

Formation and Function of Flavin Anion Radical in Cryptochrome 1 Blue-Light Photoreceptor of Monarch Butterfly*

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Sang-Hun Song^{†,1}, Nuri Öztürk^{†,1}, Tracy R. Denaro^{†,1}, N. Özlem Arat[‡], Ya-Ting Kao[§], Haisun Zhu[¶], Dongping Zhong[§], Steven M. Reppert[†], and Aziz Sancar^{†,2}

From the [†]Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, the [§]Departments of Physics, Chemistry, and Biochemistry, Ohio State University, Columbus, Ohio 43210, and the [¶]Departments of Neurobiology, University of Massachusetts Medical School, Worcester, Massachusetts 01650

The monarch butterfly (*Danaus plexippus*) cryptochrome 1 (DpCry1) belongs in the class of photosensitive insect cryptochromes. Here we purified DpCry1 expressed in a bacterial host and obtained the protein with a stoichiometric amount of the flavin cofactor in the two-electron oxidized, FAD_{ox}, form. Exposure of the purified protein to light converts the FAD_{ox} to the FAD[·] flavin anion radical by intraprotein electron transfer from a Trp residue in the apoenzyme. To test whether this novel photoreduction reaction is part of the DpCry1 physiological photocycle, we mutated the Trp residue that acts as the ultimate electron donor in flavin photoreduction. The mutation, W328F, blocked the photoreduction entirely but had no measurable effect on the light-induced degradation of DpCry1 *in vivo*. In light of this finding and the recently published action spectrum of this class of Crys, we conclude that DpCry1 and similar insect cryptochromes do not contain flavin in the FAD_{ox} form *in vivo* and that, most likely, the FAD_{ox} $\xrightarrow{h\nu}$ FAD[·] photoreduction reaction is not part of the insect cryptochrome photoreaction that results in proteolytic degradation of the photopigment.

Cryptochromes are blue-light photoreceptors that control growth and development in plants and the circadian clock in animals (1–3). They have sequence and structural similarities to DNA photolyases that repair far UV-induced DNA damage using blue light as a co-substrate. Photolyases contain two-electron reduced deprotonated FAD (FADH[·]) as the catalytic cofactor and methenyltetrahydrofolate (MTHF)³ or, in rare cases, 8-deazaflavin as a photoantenna for gathering photons and transferring excitation energy to FADH[·] to initiate catalysis (2). Cryptochromes from plant and animal sources, as well,

contain FAD and are known or presumed to contain MTHF (4–6). However, the functional redox state of flavin in cryptochromes is unknown with certainty, and there is no evidence that the flavin cofactor in cryptochrome functions as a redox catalyst (1, 3).

Arabidopsis thaliana cryptochrome 1 (AtCry1), which was the first cryptochrome to be discovered (7), has been the most extensively investigated. The AtCry1, as well as the related AtCry2, can be purified as recombinant proteins expressed in *Escherichia coli* or baculovirus/insect cell expression systems (4, 5, 8). The photopigments purified from these expression systems contain trace amounts of MTHF and stoichiometric flavin cofactor in the two-electron oxidized, FAD_{ox}, form. A number of interesting observations have been made with *Arabidopsis* cryptochromes, in particular with AtCry1, which may be relevant to their functions. First, AtCry1 binds ATP in the cavity containing the flavin cofactor (8–10). Second, AtCry1 exhibits an autophosphorylating kinase activity (9–11). Third, the FAD_{ox} of AtCry1 is photoreduced to the FADH[·] neutral radical by intraprotein electron transfer both *in vitro* and *in vivo* (4, 12). Fourth, this intraprotein electron transfer causes a conformational change in the C-terminal tail of AtCry1 (13, 14), which is known to play a key role in signal transduction. Fifth, the intraprotein electron transfer and, presumably, the accompanying conformational change activates or greatly stimulates the intrinsic kinase activity of AtCry1 (9, 11). Sixth, in support of the previous findings, it was reported that mutation of the Trp residue that functions as the electron donor in photoreduction abolished the stimulatory effect of light on the kinase activity (15). Based on these findings, a photocycle for plant cryptochromes has been proposed (12). Blue light reduces the FAD_{ox} to FADH[·], which is the signaling form of plant cryptochrome. Absorption of a green light photon by FADH[·] further reduces it to FADH² and turns off the signal. Facile oxidation of FADH² to FAD_{ox} under aerobic conditions regenerates the active form of cryptochrome and completes the photocycle.

