Flavin reduction activates *Drosophila* cryptochrome

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Entrainment of circadian rhythms in higher organisms relies on light-sensing proteins that communicate to cellular oscillators composed of delayed transcriptional feedback loops. The principal photoreceptor of the fly circadian clock, *Drosophila* cryptochrome (dCRY), contains a C-terminal tail (CTT) helix that binds beside a FAD cofactor and is essential for light signaling. Light reduces the dCRY FAD to an anionic semiquinone (ASQ) radical and increases CTT proteolytic susceptibility but does not lead to CTT chemical modification. Additional changes in proteolytic sensitivity and small-angle X-ray scattering define a conformational response of the protein to light that centers at the CTT but also involves regions remote from the flavin center. Reduction of the flavin is kinetically coupled to CTT rearrangement. Chemical reduction to either the ASQ or the fully reduced hydroquinone state produces the same conformational response as does light. The oscillator protein Timeless (TIM) contains a sequence similar to the CTT; the corresponding peptide binds dCRY in light and protects the flavin from oxidation. However, TIM mutants therein still undergo dCRY-mediated degradation. Thus, photoreduction to the ASQ releases the dCRY CTT and promotes binding to at least one region of TIM. Flavin reduction by either light or cellular reductants may be a general mechanism of CRY activation.

redox | photolyase | protein-protein interaction

Cryptochromes (CRYs) are flavoproteins found in all kingdoms of life (1–3). They regulate growth and development in plants, act as photoreceptors in some animal circadian clocks, and are necessary components of the mammalian clock (1, 4, 5). CRYs are closely related to the photolyase (PL) family of proteins that repair UV-damaged DNA in a blue light-dependent fashion (1, 3, 5). All PLs and CRYs have a FAD chromophore bound in an α-helical domain, and many also have a pteridine or deazaflavin antenna cofactor bound in an α/β-nucleotide–binding domain (5–8). Type I insect CRYs do not bind an antenna cofactor (7, 9). Compared with PLs, CRYs have varying C-terminal extensions that are essential for their function (1, 10).

When contained within members of the CRY/PL family, blue light–sensitive FAD can assume various redox states. PLs purify with a neutral flavin semiquinone (NSQ), $FADH^{\bullet}$, that light will reduce to the hydroquinone (HQ), $FADH^{-}$. In cells, energy transfer from the antenna cofactor produces an excited HQ, $FADH^{-*}$, that repairs DNA lesions by cyclical electron transfer (1, 3, 11). In plant CRYs, blue light reduces FAD to the NSQ (1), whereas in type I insect CRYs, blue light produces the anionic semiquinone (ASQ), $FAD^{\bullet-}$ (7, 12, 13). In dark aerobic conditions, the flavins reoxidize. In vitro, the electron source for flavin photoreduction is a conserved triad of Trp residues (the Trp triad), although other reductants may substitute in a cellular setting (13–15). The biological relevance of the *Drosophila* CRY (dCRY) photoreduction cycle has remained unclear.

dCRY sets the circadian clock with light (1, 16, 17). The core clock oscillator involves a feedback loop composed of the proteins Timeless (TIM) and Period, which repress their own transcription by interacting with the transcriptional activator complex Clock:Cycle (CLK:CYC). In the presence of blue light, dCRY binds to TIM and initiates its degradation through action of the E3 ubiquitin ligase, Jetlag (JET) (18–24). dCRY itself is also degraded in the presence of light with involvement of another E3 ligase, Ramshackle (BRWD3) (25). dCRY contains a C-terminal extension beyond its photolyase homology domain (PHD) that gates light-induced interactions with JET and TIM (6, 24, 26–28). dCRY purifies from insect cells with a fully oxidized FAD, which will then undergo photoreduction to the ASQ (7, 12, 13, 29). Both the photoreduced ASQ (12, 15) and a light-excited state of the ASQ (ASQ*) have been proposed as the key signaling states for initiating TIM degradation (5, 6, 30).

In dCRY, the C-terminal tail (CTT; residues 528–539) forms an α-helix that occupies a groove analogous to that where damaged DNA substrates bind in PLs (7). The sequence register of the CTT in the original dCRY structure was incorrect by two residues but has since been updated in an improved model (8) that also agrees with a more recent structure of dCRY (31). The CTT is well poised to respond to light-induced redox changes at the FAD. Indeed, proteolytic protection assays indicate that the CTT becomes more exposed upon light exposure (6, 31). Phe534 of the CTT juxtaposes the FAD isoalloxazine ring, and His378 lies between the Trp536 indole and the flavin ring. Structural elements surrounding the CTT [the CTT-coupled motif (CCM)] comprise the C-terminal lid (residues 420–446), the protrusion motif (residues 288–306), the phosphate-binding loop (residues 249–263), and the CTT base loop (residues 154–160). The CCM creates a pocket for the CTT near the flavin, and changes therein may also execute light responses (Fig. 1). Here, we apply a limited proteolysis assay in combination with MS, kinetic studies, peptide binding, and mutational analysis to probe the mechanism of light-induced conformational changes in dCRY and associate them with defined redox states of the FAD cofactor. These light-induced structural states are preferentially reactive toward regions of TIM that resemble the CTT.

