Minireview

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Regulation of the Mammalian Circadian Clock by Cryptochrome*

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Photolyase/cryptochrome blue-light photoreceptors are monomeric proteins of 50–70 kDa that contain two non-covalently bound chromophore/cofactors (1, 2). One of the cofactors is always FAD. The second chromophore is methenyltetrahydrofolate (MTHF)[1] in most organisms and 8-hydroxy-5-deazariboflavin in a few species that synthesize this cofactor. Photolyase repairs UV-induced DNA damage using violet/blue light (350–500 nm) as the energy source to initiate the reaction. Cryptochrome, which exhibits up to 50% sequence identity to some photolyases (3), regulates some of the blue-light responses including growth and development in plants and the circadian rhythm by light-independent and light-dependent mechanisms in animals (4, 5). Circadian rhythm is the oscillation in the biochemical, physiological, and behavioral functions of organisms with a periodicity of about 1 day (circa = about, dies = day). The innate rhythm is never precisely 24 h. However, it is synchronized to the 24-h solar day with light input from the environment. This light-induced synchronization (entrainment) maintains the diurnal (day dwelling) and nocturnal (night dwelling) organisms active only in their particular temporal habitats and is presumed to confer selective advantage (4).

The molecular mechanism of DNA repair by photolyase is known in considerable detail (2). In contrast, there is, at present, no information on the photochemical reaction carried out by cryptochrome. The structure-function of photolyase will be briefly reviewed to provide a mechanistic background for how cryptochrome may work. Our current understanding of the mammalian cryptochrome structure-function will then be summarized.

Structure and Function of Photolyase

UV light (200–300 nm) induces two major lesions in DNA, the cyclobutane pyrimidine dimer (Pyr<>Pyr) and the pyrimidine-pyrimidone (6–4) photoproduct. Photolyases repair these lesions by using 350–500-nm photons as a second substrate or cofactor. Photolyases that repair Pyr<>Pyr and (6–4) photoproducts are evolutionarily related but functionally distinct. Thus, a given photolyase may repair either Pyr<>Pyr or the (6–4) photoproduct, and accordingly the enzymes have been classified as cyclobutane pyrimidine photolyase and (6–4) photolyase (3). Photolyase without further qualification means cyclobutane pyrimidine photolyase, and it will be used as such in this review. Photolyase has been found in many species from all three kingdoms of life, but similarly some species from all three kingdoms lack the enzyme (see Refs. 2 and 3).

Crystal structures of photolyases from *Escherichia coli* (6) and *Anacystis nidulans* (7) have been determined. The crystal structure of *E. coli* photolyase will be summarized as representative of this family of proteins (Fig. 1). The enzyme is composed of two domains: an N-terminal α/β domain (residues 1–131) and a C-terminal α-helical domain (residues 204–471), which are connected to one another with a long loop that wraps around the α/β domain. The MTHF photoantenna is bound in a shallow cleft between the two domains, and the FAD cofactor is deeply buried within the α-helical domain. A surface potential representation of the molecule reveals a positively charged groove running the length of the molecule. A hole in the middle of this groove leads to the flavin in the core of the α-helical domain.

Photolyase recognizes the 30° kink caused in DNA by Pyr<>Pyr (8, 9); then, it "flips out" the dimer from within the duplex to the active site cavity of the enzyme to form a high stability complex. Light initiates catalysis (Fig. 2); the MTHF photoantenna absorbs a photon and transfers the energy to $FADH^-$ (the active form of flavin in photolyase) by Förster resonance energy transfer. The excited state flavin, $^1(FADH^-)^*$, transfers an electron to Pyr<>Pyr to generate a charge-separated radical pair ($FADH^\circ$ . . . $Pyr<>Pyr^{\circ-}$). The cyclobutane ring is split by ($\Pi_s^2 + \Pi_s^2$) cycloreversion, and the flavin radical is restored to the catalytically competent $FADH^-$ form by back electron transfer following splitting of the cyclobutane ring. The repaired dinucleotide no longer fits in the active site pocket and is ejected back into the duplex, and the repaired DNA dissociates from the enzyme. It is thought that the (6–4) photolyase employs essentially the same mechanism as classical photolyase (10, 11).

