blue light responses tested [14•,21•](Yang and Lin, unpublished). Nevertheless, a photoreduction cycle was proposed for Arabidopsis cryptochromes based on these results [14•,21•]. According to this photoreduction hypothesis, cryptochromes in the dark (resting state) contains oxidized FAD, which is reduced to semireduced FDAH• upon blue light absorption. As a major photoexcitation product, the semireduced FDAH• represents the signaling state of cryptochromes, which can be oxidized to regenerate the resting state oxidized flavin via dark conversion to complete the photocycle [14•,21•]. Provided the photoreduction was involved in the initial photoexcitation of cryptochromes, what would be the electron donor for the photoreduction of flavin in cryptochromes?

## The Trp-triad paradox

To address this question, researchers looked once again to photolyase for possible clues. Ironically, an answer derived from that of photolyases – three conserved tryptophan residues, referred to as Trp-triad, help transfer an electron to reduce flavin – appears to also bring skepticism to the presumed answer of the original question – whether photoreduction is the photochemical mechanism of cryptochrome photoreceptors. To better understand this paradox, one should first take a closer look at the two different photochemical reactions of photolyase. The catalytic mechanism of photolyase is peculiar in that it involves a light-dependent cyclic electron shuttle without a net gain or loss of electron [2,26]. Taking *E. coli*

photolyase as an example, our current understanding of the photolyase catalysis may be summarized in the following. First of all, *E. coli* photolyase possesses fully reduced (FADH⁻) *in vivo* and uses it as the catalytically active co-factor [2,26,27••]. Photolyase binds the cyclobutane pyrimidine dimer of DNA in a light-independent manner and flips the dimer out of helix into the active site or the FAD-cavity to make contact with the flavin, producing a stable enzyme-substrate complex. Second, exposure of the photolyase-DNA complex to light initiates catalysis. In this light-dependent catalytic process, the methenyltetrahydrofolate (MTHF) chromophore (or the other type of antenna chromophore, 8-hydroxy-7,8-didemethyl-5-deazariboflavin in some other photolyases) absorbs a photon and transfers the excitation energy to the reduced flavin chromophore (FADH⁻) by Förster-type resonance energy transfer. The excited flavin (FADH⁻)* then transfers an electron to the pyrimidine dimer, generating a pyrimidine dimer radical, which undergoes bond rearrangement and breakage to yield a repaired DNA product. Before the repaired DNA dissociates from the enzyme, it transfer an electron back to the flavin-neutral radical (FADH•) to replenish the electron lost during catalysis, resulting in regeneration of the fully reduced flavin (FADH⁻). Because there is no net gain or loss of an electron during catalysis, photolyase catalysis is not a redox reaction *per se*.

