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Invited Review

Photochemistry and Photobiology of Cryptochrome Blue-light Photopigments: The Search for a Photocycle

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ABSTRACT

Cryptochromes are flavoproteins that exhibit high sequence and structural similarity to the light-dependent DNA-repair enzyme, photolyase. Cryptochromes have lost the ability to repair DNA; instead, they use the energy from near-UV/blue light to regulate a variety of growth and adaptive processes in organisms ranging from bacteria to humans. The photocycle of cryptochrome is not yet known, although it is hypothesized that it may share some similarity to that of photolyase, which utilizes light-driven electron transfer from the catalytic flavin chromophore. In this review, we present genetic evidence for the photoreceptive role of cryptochromes and discuss recent biochemical studies that have furthered our understanding of the cryptochrome photocycle. In particular, the role of the unique C-terminal domain in cryptochrome phototransduction is discussed.

INTRODUCTION

Photolyase/cryptochrome blue-light photoreceptors comprise a class of structurally related flavoproteins found in all kingdoms of life. Photolyases repair UV-induced photoproducts in DNA, and cryptochromes (abbreviated CRY) use the energy from blue light to regulate a variety of growth and adaptive responses in organisms ranging from prokaryotes to humans. This review will focus on work performed toward identifying the mechanism of action, or the photocycle, of cryptochromes. However, the high degree of structural homology that cryptochromes share with photolyase and detailed knowledge of the photolyase photocycle necessitate a comparison of photolyase and cryptochrome structures and a description of photolyase photocycle as a potential model for cryptochrome function.

The three classes of enzymes in this family, cyclobutane pyrimidine dimer photolyase (commonly called photolyase), (6-4) photolyase, and cryptochromes, are 50–70 kDa proteins that contain two noncovalently bound chromophore/cofactors. One is always flavin adenine dinucleotide (FAD), the catalytic chromophore, and the other serves as a photoantenna and is most commonly methenyltetrahydrofolate (MTHF), or 8-hydroxy-5-deazaflavin (8-HDF) in rare organisms that synthesize this chromophore (1). Although cryptochromes and photolyases are moderately related by sequence, retaining 25–40% sequence identity on average, they are highly structurally homologous. Crystal structures of several photolyases (2–4) and the photolyase-homology region of two cryptochromes (5,6) reveal that their Cα backbones are nearly superimposable with root-mean square deviations of less than 2 Å. The structures are characterized by two modular domains: an N-terminal α/β-domain and a C-terminal α-helical domain, connected by a long, interdomain loop (Fig. 1). The catalytic FAD chromophore is bound within the α-helical domain in an unusual U-shaped conformation, with the isoalloxazine ring held in close proximity to the adenine ring, and the second chromophore is bound in a cleft located in between the two domains close to the surface of the protein. A hole of approximately 10 Å in diameter, located in the middle of the α-helical domain, allows access of solvent and oxygen to the FAD molecule and is of the right dimensions and polarity to allow entry of a pyrimidine dimer to within van der Waal's contact distance of the isoalloxazine ring of FAD (2).

A reaction scheme in terms of classic Michaelis-Menton enzymology may be written as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[h\nu]{k_3 = k_p I} E + P \qquad (1)$$

where k_p is the photolytic constant and I is light intensity (reviewed in [1]). A mechanistic description of *Escherichia coli* photolyase is in accord with the structural features of the enzyme and may be summarized as follows (1). Photolyase contains a positively charged DNA binding groove on one face of the protein, with the active site hole located in the middle of the groove. Photolyase binds the DNA around the cyclobutane dimer in a light-independent reaction and flips the dimer out into the

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Abbreviations: CCT, CRY C-termini; COP, constitutive photomorphogenic; CRY, cryptochromes; *cry*^{b}, *cry*^{baby}; CRYΔC, CRY lacking the C-terminal domain; DASH, Drosophila-Arabidopsis-Synechocystis-Human; *EDI*, early day-length insensitive; FAD, flavin adenine dinucleotide; 8-HDF, 8-hydroxy-5-deazaflavin; ipRGCs, intrinsically photosensitive retinal ganglion cells; MTHF, methenyltetrahydrofolate; PHR, photolyase-homology region; PLR, pupillary light response; RBP, retinol binding protein; *rd/rd*, mice with degeneration of the outer retina; SCN, suprachiasmatic nuclei; TIM, Timeless; VcCry1, *Vibrio cholerae* Cry1.

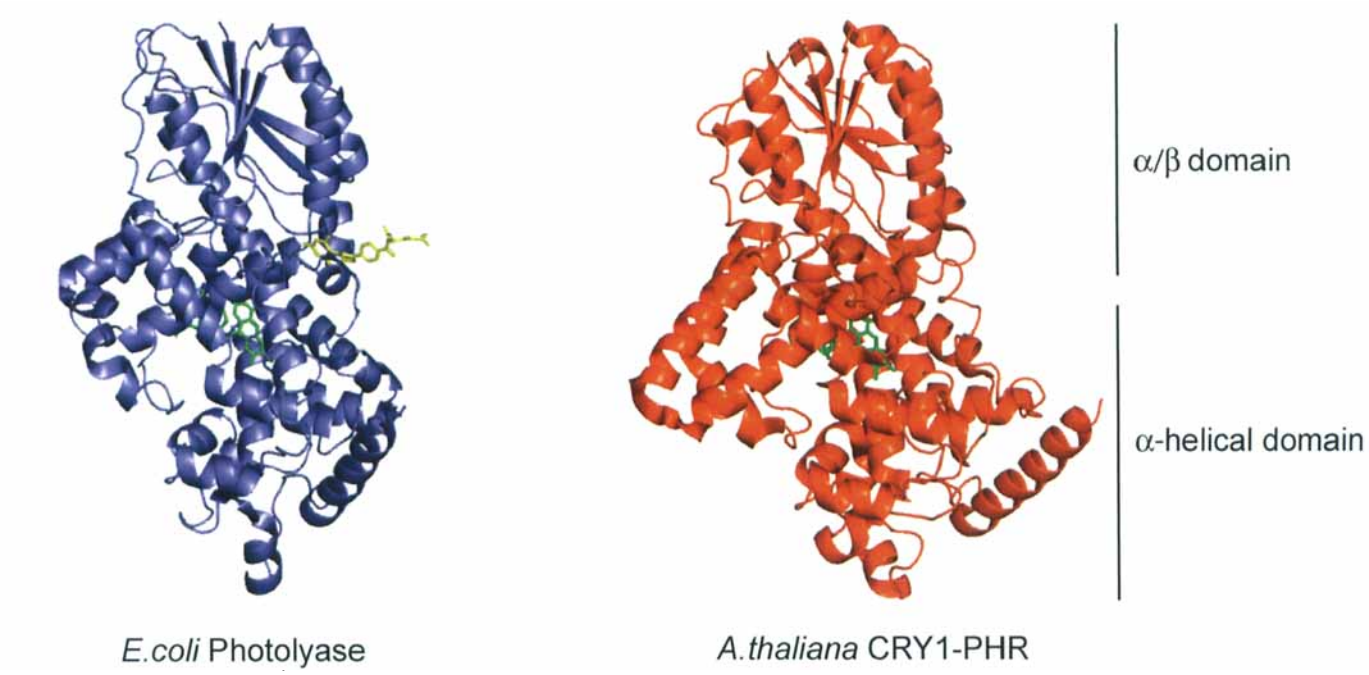

Figure 1. Crystal structures of the photolyase/cryptochrome family. Ribbon diagram representations of *E. coli* photolyase (protein data bank (PDB) code: 1DNP4, reference [2]) and the photolyase-homology region (PHR) of *A. thaliana* CRY1 (PDB: 1U3C, reference [6]). Note that the crystal structure of photolyase contains both FAD (green) and folate (yellow), whereas that of AtCRY1-PHR contains only FAD.

active site cavity to make contact with the flavin (2,7), producing a stable enzyme-substrate complex. Exposure of this complex to light initiates catalysis; the MTHF photoantenna absorbs a photon and transfers the excitation energy to the deprotonated two-electron reduced flavin by Förster dipole-dipole resonance energy transfer. The excited flavin ($^1(FADH^-)^*$) then transfers an electron to the pyrimidine dimer, generating a pyrimidine dimer radical, which undergoes bond rearrangement to yield two canonical pyrimidines. The flavin-neutral radical generated during the reaction is restored to the catalytically active form by back electron transfer from the repaired DNA, and the enzyme and product dissociate.