In contrast to the plant cryptochromes, there is rather limited information on the photochemical properties of animal cryptochromes. This is, to a large extent, due to the unavailability of animal cryptochromes with full cofactor complement and in large quantities (3, 8). Human cryptochromes purified using bacterial and insect cell expression systems contain grossly sub-

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¹ These authors contributed equally to this work.

² To whom correspondence should be addressed. Tel.: 919-962-0115; Fax: 919-966-2852; E-mail: Aziz_Sancar@med.unc.edu.

³ The abbreviations used are: MTHF, methenyltetrahydrofolate; MBP, maltose-binding protein; DpCry1, *D. plexippus* cryptochrome 1; AtCry1, *Arabidopsis thaliana* cryptochrome 1; DmCry, *Drosophila melanogaster* Cry.

stoichiometric flavin and only trace amounts of MTHF (3, 8). However, recently the monarch butterfly (*Danaus plexippus*) Cry1 (16) expressed in *E. coli* (17) and the *Drosophila melanogaster* Cry (DmCry) expressed in Sf21 insect cells (18) were found to contain near stoichiometric flavin in the form of FAD_{ox}. Interestingly, exposure of these cryptochromes to blue light led to the photoreduction of flavin to the FAD[−] flavin anion radical (17, 18) instead of the FADH[·] flavin neutral radical observed in plant cryptochromes (4, 12). The photochemically generated flavin anion radical was reoxidized to FAD_{ox} in the dark, and thus, it was proposed that, in at least these animal cryptochromes, the $\text{FAD}_{\text{ox}} \xrightleftharpoons[\text{dark}]{\text{h}\nu} \text{FAD}^{\cdot}$ constitutes the photo-cycle, and the FAD[−] form of cryptochrome represents the signaling state of the photoreceptor (18). In this study, we blocked the $\text{FAD}_{\text{ox}} \xrightleftharpoons{\text{h}\nu} \text{FAD}^{\cdot}$ transition by site-specific mutagenesis to test the biological relevance of this photochemical reaction in *Drosophila* Cry-like animal cryptochromes. Our results show that blocking the photoreduction of FAD_{ox} in DpCry1 by intramolecular electron transfer does not affect its circadian photoreception function as measured by light-induced proteolytic degradation of the cryptochrome.

EXPERIMENTAL PROCEDURES

Cloning of DpCry1 into a Bacterial Expression Vector—The DpCry1 was amplified by PCR using primers designed from the sequence that is in public databases and the pAC5.1 DpCry1 plasmid carrying the cDNA of DpCry1 as a template (16). The gene was inserted into the pMal-c2 bacterial expression vector (New England Biolabs). The plasmid, which was named pDpCry1, encodes DpCry1 fused to the C-terminal end of *E. coli* maltose-binding protein (MBP). The entire cloned gene was sequenced to ensure that no mutation was introduced into DpCry1 during PCR and the subsequent cloning manipulations. To investigate the role of photoreduction in DpCry1 activity, the W328F substitution was made in pDpCry1 and in pACS.1 DpCry for *in vitro* and *in vivo* assays, respectively. In both cases, the mutated gene was sequenced in its entirety to ensure the absence of incidental sequence alterations.