Although *E. coli* photolyase contains fully reduced (FADH⁻) *in vivo*, it is oxidized during purification to contain primarily the semireduced neutral radical (FADH•). The purified photolyase can be photoreduced *in vitro* to restore the catalytically active FADH⁻. This *in vitro* phenomenon is called "pre-illumination effect" or "photoactivation", which is distinct from the light-dependent catalysis of photolyases described above. The primary electron donor for the *in vitro* photoreduction/photoactivation of *E. coli* photolyase was found to be tryptophan 306 (W306) [28•]. The W306F mutant enzyme is incapable of photoreduction or photoactivation *in vitro*, but it is active *in vivo* and it can be chemically reduced by high concentrations of reducing agents, such as dithiothreitol (DTT), to become catalytically active *in vitro* [2,28•]. As the primary electron donor, W306 was expected to be in the vicinity of FAD. Surprisingly, when the crystal structure of *E. coli* photolyase was solved, W306 was found to locate on the surface of the enzyme, 13.4 Å away from the flavin sitting in the FAD-cavity [27••]. It was later shown by analyses of the time-resolved absorption spectroscopy and the X-ray structure of *E. coli* photolyase that W306 transfers an electron by three steps through two other tryptophan residues (W359 and W382) located between W306 and flavin [29]. These three tryptophan residues (W306, W359, and W382), referred to as Trp-triad (Fig. 2), are clearly required for the photoreduction of *E. coli* photolyase *in vitro*, because site-specific mutations of the tryptophan residues of Trp-triad abolished *in vitro* photoreduction and photoactivation of the mutant enzyme. However, none of the site-specific mutations of the three tryptophan residues of Trp-triad abolished the photolyase activity *in vivo* [28•,30]. Clearly, Trp-triad is not required for the photolyase function *in vivo*, and photoreduction is physiologically irrelevant for *E. coli* photolyase [2,26]. Consistent with this conclusion, an examination of the cellular redox environment of *E. coli* and the midpoint redox potential of *E. coli* photolyase indicate that *E. coli* photolyase needs no photoreduction to reduce its flavin *in vivo*. The cellular redox potential of *E. coli* is about −270mV [31,32], whereas the midpoint redox potential of the FADH•/FADH$_2$ (or FADH•/FADH⁻) redox couple of *E. coli* photolyase is between 16mV to −48mV [33,34]. Therefore, *E. coli* photolyase can be readily reduced *in vivo*, presumably by cellular reductants such as glutathione or thioredoxin, without a need of photoreduction.

Paradoxically, although the Trp-triad-dependent photoreduction seems biochemically unnecessary and physiologically irrelevant to the catalytic activity of photolyases, the three tryptophan residues of Trp-triad are uniformly conserved in all photolyases and cryptochromes examined [29] (Fig. 2). The conserved Trp-triad was proposed to be the electron donor for photoreduction not only of photolyases, but also of cryptochromes [29]. A

spectroscopic analysis suggests that tryptophan or tyrosine residues may be the electron donors for the *in vitro* photoreduction of Arabidopsis CRY1 [35]. Sequence alignment and structure comparison of photolyases and cryptochromes suggest that W324, W377, and W400 are the Trp-triad of Arabidopsis CRY1 [29] (Fig. 2). Possible involvements for two of those three tryptophan residues in CRY1 photoreduction *in vitro* and physiological activities *in vivo* have been tested [22]. Two site-specific mutant proteins, CRY1$^{W324F}$ and CRY1$^{W400F}$, in which the tryptophan residues (W324 and W400) were replaced with phenylalanine, were expressed and purified from insect cells. Because phenylalanine is redox-inert but structurally similar to tryptophan, the CRY1$^{W324F}$ and CRY1$^{W400F}$ were expected to abolish photoreduction with minimum structural disturbances. As expected, the CRY1$^{W324F}$ and CRY1$^{W400F}$ mutant proteins failed to undergo photoreduction *in vitro*, confirming that the Trp-triad is required for the *in vitro* photoreduction of CRY1. Moreover, transgenic plants overexpressing CRY1$^{W324F}$ and CRY1$^{W400F}$ failed to rescue the *cry1* mutant phenotypes such as hypocotyl inhibition and anthocyanin synthesis, suggesting that CRY1$^{W324F}$ and CRY1$^{W400F}$ were physiologically inactive [22]. It has been recently reported that human hCRY1 expressed in insect cells also exhibits a Trp-triad-dependent photocycle and light-dependent degradation similar to that of known photoreceptors, such as Arabidopsis CRY2 or Drosophila dCRY [36]. Although human cryptochromes, like mouse cryptochromes, are generally considered to be a photo-insensitive type 2 cryptochromes that act as the transcription suppressors of the central oscillators [37–40], it remains possible that they may also act as photoreceptors in the ganglion cells [41–43••]. These results are consistent with the hypothesis that photoreduction is associated with the function of cryptochromes and that Trp-triad is the electron donor(s) of cryptochrome photoreduction.

