Cryptochrome (CRY) is a blue-light-sensitive flavoprotein that functions as the primary circadian photoreceptor in Drosophila melanogaster. The mechanism by which it transmits the light signal to the core clock circuitry is not known. We conducted in vitro studies on the light-induced conformational change in CRY and its effect on protein–protein interaction and performed in vivo analysis of the lifetime of the signaling state of the protein to gain some insight into the mechanism of phototransduction. We find that exposure of CRY to blue light induces a conformation similar to that of the constitutively active CRY mutant with a C-terminal deletion (CRYΔ). This light-induced conformation has a half-life of ~15 min in the dark at 25 °C and is characterized by increased affinity to Jetlag E3 ligase. In vivo analysis reveals that in the Drosophila S2 cell line, the signaling state induced by a millisecond light exposure has a half-life of 27 min in the dark at 0 °C during which period it is susceptible to degradation by the ubiquitin-proteasome system. These findings lead to a plausible model for circadian photoreception/phototransduction in Drosophila.

Cryptochrome (CRY) is a flavoprotein that regulates growth and development in plants in response to blue light, functions as a circadian photoreceptor in Drosophila and other insects, and acts as a core component of the molecular clock in mammalian organisms (1–4). Despite extensive research on CRY's photosensory function in Arabidopsis and Drosophila, its mechanism of photoreception/phototransduction is poorly understood. Even the redox status of the FAD cofactor is a matter of some debate (5–8). In the phylogenetically related protein, DNA photolyase, photoinduced cyclic electron transfer from the FADH+ cofactor to a pyrimidine photodimer repairs the DNA damage and regenerates the FADH+ for new rounds of catalysis (9–11). However, there is no evidence so far for a similar reaction in either Arabidopsis CRY1 (AtCRY1) and CRY2 or Drosophila CRY, which at present are the most extensively studied CRYs. The lack of evidence for a cyclic redox reaction in CRYs has led to consideration of the mechanisms of other photosensory flavoproteins as potential models for CRYs in general and Drosophila CRY in particular.

Currently, three types of photosensory flavoproteins are known (12): the photolyase/CRY family, the LOV domain proteins such as phototropin, and the BLUF domain proteins such as the photoactivated adenylyl cyclase. Whereas photolyase, as noted above, carries out catalysis by light-induced cyclic electron transfer, LOV and BLUF domain proteins initiate photosignaling by a light-induced conformational change. Failing to obtain any evidence for CRY signaling by a photolyase-like mechanism, we considered the possibility that CRY also may carry out its light signaling by a light-induced conformational change that would affect the interaction of CRY with downstream signal transduction partners (13, 14). Indeed, a preliminary study with AtCRY1 revealed that light induced a significant conformational change in the C-terminal extension (14) of the protein that is considered to be the signaling domain of this cryptochrome (15, 16). Hence, we entertained the possibility that Drosophila CRY may also initiate photosignaling by a light-induced conformational change that would affect its interactions with known signaling partners.

Circadian clock | photocycle | proteolysis | sensory flavoprotein

Cryptochrome is the primary circadian photoreceptor in Drosophila (17). Upon exposure to light it undergoes a change that makes both CRY and its downstream partner Timeless (TIM) targets for ubiquitination (18) and proteolysis (19). TIM is a key component of the negative arm of the TTFL (Transcription-Translation-Feedback Loop) that constitutes the core circadian clock and degradation of TIM resets the clock. It has been reported that Jetlag (JET) is the E3 ligase responsible for the light-dependent ubiquitination and proteolysis of both TIM and CRY (18, 20). However, it was unclear how the absorption of a blue-light photon by the flavin cofactor made CRY a target for JET. The isolation of a constitutively active CRY mutant, CRYΔ (21–24), which is missing the C-terminal 22 amino acids, raised the possibility that light causes a conformational change in CRY and thus affects its interaction with downstream partners enabling it to transduce the signal. In this study, through analyses of partial proteolysis patterns we demonstrate that light causes a conformational change in CRY. Moreover, we find that this signaling (or “Lit”) state conformation (henceforth we will use these two terms interchangeably) that is remarkably long-lived in the dark is similar to the conformation of the constitutively active CRYΔ, and exhibits enhanced affinity for JET. Finally, we demonstrate in vivo that a light pulse of a millisecond duration induces a Lit state CRY with a half-life of 27 min at 0 °C during which period it remains susceptible to ubiquitination and degradation in the dark. Collectively, the data are consistent with a model whereby blue light induces a significant stable conformational change in CRY and this Lit state binds to JET E3 ligase with high affinity and independently of light, and promotes the ubiquitination and proteolysis of both CRY and TIM, and thus resets the clock.