Purification of DpCry1—The pDpCry1 or pDpCry1-W328F plasmids were inserted into *E. coli* BL21 (Strategene). Fresh colonies were picked and grown in Luria broth, in 1-liter batches, at 37 °C to $A_{600} = 0.5$. Then, the cultures were transferred to 15 °C and incubated for an additional 12–16 h. The cells were collected by centrifugation and resuspended in 20 ml of Buffer A per liter of culture. Buffer A contained 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol. The cell suspension was frozen in dry ice/ethanol and kept at −80 °C. To prepare cell-free extract, the cells were thawed and were then incubated on ice with lysozyme at 1 mg/ml for 30 min. After an additional freeze-thaw cycle, the cells were sonicated 10 × 10 s with a Branson sonicator. The cell debris was removed by centrifugation at 4 °C for 90 min in a Ti 45 rotor at 35,000 rpm in a Beckman L-80 centrifuge. Cell-free extract from a 12-liter culture was loaded onto a 20-ml amylose column (New England Biolabs) equilibrated with Buffer A. The column was washed with 100 ml of Buffer A, and MBP-DpCry1 was eluted with 32 ml of Buffer A containing 300 mM NaCl and 15

mm maltose. Two-ml fractions were collected, and samples were analyzed by SDS-PAGE and Coomassie Blue staining. Fractions containing MBP-DpCry1 were combined and concentrated by ultrafiltration using Vivaspin 20 (Qiagen) and centrifugation at 5,000 rpm in a Sorvall RC5B plus centrifuge. Unless otherwise indicated, the cryptochrome was dialyzed against Storage Buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol, and 50% (v/v) glycerol. Typical yield was ~2 mg of cryptochrome from a 12-liter culture.

To prevent photochemical modification of DpCry1 during purification, we undertook one of the following precautions. Either the entire purification procedure was carried out under dim yellow light, or Buffer A, and subsequent buffers used in the purification contained 20 mM potassium phosphate buffer, pH 6.8, instead of Tris-HCl buffer, and lacked reducing agents (β-mercaptoethanol or dithiothreitol) and EDTA, which also acts as a weak reductant.

Spectroscopy—Absorption and fluorescence spectra were obtained using a Shimadzu UV-601 spectrophotometer and a Shimadzu RF5000U spectrofluorometer, respectively.

Photoreduction—The wild-type DpCry1 and the W328F mutant DpCry1 in storage buffer were placed in a quartz cuvette and exposed to 366 nm light at a fluence rate of 2 milliwatts cm^{-2} using two F15T8-BLB black lights (General Electric) as an energy source. Light was filtered through a glass plate to eliminate short wavelength contaminants and through a heat-reflecting glass (Precision Glass & Optics) to prevent excessive heating.

Photoinduced Degradation of DpCry1—To test the photo-sensitivity of wild-type DpCry1 and DpCry1(W328F) to light-induced degradation (19–21), the cDNAs of these genes as well as that of DmCry (positive control) and DpCry2 (negative control) and β-galactosidase (loading control) were cloned into pAc5.1v5/HisA, a Schneider 2(S2) cell expression vector (16). The S2 cells were transiently transfected with the appropriate plasmids and incubated at 27 °C for 36 h. Then, the culture plates were exposed to 366 nm light at a rate of 1 milliwatt cm^{-2} . The dark control samples were wrapped in aluminum foil and treated similarly. The S2 cells were lysed, cellular proteins were separated by SDS-PAGE, and the levels of Crys and β-galactosidase were determined by Western blotting with monoclonal anti-V5 IgG (Invitrogen). The levels of the Crys were quantified by densitometry using ImageQuant 5.0 software (GE Healthcare) and expressed relative to the β-galactosidase internal control. Data from three experiments, with the S.E., are plotted.

