

Animal Type 1 Cryptochromes

ANALYSIS OF THE REDOX STATE OF THE FLAVIN COFACTOR BY SITE-DIRECTED MUTAGENESIS*

Received for publication, October 17, 2007, and in revised form, November 28, 2007. Published, JBC Papers in Press, December 5, 2007, DOI 10.1074/jbc.M708612200

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It has recently been realized that animal cryptochromes (CRYs) fall into two broad groups. Type 1 CRYs, the prototype of which is the *Drosophila* CRY, that is known to be a circadian photoreceptor. Type 2 CRYs, the prototypes of which are human CRY 1 and CRY 2, are known to function as core clock proteins. The mechanism of photosignaling by the Type 1 CRYs is not well understood. We recently reported that the flavin cofactor of the Type 1 CRY of the monarch butterfly may be in the form of flavin anion radical, FAD^{•-}, *in vivo*. Here we describe the purification and characterization of wild-type and mutant forms of Type 1 CRYs from fruit fly, butterfly, mosquito, and silk moth. Cryptochromes from all four sources contain FAD_{ox} when purified, and the flavin is readily reduced to FAD^{•-} by light. Interestingly, mutations that block photoreduction *in vitro* do not affect the photoreceptor activities of these CRYs, but mutations that reduce the stability of FAD^{•-} *in vitro* abolish the photoreceptor function of Type 1 CRYs *in vivo*. Collectively, our data provide strong evidence for functional similarities of Type 1 CRYs across insect species and further support the proposal that FAD^{•-} represents the ground state and not the excited state of the flavin cofactor in Type 1 CRYs.

Cryptochromes are photolyase-related flavoproteins that play important roles in regulating the circadian clock in animals and growth and development in plants (1–3). The mechanism of photosignaling by animal cryptochromes is not known. Previously, it was thought that CRYs³ in *Drosophila* and other insects function as circadian photoreceptors and in mouse and other vertebrates function as core components of the molecular clock (4). Recently, this view was revised when it was realized that some insects such as the honeybee possess only a mammalian CRY-like cryptochrome and others such as the monarch butterfly possess both *Drosophila* CRY-like and mammalian CRY-like cryptochromes (5, 6). It was proposed that *Drosophila*-like CRYs should be referred to as Type 1 CRYs and the mammalian-like CRYs should be referred to as Type 2 CRYs

(6). Furthermore, it was found that all Type 1 CRYs tested were subject to light-induced proteolysis in Schneider 2 (S2) cells and, hence, were considered to function as circadian photoreceptors in a manner analogous to DmCRY (6). Similarly, it was shown that insect Type 2 CRYs, like the mammalian CRYs, functioned as core clock proteins with no demonstrable photoreceptor activity (6).

We are interested in the photoreceptor function of CRY and specifically in the cryptochrome photocycle. Type 1 CRYs are well suited for this purpose because their photoinitiated proteolysis constitutes a convenient functional assay (7–9). Two recent studies reported that Type 1 CRYs from *Drosophila melanogaster* and the monarch butterfly (*Danaus plexippus*), purified as recombinant proteins, contained near-stoichiometric amounts of flavin in the two-electron oxidized, FAD_{ox}, form. Exposure of these CRYs to light reduced the flavin to the flavin anion semiquinone, FAD^{•-}, with high quantum yield (10, 11). Whereas it was speculated that this photoreduction reaction constituted the initial step of the DmCRY photocycle (11), experiments with DpCRY1 did not support this model as a general feature of Type 1 CRYs (10); it was found that a non-photoreducible mutant of DpCRY1 exhibited photoinduced proteolysis kinetics in S2 cells identical to that of wild-type DpCRY1 and, hence, it was proposed that FAD^{•-} actually represented the ground state of flavin in DpCRY1 and by extension in all Type 1 CRYs *in vivo*. It was, therefore, proposed that FAD^{•-} was converted to FAD_{ox} during protein purification under aerobic conditions (10).

In this study we extend the previous investigations by purifying wild-type and mutant Type 1 CRYs from four species: *D. melanogaster*, *D. plexippus* (monarch butterfly), *Anopheles gambiae* (mosquito), and *Antheraea pernyi* (Chinese oak silk moth). We find remarkable similarities among the Type 1 CRYs from these species with respect to their spectroscopic and photochemical and photobiological properties. Significantly, we extend the observation that blocking the *in vitro* photoreduction pathway does not affect the photoreceptor function *in vivo* and quite unexpectedly we find that Type 1 CRY mutants which cannot accommodate FAD^{•-} but possess FADH[•] flavin neutral radical are equally sensitive to photoinduced proteolysis as the wild-type photoreceptors.

EXPERIMENTAL PROCEDURES

Cloning of Type 1 CRYs into Bacterial and Insect Cell Expression Vectors—The cDNAs of Type 1 CRYs from *D. plexippus*, *A. gambiae*, and *A. pernyi* (6) were cloned into the pMal-c2 bacterial expression vector (New England Biolabs) by standard

* This work was supported by National Institutes of Health Grant GM31082. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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³ The abbreviations used are: S2 cells, Schneider 2 cells; CRY, cryptochrome; Tim protein, Timeless protein.

methods. The cloned genes were sequenced to ensure there were no accidental mutations. The resulting constructs expressed Type 1 CRYs as fusion proteins attached to the C-terminal end of *Escherichia coli* maltose-binding protein. Site-directed mutations in the cloned genes were introduced by standard methods using the QuikChange method (Stratagene). A viral vector for expressing *D. melanogaster* CRY with FLAG and His tags at the N terminus was prepared using the Invitrogen Bac-to-Bac Baculovirus expression system.

The cDNAs of Type 1 CRYs were also inserted into the pAc5.1v5/HisA vector for transient transfection into S2 cells to investigate photoinduced CRY degradation. Site-directed mutations of the subcloned genes were made by standard methods and verified by sequencing. In addition, the cDNA of DpCRY2 (negative control) and the β -galactosidase gene (loading control) were inserted into the same vector for co-transfection along with the Type 1 CRY clones (5).