Results

Light Induces Conformational Changes in the CCM. We applied a dCRY proteolytic assay (6) to correlate the kinetics of light-induced

Significance

Cryptochromes (CRYs) are photosensors that play central roles in the circadian rhythms of plants and animals. CRYs are related to photolyase DNA-repair enzymes, but instead of binding DNA, insect CRYs bind a C-terminal tail (CTT) α-helix in the pocket that holds the light-sensing flavin molecule. There is no consensus on how light activates *Drosophila* CRY (dCRY). We show that reduction of the flavin to the anionic semiquinone by light or chemicals releases the CTT to activate dCRY. The target of dCRY, the protein Timeless, contains a sequence similar to the CTT, and dCRY recognizes this sequence selectively in the light. This study supports a model for CRY signaling in which flavin reduction is the critical step performed by light.

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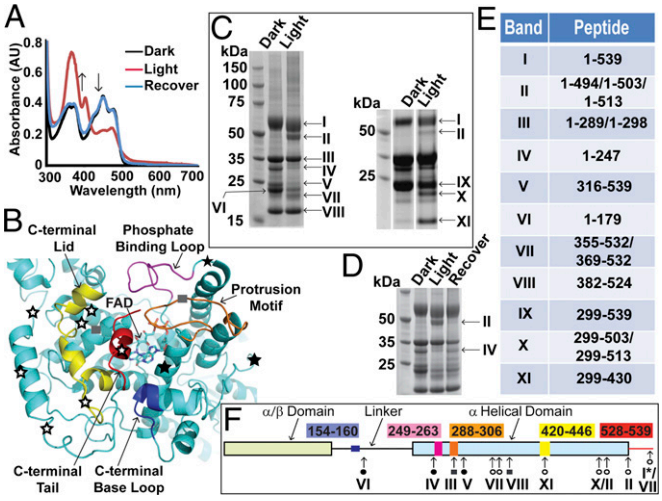

Fig. 1. Limited proteolysis of dCRY in the light vs. the dark. (*A*) Absorption spectra of dark (450-nm peak), light-exposed (367-nm and 403-nm peaks), and dark-recovered (after 1 h) samples of dCRY. AU, absorbance units. (*B*) In the crystal structure of dCRY (Protein Data Bank ID code 4GU5), elements of the CCM surround the CTT (red), which binds against the FAD (green bonds). Indicated are light-accessible tryptic sites (open stars), dark-accessible sites (closed stars), and sites found in the light and in the dark (gray squares). (*C*) SDS/PAGE of the dark and light samples after limited tryptic proteolysis under two different digestion conditions. (*D*) Digest pattern compared for light and dark recovery conditions with corresponding spectra shown in *A*. (*E*) Peptide fragments determined by MS for labeled bands in *C* and *D*. Bands II, VII, X, and XI are present only in the light-exposed samples, whereas bands IV, V, and VI are present predominantly in the dark samples. (*F*) Proteolytic cleavage patterns mapped onto the domain architecture of CRY with CCM color-coded as in *B*; residues for each motif are shown on top (arrows indicate tryptic cleavage sites, open circles indicate sites accessible to trypsin in the light, closed circles indicate accessibility in the dark, and gray squares indicate light/dark ambiguity). Light-induced cleavage at R532 (I*) could not be separated from band I of the light sample.

conformational changes with the FAD redox state. Light reduces dCRY to the ASQ, which then reoxidizes in the dark (Fig. 1*A*). Trypsin cleaves dCRY differently in light compared to in the dark, with light-activated cleavage sites mapping in the 3D structure to regions that involve the CTT and CCM (Fig. 1 *B*–*E*). Proteolytic patterns of samples that recover in the dark after light exposure closely resemble those of the dark sample (Fig. 1*D*). Peptides represented in SDS/PAGE bands II, VII, X, and XI are predominantly present in the light samples, and thereby indicate a light-induced accessibility of the corresponding cleavage sites, whereas bands IV, V, and VI are mainly observed in the dark samples, and similarly indicate a light-induced protection of these sites (Fig. 1 *C* and *D*). One light-sensitive cleavage site is in the CTT itself (R532; Fig. 1 *B* and *F*). Light protection of bands IV (R247 near the phosphate-binding loop), V (R315, helix leading to the Protrusion motif), and VI (R170, in the interdomain linker) indicates that conformational changes in dCRY are not limited to release of the the CTT and the connecting helix; rather, they involve a complex rearrangement of the protein (Fig. 1 *B*, *C*, and *F*). Band VII (light-favored) and band VI (dark-favored) also have contributions from residues outside the CCM; thus, the conformational response of the protein to light, although concentrated in the CCM, is relatively global.