RESULTS

Purification and Properties of DpCry1—Previously, it has not been possible to investigate the photochemical reactions catalyzed by animal cryptochromes such as *Drosophila* Cry, which are known to function as circadian photoreceptors (20), because these proteins were available only as recombinant proteins with 1–5% content of FAD, which is thought to be the catalytic cofactor. Recently, we discovered that of the two monarch butterfly cryptochromes, the one known to function as a photoreceptor, DpCry1 (16), can be expressed in *E. coli* as a fusion protein to a high level (17). More importantly, when

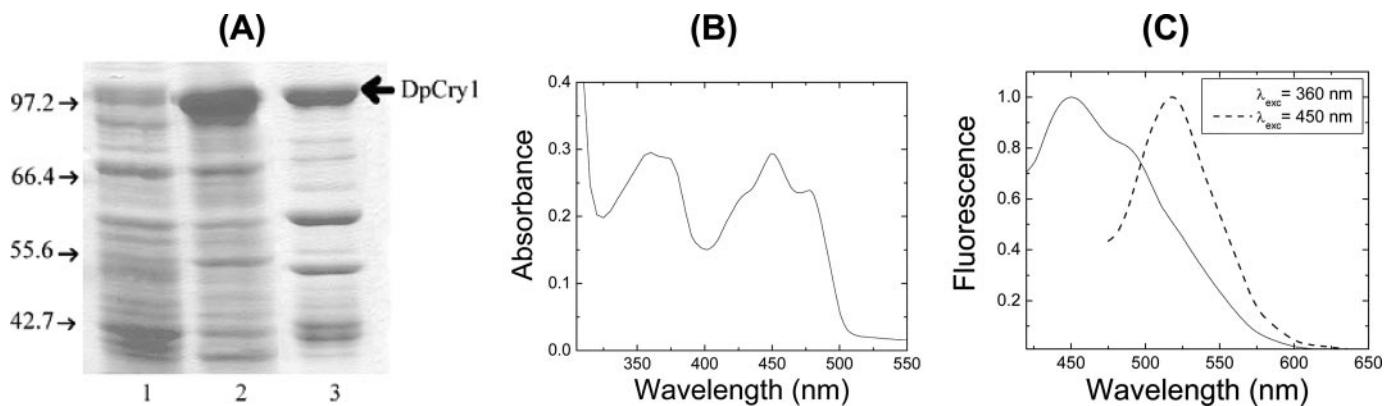


FIGURE 1. Purification and spectroscopic properties of monarch butterfly cryptochrom 1. *A*, analysis of purified protein by 10% SDS-PAGE. The numbers on the left indicate size markers. *Lane 1*, uninduced cell lysate. *Lane 2*, induced cell lysate. *Lane 3*, protein purified through amylose resin. The position of DpCry1 is indicated by an arrow. Extensive washing of the amylose resin by high salt buffer causes considerable loss of the flavin cofactor. Therefore, the bound protein was washed with a buffer of moderate ionic strength before elution with maltose, and as a consequence, the preparation contains contaminating proteins at a considerable level. However, those are not flavoprotein as ascertained from control purification schemes, and thus, they do not interfere with the analysis of the spectroscopic properties of DpCry1. *B*, absorption spectrum of DpCry1 purified as shown in *panel A*. *C*, normalized fluorescence emission spectra of DpCry1 recorded with $\lambda_{\text{exc}} = 360$ nm (solid line) and $\lambda_{\text{exc}} = 450$ nm (dashed line), respectively.

recombinant DpCry1 was purified from *E. coli* (Fig. 1A), it contained near stoichiometric amounts of flavin in the two electron-oxidized form (FAD_{ox}) (Fig. 1B). The photolyase/cryptochrom family members, in addition to flavin, are known or suspected to contain MTHF (or rarely, 8-deazaflavin) as second chromophores that are non-essential for activity but improve the efficiency of the photochemical reaction by functioning as a photoantenna that absorbs light and transfers the excitation energy to FAD (2). In the majority of photolyases and all known cryptochromes, MTHF dissociates from the apoprotein readily during purification (4–6). Thus, formal proof that Crys contain MTHF (or any other second chromophore) is lacking. Indeed, as is apparent in Fig. 1B, the UV-visible absorption spectrum of DpCry1 is typical of protein-bound flavin with no indication of an additional chromophore. We used fluorescence spectroscopy to test for the presence of MTHF at a level that is not detectable by absorption spectroscopy. As seen in Fig. 1C, excitation of DpCry1 at 360 nm results in fluorescence emission with a maximum at 480 nm and a shoulder at 505 nm. It is known that photolyase-bound MTHF has an absorption peak in the 370–415 nm range and fluorescence maximum at 460–480 nm (2). Thus, we conclude that DpCry1 does contain MTHF, albeit at grossly substoichiometric amounts in the purified pigment. Finally, excitation of DpCry1 at 450 nm where MTHF does not absorb, but FAD_{ox} does yield the characteristic flavin fluorescence emission at 520 nm. It should be noted that we measured the FAD:DpCry1 apoprotein ratio by estimating the apoprotein level by Coomassie Blue staining of SDS-PAGE gels containing known amounts of bovine serum albumin standards and the FAD level by absorption of the native protein at 450 nm. No accurate estimates can be made for the level of MTHF. Based on the absorption spectrum of DpCry1 and the known extinction coefficients of FAD_{ox} and MTHF in the 370–390 nm region (2), we estimate the MTHF content of DpCry1 to be <5%.