Photolyase model of cryptochrome function

There are several important points to keep in mind when considering the photolyase reaction mechanism as a potential model for cryptochrome function. First, the catalytically active form of flavin in photolyase is always two-electron reduced and deprotonated flavin adenine dinucleotide ($FADH^-$). This cofactor is readily oxidized *in vitro* to either the one-electron reduced flavin blue–neutral radical ($FADH^\bullet$) or the fully oxidized form (FAD_{ox}), both of which are catalytically inert (8). Photoreduction, resulting from exposure of *E. coli* photolyase containing $FADH^\bullet$ to light in the presence of a reducing agent, can convert the flavin to the active $FADH^-$ form, increasing its quantum yield for repair (9,10). However, the photochemical reactions responsible for this conversion are likely of only marginal physiological significance, because point mutations blocking the photoinduced electron transfer do not affect enzymatic activity *in vivo* (11,12). Further complicating study of the photolyase/cryptochrome family, purification of recombinant proteins from this family rarely yields stoichiometric amounts of both chromophores (13–17). Therefore, the lack of chromophore or the presence of a particular oxidation state of flavin in heterologously expressed, recombinant proteins of this family is not sufficient to make specific models regarding the *in vivo* status of the photoreceptor. Finally, and perhaps most importantly, a photon must be absorbed by photolyase while the protein is in the enzyme-substrate complex for catalysis to occur; a photon absorbed by the free enzyme generates an excited state that decays within 1–2 ns, with no lasting effect on the binding to or activity of photolyase on its substrates (1). An understanding of the cryptochrome photocycle may therefore depend on the identification of a dark-bound substrate.

Given the high degree of structural homology with photolyase, it is unclear how cryptochromes have lost the ability to repair DNA. The positively charged DNA binding groove is conserved in *Synechocystis* Cry (5) but not in *Arabidopsis* CRY1 (6), and DNA binding activity has been found in some cryptochromes *in vitro* (5,17), but the physiological relevance of this interaction has yet to be determined. What structural features of cryptochromes might explain their unique function then? Eukaryotic cryptochromes are structurally divergent from photolyases in one significant aspect; nearly all possess C-terminal domains (but not CRY1 of *S. alba*) beyond the photolyase-homology region (PHR) ranging from 30 to 350 amino acids in length. However, the sequences of these C-terminal domains are not conserved from plants to animals and, in general, prokaryotic cryptochromes lack these C-terminal extensions. Phylogenetic analysis of the photolyase/cryptochrome family from more than 100 taxa results in classification into eight subfamilies, comprised of Class I and Class II cyclobutane pyrimidine photolyases, (6-4) photolyases, plant cryptochromes, insect cryptochromes, vertebrate cryptochromes, *Drosophila-Arabidopsis-Synechocytstis*-Human (DASH) cryptochromes (found in prokaryotes and eukaryotes), and a novel subfamily of bacterial cryptochromes or photolyases (or both), which remain to be biochemically characterized (Fig. 2). Based on the clear divergence of primary sequence throughout the proteins, plant and animal cryptochromes likely evolved independently from a common

progenitor (18). However, the existence of C-terminal domains in divergent cryptochrome lineages implies structural convergence in the adaptation of the photolyase-like progenitor into a blue-light photoreceptor in higher organisms. The importance of the C-terminal domains in cryptochrome function is underscored by recent data from both plants and animals implicating them in regulation of phototransduction and will be covered in more detail in subsequent sections.

Given the difficulties in working with recombinant proteins of this family due to cofactor oxidation or loss during purification (or both), most of our understanding of cryptochrome function is based on genetic data. This *in vivo* evidence for the photoreceptive function of cryptochromes will be addressed first, followed by recapitulation of recent advances in our understanding of cryptochrome phototransduction from genetic and cell-based studies, as well as biochemical experiments using recombinant proteins.

CRYPTOCHROME GENETICS AND *IN VIVO* STUDIES

Plant cryptochromes

Cryptochromes were identified first in *Arabidopsis thaliana* in 1993 through the isolation of the *HY4* gene (19). *Arabidopsis* seedlings grown under light display a variety of photomorphogenic responses with respect to dark-grown seedlings, such as inhibition of stem elongation and stimulation of leaf expansion. In particular, seedlings grown in light have significantly shorter stems (hypocotyl) than seedlings grown in darkness; this response is mediated by blue (400–490 nm), red (600–700 nm), and far-red (700–750 nm) light. A screen for hypocotyl mutants, carried out by Koornneef and coworkers in 1980, identified several mutants (*hy*) that lost the ability to respond to one or more of these monochromatic light conditions (20). The *hy4* mutant selectively lost responsiveness to blue light, indicating that a blue-light photoreceptor or a component of the blue-light signaling pathway had been disrupted. Molecular cloning of the *HY4* gene revealed significant sequence homology to *E. coli* photolyase that was sequenced by Sancar and colleagues (19,21). However, even though the recombinant protein copurified with flavin, it lacked detectable DNA repair activity (22,23). This led to its definition as the first member in a novel class of blue-light photoreceptors called cryptochromes, which retain high sequence homology to photolyase, yet do not repair DNA.

While the loss of *Cry1* (*hy4*) has a marked effect on photomorphogenic responses under high fluence rates of blue light, the loss of a second cryptochrome gene, cloned in 1996 and named CRY2, has an effect detectable only under low fluence rates (1 μmol $m^{-2}s^{-1}$ or less) (24,25). Because the *Cry1/Cry2* double mutant has an even more pronounced phenotype than the loss of either cryptochrome, it was concluded that cryptochromes act redundantly to regulate photomorphogenic responses, with CRY1 primarily operating under bright light and CRY2 operating under dim light conditions. The differential responsiveness of CRY1 and CRY2 to lighting conditions has been explained by the rapid light-dependent degradation of CRY2 protein under moderate to high fluence rates of blue light (25). The effect of fluence rate on CRY2 protein levels is rather striking; no change in CRY2 protein levels is observed after treatment with 1 μmol $m^{-2}s^{-1}$ of blue light for up to 1 h; however, treatment with 5 μmol $m^{-2}s^{-1}$ blue light for only 15 min results in degradation of 90% of protein present in dark-grown seedlings (25,26).

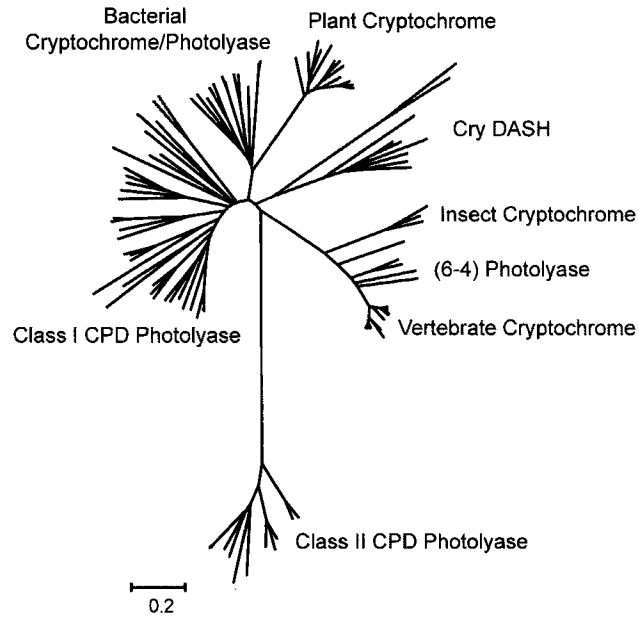

Figure 2. Phylogenetic analysis of the photolyase/cryptochrome family. An unrooted neighbor-joining (NJ) tree was constructed by the MEGA 2.1 program using 106 photolyase and cryptochrome sequences. Eight major subclasses are identified, each with bootstrap values >70% at the distinguishing nodes. The scale bar indicates amino acid substitutions per site.