The reversibility of the light-induced conformational changes suggested that flavin photochemistry was not chemically modifying the neighboring CTT. Nonetheless, we purified the trypsin-cleaved CTT before and after light exposure and analyzed it for modifications by MS. Only very minor oxidation products were discovered, and they showed no differences in light vs. dark conditions (Table S1).

FFW Motif Determines dCRY Stability. The conserved FFW motif of type 1 CRYs (F534, F535, and W536) associates closely with the PHD by binding into the flavin pocket (7, 8) (Fig. 2*A*). To study the importance of this motif in dCRY stability and conformational change, three variants were analyzed both in vitro and in cell culture: W536A, W536F, and a variant in which all three residues were mutated to Ala (named FFW) (Fig. 2 *B* and *C*). In cell culture, transfected dCRY is more stable in the dark than in the light, and it is much less stable in both without the CTT (7, 26, 28). Likewise, the FFW variant is considerably less stable in both the dark and the light (Fig. 2*C*). TIM transfected alone shows similar dark/light stability, but if dCRY is cotransfected with TIM, TIM stability increases in both the light and the dark due to interaction with dCRY (7). However, if the ubiquitin ligase JET is cotransfected along with dCRY, TIM degrades in the light (Fig. 2*C*). Unstable variants of dCRY (FFW or C10, which has the 10-residue CTT sequence changed to Ala) do not enhance TIM stability in the absence of JET, nor do they increase light-induced TIM degradation in the presence of JET (see below). In the case of dCRY FFW or C10, removal of key hydrophobic contacts provided by the FFW motif likely destabilizes the CTT against the PHD, which then leads to dCRY degradation. In contrast, the W536A and W536F variants behave similar to WT and are more stable in the dark than in the light. W536A/F levels are ∼80% of WT dCRY, and both variants effectively degrade TIM in the light with JET present. Thus, the effects of the CTT substitutions on TIM degradation largely reflect the amount of stable dCRY, which varies as WT ≥ W536F ≈ W536A >> FFW (Fig. 2*C*).

Thus, the W536F and W536A variants behave much like WT in terms of stability, trypsinolysis, and TIM degradation activity (Fig. 2 *B* and *C*). Nevertheless, the W536F protein consistently produced the sharpest light/dark band differences in the trypsin digestion assay (Fig. 2*B*); thus, this variant was used in the kinetic experiments discussed below. Ala substitution of the CTT residue closest to the flavin, Phe534, substantially destabilized dCRY to a degree where the protein could not be produced in amounts sufficient for characterization.

Rearrangement of the CCM Depends on the Lifetime of the ASQ. Bands II and IV were used to follow the kinetics of dCRY conformational changes. An increase in light exposure produced more flavin ASQ, as measured by an increase in the 403-nm peak (Fig. 3*A*), and systematically converted the protein conformation away from the dark state, as judged by the correlated appearance of band II and disappearance of band IV (Fig. 3 *B* and *C*).

The kinetics of the dCRY recovery from the light-induced ASQ to the dark oxidized FAD also correlates with changes in proteolytic accessibility. Under ambient, aerobic conditions, ASQ oxidation occurs in ∼70 min with a $t_{1/2}$ of 14 min (Fig. 3*D*). The rate and extent of recovery were greatly diminished in anaerobic conditions, with only 35% of the ASQ recovered after ∼7 h in the dark (Fig. 3*D*). Conversely, the chemical oxidant Cu-phenanthrolene oxidizes the ASQ back in 1 min in the dark (Fig. 3*E*). The recovery rates of the light-induced conformation back to the dark state (as measured by loss of band II and gain of band IV) in these three different redox conditions correlate well with loss of the ASQ (Fig. 3). Thus, dCRY conformational changes associated with CTT accessibility depend on the amount of ASQ in the sample, and reoxidation of the ASQ appears to be rate-limiting for reversion to dark-state protection of the CTT. Not all commonly used oxidants were reactive toward dCRY. For example, the redox cycling agent and inhibitor of flavoenzymes, diphenyleneiodonium chloride, was surprisingly unable to oxidize the dCRY ASQ (Fig. S1). Thus, effects of this compound on dCRY function in cellular assays are likely indirect (32, 33).