Results

Purification and Spectroscopic Properties of CRY and CRYΔ. We wished to compare the photoreactive of CRY to that of the constitutively active CRYΔ with regard to light-inducible conformational change to find out if light induced a conformation of CRY that might be similar to the CRYΔ conformation in the dark. To this end we constructed baculoviruses expressing CRY and CRYΔ carrying affinity tags either at the N- or C-termini to aid in purification and in analyzing the proteolysis patterns by immunoblotting. Fig. 1A shows that the proteins are of high purity and Fig. 1B shows that their absorption spectra in the near UV-visible range are essentially identical. Importantly, upon exposure to blue light both CRY and CRYΔ flavins undergo the same photoreduction reaction whereby FADox is converted to FADH+ through intra-protein electron transfer (5–7).

Effect of Blue Light on CRY and CRYΔ. A widely held model for CRY photoreception/phototransduction posits that light induces a con-
A light-induced conformational change in the C-terminal domain of CRY enables it to interact with downstream targets and initiate the signaling cascade (2, 13, 14). In particular, a change in the conformation of the C-terminal extension is thought to be of special significance because mutations that delete the C-terminal ~20 amino acids render CRY constitutively active (21–23). To test this model, we used CRY and CRYΔ (missing the C-terminal 22 amino acids) in an assay in which protein conformation was probed by partial proteolysis with trypsin. Purified CRY and CRYΔ, kept either in the dark or under blue light, were subjected to partial proteolysis with trypsin. Then, the products were separated by SDS-PAGE and probed with the appropriate antibodies to identify the cleavage sites. From the results shown in Fig. 2, we identify 5 cleavage sites in CRY and 4 in CRYΔ (Fig. 2) as described in SI Text. We designate these sites I, Ia–IV in CRY and I, Ia–IIIΔ in CRYΔ, as appropriate. Site I in CRY (and Ia in CRYΔ) is at K289, and the resulting fragments (intensity of cleavage at this site is unaffected by light) are detectable with both N- and C-terminal probes (Fig. 2A, lanes 2 and 3, and lanes 5 and 6, and Fig. 2B, lanes 2 and 3, and lanes 5 and 6). Site Ia and IaΔ is around 200 amino acids from the N-terminus, and the resulting fragment is detectable only with the N-terminal probe (Fig. 2B, lanes 2 and 3, and lanes 5 and 6). The intensity of cleavage at this site is unaffected by light and the fragment resulting from cleavage at this site is presumably the product of secondary cleavage of Fragment I (I) because it is not detectable with the C-terminal probe. Site II (IIΔ) is at K430. Cleavage at this site is an important conformational signature: Cleavage of CRY at this site is induced by light and cleavage of CRYΔ is very efficient and it is not affected by light (Fig. 2A, lanes 2 and 3, and lanes 5 and 6). The site II (IIΔ) cleavage is not detectable with the N-terminal probe presumably because of the much stronger cleavage efficiency at K289 site I (I) (SI Text). It is also possible that cleavage at site II promotes a secondary cleavage on the N-terminal side of the fragment. Site III (IIIΔ) cleavage at K503 is light-enhanced in CRY (Fig. 2A, lanes 2 and 3, and B, lanes 2 and 3) and constitutively trypsin-sensitive and unaffected by light in CRYΔ (Fig. 2A, lanes 5 and 6, and B, lanes 5 and 6) and therefore cleavage at this site may also be considered as a signature of the signaling state conformation. Site IV cleavage at position ~520–542 (Fig. 2B, lanes 2 and 3) represents a light-induced conformational change in the C-terminal extension of CRY. It is not detectable with the C-terminal probe because of small size of the C-terminal product and most likely further degradation of the small unstructured peptide by secondary proteolysis. Cleavage at this site is only detectable with high-resolution electrophoresis and using the N-terminal probe that reveals a light-induced proteolytic fragment of CRY that is approximately 3 kDa smaller than the full-length protein and corresponds to, essentially, the CRYΔ protein (Fig. 2B, Bottom, lanes 4–6). These cleavage sites are indicated on 1D and 3D representations of the Drosophila CRY in Fig. 2C and SI Text. Thus, the partial proteolysis experiments with CRY and CRYΔ yield complementary results consistent with the model that blue light induces a conformation in CRY that is similar to the conformation of the constitutively “on” CRYΔ.

Mechanism of the Photoinduced Conformational Change of CRY. Next, we addressed the question of the photochemical mechanism that induces the signaling state conformation. Currently, there are two models for the photosignaling reaction of Drosophila CRY (25). In one model (5, 6), the “dark state” CRY contains oxidized FAD, FADox. Upon blue-light exposure, the excited chromophore (FADox)* is converted to the FAD+· anion radical concomitant with a conformational change in CRY that generates CRY2, the
signaling or light state; reoxidation of FAD$^+$ to FAD$_{ox}$ in the dark causes the CRY conformation to change back to the dark state and turns off the signal:

Model 1: CRY(FAD$_{ox}$)$_{bu}$ $\overset{h}{\rightarrow}$ CRY(FAD$_{ox}$)$^*$ $\overset{e}{\rightarrow}$ CRY(FAD$^+$)$^*$ $\overset{h}{\rightarrow}$ CRY(FAD$_{ox}$)$_{bu}$. 