Genetic studies in *Arabidopsis* have also shown that CRY2, along with the phytochromes, regulates photoperiodic flowering (27,28). Because protein levels of CRY2 are regulated by light, CRY2 abundance in plants grown in photoperiod displays a diurnal rhythm, with low protein levels during the day and an increase in CRY2 protein during the evening. Moreover, the diurnal rhythm of CRY2 protein abundance is dependent on the photoperiod; rhythmic changes in CRY2 abundance are more pronounced in short days than in long days. Importantly, a dominant early day-length–insensitive (*EDI*) locus of the Cvi ecotype was mapped to the *Cry2* gene, caused by a single amino acid substitution (V367M) (28). The photoperiodic-insensitive early flowering of the Cvi ecotype may be due to the reduced amplitude in diurnal cycling of the mutant protein, suggesting that the diurnal rhythm of CRY2 abundance is responsible for the regulation of photoperiodic flowering.

Arabidopsis cryptochromes likely exert their largest effect on photoresponses through regulation of gene expression. Expression of approximately one-third of all *Arabidopsis* genes was found to change more than 2-fold after exposure to white light; 73% of genes differentially expressed under white light were also affected under monochromatic blue light (29). Most of the genes that were affected by blue light in wild-type plants were not differentially expressed in the *Cry1/Cry2* mutant under the same lighting conditions, indicating that cryptochromes are the major blue-light photoreceptors mediating this response, which occurs on a time scale of minutes to days (29,30). However, light-dependent regulation of gene expression in *Arabidopsis* is controlled by a broad spectrum of wavelengths; many of the genes regulated by cryptochromes are also regulated to some extent by the phytochrome family of red-light photopigments, which also absorb blue light (31).

Regulation of gene expression by cryptochrome may be mediated at least partially by its direct interaction with the E3

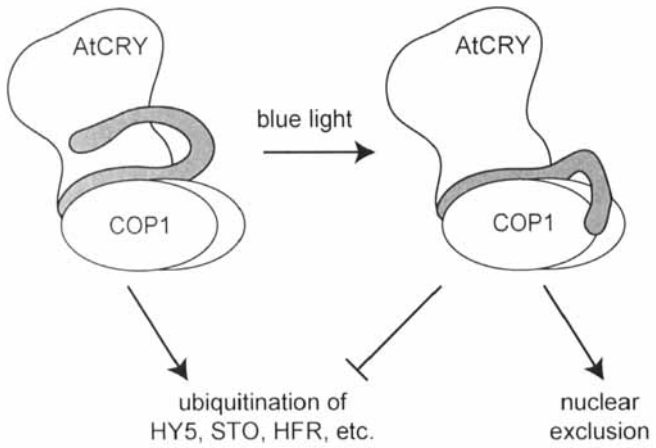

Figure 3. Model of AtCRY phototransduction. AtCRY1 and AtCRY2 interact with their effector COP1 constitutively via their C-terminal domains (shown in gray). Upon irradiation by light, the C-terminal domain of AtCRY undergoes a light-dependent conformational change that results in the rapid inhibition of COP1 E3 ubiquitin ligase activity, followed by exclusion of the AtCRY-COP1 complex from the nucleus.

ubiquitin ligase COP1 (*i.e.* constitutive photomorphogenic 1). COP1 is a zinc-finger and WD40-repeat protein that is responsible for the degradation of bZIP transcription factors such as HY5, STO, STH, and HFR in the dark (32–34). The ubiquitin ligase activity of COP1 is rapidly inhibited by light (34), followed by translocation out of the nucleus (35–37), allowing accumulation of transcription factors and initiation of the photomorphogenic transcriptional program. Both CRY1 and CRY2 interact directly with COP1 in a light-independent manner through their C-terminal domains (38,39). Because overexpression of the CRY C-termini (CCT) is sufficient to produce a constitutive "light-on" phenotype, the COP1-inhibitory region of the C-terminal domain must be repressed by the photolyase-homology region of cryptochrome in the dark (Fig. 3) (38,39). Light-dependent inhibition of COP1 by cryptochromes has been proposed to act through a two-step mechanism: COP1 is 1) rapidly down-regulated as a result of a light-dependent conformational change in C-termini of cryptochromes (34,38), and 2) it is inhibited on a longer time scale because of the light-dependent translocation of COP1 from the nucleus to the cytoplasm by CRY1 (35–37).

It has now been demonstrated that CRY1 and CRY2, along with phytochromes, can mediate photomorphogenic responses such as inhibition of stem elongation, stimulation of leaf expansion, induction of anthocyanin synthesis, control of photoperiodic flowering, entrainment of the circadian clock, and regulation of gene expression required for photomorphogenic responses (reviewed in [40,41]). A third of the cryptochrome gene has recently been identified in *Arabidopsis*, which displays more sequence similarity to prokaryotic cryptochromes than plant cryptochromes, and it lacks a C-terminal extension (5,42). Instead, CRY3 has an N-terminal extension containing chloroplast and mitochondrial import signals and has been found to localize to these organelles in plants (42). However, the function of this cryptochrome is not yet understood.

Animal cryptochromes and the circadian clock

Based on exhaustive biochemical data, it was concluded that humans and other placental mammals do not possess photolyase activity (43). Therefore, the report of a photolyase ortholog as an expressed sequence tag in the human genome database was unexpected (44). Subsequent examination of this and a second photolyase ortholog discovered by our group established that both copurified with flavin but lacked photolyase activity, and were therefore classified as cryptochromes and proposed to act as novel blue-light photoreceptors for regulating the circadian clock (45). Shortly thereafter, cryptochrome homologs were identified in many other animals, including insects, amphibians, fish, birds and other mammals (46). Currently, the *Drosophila* cryptochrome is the best characterized animal cryptochrome and therefore evidence for its photoreceptive function will be presented first.

Drosophila *cryptochrome*. Shortly after the implication of mammalian cryptochromes in circadian regulation (47,48), *Drosophila* cryptochrome was discovered in 1998 through a genetic screen for circadian rhythm mutants (49). Flies, like nearly all other metazoans, have an internal timekeeping mechanism that coordinates physiological and biochemical processes such as development and locomotor activity with the 24-h solar day. These intrinsic circadian (circa = about, dies = a day) rhythms are created by a molecular clock that functions in complete darkness to generate rhythms with a periodicity of approximately 24 h. Because the clock does not keep exact 24-h time, it must be synchronized, or entrained, daily with its surroundings; this occurs predominantly through cues from external light-dark cycles. The vitamin A-based visual opsins clearly are the major contributors to this process because genetically eyeless (50), opsin-depleted (51) or blind *Drosophila* (52) have lower sensitivities in circadian entrainment than wild-type flies. However, several studies involving dietary depletion of vitamin A suggested a role for a nonopsin photopigment in circadian photoreception (51,53). Furthermore, the maximal sensitivity for circadian entrainment in *Drosophila* occurs in blue light (51,54,55), well below wavelengths at which *Drosophila*'s rhodopsin maximally absorbs (56).

The loss of function cryptochrome mutant, named cry^{baby} (cry^{b}), is caused by a destabilizing Asp→Asn substitution at amino acid 410 in the flavin-binding domain, with mutant protein expressed at significantly reduced levels relative to wild-type protein. The molecular clock of cry^{b} mutants appears to function normally but is not sensitive to short, phase-shifting light pulses given in the dark, suggesting loss of photoreceptive input to the clock (49). In addition, cry^{b} mutant flies take more time to adjust to a shifted light/dark cycle and maintain normal, rhythmic activity under constant light, a condition that causes arrhythmicity in wild-type flies (49,57). Finally, directed expression of wild-type CRY in the pacemaker neurons of cry^{b} flies rescues the majority of the light sensitivity of behavioral rhythms, suggesting that CRY is a cell-autonomous photoreceptor sufficient for most aspects of circadian light sensitivity (58). Indeed, an important property of the *Drosophila* circadian system is that virtually every single cell in this organism, including the brain, Malphigian tubules, abdomen and wing cells are directly light-sensitive and peripheral clocks in these tissues can be reset directly by light exposure (59,60). However, cry^{b} mutant flies retain partial responses to light/dark cycles, suggesting functional redundancy between cryptochrome and opsins in circadian entrainment; generation of mutant flies eliminating all known opsin-containing photoreceptive organs ($glass^{60j}$), and cryptochrome (cry^{b}) results in flies that can no longer entrain their circadian rhythms to external light-dark cycles (61).