Purification of Type 1 CRYs—The maltose-binding protein-CRY fusion proteins were expressed in *E. coli* BL21 strain (Stratagene) and purified as described previously for DpCRY1 (10). Typical yields were about 2 mg of CRY from a 12-liter culture. The purified proteins contained essentially stoichiometric amount of FAD. They were not analyzed for the presence of the folate cofactor, which we previously reported to be present in trace amounts in DpCRY1 preparations (10). The purified proteins were kept at -80°C in storage buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM dithiothreitol, and 50% (v/v) glycerol. Purification and handling of CRYs were carried out under dim yellow light ($\lambda > 550\text{ nm}$) to prevent accidental photoreduction. To purify DmCRY, Sf9 cells were infected with the DmCRY baculovirus, and the cells were harvested 2 days later. Cells were lysed as described previously (12), and the protein was purified using anti-FLAG M2-agarose resin from Sigma. Both wild-type and mutant proteins were obtained at $\sim 0.3\text{ mg}/300\text{-ml}$ culture and contained near-stoichiometric flavin.

Photoreduction—Cryptochromes in storage buffer were irradiated with 450 nm (10-nm bandwidth) in a monochromator (150-watt xenon lamp, Photon Technology International). The fluence rate was $11.8\text{ ergs}\cdot\text{mm}^{-2}\cdot\text{s}^{-1}$. Absorption spectra were recorded using a Shimadzu UV-601 spectrophotometer. Some photoreduction experiments were carried out with 366 nm from black light (General Electric) as described previously (10).

Photoinduced Type 1 CRY Degradation—S2 cells transfected with appropriate plasmids were exposed to 366-nm light at a rate of 1 milliwatt $\cdot\text{cm}^{-2}$, and the levels of the target CRYs and control proteins were determined by Western blotting with monoclonal anti-V5 IgG (Invitrogen) as described previously (10). Mutant DmCRY (W342F) was poorly expressed and typically 5-fold more vector of this mutant was needed than the vector expressing the wild-type protein to obtain comparable levels of expression. The levels of CRYs were expressed relative to the β -galactosidase internal control and quantified using ImageQuant 5.0 software (GE Healthcare).

RESULTS

Purification of Insect Type 1 Cryptochromes—Previously, we reported the purification of monarch butterfly CRY1 (DpCRY1) expressed in *E. coli* with the maltose-binding pro-

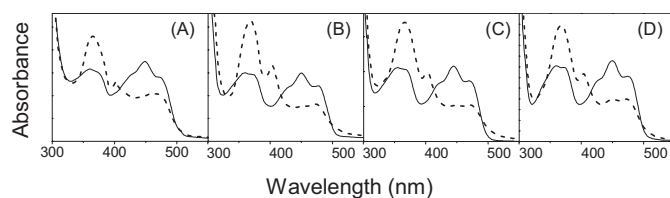


FIGURE 1. **Absorption spectra of Type 1 CRYs.** A, *Drosophila* CRY (DmCRY). B, monarch butterfly CRY1 (DpCRY1). C, silk moth CRY1 (ApCRY1). D, mosquito CRY1 (AgCRY1). Solid line, spectra of the FAD_{ox} forms of the enzymes. Dashed lines, spectra of enzymes photoreduced by exposure to 450 nm at a fluence rate of $1.18 \times 10^2\text{ erg}\cdot\text{mm}^{-2}\cdot\text{s}^{-1}$ for 5 min. The absorption spectra were normalized to absorbance at 450 nm to aid in comparison of the various enzyme preparations that were available at different concentrations.

tein tag (10). This tag aids both in solubilization and purification of the fused proteins. Hence, we expressed Type 1 CRYs from *A. gambiae* (AgCRY1) and *A. pernyi* (ApCRY1) as maltose-binding protein fusion proteins as well and purified them by affinity chromatography on amylose resin. Our previous attempt to purify *D. melanogaster* CRY (DmCRY) in this manner yielded an enzyme preparation with grossly substoichiometric flavin content (13). A recent report indicated that DmCRY expressed in a baculovirus/Sf21 vector/host system contains high levels of flavin (11). Independently, we expressed FLAG-tagged DmCRY in Sf9 cells and purified DmCRY with nearly stoichiometric content of FAD. Fig. 1 shows the absorption spectra of Type 1 CRYs of these four insect species. The absorption spectra look remarkably similar and exhibit vibrational fine structures characteristic of protein-bound flavin. Importantly, in all enzymes FAD_{ox} is converted to FAD⁻ by light exposure (Fig. 1) indicating that the similarities among these enzymes extends beyond the phylogenetic relationship and is reflected in their spectroscopic/photochemical properties as well.

Photochemical Properties of Type 1 CRYs with Mutations in the "Trp Triad" and the Flavin Binding Site—Flavin photoreduction in photolyase, plant, and animal CRYs is thought to proceed in large part by electron transport through three Trp residues that correspond to Trp-306 (distal)—Trp-359—Trp-382 (proximal) residues in *E. coli* photolyase (14–17) in which the flavin photoreduction through the Trp triad was discovered (Fig. 2A). The photoreduction reaction generates flavin neutral radical in *Arabidopsis thaliana* CRYs (18–21) and flavin anion radical in DpCRY1 and DmCRY (10, 11). It was proposed (11) that the Asp-396 of AtCRY1 opposite N5 of the isoalloxazine ring acts as a proton donor during photoreduction of FAD_{ox} through the Trp triad and, thus, generates FADH[•] by proton-coupled electron transfer (Fig. 2B). At the corresponding position, Type 1 CRYs contain a Cys residue. It was suggested that because Cys has a pK_a more than four units higher than that of Asp, it could not act as a proton donor during photoreduction, thus explaining the generation of FAD⁻ and not FADH[•] in Type 1 CRYs (11).