In the alternative model (7, 8), it is proposed that the Drosophila CRY contains FAD$^+$ in the ground state in the dark (the FAD$_{ox}$ form of CRY is presumed to be a purification artifact). According to this model, the excitation of FAD$^+$ by light to (FAD$^+$)$^*$ and the formation of FAD$^+$ causes the conformational change responsible for signaling:

Model 2: CRY(FAD$^+$)$_{bu}$ $\overset{h}{\rightarrow}$ CRY(FAD$^+$)$^*$ $\overset{e}{\rightarrow}$ CRY$^d$(FAD$^+$) $\overset{h}{\rightarrow}$ CRY(FAD$^+$)$_{bu}$. 

According to this model, the decay of the signaling state conformation, CRY$^d$, back to the ground state conformation is not mechanistically coupled with a change in the oxidation state of FAD$_{ox}$. The experiments described in Fig. 2, however, cannot discriminate between these two models because during conditions of continuous irradiation, photoreduction by the first absorbed photon is followed by absorption of a second photon and the excitation of the flavin anion radical:

FAD$_{ox}$$_{bu}$ $\overset{h}{\rightarrow}$ (FAD$_{ox}$)$^*$ $\overset{e}{\rightarrow}$ FAD$^+$ $\overset{h}{\rightarrow}$ (FAD$^+$)$^*$. 

As a consequence, with the Fig. 2A experimental design it cannot be determined whether the signaling state conformation was induced by the FAD$_{ox}$ $\overset{h}{\rightarrow}$ FAD$^+$ photoreduction or by the FAD$^+$ $\overset{h}{\rightarrow}$ (FAD$^+$)$^*$ photoexcitation. To discriminate between models 1 and 2, we chemically reduced the flavin to FAD$^+$ by incubation with dithionite (26) and then carried out comparative partial proteolysis of CRY (FAD$_{ox}$), CRY(FAD$^+$), and CRY(FAD$^+$)$_{bu}$ exposed to light using the readily quantifiable light-enhanced site III cleavage to monitor conformational change. The results of these experiments are shown in Fig. 3. The following conclusions emerge from this figure. First, dithionite reduces the flavin in CRY to FAD$^+$ (Fig. 3A, Top) in a manner indistinguishable from photo-reduction (Fig. 1B). Second, the proteolysis pattern of CRY as probed by the sensitivity of K503 (site III) to trypsin is not affected by the FAD$_{ox}$ $\overset{h}{\rightarrow}$ FAD$^+$ reduction (Fig. 3A, Bottom). Third, exposure of dithionite-reduced CRY to light does not cause further change in the absorption spectrum of the flavin co-factor (Fig. 3B, Top) but it causes a conformational change in CRY that sensitizes K503 to proteolytic attack (Fig. 3B, Bottom). These data are consistent with Model 2.

**Lifetime of the Signaling State of CRY.** The proposed model for the action mechanism of CRY that the light-activated state must have a longer lifetime than the photochemically excited state of flavin because the lifetime of the flavin excited state is in the nanosecond range (26, 27), and considering the intracellular concentrations of CRY and its known protein targets, JET and TIM, the probability of encounter within the lifetime of the excited state is negligible. To measure the lifetime of the signaling state conformation we exposed CRY to a light pulse and then kept it in the dark for various periods of time before subjecting it to trypsin proteolysis and monitoring the rate of disappearance of light-enhanced cleavage at K503 (site III) of CRY. Fig. 4A shows an immunoblot used to measure the rate of decay of the light-promoted hypersensitivity to trypsin cleavage at K503.

Quantitative analysis of the data from this and two additional experiments carried out under identical conditions is shown in Fig. 4B. From this figure, a half-life of 15 min is calculated that is in the range of half-lives of other flavin-based sensory photoreceptors such as (12, 28).

**Effect of Light-induced Conformational Change on CRY–JET Interaction.** If the conformational change detected by partial proteolysis is relevant to CRY function, then it is expected that light would promote CRY-downstream target interaction whereas CRY$_{Δ}$ would be expected to exhibit these interactions strongly and independently of light. To test these predictions CRY or CRY$_{Δ}$ were added to JET bound to sepharose beads, and the mixtures were either kept in the dark or exposed to light for 10 min. Then, the beads were collected by centrifugation, washed extensively, and the bound proteins were visualized by immunoblotting. The results of such an experiment are shown in Fig. 5. As is apparent from the figure, the binding of CRY to JET is essentially light-dependent (Fig. 5, lanes 5 and 7). In contrast, CRY$_{Δ}$ bound to JET strongly, irrespective of light (Fig. 5, lanes 6 and 8). These data are consistent with the model that deletion of the C-terminal 20 amino acids causes a conformational change in CRY that enables it to bind to JET at a maximal level (20) and that exposure of CRY to blue light induces a conformation similar to that of CRY$_{Δ}$ with the consequent promotion of high affinity CRY–JET interaction. Next, we wished to determine if the half-life of the signaling state defined by the partial proteolysis profile matched the half-life determined by CRY–JET binding. To this
end, we irradiated CRY with blue light and then kept it in the dark for various periods of time before adding it to JET-GST beads and measuring the level of CRY–JET binding by immunoblotting. The results of such an experiment are shown in Fig. 6A. As is apparent from this figure, light greatly enhances the CRY–JET interaction even when CRY is preirradiated with blue light prior to mixing with JET. Importantly, this light-enhanced affinity of CRY for JET decays with a half-life of ∼15 min (Fig. 6A and B). This half-life is essentially the same as the half-life of blue-light-induced conformational change as probed by proteolysis, indicating that the two observations are intimately linked and reflect different facets of the same phenomenon. In the next series of experiments we tested the in vivo implications of these observations.