However, whether the Trp-triad-dependent photoreduction is directly associated with cryptochrome photoexcitation *in vivo* has been questioned. First, although the Trp-triad is conserved in all cryptochromes and the three tryptophan residues are required for the *in vitro* photoreduction of all photolyases and cryptochromes tested, the physiological relevance of the Trp-triad has so far been reported only for Arabidopsis CRY1. For example, the Trp-triad mutations have been prepared and tested for several insect type 1 cryptochromes (Drosophila, monarch butterfly, mosquito, and silk moth) that act as photoreceptors. All those Trp-triad mutations abolished *in vitro* photoreduction of the purified proteins, but none of those Trp-triad mutations abolished the *in vivo* physiological or photobiochemical activity of the respective cryptochromes tested, including light-dependent magnetoreception, light-dependent transcription regulation, and light-induced proteolysis [44–47]. For example, it has been shown recently that the Trp-triad-dependent photoreduction is not required for the photoentrainment of the circadian clock or blue light-dependent magnetoreception of insect cryptochromes [44]. Similarly, we have recently found that the Trp-triad-dependent photoreduction is not required for the *in vivo* photobiochemical or physiological activities of Arabidopsis CRY2 (Yu and Lin, unpublished). Therefore, similar to photolyase, the Trp-triad-dependent photoreduction is most likely not required for the functions of both animal and plant cryptochromes. Second, the high resolution time-resolved spectroscopic studies of photolyases and the type 1 photo-sensitive cryptochromes suggested a photolyase-like photochemistry of the insect photoreceptors [20•,48,49]. It was hypothesized that the resting state of the flavin cofactor is semireduced flavin, such as $FAD^{\bullet-}$, in insect cryptochromes. Upon photoexcitation, the excited $FAD^{\bullet-}$ donates one electron to an unidentified acceptor to induce a conformational change through the primary electron transfer. Although the fate of the transferred electron remains unclear at present, this model suggests that the insect type 1 cryptochromes may have the photolyase-like photocycle that do not require the Trp-triad-dependent photoreduction for *in vivo* function [20•,48,49]. Third, the positive correlation of the abolished photoreduction activity *in vitro* and physiological activity *in vivo* of the CRY1$^{W324F}$ and CRY1$^{W400F}$ mutations appears insufficient to establish a causal relationship between the two activities, because it cannot exclude a possibility that the two

mutant proteins may be structurally compromised. The three tryptophan residues of Trp-triad located in the structurally important FAD-binding pocket of photolyases and cryptochromes are not the only residues conserved in this highly conserved region. In addition to the three Trp-triad residues of the Trp-triad, two other tryptophan residues in this region are also perfectly conserved in proteins from *E. coli* photolyase to cryptochromes in Arabidopsis, Drosophila, and human (Fig. 2A). The possibility that Trp-triad may be structurally important but not photochemically critical to cryptochromes can be illustrated by a study of two frog (*Xenopus laevis*) cryptochromes, xCRY1 and xCRY2b [50•]. *Xenopus* xCRY1 and xCRY2b are photo-insensitive type 2 animal cryptochromes that showed similar activity suppressing the bMAL1/CLK-dependent transcription of the reporter gene in the COS-7 cells. The transcription suppressor activities of xCRY1 and xCRY2b are independent of light. However, mutation of any of the Trp-triad residues of xCRY2b severely reduced the transcription suppressor activity of the xCRY2b mutant proteins, whereas only one of the three mutations of xCRY1 affected its activity significantly [50•]. The fact that the light-independent activity of two type 2 cryptochromes was differentially affected by Trp-triad mutations argues strongly that those mutations simply upset the structural integrity of the respective non-photoreceptor cryptochromes rather than affected the fundamental photochemistry of cryptochromes.