To test these models for the pathways of electron and proton transfer during photoreduction, we performed site-directed mutagenesis in Type 1 CRYs to either block the proposed electron transport path or open the proposed proton transport route. It should be noted that the mutants of all Type 1 CRYs were not equally expressed or equally soluble. Hence, for our

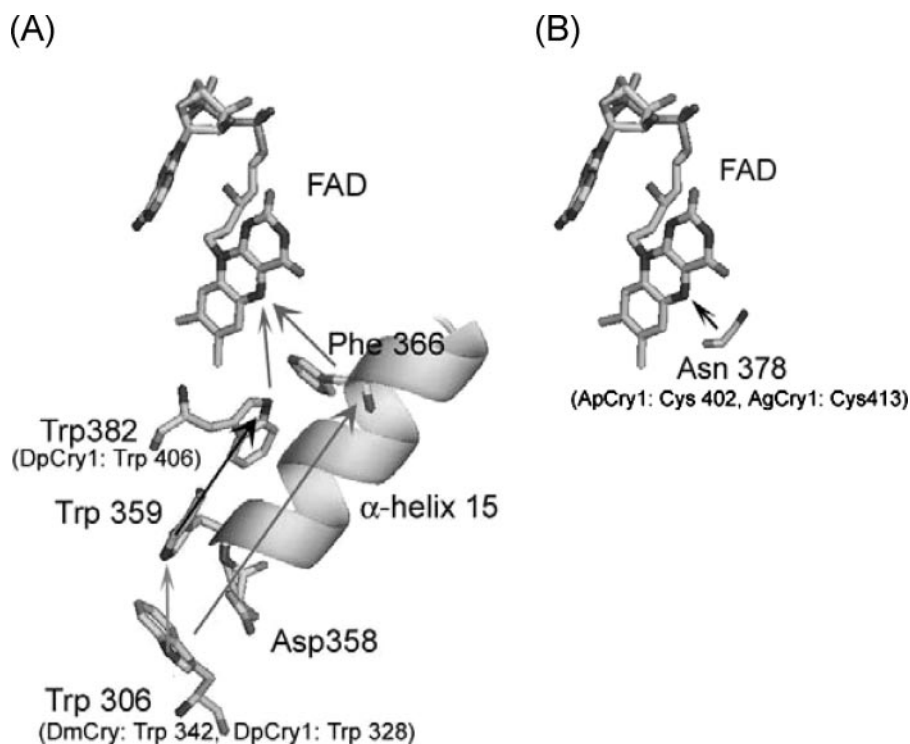


FIGURE 2. **Trp triad and flavin binding site models for photolyase/cryptochrome family.** The structures shown are those of *E. coli* photolyase (16), and only the amino acid residues that have been implicated in affecting the redox and protonation states of FAD are represented. *A*, the Trp triad. The arrows show the two pathways of photoreduction (16, 17); that is, electron hopping through the Trp triad and electron tunneling through α -helix 15 and Phe-366. The Trp triad residues in Type 1 CRYs that correspond to the *E. coli* Trp triad and which were mutated in this study are indicated in parentheses. *B*, the FAD cofactor and the amino acid residue (Asn-378) implicated in interacting with the N5 of the isoalloxazine ring. In parentheses are the corresponding residues in Type 1 CRYs that were mutated in this study.

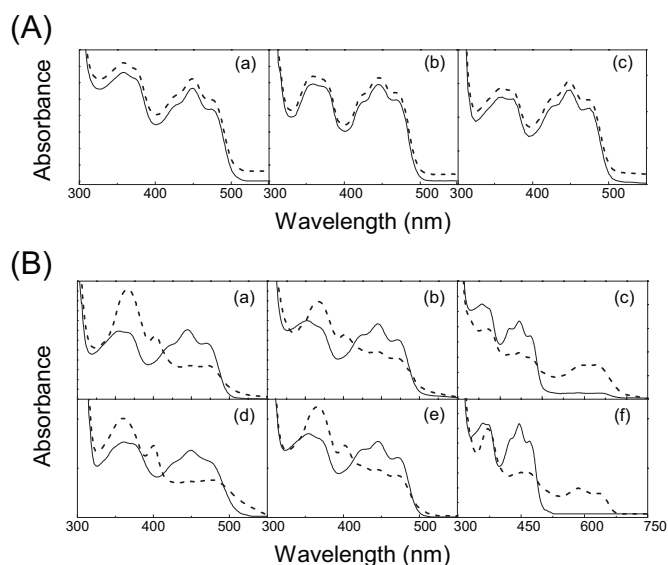


FIGURE 3. **Effect of mutations in the Trp triad and the Cys residue in the flavin binding pocket on photoreduction of the flavin cofactor in Type 1 CRYs.** *A*, Trp triad mutants. *a*, DmCRY-W342F. *b*, DpCRY1-W328F. *c*, DpCRY1-W406F. *B*, active site Cys mutants. *a*, ApCRY1 wild type (reference). *b*, ApCRY1-C402A. *c*, ApCRY1-C402N. *d*, AgCRY1-C413D. *e*, AgCRY1-C413A. *f*, ApCRY1-C413N. Solid line, dark spectra. Dashed line, spectra after irradiation at 450 nm with a fluence rate of $118 \text{ erg} \cdot \text{mm}^{-2} \cdot \text{s}^{-1}$ for 5 min.

analysis we chose those enzymes that tolerated a given mutation the best as evidenced by their level of overproduction, solubility, and absorption spectra.

The Trp Triad and Photo-reduction—We previously reported that the DpCRY1-W328F mutant was not photoreducible, supporting the role of the Trp triad in Type 1 CRY photoreduction (10). To generalize this observation we made the corresponding mutation in DmCRY (DmCRY-W342F) as well as the DpCRY1-W406F (proximal electron donor) and tested them for photoreduction. The results are shown in Fig. 3A. The replacement of Trp with redox inactive Phe in either the proximal (DpCRY1-Trp-406) or ultimate (DpCRY1-Trp-328 and DmCRY-Trp 342) electron donor blocked photoreduction, supporting the model of electron transfer through the Trp triad. It must be noted, however, that high doses of irradiation photoreduced 5–10% of DmCRY-W342F, suggesting a minor alternative pathway of photoreduction (data not shown). When the Trp residue corresponding to the middle Trp of the triad (W359 in *E. coli* photolyase) was changed to Phe, the mutant proteins of all Type 1 CRYs tested lacked flavin, presumably because of misfold-

ing, and hence, the contribution of this Trp to electron transport during photoreduction could not be tested. With this caveat, we believe our data strongly support the notion that the Trp triad is the major route for photoreduction in Type 1 CRYs.