**Lifetime of the Signaling State of CRY in Vivo.** Upon exposure of *Drosophila* S2 cells to light CRY is proteolytically degraded by the UPS (Ubiquitin/Proteasome system) (19, 29). It was reported that proteolytic degradation of CRY stopped when the light was turned off (23), suggesting that CRY had to be in a photochemically excited state for enzymatic modification that ultimately leads to its proteolysis. However, a subsequent study using camera flash photolysis revealed that proteolysis of CRY continues for 60 min after the light pulse (30), leading to the conclusion that light induces a long-lived signaling state conformation (Lit state) that continues to interact with downstream partners including ubiquitin ligase so that CRY could be marked for proteolysis. However, in that study the kinetics of conversion of the Lit state to the Dark state could not be determined because following the light flash, cells were incubated at 25 °C, and during incubation in the dark CRY was continuously being degraded. To overcome this problem we used the experimental design illustrated in Fig. 7A: S2 cells expressing CRY were placed in an ice bath and exposed to a camera flash lasting ∼1 ms. The cells were then kept on ice in the dark for various periods before moving to a 25 °C bath to allow enzymatic modification and proteolysis. This approach is based on the fact that whereas photochemical reactions in general are insensitive to temperature over a wide temperature range, most chemical reactions, including enzymatic reactions, are inhibited at 0°C. Thus, to determine the half-life of the Lit state of CRY both the control (no light exposure) and the test (∼1 ms flash) samples were kept on ice and at time intervals samples were removed from the ice bath and placed at 25 °C and incubated for 60 min. Then, the S2 cells were lysed and the proteolytic degradation of CRY was assessed by immunoblotting. The results of a representative experiment are shown in Fig. 7B and data points from 2 experiments are plotted in Fig. 7C. As is apparent from this figure, if a flashed sample is kept on ice for the duration of the experiment, no CRY degradation occurs (Fig. 7B, lanes 1 and 10). In contrast, if immediately following the flash the cells are incubated at 25 °C for 60 min nearly all CRY is proteolyzed (Fig. 7B, lanes 1 and 2). However, the sensitivity of photoflushed CRY to proteolysis decays in proportion to the time the sample is kept on ice before transfer to 25 °C. From the plot in Fig. 7C we calculate the half-life of the Lit state (signaling state) to be 27 min. This value is in remarkable agreement with the CRY Lit state values determined from partial proteolysis (Fig. 4) and CRY–JET binding (Fig. 6) experiments. Taken together, the decay data along with previous ultrafast kinetics on CRY (26) are consistent with the following reaction scheme:

\[
CRY_D(FAD^+) \xrightarrow{lu} \overset{10^4 \text{ s}^{-1}}{\xrightarrow{10^{-3} \text{ s}^{-1}}} CRY_D(FAD^+) + \nu \\
\rightarrow CRY^2(FAD^+) \rightarrow CRY_D(FAD^+).
\]

Where (\(\nu\)), (\(\nu\)), and (\(\nu\)) represent “dark state,” “excited state,” and the decay-constant, respectively.
and “signaling (Lit) state” CRY, respectively, and the values below the reaction arrows indicate the approximate values for the first-order rate constants of the respective reactions.

Some caution is warranted in interpreting light-induced conformational changes and their mechanistic relevance. For example, flavodoxin undergoes light-induced photooxidation with an accompanying conformational change (31). However, flavodoxin has no known photosensory function. Conversely, not all flavoproteins that carry out photosignaling exhibit a detectable light-induced conformational change (31). For example, as shown in SI Text, we find that the zebrafish CRY4 does not exhibit any light-induced conformational change under the conditions of partial proteolysis used with Drosophila CRY. This is in spite of the fact that zebrafish CRY4 is thought to be a photosensory CRY and the purified protein possesses a full complement of flavin that is photoreduced in vitro. The results with zebrafish CRY4 show that the light-induced conformational change in Drosophila CRY is not a response that is common to all CRYs and support the conclusion that a light-induced conformational change is the mechanistic basis for the photosensory role of Drosophila CRY. However, the greatest support for this conclusion is provided by the structural similarity between the constitutively active CRYΔ and the Lit state CRY2, the light-dependent (CRY) and -independent (CRYΔ) interactions with JET, and in addition the finding that not only is a long-lived, Lit state of CRY observed in vitro, but a long-lived, active state of CRY, which is a substrate for the UPS system, is formed by light in vivo.