Photochemical Properties of DpCry1—Exposure of *Arabidopsis* Cry1 to blue light reduces FAD_{ox} to FADH^{\cdot} neutral radical, and this reaction has been considered to be the key step in the photocycle of AtCry1 (15, 18, 22). Thus, we investigated the effect of blue light on DpCry1. Unexpectedly, we found that

FAD_{ox} in DpCry1 was photoreduced to the flavin anion radical, FAD^{\cdot} , and not to FADH^{\cdot} , as is seen in *Arabidopsis* (Fig. 2A). Moreover, the quantum yield of the photoreduction was extremely high, in the range of 0.4–0.5 (Fig. 2C). This contrasts with the quantum yield of 0.01 for photoreduction of FAD_{ox} to FADH^{\cdot} in *Arabidopsis* Cry1 (15, 18). In fact, although AtCry1 can be purified with FAD_{ox} under normal laboratory lighting conditions, when DpCry1 is purified under the same condition, all of the flavin is in the FAD_2^{\cdot} form (17). To prevent photoreduction during purification, DpCry1 must be purified either under yellow light ($\lambda > 550$ nm), or reducing agents such as dithiothreitol, β -mercaptoethanol, and even EDTA must be excluded from the purification buffers. DpCry1(FAD^{\cdot}) is relatively stable and reoxidizes back to DpCry1(FAD_{ox}) under aerobic conditions with a life time of 54 s under our reaction conditions (Fig. 2C). It must also be noted that under our experimental conditions, even very long irradiation of DpCry1 failed to convert the flavin to the two-electron reduced FADH^{\cdot} form (data not shown).

Physiologic Role of Photoreduction—In *E. coli* photolyase, the Trp triad, $\text{FADH}^{\cdot} \leftarrow \text{Trp-382} \leftarrow \text{Trp-359} \leftarrow \text{Trp-306}$, is the photoreduction pathway with Trp-306 being the ultimate electron donor (23–25). Interestingly, this Trp triad is conserved in most DNA photolyases and cryptochromes (1, 3, 15). It has been shown that this pathway plays no role in the photocycle of photolyase (23, 24), which contains the flavin in the FADH^{\cdot} form *in vivo* (2, 24). In contrast, it has been reported that *Arabidopsis* Cry1 contains the flavin in the FAD_{ox} form *in vivo*, that the photoreduction is part of the catalytic photocycle, and that the photosignaling activity of AtCry1 is eliminated by the Trp \rightarrow Phe mutation of the Trp residue corresponding to the *E. coli* Trp-306 (15). We wished to find out whether photoreduction of FAD_{ox} in DpCry1 was a key reaction in the photocycle of this cryptochrom as well. To this end, we mutated Trp-328 in DpCry1, which is the structural counterpart of Trp-306 of *E. coli* photolyase. Mutating the Trp residue corresponding to Trp-306 of *E. coli* photolyase to investigate intraprotein electron transfer and its role in cryptochrom function is preferable to mutating the Trp residues that correspond to Trp-359 and Trp-