Chemical Reduction of dCRY Produces the Light-Induced Conformation. If light-induced ASQ formation releases the CTT, chemical reduction of the FAD should generate similar conformational changes in dCRY. In the dark, dithionite (DT) reduces dCRY

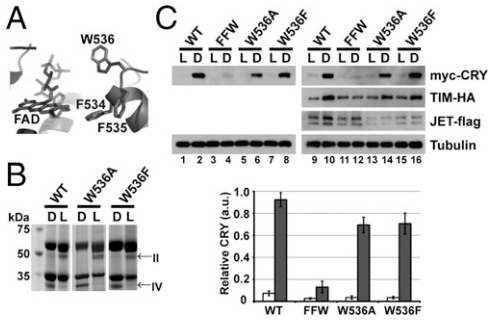

Fig. 2. Role of W536 and the FFW motif in light responses. (*A*) FFW motif (F534, F535, and W536) of the CTT binds adjacent to the FAD in the crystal structure of dCRY. (*B*) SDS/PAGE gels of the dark and light samples of WT CRY, W536F, and W536A variants treated by limited tryptic proteolysis. The appearance of band II and disappearance of band IV correlate with the dark-to-light transition. (*C*) dCRY and the W536F and W536A variants stimulate TIM degradation in the light when JET is present. (*Upper*) Western blot analysis of dCRY expressed in S2 cells alone (*Left*) and cotransfected with TIM and JET (*Right*; JET produces a secondary band during isolation) is shown. (*Lower*) Load control cells were exposed to the light for 1 h before processing; dark cells were processed under red light. Quantification of relative protein levels (corresponding to *Upper Right*) is shown. a.u., absorbance units; D, dark; FFW, Ala mutation of amino acids 534–536; L, light.

to the ASQ, the concentration of which peaks at 2 min after addition (Fig. 4*A*). Proteolytic digestion of the DT-reduced protein reveals that dCRY adopts a conformation that closely resembles that resulting from illumination (Fig. 1 and Fig. 4 *A* and *B*). Over the course of 2 h, DT completely reduces the FAD to a two-electron HQ ($FADH^-$), which also adopts a conformation similar to that seen with the ASQ state, albeit the conversion proceeds to a lesser extent, as indicated by the lower intensity of band II (Fig. 4 *A* and *B*). Upon reoxidation in the dark (for 1 h), the protein recovered fully to the dark-state conformation (Fig. 4). In agreement with previous studies, DTT and β-mercaptoethanol (BME) did not affect the redox state of the dCRY FAD (12). The very low potential reductant Cr:EDTA [< −1.0 V vs. normal hydrogen electrode (34)] also reduces the FAD to an ASQ in 2 min and to the HQ in 2 h, with conversion to the same conformation observed with light in each case (Fig. 4 *C* and *D*). As with DT, oxidation of Cr:EDTA-reduced dCRY recapitulates the dark-state conformation and light exposure of the reoxidized sample produces the light-induced conformation again (Fig. 4 and Figs. S2 and S3). Thus, dCRY conformational changes monitored by proteolysis and caused by light exposure also result from chemical reduction to the ASQ. These conformational changes persist in the HQ state, which forms very slowly, probably due to a rate-limiting protonation of the flavin. Notably, prolonged light exposure of dCRY for many hours will not reduce the protein beyond the ASQ (Fig. S4).

TIM–CRY Interaction. dCRY binds TIM in a light-dependent fashion gated by the CTT (22, 26–28). Unfortunately, biochemical studies of TIM have been hampered by the inability to overexpress the full length or even domains of this ∼1,400-residue protein. However, the TIM sequence contains a 10-residue segment very similar to that of the dCRY CTT (Fig. 5*A*). When synthesized with a biotin tag, this peptide [referred to as the TIM C-terminal tail-like (CTL)] pulled down much more dCRY in the light than in the dark (Fig. 5 *B* and *C*). dCRY reduced to the ASQ in the dark by Cr:EDTA binds the TIM-CTL as strongly compared with dCRY reduced in the light (Fig. 5*B*). Thus, interaction with the TIM-CTL differentiates the light state from the dark state in a manner similar to that seen with the proteolytic assay (Figs. 3 and 5). Dark recovery reduces the affinity of the TIM-CTL for dCRY (Fig. 5*B*). Interestingly, if the TIM-CTL is present when dCRY recovers, some interaction is maintained as the ASQ reoxidizes (Fig. 5*B*). This may indicate that the TIM-CTL binds in place of the dCRY CTT and that its release is slow. Indeed, the TIM-CTL substantially slows reoxidation of the ASQ, perhaps because the peptide blocks the flavin from oxygen (Fig. 5*C*). A TIM-CTL with the FFW motif changed to AAA has greatly reduced interaction with dCRY in both the dark and the light; consequently, it does not affect the redox kinetics (Fig. 5 *B* and *C*). A peptide corresponding to the dCRY CTT shows no binding to dCRY in the dark and only minimal binding in the light (Fig. S5).