## A redox potential problem

In addition to the photoreduction controversy, there seems another redox puzzle remaining unsolved for the cryptochrome photoreceptors. This puzzle concerns the resting state of flavin. We have asserted in the previous section that for cryptochromes to absorb blue light, the chromophore flavin should be in its oxidized form, because oxidized FAD is the redox species of flavin that absorbs blue light most effectively [12•]. This assumption, however, has never been proven. Many factors affect the redox states of a flavoprotein, including the midpoint redox potentials of the respective flavoprotein and redox potential of the cell. For example, the midpoint potential for the $FADH^{\bullet}/FADH^{-}$ redox couple of *E. coli* photolyase has been measured to be between 16mV to −48mV [33,34,51]. Therefore, photolyase should contain mostly reduced flavin ($FADH^{-}$) in a cellular environment with a redox potential more negative (reduced) than −48mV. Indeed, it has been known for a long time that *E. coli* photolyase exists as the fully reduced form *in vivo* without a need of photoreduction [2], presumably because *E. coli* cells have the redox environment of about −280mV [32]. The midpoint redox potentials of Arabidopsis CRY1 have also been measured, by at least two independent experiments, to be approximately −143mV to −153mV for the $FAD/FADH^{\bullet}$ couple and −161mV to −181mV for the $FADH^{\bullet}/FADH_2$ couple [12•,34]. It means that in a solution of about −143/−153mV, approximately half of the CRY1 molecules contain semireduced $FADH^{\bullet}$ (or $FAD^{\bullet-}$) and the other half oxidized FAD. Only in a solution that is less negative than −143/−153mV would more than half of CRY1 contain the blue light-absorbing oxidized FAD. By the same token, in a solution that is more negative than (or FADH−) that absorbs −161/−181mV, more than half of CRY1 would contain $FADH_2$ little blue light. It is difficult to accurately measure the cellular redox potential, because cells, especially plant cells undergoing photosynthesis, are usually not in the equilibrium state and it is difficult to estimate the net potentials of all redox species inside the cell. However, the cellular redox potentials have been roughly approximated from the redox potentials of a few primary redox regulators, such as glutathione, NAD(P), ascorbate, or thioredoxin couples [52,53]. For example, based on the redox potentials of glutathione or thioredoxin, the redox potentials of cultured mouse cytosol and *E. coli* cells were estimated to be approximately −260mV and −280mV, respectively [31,32]. The redox potential of Arabidopsis cells (cytosol) has been recently measured *in vivo*, using transgenic plants expressing redox-sensitive GFP, to be approximately −320mV [54,55]. This estimation seems consistent with the redox potentials of mammalian (ca. −260mV), bacterial (ca. −280mV), or plant cells (ca.

-300mV) measured previously by other methods [31,32,56,57](G. Noctor, personal communication). Assuming the redox potential of the nucleus is also about −300mV, which is significantly more negative than the middle point potential of the $FADH^{\bullet}/FADH_2$ couple, the flavin chromophore of an Arabidopsis cryptochrome should theoretically exist *in vivo* as the fully reduced form ($FADH_2$) that absorbs little blue light! This apparent discrepancy between the theoretical redox equilibrium of cryptochromes under the presently known cellular redox environment and our expectation that cryptochromes should contain flavin in the oxidized state to absorb blue light presents an inescapable challenge to the study of cryptochrome photochemistry. It should be pointed out that the in vivo absorption spectrum of cryptochromes is likely to involve not only flavin but also the putative second chromophore, which is likely MTHF (5,10-methenyltetrahydrofolate). However, MTHF does not seem to be very helpful in this issue, because it also absorbs little blue light. One potential explanation of this problem may be that cryptochromes in the nucleus interact with other proteins to form a protein complex to provide a more oxidative microenvironment such that the ground-state cryptochromes may exist as a less reduced form, such as FAD or $FAD^-$, to absorb blue light.