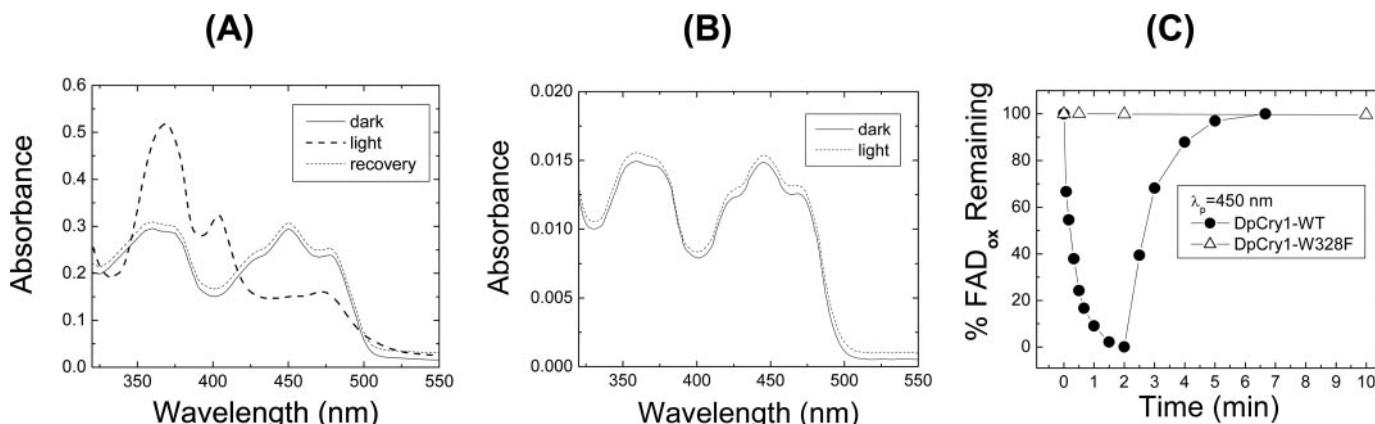


FIGURE 2. Photoreduction of DpCry1 wild-type and W328F mutant proteins. *A*, absorption spectra of DpCry1 in the dark and after light exposure. *Solid line*, spectrum before light exposure. *Dashed line*, spectrum after exposure to 366 nm at a rate of $200 \text{ J m}^{-2} \text{ s}^{-1}$ for 10 min. This is a characteristic anion radical spectrum (29). *Dotted line*, the anion radical was incubated in dark and aerobic conditions for 10 min. As seen, the radical is reoxidized back to the FAD_{ox} form. *B*, absorption spectrum of DpCry1 (W328F). *Solid line*, before light exposure. *Dotted line*, after exposure to 366 nm at a rate of $200 \text{ J m}^{-2} \text{ s}^{-1}$ for 10 min. *C*, kinetics of photoreduction of DpCry1 and of DpCry1 (W328F) by 366 nm light. *Filled circle*, DpCry1. *Open triangle*, DpCry1 (W328F). The wild-type protein was irradiated with 366 nm at a rate of $200 \text{ J m}^{-2} \text{ s}^{-1}$ for 2 min and then placed in the dark, and absorption spectra were recorded at the indicated times. The mutant was irradiated under the same conditions for 10 min, and absorption spectra were recorded at the indicated time intervals. The level of the FAD_{ox} \rightarrow FAD⁻ conversion, achieved at 10 min with the wild-type enzyme, was considered to be 100%, and the values at other time points are expressed relative to this value as determined by $(\Delta A_{450})_t / (\Delta A_{450})_{t=10 \text{ min}}$.