Cryptochrome entrains the molecular clock in *Drosophila* through its light-dependent interaction with the integral clock protein Timeless (TIM). In yeast two-hybrid studies, the interaction of CRY with TIM was found to occur only in the light (62,63).

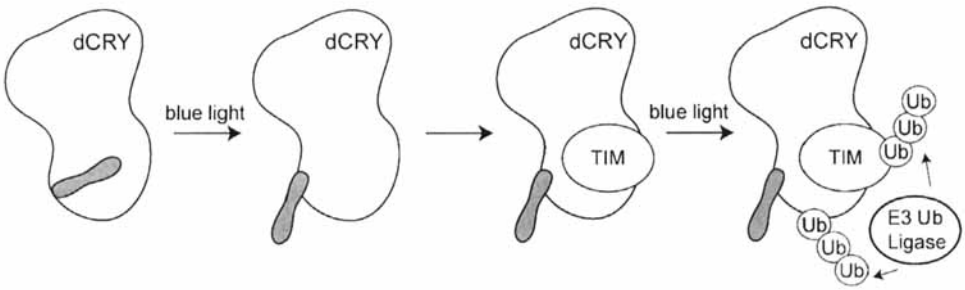

Figure 4. Model of dCRY phototransduction. Irradiation of dCRY with light releases the short C-terminal domain from the photolyase-homology region, allowing the circadian clock protein TIM to bind. In a distinct light-dependent reaction, dCRY primes TIM (and itself) for ubiquitin-mediated degradation by an unknown mechanism.

Because yeast does not have photosensory capability, heterologously expressed CRY must form a photopigment in yeast, capable of undergoing the photochemical reaction required to mediate the TIM interaction. Deletion of the C-terminal 20 amino acids of CRY by truncation (63,64) or random mutagenesis (65) leads to CRY-TIM interaction in a light-independent manner. This has been interpreted to mean that the C-terminal domain specifically occludes binding of TIM to CRY in the dark. Synchronization of the molecular clock with the light cycle most likely occurs as cryptochrome induces the rapid, ubiquitin-mediated degradation of TIM (66–68), which destabilizes the transcriptional repressor period (PER) and allows transcription by the CYCLE/CLOCK heterodimer to occur (69). Furthermore, the light-dependent degradation of TIM is absent in larval lateral neurons and adult Malphigian tubules of cry^b flies, indicating the absolute requirement of cryptochrome for TIM degradation (60). Cryptochrome may also contribute to entrainment by regulating the subcellular localization of a presumably inactive CRY/PER/TIM complex; irradiation of *Drosophila* S2 cells with light has been reported to result in a significant increase in nuclear CRY in the presence of PER and TIM relative to dark-grown cells (62).

Although *cry* messenger RNA (mRNA) is rhythmically expressed with a peak in the early morning, CRY protein cycles only under light/dark conditions because of its rapid, light-dependent degradation, and accumulates under constant darkness (58,68). The light-dependent degradation of CRY is regulated by the 20–amino acid C-terminal domain; loss of the C-terminus (CRYΔC) results in constitutive degradation of the protein. CRYΔC is undetectable by Western blotting (65), and overexpression by the upstream activation sequence (UAS) promoter in transgenic flies (64) results in barely detectable protein levels that appear to be degraded by the proteosome regardless of lighting conditions. The absence of the C-terminal domain results in CRY protein that interacts constitutively with TIM (63). Despite this, CRYΔC transgenic flies exhibit oscillations in PER and TIM proteins and respond to pulses of light that shift the circadian phase of behavior, although with reduced amplitudes relative to wild-type flies (64,65). These data indicate that the light dependence of TIM degradation by cryptochromes is mediated by two separate events: first, light is required to release the repressive C-terminal domain in full-length CRY to allow for TIM binding, which is necessary but not sufficient for phototransduction; second, TIM is modified for proteosomal targeting by a cryptochrome-dependent signal that requires light, which can occur in the absence of the C-terminal domain because the photolyase-homology region is sufficient for the photoreceptive function of cryptochrome (Fig. 4) (65).

This mechanism of action shares both similarities with and clear differences from the proposed mechanism of action of plant cryptochromes (see below). In both plant and animal cryptochromes, light irradiation appears to result in derepression of a signal transduction domain, presumably through conformational rearrangement. Light-dependent initiation of signaling by all cryptochromes absolutely requires the photolyase-homology region; however, signaling by plant cryptochromes appears to be mediated entirely by the C-terminal domain, because overexpression of this domain is sufficient to generate a constitutive photomorphogenic response (38,39). In contrast, the photolyase homology region of *Drosophila* cryptochrome appears to require light for two distinct steps in its signal transduction: 1) light-dependent release of the C-terminal domain with concomitant binding of TIM, and 2) light-dependent proteosomal targeting of the TIM protein (64,65). In summary, cryptochrome functions in the *Drosophila* circadian clock by acting as a cell-autonomous photopigment, conveying light information directly to core clock components through protein–protein interactions and light-regulated protein degradation.

Mammalian cryptochromes. The entrainment of circadian rhythms in mammals is fundamentally different from that in *Drosophila*, because all photoreception in mammals occurs through the retina (46) and is communicated to the master circadian clock in the suprachiasmatic nuclei (SCN) of the hypothalamus via the retinohypothalamic tract (70). As in *Drosophila*, early studies of mammalian circadian photoreception identified the apparent role of a nonvisual photopigment in the entrainment of mice, because mice with degeneration of the outer retina (*rd/rd*), thus lacking the classical visual photoreceptors, still entrain their rhythms normally (71). After the discovery and characterization of mammalian cryptochromes in 1996, it was proposed that they might be the elusive nonvisual photopigment (45). Indeed, histochemical studies have revealed that both cryptochromes are highly expressed in the ganglion cells of the inner retina of mice (47,72) and humans (73) (Fig. 5).

The potential role of mammalian cryptochromes in circadian photoreception has been studied primarily through the use of mouse genetics. Although cryptochromes were introduced into the field of circadian biology as putative blue-light photopigments, current evidence puts them, interestingly, squarely in the core of the circadian clock machinery (see [74]). The free-running circadian periods of both *Cry1–/–* and *Cry2–/–* mice are shorter and longer, respectively, than wild-type mice, revealing a role for cryptochromes in the molecular clock independent of any presumptive photoreceptive function (48,75). Furthermore, *Cry1–/–Cry2–/–* mice are

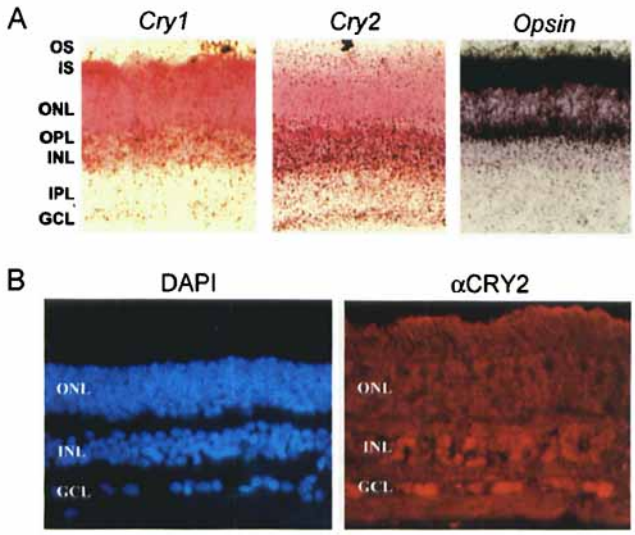

Figure 5. Expression of cryptochromes in the mammalian retina. (A) Expression of *Cry1, Cry2,* and *opsin* mRNA in the mouse retina by *in situ* hybridization (reference [43]). Note predominant expression of *Cry2* in the inner nuclear layer and ganglion cell layer, as opposed to rhodopsin, which is expressed exclusively in the outer retina. (B) Expression of CRY2 protein in the human retina by immunofluorescence (reference [69]). Left panel, 4′,6′,-diamidino-2-phenylidole (DAPI) staining for nuclei (blue); right panel, immunostaining for CRY2 (red). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

arrhythmic under constant darkness, underscoring the fact that cryptochromes are required for the molecular clock to function in mammals (75,76). These animals do respond behaviorally to light/dark cycles; however, this is due to "masking," a direct repressive effect of light on locomotor behavior independent of the clock (77). Cryptochromes were later characterized as essential transcriptional repressors in the feedback loop that generates the molecular clock (78,79). Because mutations in cryptochromes affect locomotor activity independently of light, behavior does not provide an accurate readout of photoresponse in these mice. The most quantitative demonstration of the effect of cryptochromes on photoreceptive input to the clock is measured through light-dependent gene induction in the SCN. Light given in the middle of the night induces rapid and robust induction of *Period* clock gene mRNA (80), as well as the immediate-early gene *c-fos* (81,82). Because the loss of cryptochrome affects steady-state levels of *Per* mRNA due to its role in the clock, the contribution of cryptochrome to circadian phototransduction is most accurately measured through induction of *c-fos* in the SCN.