## An alternative photocycle of cryptochromes

In summary, the prevailing Trp-triad-dependent photoreduction hypothesis of cryptochrome photochemistry (Fig. 3A) has several difficulties. First, it does not explain why elimination of flavin photoreduction fails to abolish the photobiochemical or photophysiologically activities of photolyases and most cryptochrome photoreceptors studied *in vivo*. Second, the tryptophan residues of Trp-triad required for photoreduction *in vitro* are only three of the five conserved tryptophan residues in the FAD-binding region of photolyases/ cryptochromes; and they may be more important for the structural integrity than for the photochemistry *per se* of photolyase/cryptochrome. Third, the redox potential of plant cells appears unfavorable for the oxidized FAD to exist as the resting-state chromophore of cryptochromes.

If the Trp-triad-dependent photoreduction mechanism did not explain the photochemistry of cryptochromes, what are alternatives mechanisms? In this regard, the very similar structure of photolyases and cryptochromes and the unusual U-shaped configuration of FAD (Fig. 2B) commonly found in photolyase and cryptochromes might offer some clues. We propose that the photochemistry of cryptochromes involves a photolyase-like photocycle that is dependent on a cyclic electron shuttle but not on the Trp-triad-dependent photoreduction (Fig. 3B). According to this proposition, the resting state cryptochrome contains anion radical semiquinone $FAD^{\bullet-}$, which apparently absorbs blue light with the efficiency of at least half that of FAD (Fig. 1B), but it is more likely to exist than FAD under reducing redox environment of the cell (see above). Absorption of a photon excites flavin to trigger an intermolecular electron transfer from flavin to an acceptor, such as ATP bound to cryptochromes or an unknown residue of cryptochromes. Given that pyrimidine-dimer is the electron acceptor for photolyase and that the location of the ATP-binding site at cryptochrome is equivalent to the pyrimidine-dimer binding site in photolyases [58], it is conceivable that ATP may act as an electron acceptor for cryptochromes. Electron transfer to ATP may promote a phosphotransfer reaction to "autophosphorylate" the cryptochrome protein, leading to further phosphorylation and conformational change of the photoreceptor. Upon phosphotransfer, the electron is shuttled back from ATP to flavin to complete the photocycle. The phosphorylated cryptochrome adopts an open conformation to interact with signaling proteins, resulting in gene expression changes and physiological responses [17,59•]. One attractive feature of the photolyase-like model is that it explains why the overall structure of the PHR domain of cryptochromes has deviated little from that of photolyases during evolution and what triggers the light-dependent autophosphorylation of

cryptochromes. On the other hand, the photolyase-like hypothesis has its own problems. For example, it does not seem to help solve the discrepancy between the redox potentials of cryptochromes and the cellular environment. Continuous investigation of both the Trp-triad-dependent photoreduction and the photolyase-like hypotheses will shed more light to the photoexcitation mechanism of this arguably most widely distributed photoreceptors in nature.