Cryptochromes

For over 125 years it has been known that blue light elicits several responses in plants including phototropism, photoperiodism (measuring day length), and growth and development (12). The nature of the blue-light receptor remained cryptic for a long time and the term "cryptochrome" was coined to refer to this mysterious pigment responsible for blue light responses (13). Now it is known that plants contain at least three types of flavoproteins that would fit the original definition of cryptochrome: phototropin (14), FKF1 (15), and the protein that is encoded by the HY4 gene of *Arabidopsis thaliana* that exhibits high similarity to DNA photolyase (16). The HY4-encoded blue-light photoreceptor was the first putative plant blue-light photoreceptor discovered and hence it was assumed to be the elusive "cryptochrome" and was renamed as such (17). Now, by convention any protein in plants, animals, or bacteria with similarity to photolyase and with no repair function is called a cryptochrome and is presumed to perform a blue-light receptor function (1–5). Cryptochromes exhibit 25–50% sequence identity to photolyase (18) and, like photolyases, contain both FAD and folate as cofactors (17, 19). Most cryptochromes, including the human cryptochromes, have C-terminal extensions of 20–200 amino acids beyond the photolyase homology region. Of all cryptochromes identified to date, the *Arabidopsis* cryptochromes are the best characterized. However, despite numerous findings indicative of a photoreceptor function of cryptochromes in *Arabidopsis*, the photochemical reaction in blue-light signaling by cryptochromes is not known and still the strongest evidence that *Arabidopsis* cryptochromes are blue-light receptors is their high degree of similarity to photolyase (1, 2, 12).

Mammalian Cryptochromes

Discovery of Mammalian Cryptochromes—Based on exhaustive biochemical data, it has been concluded that humans and other placental mammals do not have photolyase (20). Therefore, the report of a photolyase ortholog as an expressed sequence tag in the human genome data base in 1995 (21) was unexpected and led to a re-evaluation of the previous conclusion. Hsu *et al.* (22) found that neither this protein nor a second ortholog they subsequently discovered had photolyase activity and suggested that these proteins must therefore perform other blue-light-dependent functions in human cells and named them human cryptochrome 1 and cryptochrome 2 (hCRY1 and hCRY2). Taking into account the informa-

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[1] The abbreviations used are: MTHF, methenyltetrahydrofolate; SCN, suprachiasmatic nuclei.

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tion available on circadian photoreception at the time, it was proposed that these proteins may function as circadian photoreceptors (22).

In mice and humans, the outer (back) retina that contains rods and cones is required for vision but not for entrainment of the circadian clock. The inner (front) retina contains the ganglion cells, the axons of which make up the optic nerve, and is sufficient to entrain the circadian clock in the absence of the outer retina (23–25). For this reason, humans and mice that become blind because of retinal degeneration diseases that destroy the outer retina can still synchronize their circadian clock to the daily light-dark cycles (23, 26). Indeed, even the brain centers for vision and circadian photoreception are separate (27, 28). The visual center is located in the cortex whereas the circadian center is located in the midbrain in two clusters of neurons above the optic chiasma called the suprachiasmatic nuclei (SCN).

To test the hypothesis that cryptochromes function as circadian photoreceptors, the expression pattern of Cry1 and Cry2 was analyzed. Cryptochromes were found to be expressed in all tissues; however, expression was high in the retina and restricted to the inner retina in both mice and humans (29–31). In the brain, mCry1 was highly expressed in the SCN, and expression exhibited a daily oscillation, peaking at about 2:00 p.m. and reaching its nadir at around 2:00 a.m. (29, 30). Thus, it appeared that cryptochrome was expressed in the appropriate places for setting the clock and for running it and gave credence not only to the hypothesis that cryptochrome is a circadian photoreceptor but also raised the possibility that cryptochrome is a component of the molecular clock (29). Subsequently, cryptochromes were found in *Drosophila* and all other insects tested as well as *Xenopus* and all other vertebrate animals whose genomes have been sequenced (3–5). The most extensive studies on animal cryptochromes have been carried out with the mammalian and *Drosophila* cryptochromes. Below, our current understanding of the mammalian cryptochrome will be summarized and where necessary reference will be made to the single *Drosophila* cryptochrome.