**Discussion**

Currently, 3 classes of photosensory flavoproteins are known (12, 28, 31): Cryptochrome, LOV domain photoreceptors, and BLUF domain photoreceptors. Although CRY was the first flavin-based sensory photoreceptor to be discovered, more progress has been made in understanding the signaling mechanisms of LOV and BLUF proteins.

In LOV domain proteins blue light generates an excited singlet state of FMN that by intersystem crossing produces a long-lived (1–2 μs) flavin triplet state. The 3FMN reacts with a cysteine in the flavin binding site, leading to the formation of a covalent cysteinylligadduct of flavin in which the flavin is formally in a two-electron reduced state. This adduct formation is accompanied by a blue shift of the absorption maximum from 450 to 390 nm. This change in the redox state and the covalent structure of FMN, and the accompanying conformational change of the cofactor is transmitted through a change in the H-bonding network leading to the displacement of an α-helix folded upon the LOV domain (32, 33). The resulting conformational change signal is transmitted to the rest of the molecule with functional consequences such as inducing the latent kinase activity associated with the photoreceptor polypeptide, changing the quaternary structure of the photoreceptor and potentially changing its interacting partners, and the subcellular localization of the protein.

In BLUF domain proteins, the FAD excited state singlet that is formed by absorption of a blue-light photon abstracts an electron from a nearby Trp residue to form FADH*, which rapidly takes up a proton to form FADH++. Finally, a H-atom abstraction from the latter generates the long-lived (3–2,000 s) flavin that is, like the ground-state flavin, 2-electron oxidized; but now is 10-nm red-shifted relative to the ground-state flavin (28). The formation of this red-shifted flavin, FADoxED, is associated with H-bond rearrangement around the FAD binding site and causes a conformational change in the photoreceptor that may induce a latent enzymatic activity (adenyl cyclase) or alter protein–protein interactions that are involved in gene regulation (AppA).

In contrast to the photophysics/photochemistry of LOV and BLUF proteins we have only limited knowledge of CRY photo-reception/phototransduction mechanisms. If we don’t take into account the so-called CRY-DASH proteins, which turned out to be photolyases specific for pyrimidine dimers in single-stranded DNA (34), the current state of knowledge regarding CRY action mechanism might be summarized as follows: (i) Photolyase, which evolutionarily is related to CRY contains the flavin cofactor in the two-electron reduced and deprotonated FADH* form and carries out catalysis by a nonreductive cyclic electron transfer reaction (9, 35). In contrast, the redox state of FADH* in CRY is not known with certainty. Although when plant and insect CRYs, expressed in heterologous systems, are purified they contain flavin in the two-electron oxidized state (36, 37) they are readily reduced by light to FADH* (36) and FADH2 (5–7) and there are some experimental data indicating that the FADoxED form is an artifact generated by exposure to air during purification (8). (ii) Some CRYs (Arabidopsis CRY1 and human CRY1 and CRY2) but not others (Arabidopsis CRY2 and insect Type 1 CRYs) have autokinase activity (30, 38–40). Furthermore, the crystal structure of Arabidopsis CRY1 crystallized in the presence of ATP reveals that the ATP is located in the cavity leading to flavin, which corresponds to the photodimer binding site in DNA photolyases (41, 42). Whether the autokinase activity is stimulated by light
and whether the ATP detected in the crystal structure is the one used for the kinase reaction are not known with any certainty. (iii) In Arabidopsis, CRY binds to COP1 E3 ligase (which ubiquiti-nates and inactivates transcription factors) independently of light but affects (inhibits) COP1 activity only under light exposure (15, 16). In contrast, in Drosophila, CRY activates/binds to JET E3 ligase essentially in a light-dependent manner (20) and light activates the ubiquitination and destruction of CRY and its binding partner TIM (17). (iv) All bona fide CRYs have C-terminal extensions beyond the photolyase homology region (PHR), which range from ~20 amino acids in Drosophila CRY to ~250 amino acids in Arabidopsis CRY1 (2, 14, 36). (v) Light causes a significant conformational change in the C-terminal extensions of Arabidopsis CRY1 (14, 43) and Drosophila CRY (this work) as revealed by the effect of light on the partial proteolysis profile. (vi) The C-terminal extensions of Arabidopsis CRYs when separated from the PHR domain confer a constitutive “light-on” phenotype (15). In contrast, the Drosophila CRY PHR domain when separated from the C-terminal 20 amino acid extension confers as a C-terminal cap folded upon the PHR domain in a manner analogous to the N-terminal cap of the LOV domain photoreceptor VIVID (44, 45). Upon absorption of a blue-light photon the FAD− goes through its photophy-sical cycle in <1 ns; however, the excited (FAD−)∗ doublet or quartet state causes a modest bending motion around the N5−N10 axis (27, 31) leading to a significant change in the H-bonding network around the flavin binding site that is transmitted to the C-terminal cap, causing “opening” of the cap (as evidenced by the increased sensitivity of the C-terminal hinge region to trypsin). This conformation is relatively stable and it is retained even after the flavin excited state is deactivated by internal conversion in <1 ns. The active (signaling state) conformation of the protein decays with a much longer half-life of approximately 15 min, during which time CRY does interact with JET strongly as shown here and with TIM as has been shown in previous studies (21, 46). These interactions lead to the ubiquitination and eventual proteolytic degradation of both CRY and TIM, relieving the repression of clock controlled genes and thus resetting the clock.