Flavin Binding Site and Photoreduction—Photoreduction of FAD_{ox} in AtCRY1 and AtCRY2 first generates the FADH^{\cdot} neutral radical, which is quite stable (20, 21). Further illumination produces the two-electron reduced and presumably deprotonated flavin, FADH^{-} (20). The latter is rather unstable under aerobic conditions and rapidly re-oxidizes to the FADH^{\cdot} and more slowly to the FAD_{ox} form. It was rather surprising, therefore, to find that photoreduction of Type 1 CRYs generates the one-electron reduced and deprotonated flavin, $\text{FAD}^{\cdot-}$. It has been suggested that the reason for this difference between plant CRYs and animal Type 1 CRYs is that in plant CRYs an Asp is located opposite the N5 of the isoalloxazine ring, whereas in Type 1 CRYs there is a Cys in that position, and Asp can donate a proton to reduced flavin because of its low $\text{p}K_a$ (~ 3.9), whereas Cys cannot because of its much higher $\text{p}K_a$ (~ 8.3) (11). To test this model we replaced the Cys residue with Asp, Ala, or Asn in AgCRY1 and with Ala or Asn in ApCRY1 and analyzed the mutants for photoreduction. To our surprise the FAD in AgCRY1 with the Cys \rightarrow Asp replacement was reduced to $\text{FAD}^{\cdot-}$ and not to FADH^{\cdot} as predicted by the model (Fig. 3B). This finding suggests that the Asp in the flavin binding pocket is not the proton donor in the proton-coupled electron transfer reaction during photoreduction of FAD_{ox} to FADH^{\cdot} . In fact,

even the Cys → Ala mutants of both AgCRY1 and ApCRY1 were photoreduced to FAD^{•-}, suggesting that so far as the amino acid residue opposite N5 of the isoalloxazine ring is concerned there is no difference among the three amino acids (Cys, Asp, and Ala) with vastly different proton donating potentials. In contrast and quite unexpectedly, replacement of Cys by Asn led to formation of FADH[•] by photoreduction, consistent with electron transfer through the Trp triad coupled with a proton transfer from an amino acid residue at the flavin binding pocket. However, this amino acid is unlikely to be the Asn because the pK_a of Asn (~17) is nearly twice the pK_a of Cys (~8.3), which cannot act as proton donor. It is noteworthy that at the corresponding position *E. coli* photolyase contains Asn (16). However, in *E. coli* photolyase the flavin is in the FADH[•] form before photoreduction, and photoreduction further reduces the flavin to the FADH[•] form (14). In light of these findings we conclude that the reduced flavin takes up a proton not from an amino acid but from an acidic water molecule in the water network within the close confines of the active site of the photolyase/cryptochrome family.

Quantitative Analysis of Photoreduction and Reoxidation—Next we analyzed the photoreduction of wild-type and mutant CRYs as a function of light dose to obtain the quantum yields for the photoreactions as well as the rates of oxidation of reduced CRYs so as to be able to correlate the flavin reduction/oxidation thermodynamics and kinetics with the biological responses of the mutant proteins.

The quantum yield of photoreduction is obtained from the Rupert plot (22, 23) in which the fraction of remaining substrate, in this case FAD_{ox}, is plotted as a function of the light dose on a semi-log plot (Rupert plot),

$$\ln([FAD_{ox}]_t/[FAD_{ox}]_0) = -k_p L \quad (\text{Eq. 1})$$

where L is the light dose in $\text{erg}\cdot\text{mm}^{-2}$, and k_p is the photolytic constant, which is related to the quantum yield of the photoreaction by $\epsilon\phi$ ($\text{M}^{-1}\text{cm}^{-1}$) = $5.2 \times 10^9 \cdot k_p$ ($\text{mm}^2 \text{erg}^{-1}$) $\cdot \lambda^{-1}$ (nm), where ϵ = molar extinction coefficient, ϕ = quantum yield, and λ = the wavelength of irradiation. Rupert plots for photoreduction of the Trp triad mutants are shown in Fig. 4, A and B, and the plots for photoreduction of the flavin binding site mutants are shown in Fig. 4, C and D. The photolytic cross section and quantum yield values are listed in Table 1. The following conclusions can be made from these data. First, all of the Type 1 CRYs tested are photoreduced with quantum yields of $\phi = 0.16$ – 0.18 . These are considerably higher than the quantum yields of photoreduction of photolyase (14) and AtCRYs (20, 21). Second, the Trp triad mutations with non-redox active amino acids essentially eliminate photoreduction, although at high light doses some residual photoreduction is observed in DmCRY-W342F (data not shown) as has been observed with *E. coli* photolyase W306F mutant (14, 15). Third, mutation of the Cys residue in the flavin binding pocket affects the photoreduction quantum yield in an interesting pattern; Cys → Asp mutation does not significantly affect ϕ , Cys → Ala mutation reduces the quantum yields by about a factor of 2, and the Cys → Asn mutation increases the quantum yield by approximately a factor of 2. However, it must be noted that photore-