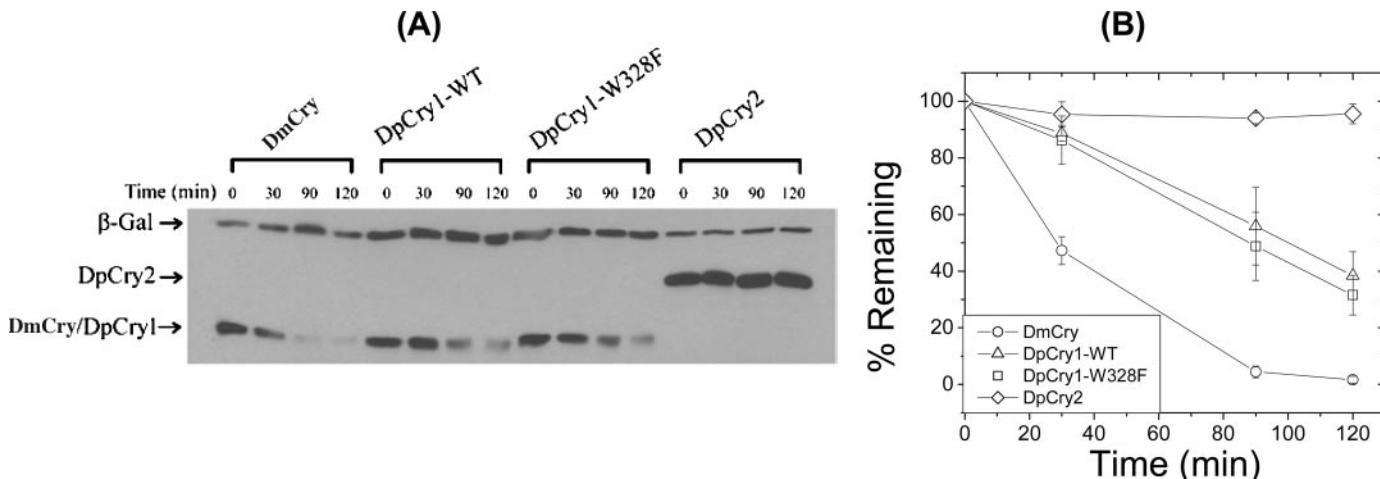


FIGURE 3. Photoinduced degradation of DmCry, DpCry1, DpCry1 (W328F), and DpCry2 in S2 cells. Cells were transfected with plasmids encoding the indicated cryptochromes and then exposed to 366 nm light for the indicated times. The cell lysates were analyzed by Western blotting. *A*, representative Western blot. β -gal, β -galactosidase. *B*, quantitative analysis of photoinduced degradation as a function of light dose. Averages from three experiments including the one shown in panel *A* are plotted. Bars indicate the S.E. Circle, DmCry; triangle, DpCry1; square, DpCry1 (W328F); diamond, DpCry2. The slower rate of degradation of DpCry1 relative to DmCry is consistent with a previous report (21) and may be due to the use of a heterologous cell line (S2) as a host for DpCry1.

382 in photolyase because Trp-306 and its counterparts in cryptochromes are located on the surface of the proteins (10, 24), and mutating these residues does not destabilize the proteins, whereas mutating Trp-359 and Trp-382, which are located in the core of the apoenzymes, causes significant destabilization (23), making the photochemical properties of these mutants open to alternative interpretations. Thus, we confined our analysis to the DpCry1 (W328F) mutant.

We tested the DpCry1 (W328F) mutant protein *in vitro* for photoreduction and *in vivo* for photosignaling as measured by light-induced DpCry1 degradation. Fig. 2, *B* and *C*, show that the W328F mutation blocked photoreduction of FAD_{ox} in DpCry1 as expected. Interestingly, however, this mutation had no effect on photodegradation of DpCry1 in S2 insect cells (Fig. 3), leading us to conclude that in DpCry1 and other insect Cry1 type (*Drosophila*-like) cryptochromes, the photogeneration of

FAD⁻ anion radical is not required for the photoreceptor function of cryptochrome.