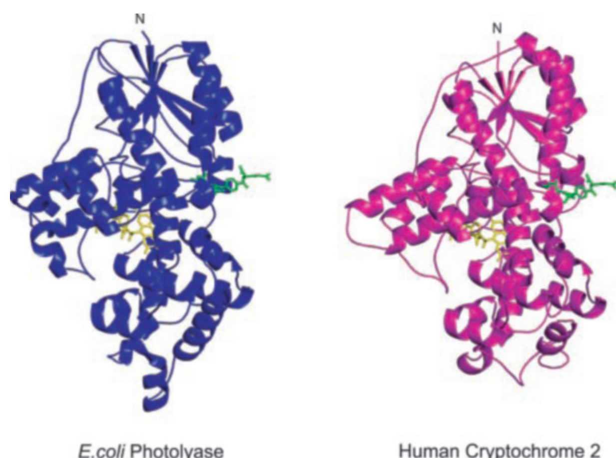

FIG. 1. **Structures of photolyase and cryptochrome.** *Left*, crystal structure of *E. coli* photolyase (6); *right*, the computer-generated structure of human CRY2 (32). The C-terminal 106 amino acids of CRY2 are not shown. *Yellow*, FAD; *green*, MTHF.

Biochemical Properties—Both human cryptochromes have been purified from HeLa cells expressing the *Cry* genes ectopically (32) and from *E. coli* as recombinant proteins (22). Proteins isolated from both sources contain FAD and a pterin, presumably in the form of MTHF. However, both cofactors are at grossly substoichiometric levels relative to the apoenzyme precluding extensive characterization of their biophysical properties, including the redox status of the flavin in the native enzyme. The hCRY1 and hCRY2 purified from *E. coli* exhibit a near-UV absorption peak at 420 nm (22). The *Drosophila* cryptochrome exhibits properties similar to those of human cryptochromes with a 420 nm near-UV absorption peak and 1–5% cofactor content (4, 5).

At present, there is no crystal structure of any animal cryptochrome. Molecular modeling of the hCRY2 photolyase homology region reveals a photolyase-like structure including the positively charged DNA-binding groove (Fig. 1) (32). Both hCRY1 and hCRY2 bind with moderate affinity to DNA and with higher affinity to UV light-damaged DNA but have no repair activity (32). The DNA binding activity of cryptochrome might be an evolutionary relic of its common ancestry with photolyase. As in the case of AtCry1 (33, 34), it has been reported that hCRY1 has an autophosphorylating kinase activity; however, this activity is not light-dependent (33) and its relevance to a putative cryptochrome photocycle is unknown. In contrast to these biochemical activities of obscure significance, human cryptochromes interact strongly with several "clock proteins" to generate a transcriptional feedback loop called the molecular clock, summarized below (27, 28).

Function of Mammalian Cryptochromes

Clock Function—The first direct evidence that cryptochrome plays a role in the circadian clock came from the analysis of mice in which the *Cry2* gene was knocked out; wild type mice in constant darkness exhibit a circadian rhythm of behavior with an intrinsic period of 23.7 h. The *Cry2*−/− mice exhibited a period about 1 h longer than wild type mice (Fig. 3) (35). These data led to the conclusion that Cry2, in addition to any putative photoreceptor function, must have a light-independent role in the maintenance of