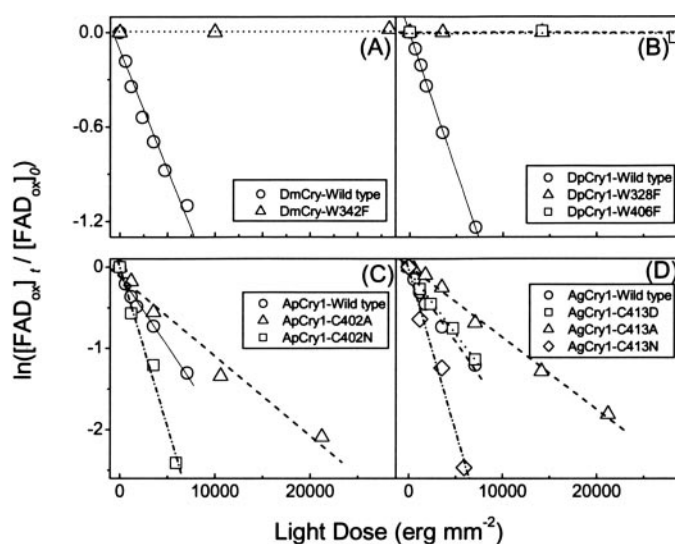


FIGURE 4. Rupert plots for photoreduction of Type 1 CRYs. Top panels, Trp triad mutants. A, DmCRY; circle, wild type; triangle, DmCRY-W342F. B, DpCRY; circle, wild type; triangle, W328F; square, W406F. Bottom panels, active site Cys mutants. C, ApCRY1; circle, wild type; triangle, C402A; square, C402N. D, AgCRY1; circle, wild type; triangle, C413A; square, C413D; diamond, C413N. The samples were irradiated with 450 nm at a fluence rate of $118 \text{ erg}\cdot\text{s}^{-1}\cdot\text{mm}^{-2}$.

TABLE 1

Photoreduction of FAD_{ox} and reoxidation in select members of the Type1 CRYs and their mutants

The quantum yield is calculated based on the extinction coefficient of $1.1 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 450 nm for FAD_{ox}. τ = life time of the reduced form under aerobic conditions. There was no measurable photoreduction of DmCRY-W342F, DpCRY1-W328F, and DpCRY1-W406F under our irradiation conditions.

Protein	Photoreduction		Reoxidation, τ <i>min</i>
	k_p	ϕ	
DmCry-wild type	1.52×10^{-4}	0.159	4.5
DmCry-W342F			
DpCry1-wild type	1.74×10^{-4}	0.183	0.91
DpCry1-W328F			
DpCry1-W406F			
ApCry1-wild type	1.73×10^{-4}	0.182	1.8
ApCry1-C402A	9.78×10^{-5}	0.102	0.21
ApCry1-C402N	3.92×10^{-4}	0.411	FAD ^{•-} , 1.1 FADH [•] , overnight
AgCry1-wild type	1.66×10^{-4}	0.174	4
AgCry1-C413D	1.54×10^{-4}	0.162	22
AgCry1-C413A	8.80×10^{-5}	0.093	0.25
AgCry1-C413N	3.97×10^{-4}	0.417	FAD ^{•-} , 1.3 FADH [•] , overnight

duction of the Cys → Asn mutants generates the flavin neutral radical, FADH[•], and not the FAD^{•-} observed in the wild-type, the Cys → Asp, and the Cys → Ala mutants. Conceivably, H-bonding between the N5 of the isoalloxazine ring and the NH₂ group of Asn increases the quantum yield by stabilizing the neutral radical and reducing the rate of back electron transfer.

Next we determined the rates of reoxidation of wild-type and mutant CRYs that had been photoreduced (Fig. 5). Interestingly, the wild-type proteins differed in their reoxidation rates by as much as a factor of 5, with DpCRY1 having the fastest (life time (τ) = 0.9 min) and DmCRY having the slowest (τ = 4.5 min) reoxidation rates (Table 1). Of equal significance, mutations of the Cys residue in the flavin binding pocket have drastic effects on the rates of reoxidation (Table 1); in AgCRY1 FAD^{•-} has a lifetime of 4 min, which increases to 22 min in the C413D

Photochemistry of Type 1 Cryptochromes

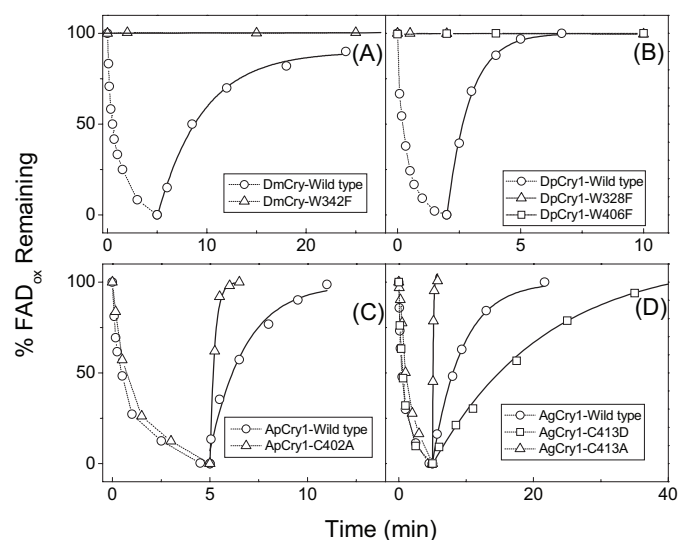


FIGURE 5. **Re-oxidation kinetics of photoreduced Type 1 CRYs.** Top panels, Trp triad mutants. A, DmCry; circle, wild type; triangle, DmCry-W342F. B, DpCRY1; circle, wild type; triangle, DpCRY1-W328F; square, DpCRY1-W406F. Bottom panels, active site Cys mutants. C, ApCRY1; circle, wild type; triangle, ApCRY1-C402A. D, AgCRY1; circle, wild-type; triangle, AgCRY1-C413A; square, AgCRY1-C402D. The samples were photoreduced with 450 nm and then kept in the dark under aerobic conditions. Recovery of FAD_{ox} was monitored by measuring absorbance at 450 nm.

mutant and decreases by 16-fold to 0.25 min in the C413A mutant. The latter value may explain the apparent low quantum yield of photoreduction of this mutant as during photoreduction there is significant reoxidation on the time scale of the photoreduction treatment. Most strikingly, the lifetime of FADH[•] in both AgCRY1-C413N and ApCRY1-C402N is ~12 h under our assay conditions, although a small fraction of the flavin that is in the form of FAD^{•-} after photoreduction in these mutants reoxidizes with rates comparable with those of the wild-type enzymes.