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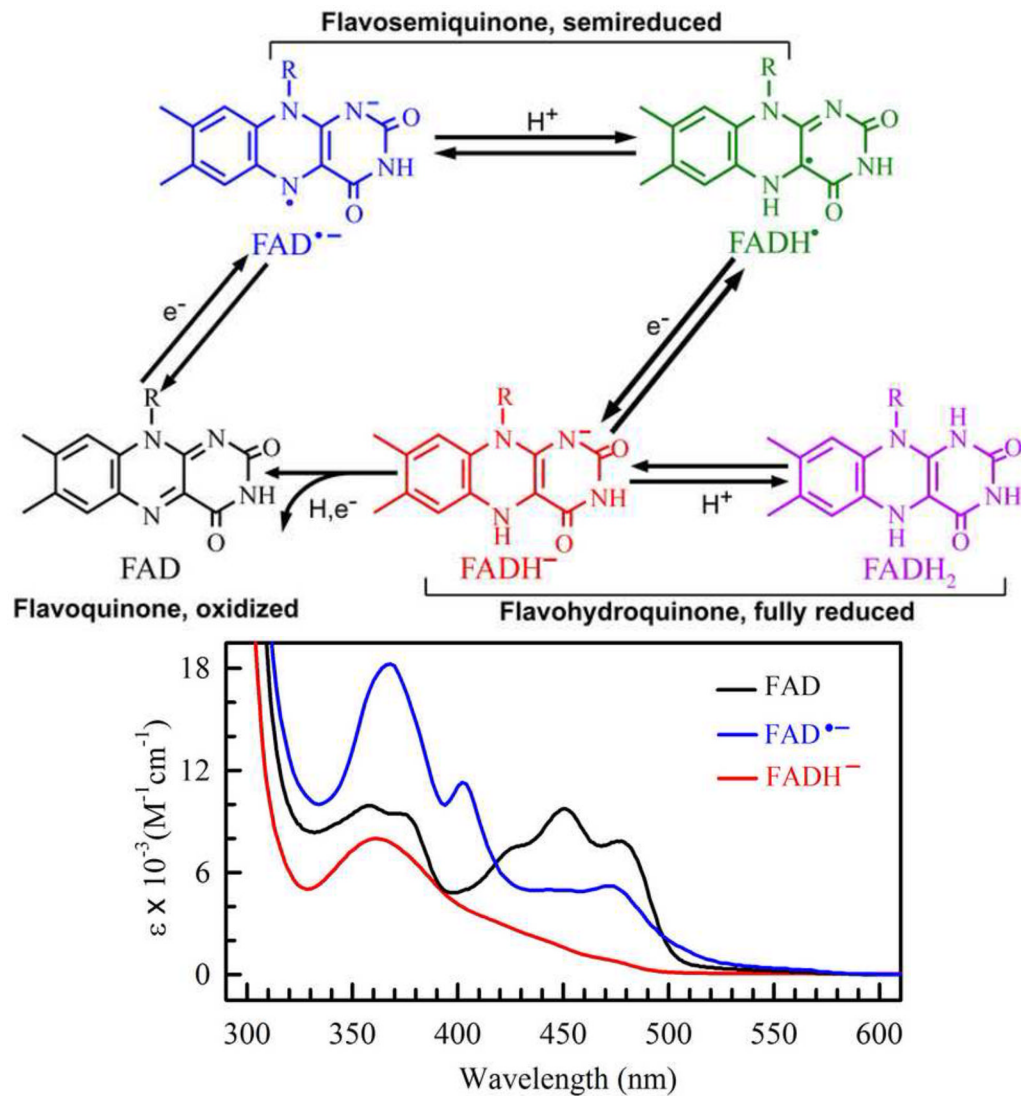
**Fig. 1. Oxidoreduction of flavins**
**A.** Five possible redox forms of flavins are shown. R indicates different side groups in different flavins. The two different forms of semiquinone radicals: anion radical (e.g. $FAD^{\bullet-}$) and neutral radical (e.g. $FADH^{\bullet}$), and two forms of reduced flavins: protonated hydroquinone (e.g. $FADH_2$) and anionic hydroquinone (e.g. $FADH^-$) are shown.
**B.** Absorption spectra and extinction coefficients ($\varepsilon$) of different redox forms of selected cryptochrome/photolyase [4]. Mosquito AgCRY1 (*Anopheles gambiae*) containing oxidized FAD (black line) or anion radical semiquinone ($FAD^{\bullet-}$, blue line), and *E. coli* photolyase containing neutral radical semiquinone ($FADH^{\bullet}$, green line) or fully reduced flavin ($FADH^-$, red line). The absorption spectra of Arabidopsis cryptochromes with the respective redox forms of flavin are similar to those shown here.
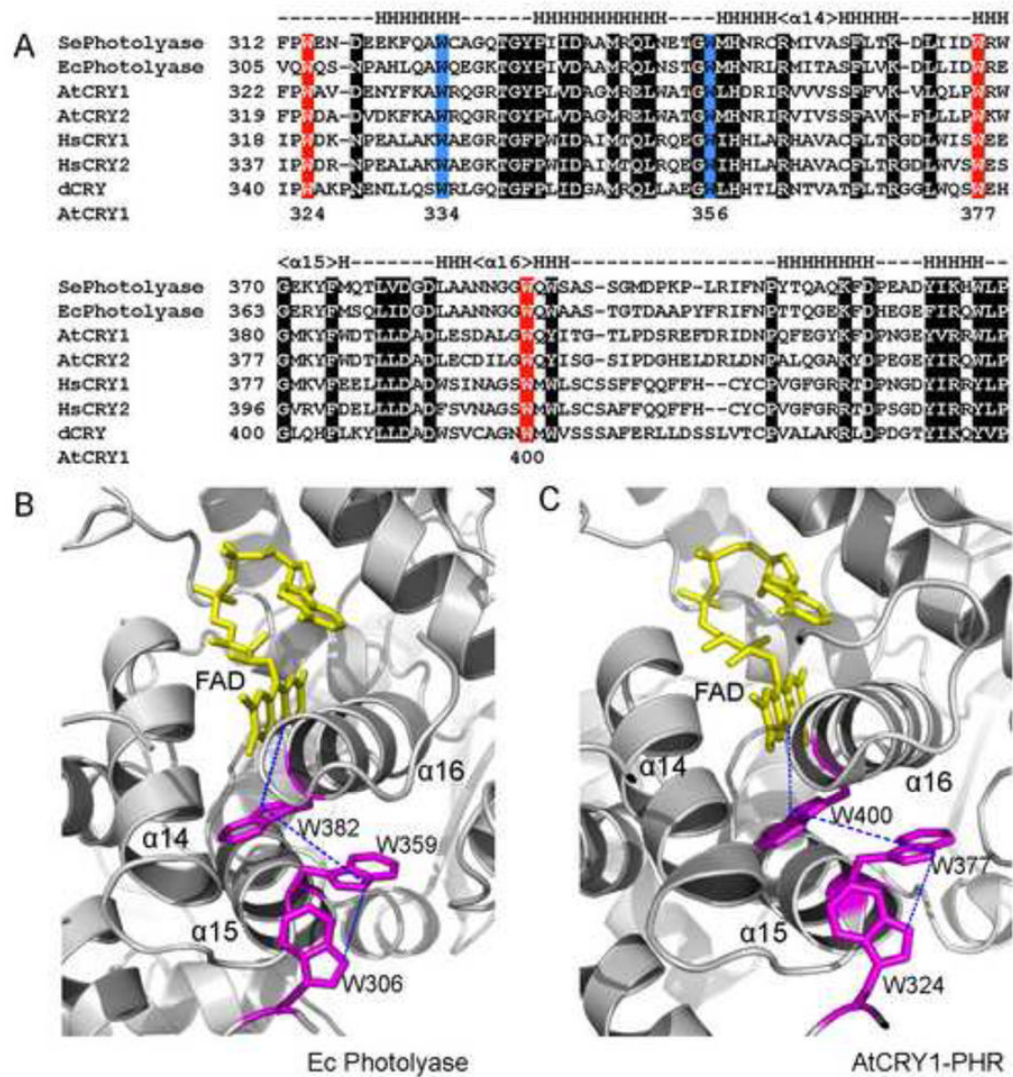