DISCUSSION

Excitation of flavin by blue light in the majority of flavoproteins leads to quenching of the excited state at a much faster rate than that of free flavin (2). This is, almost invariably, due to electron transfer from neighboring redox active amino acids such as tryptophan, tyrosine, and histidine to the excited singlet state flavin, which is a potent oxidant. This property of flavoproteins has been extensively used to study the physical-chemical aspects of intra-protein electron transfer (26). Naturally, in enzymes that utilize ground state flavin to catalyze redox reactions, such as glucose oxidase or cholesterol oxidase that carry out catalysis independently of light, the photoinduced reduction of flavin has no bearing on enzyme activity under physiological conditions.

The significance, or the lack of, photoinduced electron transfer from aromatic amino acids to the flavin in photoreceptor flavoproteins such as photolyase and cryptochromes is more difficult to ascertain. Stringent criteria need to be applied to determine whether photoreduction of the flavin is a side reaction with no biological relevance or a key step in the photocycle of these flavin-based blue-light photoreceptors. In the case of DNA photolyases, overwhelming evidence indicates that the native form of the enzyme contains flavin in the FADH^- form, which, when excited by light directly or by energy transfer, is incapable of abstracting electrons from neighboring amino acids because of its high reduction potential. Instead, it initiates DNA repair by donating an electron to DNA photoproducts and catalyzes repair by a cyclic electron transfer reaction (2).

Our understanding of the cryptochrome photocycle, in contrast, is rather limited. There is no known redox reaction catalyzed by cryptochromes. As a consequence, alternative roles for flavin must be considered, and any light-induced reaction in these photopigments must be seriously considered as a potential candidate for the photocycle. Light has been shown to induce a conformational change in the C-terminal extension of AtCry1 (13, 14) and to stimulate the kinase activity of AtCry1 (9, 11). Moreover, this stimulation was reported to be caused by electron transfer through the Trp triad of AtCry1, and it was claimed that the blockage of intraprotein electron transfer through the triad abolished the photoreceptor function of AtCry1 *in vivo* (15). The model that has emerged from these studies is that blue light induces electron transfer from a specific Trp residue in the apoprotein to flavin to generate FADH^\bullet , and in the process, to cause a conformational change in the protein that activates its kinase function (9, 12, 15). The activated kinase, in turn, modulates the interaction of AtCry1 with the COP1 ubiquitin-protein isopeptide ligase (E3) ligase and inhibits the ubiquitination of HY5 and related light-responsive transcription factors by the ligase, prevents their proteolytic degradation, and thus, turns on blue-light response genes (1, 3). The findings reported here and elsewhere (17, 18), showing that photoreduction does occur in *Drosophila* Cry-type insect cryptochromes, have raised the possibility that these animal cryptochromes as well may have a somewhat similar photocycle. However, the model for the AtCry1 photocycle has encountered some serious difficulties. First, the light-induced autokinase activity has not been consistently reproducible (8). Second, AtCry2, which also functions as a blue-light photoreceptor exhibits no autokinase activity with or without blue light (8). Finally, the site-specific mutations that block intraprotein electron transfer in AtCry1 reduce not only the light-stimulated kinase but also the basal light-independent kinase activity (15), suggesting that they may exert their effects by causing a nonspecific overall protein misfolding rather than disruption of a specific pathway.

When considered in light of these findings in plant cryptochromes and other biochemical and photochemical findings in animal cryptochromes, it appears that the photoreduction of DpCry1, and by extension of other insect cryptochromes of the same class (27), is not part of the cryptochrome photocycle. Of particular significance, the action spectrum of *Drosophila* Cry, which exhibits a major peak in the 350–400 nm range and a plateau in the 430–450 nm range, does not match the absorption spectrum of FAD_ox (28), as would be expected from the Trp-me-

diated intraprotein photoreduction model. Finally, as we show here, blocking photoreduction of DpCry1 by site-specific mutagenesis does not affect its photoreceptor activity, at least not the photochemical reaction that promotes DpCry1 degradation. These latter two findings raise the possibility that Cry1-type insect blue-light photoreceptors may contain not FAD_ox but rather, FAD^\bullet as the catalytic cofactor and may mediate photoinduced redox reactions as part of their photocycle.

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