Photoreceptor Functions of Type 1 CRYs with Mutations in the Trp Triad and the Flavin Binding Site—Having analyzed some of the factors that affect the photoreduction of and the stability of the photoreduced flavin, we then proceeded to measure the effect of these factors on the photoreceptor function of cryptochromes. Currently, there are two assays for the photoreceptor functions of Type 1 CRYs; that is, photoinduced degradation of CRY itself and photoinduced degradation of the Timeless (Tim) protein (8, 24). Although Tim proteolysis, by virtue of the photochemical reaction, is CRY-dependent, the photoinduced degradation of CRY is independent of Tim (24, 25) and, hence, can be used as a convenient assay to test the relevance of certain photochemical reactions and of the mutations that affect these reactions on the photoreceptor function of Type 1 CRYs. Hence, we used light-induced CRY degradation as a functional end point in our study.

The Trp Triad and Photoreception—S2 cells were transfected with expression vectors to produce representative wild-type and mutant Type 1 CRYs. Cultures were then exposed to various light doses, and then the cells were lysed, and the levels of CRYs were determined by immunoblotting. The results obtained from these experiments are shown in Fig. 6, and the quantum yields obtained from this figure are presented in Table 2. The following facts with some bearing on the mechanism of

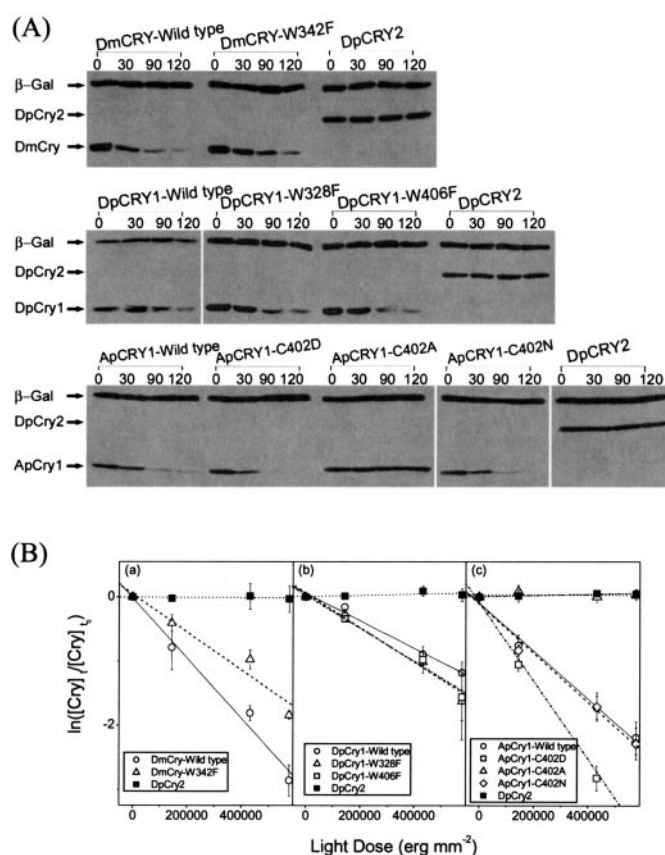


FIGURE 6. **Photoinduced proteolysis of Type 1 CRYs.** S2 cells transfected with plasmids expressing the indicated proteins were exposed to 366 nm at a fluence rate of 80 erg·s⁻¹·mm⁻² for the indicated periods. Two hours after the start of the irradiation cells were lysed, and cell extracts were analyzed by immunoblotting for the indicated proteins. β -Galactosidase was used as a loading control, and DpCRY2 was used as a negative control for light-induced proteolysis. S.E. were plotted as error bars. A, immunoblots from representative experiments. B, Rupert plot analysis of the data shown in panel A and at least one more experiment carried out under identical conditions. a, DmCRY proteolysis; circle, wild type; triangle, DmCRY-W342F; filled square, DpCRY2 as the negative control. b, DpCRY1; circle, wild type; triangle, DpCRY1-W328F; square, DpCRY1-W406F; filled square, DpCRY2 negative control. c, ApCRY1; circle, wild type; triangle, ApCRY1-C402A; square, ApCRY1-C402D; diamond, ApCRY1-C402N; filled square, DpCRY2 negative control. Data points are the averages of 2–3 experiments, and bars indicate S.E.

TABLE 2
Quantum yields of photoinduced proteolysis of wild-type and mutant CRYs

Quantum yields are based on $\epsilon_{450} = 1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for FAD_{ox}. The numbers in parentheses are based on the assumption of the presence of stoichiometric amount of the folate cofactor *in vivo* with the combined folate plus flavin extinction coefficient of $\epsilon_{450} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (see Ref. 14).

Protein	k_p	ϕ
DmCry-wild type	4.68×10^{-6}	4.75×10^{-3} (1.71×10^{-3})
DmCry-W342F	2.95×10^{-6}	2.99×10^{-3} (1.07×10^{-3})
DpCry2	0	0
DpCry1-wild type	2.15×10^{-6}	2.18×10^{-3} (7.81×10^{-4})
DpCry1-W328F	2.69×10^{-6}	2.73×10^{-3} (9.82×10^{-4})
DpCry1-W406F	2.58×10^{-6}	2.73×10^{-3} (9.82×10^{-4})
DpCry2	0	0
ApCry1-wild type	3.73×10^{-6}	3.79×10^{-3} (1.36×10^{-3})
ApCry1-C402D	6.09×10^{-6}	6.19×10^{-3} (2.22×10^{-3})
ApCry1-C402A	0	0
ApCry1-C402N	3.82×10^{-6}	3.88×10^{-3} (1.39×10^{-3})
DpCry2	0	0

photoreception emerge from these data. First, as previously reported, DpCRY1-W328F, which is unable to be photoreduced *in vitro* because of the Trp → Phe mutation in the ul-