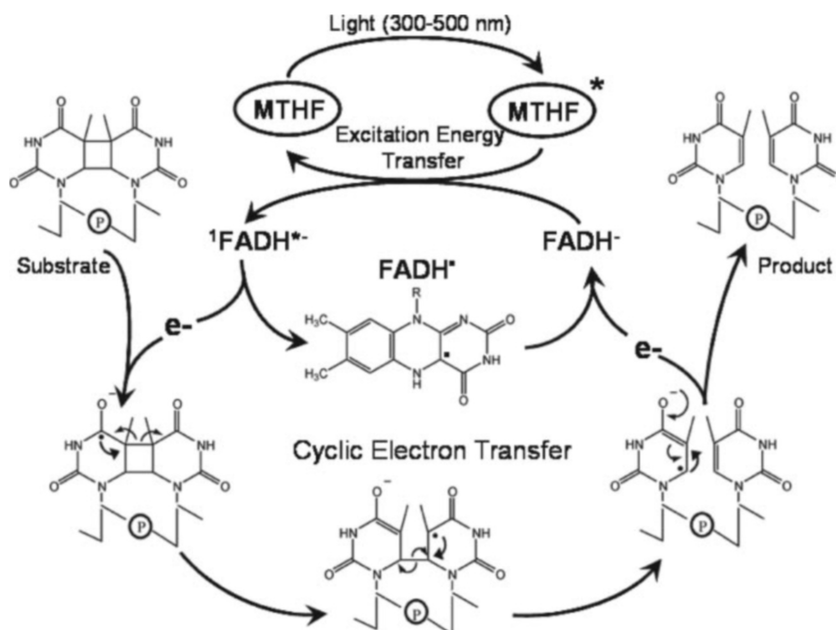

FIG. 2. **Photolyase reaction mechanism.** The enzyme contacts the DNA backbone of the damaged strand and thermally flips out the cyclobutane dimer from the duplex into the active site cavity; the dimer is split photochemically. The figure shows the steps in the photochemical reaction. The MTHF photoantenna absorbs a photon and transfers the excitation energy to $FADH^-$, which in turn transfers an electron to Pyr<>Pyr; the resulting dimer radical splits to two normal bases concomitant with back electron transfer to restore the flavin neutral radical to the catalytically competent $FADH^-$ form (2). Following repair, the dinucleotide flips out of the enzyme and into the DNA, and the enzyme dissociates from repaired DNA.

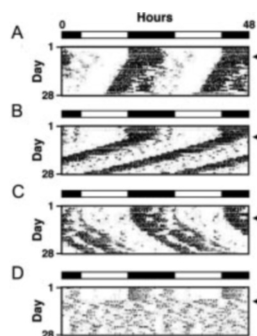

FIG. 3. **Effect of cryptochrome mutation on the circadian behavior of mice (35, 37).** The circadian behavior was assessed by measuring the wheel running activity as a function of time of day. The rpm of the running wheel is plotted on the *y* axis, and the time of day is plotted on the *x* axis. The activity is plotted twice from the 2nd day on to make a comparison of activities in successive days easier. The *bar* on *top* indicates the dark (*closed rectangle*) and light (*open rectangle*) phases of day. These graphs (actograms) show activity profiles of four mice for a 28-day period. At the day indicated by *arrows* the animals were switched from a 12 h light:12 h dark (LD) cycle into constant darkness (DD). Note that under LD, all four mice exhibit 12-h activity and 12-h rest phases with a 24-h daily periodicity. In DD, the mice exhibit activity/rest phases with periodicities (τ) imposed by their intrinsic clock (free running), and the mutants behave differently from the wild type. *A*, wild type, τ = 23.7 h; *B*, *Cry1*−/−, τ = 22.7 h; *C*, *Cry2*−/−; τ = 24.7 h; *D*, *Cry1*−/−;*Cry2*−/−, arrhythmic.

the normal rhythm (35). This conclusion was strengthened and extended by the analysis of mice lacking Cry1 or both Cry1 and Cry2 (36, 37); *Cry1*−/− mice have a period 1 h shorter than wild type mice and, most strikingly, *Cry1*−/−; *Cry2*−/− animals are arrhythmic (Fig. 3). The cause of the drastic effect of the loss of cryptochromes became apparent when the molecular basis of the mammalian circadian rhythm was elucidated (see Refs. 27 and 28).

The molecular clock is generated by an autoregulatory transcriptional loop with ~24-h periodicity (Fig. 4*A*) composed of several integral clock proteins. The key proteins that make up the molecular clock in mammals are Clock, Bmal1, Cry1, Cry2, Per1, and Per2. In addition, other clock proteins including Per3, Tim, Rev/Erbα, and CKIϵ make important contributions in the maintenance of robust oscillatory amplitude (27, 28).

The core of the molecular clock machinery may be summarized as follows (Fig. 4*A*). The Clock and Bmal1 proteins are transcription factors that function as a heterodimer, binding E-box motifs in the promoters of the *Bmal1*, *Cry1*, *Cry2*, *Per1*, and *Per2* genes to stimulate their transcription. The Cry and Per proteins make combinatorial heterodimers and enter the nucleus, disrupting the Clock-Bmal1 complex and down-regulating the transcription of the genes driven by this complex, including their own. The ensuing decline in Cry and Per protein levels eventually leads to reactivation of the Clock-Bmal1-regulated promoters and reinitiation of the cycle. The clock transcriptional loop (oscillatory) differs from steady state negative feedback transcriptional loops (homeostatic) because of the concerted activity of the post-translational modifications (including phosphorylation of Per by CKIϵ) (38) regulating proteolytic degradation and nucleocytoplasmic shuttling of the clock proteins. These events add a lag phase between the synthesis of the transcriptional inhibitors and their availability to act on their target within the nucleus, such that the time interval between the maximum transcription of the negative regulators and their actions on the target transcription factors within the nucleus is about 24 h.