To determine whether cryptochromes mediate circadian photoreception in the absence of visual photoreceptors, photoinduction of *c-fos* was examined in the *rd/rd* background. Although *rd/rd* mice exhibit normal levels of *c-fos* induction by light (83–85), light induction of *c-fos* in *rd/rd Cry1–/–Cry2–/–* mice is reduced approximately 3000-fold under limiting light (85,86). Even in the presence of all other photoreceptors, the loss of cryptochromes significantly reduces circadian photosensitivity. Mice lacking *Cry2*, the cryptochrome expressed most predominantly in the mouse retina, have a 2-fold decrease in photoinduction of *c-fos* (48), and mice lacking both cryptochromes exhibit a 10- to 20-fold reduction in sensitivity (85). Reduced responses in cryptochrome-less mice are not likely due to secondary or nonspecific changes in expression of signaling intermediates on the immediate-early gene induction pathway, because the magnitude and time course of *c-fos* induction by serum or forskolin in dermal fibroblast cell lines derived from *Cry1–/–Cry2–/–* and wild-type mice are similar (86). In addition, the pupillary light response of *rd/rd Cry1–/–Cry2–/–* mice is reduced 20-fold with respect to *rd/rd* mice, implicating cryptochromes in this nonvisual photoresponse as well (87). However, the presence of residual photoresponses in *rd/rd Cry1–/–Cry2–/–* mice, both in gene induction and pupillary response, led to the conclusion that yet another nonvisual photoreceptor must exist in the inner retina that remains intact in *rd/rd* mice (85,87). Indeed, in an independent approach, Provencio and colleagues discovered a novel opsin, called melanopsin, which is expressed exclusively in the retinal ganglion cells of the retina, are implicated in nonvisual phototransduction, such as circadian photoreception and the pupillary light response (88). Following up on this pioneering discovery, later work showed that these ganglion cells were intrinsically photosensitive and were named intrinsically photosensitive retinal ganglion cells (ipRGCs) (89,90). Although genetic deletion of this invertebrate-like opsin alone has only minor effects on nonvisual photoresponses (91,92), the elimination of visual photoreceptors and melanopsin results in mice that lose all nonvisual photoresponses (93,94).

These findings raised serious doubts about a photoreceptive role for cryptochrome in mammalian nonvisual photoresponses and forced a re-evaluation of the experiments indicative of such a role in mammals. Another genetic approach was taken to address this issue using mice lacking retinol binding protein (RBP). Elimination of dietary vitamin A in the *RBP–/–* background depletes ocular retinaldehyde, the required cofactor of all opsin photopigments, to undetectable levels by 10 months of age (95). Surprisingly, *RBP–/–* mice raised on a vitamin A–free diet are blind (95) yet retain essentially normal retinohypothalamic photoreception/phototransduction as measured by gene induction in the SCN (96,97). Importantly, *RBP–/– Cry1–/–Cry2–/–* mice raised on the vitamin A–free diet fail to induce *c-fos* in the SCN by light, indicating that inactivation of opsins in combination with the loss of cryptochromes is sufficient to eliminate phototransduction to the SCN (97). It is unclear why genetic ablation of opsin function (*rd/rdOpn4–/–* (93) or *Gnat1–/–Cnga–/–Opn4–/–* [94]) and inactivation of opsins via cofactor depletion (*RBP–/–* mice raised on a vitamin A–free diet [96]), which leaves the opsin apoproteins intact, produce different phenotypes in circadian photoreception. Conceivably, opsin apoproteins, which are clearly required to generate an action potential through the retinohypothalamic tract, can act as G-protein–coupled receptors to transmit a signal generated by cryptochrome.

In summary, the available data on the role of mammalian cryptochromes as circadian photopigments indicate that they are neither necessary nor sufficient for generating an action potential through the retinohypothalamic tract to the SCN. However, in their absence, circadian phototransduction to the SCN is significantly compromised, indicating that cryptochromes may generate a signal that is converted to an action potential by melanopsin, or they may amplify or prolong the signal generated by opsins. Given that the phototransduction cascade of melanopsin is highly unusual in mammals, resembling that of invertebrate opsins (98–100), we can only postulate how cryptochromes might regulate the phototransduction pathway (Fig. 6). Using genetic approaches, it is not possible to determine whether they act as photopigments or light-independent signal transducers in this pathway. It is clear

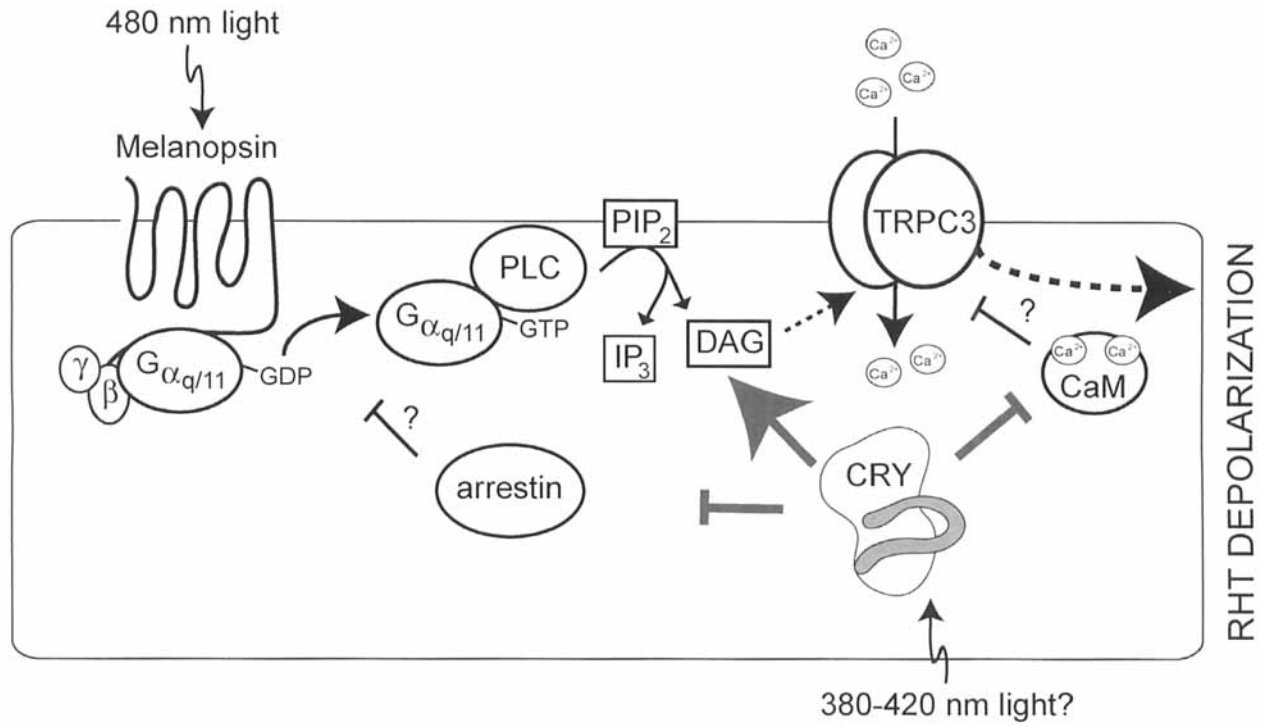

Figure 6. Potential cryptochrome signaling pathways in intrinsically photosensitive retinal ganglion cells. The initial steps in melanopsin phototransduction have been elucidated and resemble that of invertebrate opsins (references 93–95). Light (480 nm) activates melanopsin, which promotes guanosine triphosphate binding and activation of $G\alpha_{q/11}$ and subsequent activation of phospholipase C. Based on invertebrate phototransduction pathways in *Drosophila*, diacylglycerol (DAG) is a key signaling intermediate in activation of transient receptor potential (TRP) channels. Cryptochrome could amplify or prolong melanopsin signaling by inhibiting proteins that potentially down-regulate melanopsin phototransduction, like calmodulin or arrestin, or by stabilizing signaling intermediates such as DAG. It is not known whether the involvement of cryptochrome in this pathway is light-dependent.