mate electron donor of the Trp triad, exhibits normal light-induced proteolysis *in vivo*, suggesting that the Trp triad is not relevant to the photoreceptor function of Type 1 CRYs (10). Here we extend this work by showing that the W406F mutation in the proximal electron donor of the Trp triad of DpCRY1 also blocks *in vitro* photoreduction but has no measurable effect on the photoinduced proteolysis of the cryptochrome *in vivo*. The same also holds true for DmCRY, in agreement with a previous study (26) which showed that DmCRY-W342F was subject to photoinduced proteolysis like the wild-type protein. However, in that study the protein was not analyzed *in vitro*, and it was assumed that the mutation did not affect photoreduction. Here we show that the Trp → Phe mutation at this position (the ultimate electron donor in the Trp triad) does block photoreduction, as it does in all other CRYs tested to date. It should be noted, however, that it appears that in Fig. 6 and Table 2 DmCRY-W342F is less sensitive to photodegradation than the wild-type protein. In fact, it was observed in a previous study (26), and we confirmed in our own investigation (data not shown) that this mutant is produced at a significantly lower level than wild-type protein, possibly because of misfolding, and hence, a significant fraction of the protein may lack flavin and, therefore, be resistant to photodegradation. With this caveat and taking into account all the data on Type 1 CRY photo-induced proteolysis, we believe the data collectively reinforces an earlier conclusion that photoreduction through the Trp triad does not take place *in vivo* because the flavin in Type 1 CRYs is in the form of FAD⁻ in ground state (10). This is in contrast to the model proposed for *Arabidopsis* cryptochromes, which posits that photoreduction of AtCRY1 and AtCRY2 through the Trp triad is the initial reaction in cryptochrome photocycle (19–21).

Flavin Binding Site and Photoreception—Considering the drastic effects some of the flavin binding site mutations had on the photophysical/photochemical properties of the cofactor, we expected that these mutations would seriously affect the photoreceptor function of cryptochrome. The photoinduced proteolysis assay did indeed reveal some interesting findings. First, wild-type ApCRY1 and ApCRY1-C402N, which in reduced state contain FAD⁻ and FADH[•], respectively, exhibit essentially the same photoinduced degradation kinetics (Fig. 6 and Table 2), indicating that the deprotonated flavin anionic radical and the flavin blue neutral radical are equally efficient in initiating CRY photosignaling. Second, compared with wild type, ApCRY1-C402D, which based on data from AgCRY1-C413D is expected to be more resistant to oxidation of the FAD⁻ cofactor (see Table 1), consistently exhibited higher efficiency of photoinduced proteolysis, suggesting that the intrinsic stability of FAD⁻ in a given Type 1 CRY may dictate its quantum yield of photosignaling. Indeed, in support of this idea we find that ApCRY1-C402A, which is photoreduced with reasonable efficiency but is very sensitive to oxygen (Table 1), is also resistant to photoinduced proteolysis (Table 2), and the mutant may in fact contain the flavin in the FAD_{ox} form *in vivo*. Third, as has been noted before (5, 6), the quantum yield of photoinduced degradation of DmCRY is higher than those of other Type 1 CRYs. This is most likely because the S2 cells are derived from *Drosophila* and, hence, the DmCRY has higher

affinity interaction with the S2 cells proteolytic degradation machinery than the other Type 1 CRYs expressed in this heterologous system (5). Finally, the very low quantum yield of photoinduced proteolysis of all Type 1 CRYs tested ($\phi \sim 2\text{--}4 \times 10^{-3}$, see Table 2) deserves some comment. This is 200–400-fold lower than the quantum yield of cyclobutane photolyases, which repair DNA typically with quantum yield of $\phi \sim 0.7\text{--}0.9$ (13). However, it must be noted that in the case of photolyase, the reaction is a pseudo-first order photo-induced cyclic redox reaction in a simple E·S complex. In contrast, at present we have no knowledge about the events that take place between light excitation of Type 1 CRYs and their degradation. It is conceivable that the initial photochemical reaction does have a high quantum yield similar to that of photolyase but that the subsequent thermal (chemical) steps are much less efficient, resulting in the very low quantum yield of the overall reaction. Indeed, the 6-4 photolyase, which carries out both thermal (closing of the oxetane ring) and photochemical (splitting of the oxetane ring by photoinduced electron transfer) reactions, also has a quantum yield an order of magnitude smaller than that of the cyclobutane photolyase (27, 28). Clearly, there is a need to understand the steps between flavin photoexcitation and the proteolytic attack on Type 1 CRYs to develop a mechanistic explanation for the low quantum yield of Type 1 CRY proteolysis.

DISCUSSION

In this paper we have extended previous studies to demonstrate that Type 1 insect cryptochromes contain the deprotonated flavin anion radical, FAD⁻, as a cofactor. We have shown that mutations in the Trp triad that block photoreduction of FAD_{ox} to FAD⁻ *in vitro*, and a mutation in the flavin binding pocket that leads to formation of FADH[•] neutral radical instead of FAD⁻ do not affect the photoreceptor function of Type 1 CRYs. In light of these findings and of recent data from several groups, we discuss below potential models for photoreception/phototransduction by cryptochromes.

The Flavin Redox State and the Primary Photochemical Reactions in CRYs—Currently there are two models for photoreception/photosignaling mechanisms of CRYs (Fig. 7). These models differ fundamentally in the presumed redox status of the flavin cofactor in the dark phase. In one model, which we have designated the “phototropin model” (29), it is assumed that CRYs contain FAD_{ox} in ground state. Light exposure leads to photoreduction through the Trp triad to either FADH[•] (plant CRYs) or FAD⁻ (Type 1 CRYs) forms of CRYs, which are the signaling forms (29, 30). In the second model, which we refer to as the “photolyase model,” it is proposed that the flavin cofactor is actually in the form of FAD⁻ (or FADH⁻) *in vivo* and the FAD_{ox} observed in purified CRYs is an *in vitro* artifact resulting from oxidation of the reduced cofactor during purification under aerobic conditions. In support of this model we show that mutations that block FAD_{ox} $\xrightarrow{h\nu}$ FAD⁻ photoreduction *in vitro* do not affect the photoreceptor function of Type 1 CRYs *in vivo*. Similarly, we show that a mutation in the flavin binding site of a Type 1 CRY that leads to formation of FADH[•] instead of FAD⁻ upon photoreduction does not affect the photoreceptor