The molecular clock is present in virtually all mammalian tissues. The master circadian clock, located in the SCN, performs two fundamental functions for the manifestation of macroscopic (physiological and behavioral) rhythms (28). First, it receives the light signal from the retina, which stimulates transcription of *Per1* and *Per2* genes, thus resetting the phase of the rhythm. Second, the master clock synchronizes the peripheral clocks through neural and humoral communication so as to achieve rhythm at the organism level. The circadian rhythm at the organism level is achieved by the action of Clock-Bmal1 and other components of the molecular clockwork on the transcription of effector clock-controlled genes, such as those involved in melatonin and corticosteroid synthesis. Cryptochromes clearly play a central role in the inhibitory branch of the autoregulatory transcriptional loop that makes up the clock (39). As

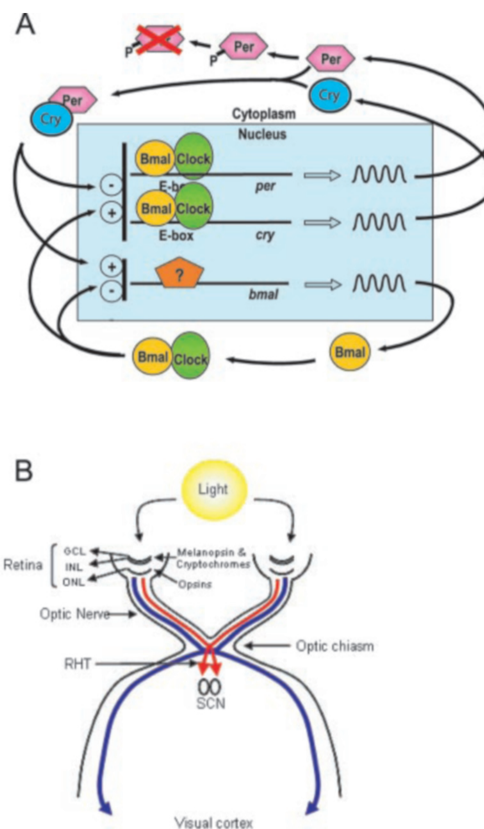

FIG. 4. **Dark and light functions of mammalian cryptochrome.** *A*, dark function. This is a simplified scheme for the mammalian circadian clock. Clock and Bmal1 form a heterodimer that binds to the E-box sequences of target genes and stimulates their transcription. Crys and Pers also make heterodimers in the cytoplasm, translocate into the nucleus, and inhibit Clock-Bmal1-activated transcription (27, 28). *B*, light function. The light signal received by the opsins in rods and cones in the outer (back) retina is transmitted to the visual cortex by the optic nerve (*blue*). The light signal received by both the rods and cones in the outer retina and by the cryptochromes and melanopsin in the inner retina (front) is transmitted to the master circadian clock, the SCN, by a specialized group of retinal ganglion cells constituting the retinohypothalamic tract (*red*) (4, 5).

a consequence, elimination of both cryptochromes results in constitutively elevated levels of Clock-Bmal1-controlled transcripts (37, 39) and hence molecular and behavioral arrhythmicity.

Cryptochrome as a Circadian Photoreceptor—Ironically, even though cryptochrome was introduced into the field of circadian biology as a putative circadian photoreceptor in the mammalian circadian clock (22, 29), it is now securely established as a key clock protein, but its role as a mammalian blue-light photoreceptor is a matter of considerable debate and is far from proven. In fact, one widely held view is that cryptochrome functions as a circadian photoreceptor in *Drosophila* but it has lost its photoreceptive function in mammals and has become just another "cog" in the wheel of the molecular clock (40, 41). Some of the findings that have led to this view will be discussed. First, in yeast two-hybrid assays it has been found that *Drosophila* Cry binds to the clock protein dTim in a light-dependent manner (42, 43), but the human cryptochromes bind to human Per1, Per2, Clock, and Tim proteins independently of light (44). Second, *Drosophila* lacking all opsins and cryptochrome are circadian blind (45, 46), whereas mice lacking rods, cones, and cryptochromes exhibit residual photoresponses (47). Finally, the clock in *Drosophila* peripheral organs (that contain no opsins but express cryptochrome) can be reset by light (48). In contrast, photoreception through the eye is essential for resetting the clock in mammals even though cryptochromes are expressed in all tissues (49, 50).