that expression of mammalian cryptochromes is not necessarily sufficient to confer photoresponsiveness in a tissue, because the SCN expresses high levels of cryptochromes yet does not respond directly to light (101). Whether cryptochromes act as photopigments or light-independent signal transducers in a given tissue is likely dependent on the cellular context in which they are expressed. This is a common property of receptors, which can manifest their effector function only when the cell expresses the appropriate adapters/transducers as well. For instance, the detection of opsin photoreceptive activity in heterologous systems (such as *Xenopus* oocytes or COS7 cells) requires coexpression of specific G-protein transducers or ion channels (or both) to generate a signal (98).

Other vertebrate cryptochromes. Unlike mammalian organisms, which rely strictly on communication between ocular photoreceptors and the brain to detect light, a number of other vertebrate animals employ extraocular or cell-autonomous photoreception, thereby providing more tractable systems in which to investigate cryptochrome photoreception at a molecular level. In particular, it has been found that peripheral organs in zebrafish, such as the heart and kidney, are directly photosensitive and have light-entrainable circadian clocks (102). Embryonic cell lines derived from zebrafish possess circadian clocks that are responsive to light/dark cycles (103,104). Action spectrum analysis of the Z3 cell line revealed that light in the near UV/blue range (λ_{max} ~380 nm) is most effective for light-dependent clock gene induction; these data, and the apparent lack of detectable retinal in the cell line, are consistent with photoreception by cryptochromes (105). While the cell line expresses all six of the zebrafish cryptochromes, a more detailed analysis will be required to make the absolute determination of the identity of the blue light photoreceptor mediating this response (105). The use of RNA interference (RNAi) and small molecule inhibitors of signaling pathways in cell culture provides a promising system for the investigation of cryptochrome photoreception in zebrafish.

The investigation of cryptochrome function in vertebrates has also been advanced significantly in studies of cell-autonomous photoreception in isolated tissue samples. In contrast to the mammalian iris and adult chicken iris, the embryonic chick iris is directly photosensitive to light, and the pupil constricts as it does in the whole animal when the iris is exposed to light *ex vivo*. While melanopsin and both cryptochromes 1 and 2 are expressed in the iris, destruction of the opsin cofactor retinaldehyde by bright light has no effect on the pupillary light response (PLR), indicating that cryptochromes may mediate this response (106). Partial down-regulation of melanopsin by treating the isolated iris with short interfering RNA (siRNA) has no effect on PLR photosensitivity, but down-regulation of either cryptochrome by 50% decreases PLR photosensitivity by a comparable amount (106). Action spectrum analysis of the PLR in the isolated iris does not fit the absorption spectrum of any known opsin; instead, it most closely resembles the absorption spectrum of recombinant cryptochromes, with a peak at 420 nm and higher sensitivity at shorter wavelengths (17,45,106). These findings strongly suggest that cryptochrome is the photopigment responsible for the PLR in the embryonic chick iris, providing the most compelling evidence to date that cryptochromes function as photosensory pigments in vertebrate animals.

CRYPTOCHROME PHOTOCHEMISTRY AND *IN VITRO* STUDIES

There are currently only limited studies on the biochemical properties of cryptochromes due to difficulties in the process of purifying cryptochromes from native sources in preparative quantities containing chromophore in the quantities required for biochemical analysis. The following section summarizes the current body of work on the biochemical properties of cryptochromes, from bacteria to humans.

Interchromophore energy transfer

In contrast to most other cryptochromes, purification of recombinant *Vibrio cholerae* Cry1 (VcCry1) from *E. coli* yields large quantities of protein containing near stoichiometric amounts of both the $FADH^-$ and MTHF chromophores, making it highly suitable for photochemical studies (107). The dynamics of resonance energy transfer from the photoantenna MTHF to the $FADH^-$ cofactor have recently been studied in VcCry1 with femtosecond resolution (108). The resonance energy transfer process from MTHF* to $FADH^-$ occurs with a lifetime of 60 ps, approximately five times faster than in *E. coli* photolyase. Furthermore, the fluorescence lifetime of MTHF* is 845 ps, more than twice as long as that in *E. coli* photolyase (108,109). While there are mechanistic similarities of ultrafast resonance energy transfer between *E. coli* photolyase and *V. cholerae* cryptochrome, interchromophore energy transfer appears to be more efficient in cryptochrome than in photolyase, suggesting a shorter interchromophore distance or a more favorable orientation of the two chromophores. Because the two crystal structures of cryptochromes solved to date lack the MTHF chromophore, further studies will be required to understand the structural basis of the different photophysical properties of VcCry1 and *E. coli* photolyase.

Photoinduced intraprotein electron transfer

It has been found that in many flavoproteins, such as glucose oxidase or cholesterol oxidase, flavin fluorescence is partly quenched by electron transfer from neighboring Trp and Tyr residues to the flavin-excited singlet state, which has a high oxidizing potential (110). Thus, flavoproteins are convenient systems for investigation of the kinetics and mechanisms of intraprotein electron transfer, although these reactions have no relevance to the biological functions of the particular proteins. As previously mentioned, the catalytically active form of flavin in photolyase is always two-electron reduced and deprotonated flavin adenine dinucleotide ($FADH^-$). Purification of photolyase yields protein containing either the semireduced flavin radical ($FADH^\bullet$) or the fully oxidized flavin (FAD_{ox}), which must be reduced *in vitro* by photoexcitation of the protein in the presence of reducing agents (11,109). Photoreduction in *E. coli* photolyase occurs in part through intraprotein electron transfer along a chain of three tryptophan residues, W382-W359-W306, and in part through W306 and α-helix 15 (2,11,108,109,111). However, this photoreduction through intraprotein electron transfer has little or no physiological significance for the activity of photolyase, because mutations abolishing electron transfer have no effect on the enzymatic activity of photolyase *in vivo* (11,12).

Because the tryptophans of the "Trp triad" are conserved in cryptochromes from plants to animals, it has been speculated that intraprotein electron transfer may play a role in the cryptochrome photocycle. Several studies investigating the role of tryptophans corresponding to the Trp triad have implicated these residues in regulation of both light-dependent and light-independent reactions mediated by animal cryptochromes (112,113). Mutation of two tryptophans in the Trp triad of *Drosophila* cryptochrome (W342 and W397, corresponding to W306 and W359, respectively, in *E. coli* photolyase) to alanine results in a loss of light stimulation in the regulation of CRY protein stability and repression of PER/TIM-mediated transcriptional inhibition (113). However, substitution of these tryptophans with either tyrosine (redox-active) or phenylalanine (redox-inactive) has no effect on these activities. Because phenylalanine is incapable of initiating electron transfer to excited-state flavin, it appears that the effect, or lack thereof, of Trp triad mutations in *Drosophila* CRY is due to the effect of the mutations on protein folding rather than the involvement of these residues in cryptochrome photochemistry. Indeed, in this study it was found that overall protein stability in the alanine mutants was significantly reduced, indicating that substitution of tryptophan with a structurally dissimilar amino acid results in loss of protein stability and therefore, signaling capability. In line with this interpretation, mutations of the Trp triad also affect the light-independent transcriptional regulatory activity of mouse and *Xenopus* cryptochromes. Because tryptophan cannot carry out a redox reaction with ground-state flavin, these studies again suggest that the Trp triad has a role in maintaining the structural integrity necessary for function, rather than participating in the photocycle, of animal cryptochromes (112,113).