Materials and Methods

The plasmids used in this study, protein expression, purification, and immunoblotting as well as detailed description of biochemical and spectroscopic experiments are given in SI Text.

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Supporting Information

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SI Materials and Methods

Plasmids. The plasmids used in this study and abbreviated designations are as follows (all CRYs are DmCRY unless designated otherwise): pAc5.1-dCRY-V5/HisA, pAc5.1-Δ-β-Gal-V5/HisA, pFast-Flag-His-DmCRY (FH-DmCRY), pFast-Flag-His-DmCRYΔ (FH-CRYΔ), pFast-DmCRY-V5His (CRY-V5H), pFast-DmCRYΔ-V5His (CRYΔ-V5H), pFast-ZiCRY4-V5His (ZiCRY4-V5H), and pGEXT4T1-His-Jetag (JET). pAc5.1-dCRY-V5/HisA, pAc5.1-Δ-β-Gal-V5/HisA (1), and pFast-Flag-His-DmCRY (2) have been described previously. pFast-ZiCRY4-V5His was constructed by inserting the ZiCRY4-V5His coding sequence from pAc5.1-ZiCRY4-V5HisA (3) into pFastBac1 (Invitrogen). pFast-DmCRY-V5H was constructed by inserting the DmCRY-V5His coding sequence from pAc5.1-Δ-ZiCRY4-V5HisA into pFastBac1 (Invitrogen). pGEXT4T1-His-Jetag was constructed by inserting the amplified Jetag coding sequence (6 x His tag was included in Forward oligo in frame) from yellow-white (yw) Drosophila embryonic cDNA (kindly provided by Maki Asano, Ohio State University) into pGEXT4T1 (GE Healthcare) and L247 was converted to V using site-directed mutagenesis to restore the wild-type sequence. pFast-Flag-His-DmCRYA and pFast-DmCRYΔ-V5His were constructed by deleting residues 521–542 using conventional cloning methods.

Cell Lines. The Sf21 insect cell line used for virus construction/amplification and protein purification was obtained from Stratagene. The Drosophila Schneider S2 cell line was obtained from Steven M. Reppert (University of Massachusetts), and was cultured and transfected as described previously (3).

Baculoviruses. Baculoviruses were prepared using the Gibco BRL Bac-to-Bac baculovirus expression system (Invitrogen). Briefly, pFastBac1 based plasmids containing target coding sequences were transformed into Escherichia coli DH10Bac. Recombinant, bacmid vectors were isolated from 1 mL of bacterial cultures grown from colonies of transformants. For recombinant virus generation, Sf21 cells in six-well plates were transfected with 1 μg of bacmids using 6 μL of Cellfectin reagent (Invitrogen), and parental virus was collected after 72 hours. After three more 72-hour virus amplification steps, the fourth passage (P4) high titer stock was obtained and used to infect S2 cells for large-scale expression of protein.

Protein Purification. To purify recombinant proteins from insect cells, 1 liter of Sf21 cells (106 per mL) growing at 27 °C in spinner flasks was infected with the P4 high titer virus at 1:100 (v/v) ratio, and the cells were harvested 2 days later and lysed in 30 mL of lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP40, 0.5% Triton-X100 with 10× of sonication for 10 sec each time on ice. The cell lysates were cleared by centrifugation at 17000 × g for 1 hour. The flag tagged proteins were purified using antiFLAG M2-agarose beads (Sigma). Briefly, 400 μL of Flag-agarose beads was incubated with 30 mL of cleared cell lysate for 2 hours and then washed three times with 15 mL of 1 × TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) to remove unbound proteins. Recombinant proteins were eluted from Flag-agarose beads in 4 mL of 1 × TBS containing 100 μg/mL Flag peptide (Sigma). Eluted proteins were dialyzed against Storage Buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol, and 50% (v/v) glycerol. Recombinant proteins containing the V5His tags were purified using nickel affinity chromatography. The cell pellets from 1 liter cultures were lysed in 30 mL lysis buffer with 10 times of sonication for 10 sec each time on ice. The cell lysates were cleared by centrifugation at 17000 × g for 1 hour and 30 mM imidazol was added to prevent nonspecific binding to Ni-agarose beads. 500 μL of Ni-agarose beads was incubated with each cell lysate at 4 °C for 2 hours, and unbound proteins were washed away with 3 washes of lysis buffer containing 30 mM imidazol. Recombinant proteins were eluted from the beads with 1 × TBS containing 250 mM imidazol for 1 hour and then dialyzed against Storage Buffer. Typical yields were about 2 mg of CRY and 0.5 mg of CRY A from 1 l of culture. The purified proteins contained an essentially stoichiometric amount of FAD as determined by the absorbance at 440 nm/absorbance at 280 nm ratio as described previously (2).