**Fig. 2. The Trp-triad of photolyases and cryptochromes**
**A.** A partial sequence alignment of the region of photolyases and cryptochromes highly conserved and important for FAD-binding. *Synechococcus elongatus* (SePhotolyse) and *Escherichia coli* photolyases (Ecphotolyase), Arapidopsis cryptochrome 1 and 2 (AtCRY1, AtCRY2), human cryptochrome 1 and 2 (HsCRY1, HsCRY2), and Drosophila cryptochrome (dCRY) are compared. Symbols above indicate secondary structures, with dashed-lines representing loops and "H" representing alpha-helices. Numbers beneath indicate positions of the tryptophan residues of Arabidopsis CRY1. The three tryptophan residues of the Trp-triad are highlighted in red, the two tryptophan residues not part of Trp-triad are highlighted in blue.
**B–C.** The structure of FAD–binding pocket, FAD (yellow), the tryptophan residues of the Trp-triad (pink), and the path of electron movement from Trp-triad to FAD by photoreduction (blue dashed-line) of *E. coli* photolyase (B) and the PHR domain of Arabidopsis CRY1 (C) are indicated by blue dashed lines.
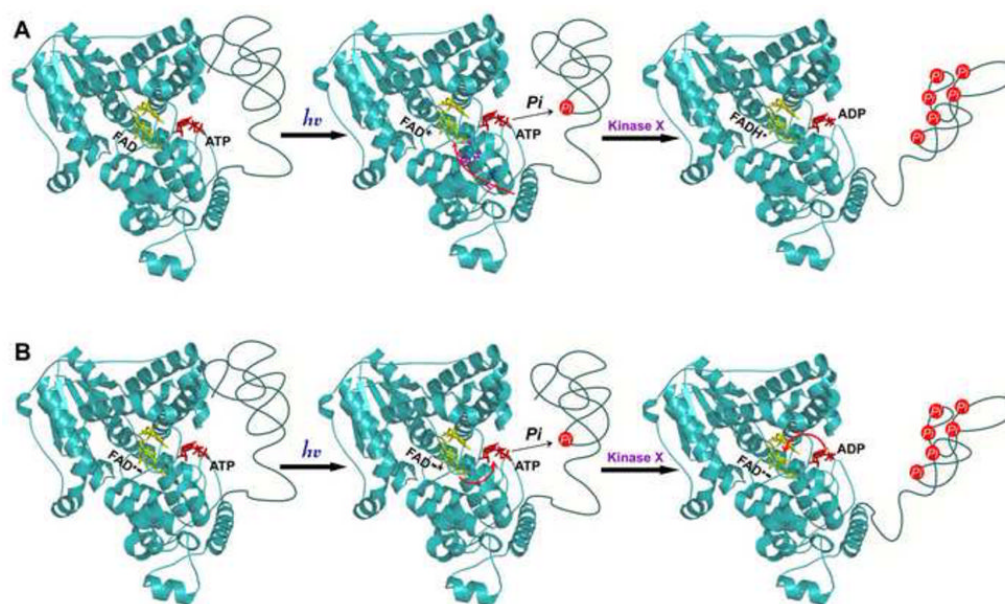
**Fig. 3. Alternative models of cryptochrome photochemistry**

**A.** The Trp-triad-dependent photoreduction model. In this model, the resting state of a cryptochrome contains the oxidized FAD. Upon photon absorption, the excited FAD* gains an electron from tryptophan residues of Trp-triad (see Fig. 2) and a proton from an unknown source to become the active neutral radical semiquinone, FADH$^\bullet$. How the Trp-triad-dependent photoreduction triggers autophosphorylation and conformational change is not clear.

**B.** The photolyase-like cyclic electron shuttle model. In this model, the resting state of a cryptochrome contains the anion radical semiquinone (FAD$^-$). Upon photon absorption, the excited FAD$^-$* transfers an electron to ATP, triggering phosphotransfer and autophosphorylation of the cryptochrome. The electron is subsequently transferred back to flavin to complete the cycle. Autophosphorylation of cryptochromes facilitates further phosphorylation by an unidentified kinase (kinase X), resulting in separation of the CCE domain from the PHR domain and formation of the open conformation. Cryptochromes with the open conformation interacts with signaling partners to trigger signal transduction. The locations of FAD and ATP are indicated by the yellow and red structures, respectively. The putative locations of phosphorous group (red circle) and electron transfer path (red arrows) are indicated.