The intraprotein electron transfer pathway has also been implicated in the photoreduction of plant cryptochromes. Irradiation of recombinant AtCRY1 containing fully oxidized flavin in the presence of β-mercaptoethanol results in transient absorption spectra consistent with the formation of a semireduced radical ($FADH^\circ$) and concomitant production of a neutral tryptophan radical, which presumably abstracts an electron from one of four nearby tyrosine residues (114). Therefore, the FAD cofactor can be photoreduced in a plant cryptochrome, just as in photolyase or cholesterol oxidase, in a process involving tryptophan radicals. Even though it has been shown that the intraprotein electron transfer from Trp to flavin is not part of the photolyase photocycle because the enzyme contains two-electron reduced flavin before and after the photoenzymatic repair reaction (1,12), the same cannot be said for cryptochromes because the redox state of the flavin in cryptochromes *in vivo* is not known. Purified cryptochromes usually contain two-electron oxidized flavin and several recent studies (114,115) suggest that photoreduction of FAD_{ox} by intraprotein transfer is the primary photochemical step in the cryptochrome photocycle in plants.

The most recent study addressed the role of photoinduced electron transfer through the Trp triad as part of the CRY photocycle by examining the effect of tryptophan mutations on the light-stimulated kinase activity of AtCRY1 (see below). It was found that mutation of either W400 or W324, analogous to W382 and W306 in *E. coli*, to redox-inactive phenylalanine results in a loss of photoreduction of the oxidized mutant protein, consistent with interruption of an electron transfer pathway (115). Significantly, both the W400F and W324F mutations abolish the light stimulation of kinase activity seen in wild-type protein and plants expressing the mutant cryptochromes appear to lack cryptochrome activity (115). However, mutations disrupting the intraprotein electron transfer pathway should theoretically affect only the light-

stimulated activity; instead, both W400F and W324F have drastic effects on the basal (light-independent) kinase activity of AtCRY1, indicating that structural perturbations caused by these mutations (as in the case of mouse, *Xenopus* and *Drosophila* cryptochromes) may be responsible for disrupting the light-independent and light-dependent kinase activity of cryptochrome, thus casting doubt about the relevance of "Trp triad" photochemistry in the photoreceptive function of *Arabidopsis* cryptochromes. The identification of the *in vivo* oxidation state of native cryptochromes will help resolve the questions regarding a putative role of redox active amino acids in the cryptochrome photocycle.

Light-stimulated kinase activity

Phosphorylation is a key regulatory mechanism for nearly all photopigments (116–118). The C-termini of both plant cryptochromes are phosphorylated *in vivo* in response to light; AtCRY1 is phosphorylated by phytochrome A in a red light–dependent manner (119) and AtCRY2 is phosphorylated by an as-yet unidentified kinase in a blue light–dependent manner (120). In addition, an ATP-binding and autophosphorylation activity was recently described for AtCRY1 (6,121,122).

Adenosine triphosphate binding by AtCRY1 occurs with a 1:1 stoichiometry (6,121) and autophosphorylation occurs primarily on serine residues (121). The kinase activity was reported to be dependent on the presence of flavin and a reducing agent, and stimulated 2- to 3-fold or more *in vitro* in the presence of white light (121,122). Treatment of recombinant AtCRY1 with flavin antagonists such as potassium iodide (shown to inhibit redox reactions mediated by other flavoproteins), or an oxidizing agent, H_2O_2, abolishes the light stimulation, indicating that AtCRY1 autophosphorylation is regulated by both redox state and light status (121). Blue light–dependent autophosphorlyation of AtCRY1 may represent a significant means of regulation, because the magnitude of blue light–dependent phosphorylation *in vivo* far exceeds that observed in response to red light and is rapidly reversed, virtually absent after only 15 min in the dark (122).

It is not currently known on which residue(s) the autophosphorylation occurs, but it was reported that the photolyase-homology region of AtCRY1 is sufficient to autophosphorylate (121). Furthermore, the crystal structure of the photolyase-homology region of AtCRY1 reveals a single molecule of adenosine triphosphate bound in the cavity that corresponds to the active site in photolyase (6). Unexpectedly, however, the absence of serine residues within 11 Å of the cavity and lack of homology to canonical kinase domains raise questions as to whether the ATP bound in the flavin cavity participates in the kinase reaction (6). ATP binding and autophosphorylation under light conditions were also demonstrated for human CRY1 but the flavin requirement and light dependence of this reaction were not analyzed (121). Light stimulation of hCRY1 kinase activity *in vitro* is highly unlikely given that recombinant preparations of this protein typically contain no or grossly substoichiometric chromophore (17,45).

In fact, a recent study of kinase activity by both plant and animal cryptochromes raises some reservations about earlier conclusions regarding the requirement of flavin for kinase activity (123). The *in vitro* kinase activity of AtCRY1, human CRY1 and CRY2, was not stimulated significantly by white light and did not correlate with flavin content. AtCRY1 and AtCRY2 produced in the baculovirus/insect cell system both contain stoichiometric flavin, and yet AtCRY1 has kinase activity and AtCRY2 does not. Moreover, there was little or no light stimulation of AtCRY1 kinase activity. In addition, human CRY1 and CRY2 produced in this system contain no flavin but have kinase activity similar to that of AtCRY1 (123). Several key questions regarding the kinase activity of cryptochromes emerge: What is the determining factor for kinase activity in cryptochromes in the absence of a canonical kinase domain or apparent dependence on flavin? Why do both AtCRY1 and the human cryptochromes possess the ability to autophosphorylate, but AtCRY2 does not? Does the ability to autophosphorylate represent another convergence of function in divergent cryptochrome lineages? It is conceivable that the autophosphorylation of cryptochrome, as in the case of phytochromes, may have only minor significance to the photocycle (124). Clearly, the potential role of autophosphorylation in the cryptochrome photocycle is intriguing and will require more in-depth study.

Photoinduced domain movement

Transduction of the light signal in most, if not all, photopigments involves light-stimulated domain movement (125–128). While *in vivo* evidence from *Arabidopsis* and *Drosophila* has supported the hypothesis of light-stimulated conformational rearrangement in cryptochromes, biochemical support for this model was lacking until recently (38,64,65). The C-terminal domains of both plant and animal cryptochromes interact stably with their cognate photolyase-homology regions in the dark or in the absence of flavin (129). Using limited proteolysis to probe the tertiary structure of AtCRY1, it was found that three structurally distinct regions exist (Fig. 7A,B): 1) a region highly stable to proteolytic degradation, including the PHR and the first 50 amino acids of the C-terminal domain; 2) a region of approximately 70 amino acids that is semistable to digestion, indicating moderate backbone flexibility; and 3) the extreme C-terminal 50 amino acids, which are semistable to digestion in the dark but highly sensitive to digestion in the light (122). This light-dependent conformational change is localized to a region of approximately 35 amino acids that includes the highly conserved STAESSS motif found in plant cryptochromes (40,129). Release of this conserved region may create a secondary interaction of AtCRY with its effector, COP1, resulting in the rapid, light-dependent inhibition of COP1 ubiquitin ligase activity (34,38). This model for cryptochrome signaling suggests that the high energy state created by photoexcited flavin may be resolved by inducing a relatively long-lived, reversible conformational rearrangement. This satisfies one of the suppositions of the photolyase model of cryptochrome function; namely, the requirement that a substrate must be bound in the dark, because the excited state generated by absorption of a photon by flavin decays within 1–2 ns (1). The substrate in this model is the cryptochrome protein itself, undergoing some form of transient modification by the photoexcited flavin that results in a conformational rearrangement of the protein and release of a region of the C-terminal domain from the photolyase-homology region.