Light-Induced Degradation of CRY in S2 Cells. The methods used to transfect S2 cells, to express CRY-V5H and beta-galactosidase, irradiate transfected cells, incubation media, and the measurement of CRY-V5H and beta-galactosidase proteins were described in detail previously (3). In this study, transfected S2 cells, in 35-mm diameter tissue culture dishes, were maintained in the dark, and were cooled by floating on ice water before irradiation. Dishes floating on ice water were individually exposed (controls remained in the dark) to a single flash (white light) from a camera attachment (Wildlar). Cells were irradiated through a glass slide, and the lids of the tissue culture dishes were removed for this treatment. After the flash, dishes were incubated for various times on ice water in the dark. Each dish was then incubated at room temperature for 1 hour. Room temperature was achieved by floating dishes on top of a volume (about 1.5 liters) of water (which was stirred) at room temperature. Cells were then harvested and the levels of CRY and beta-galactosidase were analyzed by Western blot.

Partial proteolysis. For the trypsin titration, 5 μg of DmCry-V5His and ZiCRY4-V5His were aliquoted into eppendorf tubes containing 1× PBS (138 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4) pH 7.4 in a total volume of 50 μL, and reactions were initiated with the addition of 5 μL of sequencing grade trypsin (Promega) at final trypsin:protein ratios of 1:3200, 1:1600, 1:800, 1:400, 1:100, and 1:25 (w:w). Tubes were exposed to blue-light at 1 mW/cm2 without (light) or with (dark) aluminum foil covers. The blue-light source consisted of two F15T8-2BL Black Lights (General Electric) with emission maximum at 366 nm. Reactions were stopped with 15 μL of 5× SDS buffer (250 mM Tris, pH 6.8, 25 mM EDTA, 10% SDS, 5% β-mercaptoethanol, 30% glycerol) after 30 min at 25 °C. Then, 15 μL of each reaction was resolved by a 4 to 12% SDS-PAGE, and then proteins were transferred onto a PDVF membrane and immunoblotting was performed. A trypsin/protein ratio of 1:800 (w:w) was selected for further experiments because the best visualization of light-dependent proteolysis was obtained at this concentration. For decay of the active conformation and chemical reduction/trypsinization experiments, samples were trypsinized at 1:100 (w:w) ratio for 10 min because these experiments required shorter time points. Notice that the light-induced fragment was not visible using these conditions. Densitometric quantification of antibody-reactive bands or total protein was performed with ImageQuant 5.0 software (GE Healthcare) and expressed relative to undigested controls in each experiment. Data from at least 3 independent experiments are plotted and standard error of the mean (SEM) is shown.
**In vitro pull down assays.** GST-His-Jetlag was expressed in 1 liter of *E. coli* BL21 strain (Stratagene) with 0.3 mM IPTG (Promega) for 5 hours and adsorbed to Glutathione Sepharose 4B according to manufacturer’s instructions (GE Healthcare). GST-His-Jetlag-sepharose complexes, which contained approximately 10 mg protein per mL sepharose, were kept at 4 °C in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl. Purified CRY (5 μg) was added to 10 μL of GST-His-JET-sepharose beads containing 10 μg/μL of GST-His-JET in 50 μL of 1× TBS containing 100 ng/μL BSA. Eppendorf tubes were incubated for 10 min under μ of GST-His-JET in 50 μL of pH 6.8, 10 mM EDTA, 4% SDS, 2% reduction, CRY at were measured immediately after photoreduction. For chemical (Precision Glass and Optics) to prevent excessive heating. Spectra wavelengths contaminants and through a heat-reflecting glass at 25 °C. Light was filtered through a glass plate to eliminate short-

**Immunoblot.** Samples separated on SDS-PAGE were transferred onto 0.2 μm PVDF membranes (Sigma) for 50 min at 12 volts. PDVF membranes were treated with blocking solution (1× TBS containing 0.1% Tween-20, 5% nonfat dry milk) for 1 hour at room temperature and incubated with primary antibodies overnight at 4 °C, washed 4 times with TBS containing 0.1% Tween-20 (TBS-T), and incubated with HRP-conjugated antismouse antibo-

**Absorption spectra.** Absorption spectra were obtained using a Shimadzu UV-601 spectrophotometer. For photoreduction, CRY at μL of PBS containing 5 mM DTT was placed in a quartz cuvette. The cuvette was sealed and a stream of argon was passed through the sample for 5 min at room temperature to obtain an anaerobic environment. Trypsin in 100 μL of PBS or only PBS was injected into the solution in the quartz cuvette. Irradiation of the reduced and mock-reduced samples began upon addition of trypsin. The reaction was stopped with SDS loading buffer.