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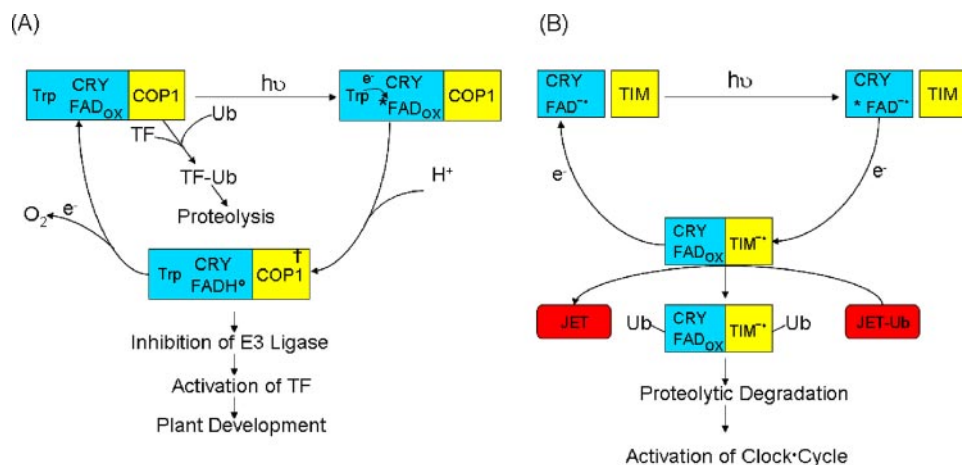


FIGURE 7. Two models for the CRY photocycle. *A*, conformational change model (phototropin model). AtCRY1 is in a complex with COP1 E3 ligase and contains FAD_{ox} as a cofactor. In the dark, COP1 ubiquitylates (*Ub*) transcription factors (*TF*) of light-responsive genes, leading to their proteolysis, and keeps transcription off. Light exposure induces electron transfer from the Trp triad of the CRY to FAD_{ox} to generate FADH[•], causing a conformational change in the CRY-COP1 complex, inhibition of COP1 activity, accumulation of transcription factors, and transcriptional activation of blue light-responsive genes. *B*, cyclic electron transfer model (photolyase model). DmCRY (or DpCRY1) is in a loose complex with the transcriptional inhibitor TIM in the dark and contains the flavin cofactor in the FAD^{•-} (or FADH⁻) form. Light exposure initiates an intra- or interprotein (shown) electron transfer reaction, leading to formation of a stable CRY-TIM complex. Within this complex TIM becomes a target for the JET E3 ligase, which ubiquitylates TIM, resulting in its degradation and removal of its inhibitory effect on the Clock-Cycle circadian transcriptional activator. CRY also may be ubiquitylated and degraded to allow eventual accumulation of TIM to restart the circadian cycle.

function of the CRY, indicating the functional equivalency of unprotonated and protonated 1-electron reduced flavins.

Cryptochrome Photocycle—Currently two classes of CRYs have been shown to function as photoreceptors; that is, *Arabidopsis* CRY1 and CRY2 and insect Type 1 CRYs. Although the precise sequence of events leading from absorption of a photon to gene regulation mediated by these CRYs is not known, sufficient information is available to formulate some specific models. In developing such models, however, some salient facts must be considered. Most importantly, the majority of known photosensory CRYs including all Type 1 CRYs tested and AtCRY2 are proteolytically degraded after light exposure *in vivo*, but AtCRY1 is not. This property of photosensory CRYs raises some interesting questions regarding the CRY photocycle. Specifically, if CRY is degraded during each photoreception/phototransduction reaction, can one speak of a photocycle? No definitive answer to this question can be given at present. However, considering the low quantum yield of photoinduced proteolysis of Type 1 CRYs we report in this paper it is conceivable that Type 1 CRYs carry out several rounds of phototransduction before being degraded by proteolysis. This model is similar to the “black widow model” for some transcriptional activators (31, 32). This class of transcriptional activators bind to the promoters of the cognate genes and recruit the general transcription factors and RNA polymerase to the promoter to carry out transcription. After transcription initiation, the activators become phosphorylated by one or more of the transcription initiation machinery and are targeted for proteolysis with the consequent down-regulation of transcription. It is presumed that the proteolytic degradation of the activator is stochastic and, therefore, on average each activator may initiate several rounds of transcription before being degraded. In support of this model for CRYs, it was

recently reported that a specific glycogen synthase kinase-3 β inhibitor blocked photoinduced degradation of the monarch butterfly CRY1 but not that of Tim, indicating that DpCRY1 can promote light-induced DpTim proteolysis without being degraded itself (33). In line with this notion it must be noted that the first identified photosensory cryptochrome, AtCRY1, is not degraded by light and, hence, it must have a photocycle.

With these considerations in mind and with the currently available data, the two models for the cryptochrome photocycle are summarized in Fig. 7. In the phototropin model (light-induced conformational change initiating the photocycle; Fig. 7A) light induces intra-protein electron transfer to reduce FAD_{ox} to FADH[•], causing a conformational change that affects CRYs

activity. In support of this model it has been reported that blocking photoreduction of FAD_{ox} to FADH[•] by a mutation in the Trp triad inactivates photoreceptor function of AtCRY1 *in vivo* (19). In the photolyase model (photoinduced cyclic electron transfer), the flavin is either in the FAD^{•-} or FADH⁻ form. Light induces a cyclic intra- or intermolecular electron transfer that initiates the sequence of events that leads to the proteolysis of the target protein. This model is supported by data presented in this paper for insect Type 1 CRYs. Whether or not plant and animal photosensory CRYs employ different types of photocycles remains to be determined by further mechanistic studies.

Acknowledgment—We thank Steven M. Reppert for providing the plasmids carrying the cDNAs of the Type 1 CRYs and the pAc5.1v5/HisA vector used in our study.

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