A recent study suggests that AtCRY1 forms a constitutive homodimer in plants, mediated by the N-terminal photolyase-homology region (130). Dimerization of the protein appears to be required for full activation by light; the COP phenotype reported after overexpression of GUS-CCT (Cry C-terminal) fusion proteins (38) may have been promoted by multimerization of the GUS fusion protein, because overexpression of CCTs fused to

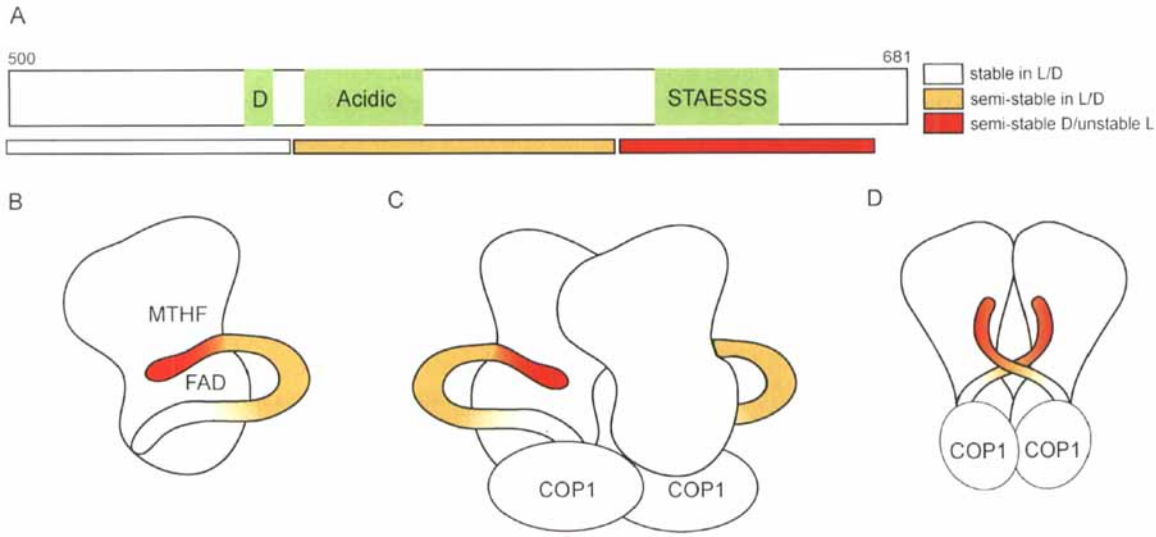

Figure 7. Model of photoinduced domain movement in AtCRY1. (A) Schematic of AtCRY1 C-terminal domain, from amino acids 500–681. The location of the highly conserved DAS motif (DQXVP-acidic-STAESSS) is indicated. Three structurally distinct regions of the C-terminus, determined by sensitivity to limiting proteolysis, are highlighted. (B) Proteolytic sensitivity of small region in the extreme C-terminus, localized to the STAESSS motif, is increased in response to light, indicating release of this region from the photolyase-homology region after light. (C) Dimerization of AtCRYs may facilitate interaction with dimeric COP1. (D) Dimerization of AtCRYs may occur through swapping of the C-terminal domains, which may create a unique C-terminal conformation upon exposure to light.

monomeric proteins does not cause a COP phenotype (130). Dimerization of CRY as a simple homodimer may stabilize its interaction with the dimeric COP1 (Fig. 7C) (131), or a unique C-terminal conformation may be created as a result of domain-swapped cryptochrome dimers (Fig. 7D).

CRYPTOCHROME AND MAGNETORECEPTION

Recently, photoexcited cryptochrome has been implicated in magnetoreception in birds and insects. Migratory animals use the earth's magnetic field to orient themselves while migrating or homing over vast distances (132). The physicochemical mechanism enabling birds to sense the reference direction given by the earth's magnetic field is not known, although it is known that the eye is required (133) and that magnetoreception is dependent on light in the blue-green range (134). Two forms of information can be derived from the geomagnetic field (132): 1) a directional (or compass) sense allows an animal to orient its movements with respect to the geomagnetic field, and 2) a positional (or map) sense provides information on where an animal is with respect to its destination by analyzing the intensity and inclination of magnetic field lines, which vary across the earth's surface and can be used to estimate position. There is evidence that both mechanisms are employed by animals, some relying on one or both senses (135). Furthermore, these two guidance systems rely on separate magnetoreceptors with different mechanisms, based on either 1) biogenic magnetite ("physical model") existing as either single-domain particles (136), fixed superparamagnetic particles (137) or as a liquid crystal (138); or 2) a magnetically sensitive radical-pair reaction ("chemical model") (139–141). Current data suggest a role for magnetite-based magnetoreception in generation of the map sense (142) and the radical-pair mechanism in generation of the compass sense (141,143).

Cryptochromes have been suggested as candidate receptors mediating the radical-pair mechanism (140,144,145). In this mechanism, the magnetoreceptor must possess an excited state in which photon absorption generates singlet-excited states and subsequent radical pairs (140). Singlet pairs may be converted to triplet pairs, with varying yield depending on the alignment of the receptor in the geomagnetic field. By analyzing the triplet yield over the fixed surface of the retina, animals could obtain directional information from the magnetic field (144). The strongest evidence to date for this mechanism in the compass sense of birds was demonstrated by using oscillating magnetic fields to perturb the magnetic orientation behavior of European robins (141). A weak oscillating field that is in resonance with the splitting between the radical-pair states could perturb the signaling mechanism by directly driving singlet-triplet transitions, but is not predicted to affect a magnetite-based receptor because the cellular environment would prevent magnetite particles from tracking weak radio-frequency magnetic fields (141).

In order for a candidate receptor to participate in the radical-pair mechanism, several important criteria must be met. First, the receptor must be expressed in the retina, specifically in neurons that signal geomagnetic information to the brain. Second, the receptor should be localized in an ordered lattice with fixed orientation. The receptor must create a radical-pair as a result of photon absorption, and have a long enough lifetime (~100 ns) to allow the ambient magnetic field to alter the spin correlation, but not slow enough to allow stochastic fluctuations in spin states to dominate the signaling species. Based on evidence presented to date, cryptochromes satisfy several of these criteria, suggesting that they may act as magnetoreceptors for directional sense in animals. Cryptochrome expression in magnetically orienting garden warblers was identified in the subset of retinal ganglion cells that project to the nucleus of the basal optic root (145), where magnetically sensitive neurons have been reported (133) and the visual flow-fields arising from self-motion are processed (146). Moreover, CRY1-expressing ganglion cells in these birds show high levels of neuronal activity at night during magnetic orientation (145). Retinal CRY1 expression is predominantly cytoplasmic

and is therefore more likely to be maintained in a specific orientation via structural proteins than nuclear protein. Striking differences in the expression of CRY in migratory and non-migratory birds also supports a unique role for cryptochromes in magnetoreception (145). This model clearly depends on the formation of a cryptochrome radical state *in vivo;* although the physiological relevance of such a state is not known, a plant cryptochrome has been shown to generate a radical pair upon photon absorption *in vitro* (114). These studies therefore provide a correlative link between cryptochromes and magnetoreception in animals.

Cryptochromes have also been implicated in the generation of a time-compensated sun compass in monarch butterflies (147). Cryptochrome expression was found in fibers connecting the clock neurons of the dorsolateral region to the optic medulla, where the dorsal rim photoreceptors (sensitive to polarized light from the sun) terminate (147). This pathway may provide a link between the circadian clock and sun compass input into the brain by providing photoperiodic information used to regulate migratory behavior (148).

CONCLUSION

It is noteworthy that the photocycle of another flavin-based blue-light photopigment, phototropin, discovered 5 years after cryptochromes (149), is now understood in considerable detail (126,150–152). Twelve years after the discovery of cryptochromes in plants and 8 years after its discovery in animals, we still do not have even a general idea about the cryptochrome photocycle. Significant progress in determining the cryptochrome photocycle is most likely to come when the protein can either be purified with near-stoichiometric chromophore and in large quantities, or in cell culture systems where RNAi and small molecule inhibitors can be used. The recent advances highlighted in this review are stimulating new hypotheses in the study of cryptochrome photochemistry, yet many questions remain to be answered: What is the oxidation state of catalytically active flavin in cryptochromes, and is it similar in divergent cryptochromes from plants, bacteria, and animals? In what photochemistry does the flavin participate, and is the cryptochrome photocycle, like that of photolyase, based on cyclic electron transfer, or is it based on redox modification of a substrate molecule? The identification of cryptochrome substrates, whether they are effector proteins, DNA or even regions of the cryptochrome protein itself, will be a crucial step in understanding the photocycle of cryptochromes.

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