**Molecular Modeling.** For the structural modeling of CRY, we used the LOOPP (Learning, Observing and Outputting Protein Patterns) server (http://cbuapps.tc.cornell.edu/loopp.aspx) and formed the closest match with the crystal structure of DNA photolyase from *Anacystis nidulans* (PDB ID: 1QNF). The C-terminal 25 amino acids of CRY (total length, 542 amino acids) that constitutes a separate domain with no homology to photolyases was not included in the modeling operation. The alignment of CRY and *A. nidulans* photolyase was performed and visualization of the predicted structure of CRY was performed using PyMOL molecular visualization system (http://www.pymol.org). The secondary structure of the C-terminus of CRY was predicted using SOPMA Secondary Structure Prediction Method at (http://npsa-pbil.ibcp.fr/).

**Mass Spectroscopy and Identification of Tryptic Sites.** Partially trypsinized CRY-V5His was resolved on a 4–12% NuPAGE Bis–Tris SDS-PAGE (Invitrogen), and visualized by Coomassie Blue Staining. Mass spectrometric analysis was performed by the University of North Carolina Mass Spectrometry Core Facility. This analysis identified the fragments 1–289 (N-terminus fragment of light insensitive of Site I cleavage), and 1–503 (N-terminus fragment of light enhanced Site III cleavage). The location of the light-induced Site II cleavage was determined as R430 using the following criteria. The approximate cut region was determined using the fragments size. Then, the possible trypsin cuts sites were located on the 3D modeled structure of CRY, and the R430 was selected because it was located at an exposed surface that makes it a strong candidate for trypsin attack. The light insensitive trypsin cut (Site Ia) was estimated using the molecular size of the N-terminal fragment. There are two possible sites for trypsin digestion in the C-terminus, at R524 and R532. It is unclear which of these sites is digested resulting in the Site IV seen in our gels.

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**Fig. S1.** Comparison of partial trypsinization sites using CRY-V5H. (Left) The conformations were probed by partial proteolysis with trypsin at 25 °C for 30 min and 1:800 (trypsin: CRY) ratio (v:v). The digested proteins were separated by 4–12% gradient SDS-PAGE and probed with the appropriate antibodies against the V5 tag at the C-termini. The longer exposure of the lower panel on the left shows that Site III is light enhanced, that is, there is a low level of digestion even in the dark. (Right) The entire immunoblot of the same samples was presented to display the overall partial proteolysis pattern. The right panel shows that Site I is far more sensitive to trypsin than Site II. C, control sample with no protease treatment; D, protein that was treated with trypsin in the dark; L, protein treated with trypsin under blue-light.
Fig. S2. Effect of blue light on the absorption spectrum and conformation of ZfCRY4-V5H. The protein was treated as described in Fig. 1B and Fig. 2A. (A) Spectroscopic and photochemical properties of ZfCRY4-V5H. The near UV-visible spectrum is shown. Solid line, purified protein kept in the dark exhibits characteristic FADox spectrum. Dashed line, absorption spectra after exposure to blue-light reveals that most of ZfCRY4-V5H is converted to the FADH\(^{-}\) reduced form. (B) The conformations were probed by partial proteolysis with trypsin at 25 °C for 30 min and 1:800 (trypsin:CRY) ratio (w:w). The digested protein was separated by 4–12% gradient SDS-PAGE and probed with the appropriate antibodies against the V5 tag at the C-terminus. Partial trypsinization of ZfCRY4-V5H was not affected by light. C, control sample with no protease treatment; D, protein that was treated with trypsin in dark; L, protein treated with trypsin under blue light.

Fig. S3. Tryptic cleavage sites and positions of amino acids in contact with the FAD cofactor of CRY. Potential flavin binding contacts based on *E. coli* photolyase flavin binding contacts (17, 42) (underlined red letters) and trypsin digestion sites (blue letters indicated by black arrows, this work) are labeled. There are two potential Arginines for Site IV cleavage, as indicated. The fragment deleted to construct CRY\(\Delta\) is underlined and labeled in blue.
Fig. S4. Model of CRY function and light-induced conformational change. In the dark, JET binds to CRYΔ, but the C-terminus of full-length CRY blocks a stable CRY–JET interaction. Exposure to light has no effect on the structure of CRYΔ, but light changes the conformation of full-length CRY to an open structure that exposes its JET binding site.

Fig. S5. Computational 3D model of CRY based on the crystal structure of *Anacystis nidulans* photolyase (1). The centrally located flavin is blue. The red, yellow, and green colors represent helix, sheet, and loop secondary structures, respectively. The trypsin cleavage sites of interest are marked by arrows. Because the C-terminal 22-amino acid extension has no counterpart in photolyase this region was not included in the model; instead it is shown in the textbox in the bottom with secondary structure prediction: h, helix; c, coil; e, extended strand.