Perhaps of more relevance to cryptochrome's putative photoreceptive role is the presence of a newly discovered opsin called melanopsin in the mammalian inner retina with a clear role in circadian photoreception (51). Melanopsin is expressed in a small fraction (~1%) of the mouse retinal ganglion cells that innervate the SCN (retinohypothalamic tract) and brain regions involved in other non-visual photoreceptive tasks such as pupillary light re-

sponse (52–54). Elimination of melanopsin by gene knockout causes only a modest reduction in circadian photoresponse (55–57). However, genetic ablation of rods and cones and melanopsin eliminates both visual and circadian phototransduction, and these mice are completely insensitive to light (58, 59). This last finding has given credence to the notion that classical opsins and melanopsin play redundant roles in circadian photoreception and are necessary and sufficient for all mammalian photoresponses. This model, however, is inconsistent with several observations, listed below, indicating a photoreceptive role of cryptochrome in mammals.

Genetic analysis of photoreceptor contribution is most quantitatively performed at the molecular level using gene induction in the SCN in response to light given in the dark cycle of the animals. Mice lacking *Cry2*, the predominant cryptochrome in the mouse retina, have 2-fold reduced sensitivity for photoinduction of genes in the SCN even in the presence of the visual photoreceptors (35), and mice lacking both *Cry1* and *Cry2* exhibit 10–20-fold reduced sensitivity (47). Most strikingly, mice of the *rd/rd*;*Cry1−/−*; *Cry2−/−* genotype, which have no classical opsins or cryptochromes but retain melanopsin, exhibit nearly 3000-fold reduction in circadian photosensitivity (47, 60). In addition, the pupillary responses of rodless and coneless mice lacking cryptochromes are 20-fold less sensitive to blue light than rodless and coneless mice (24). Finally, severe depletion of ocular retinaldehyde, the cofactor of all opsins, was achieved by maintaining mice on a vitamin A-free diet; this causes visual blindness and a 10,000-fold reduction in pupillary light response (61) relative to wild type mice but has only a modest effect on retinohypothalamic phototransduction as measured by light induction of genes in the SCN (61, 62). Importantly, when ocular retinaldehyde is depleted in cryptochromeless mice, in the majority of animals there is no gene induction in SCN and no behavioral rhythmicity under 12 h light:12 h dark cycles as measured by wheel running activity (61), again indicating a role for cryptochrome in circadian photoreception.

At face value, the retinal depletion data show that cryptochrome can mediate circadian photoreception in the eye in the absence of functional opsins. However, this conclusion is at odds with the finding that elimination of opsins by genetic ablation abolishes all visual and non-visual photoresponses. Clearly, in the absence of opsins, cryptochrome cannot generate an action potential for neurotransmission from the retina to the SCN (58, 59). One way to reconcile these seemingly contradictory data is to assume that, independently of the classical opsins, cryptochromes and melanopsin work cooperatively to generate a robust retinohypothalamic photosignal (Fig. 4*B*). At present, no specific models can be advanced on how this might occur because neither the photochemical reaction carried out by cryptochrome nor the downstream signaling events initiated by melanopsin are known. It is clear, however, that none of the mammalian photopigments identified to date (rod and cone opsins, melanopsin, or cryptochromes) are independently essential for circadian phototransduction. This conclusion, in turn, brings into focus the most central question of cryptochrome research not just for mammalian cryptochromes but for all cryptochromes in plants, animals, and bacteria: what is the primary photochemical reaction carried out by cryptochrome? Despite the wealth of genetic evidence that cryptochrome is a photoreceptor in *Arabidopsis*, *Drosophila*, and mice, until this question is answered there will always be reservations about whether cryptochrome is a photoreceptor or a molecule downstream of the actual photoreceptor.

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