Despite their sequence similarity, the dCRY CTT and TIM-CTL are not interchangeable, which is consistent with dCRY favoring one sequence in the dark and the other in the light. Chimeras in which the dCRY CTT is swapped for the TIM-CTL, and vice versa, have reduced stability when expressed alone, especially in the case of dCRY (Fig. S6). However, if the TIM-CTL is swapped for the dCRY CTT and the TIM chimera is expressed with intact dCRY, both proteins are stabilized (Fig. S6).

In cells, the light-induced interaction of TIM with dCRY leads to TIM degradation via the E3 ubiquitin ligase JET. Changing the 10 residues in the dCRY CTT to Ala (C10) destabilizes the protein under all conditions (Fig. S7) and produces behavior similar to complete removal of the motif (7). Likewise, changing the 10 residues of the TIM-CTL (T10) to Ala also destabilizes

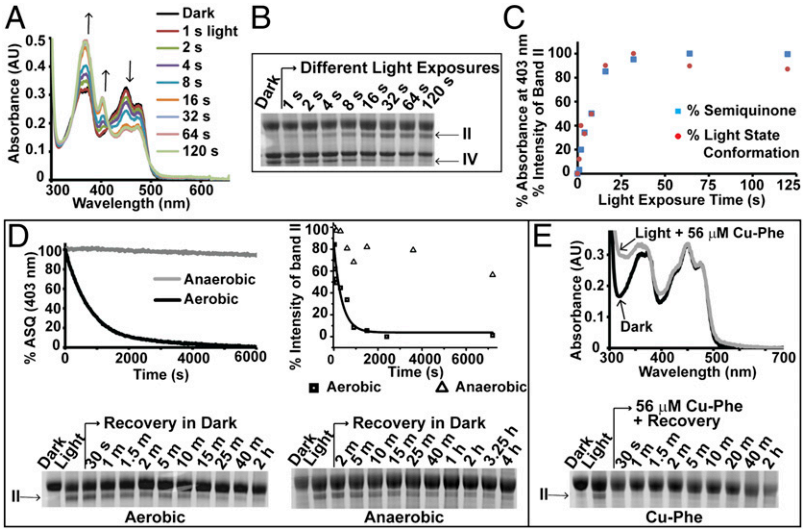

Fig. 3. ASQ formation correlates with light-induced conformational changes. (*A*) Increasing light exposure generates more ASQ, as indicated by the rise in the 403-nm peak. (*B*) Trypsin digestion of dCRY with increasing light exposure, at times corresponding to those in *A*. (*C*) Changes in proteolysis (band II intensity) and optical spectra (403 nm) correlate until saturation. (*D*) ASQ recovery kinetics and reversion to the dark conformation correspond to aerobic and anaerobic conditions. (*E*) Cu-phenanthrolene (Cu-Phe) oxidizes the ASQ within 1 min, which corresponds to the lifetime of the light-induced conformation indicated by band II.

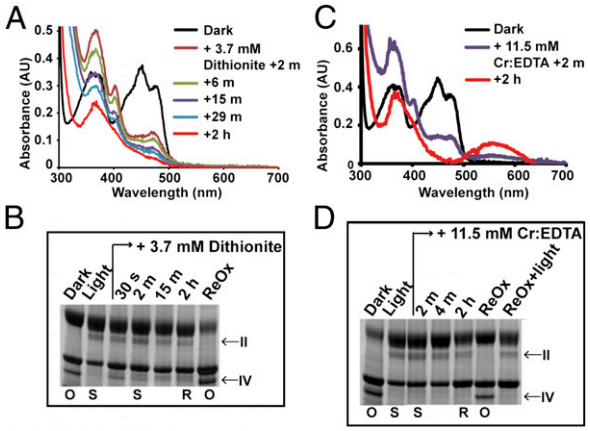

Fig. 4. Chemical reduction of dCRY induces the same conformational changes as light. Reduction of dCRY (W536F) by DT (*A*) and by Cr:EDTA (*C*) to the ASQ (2 min; 367 nm and 403 nm, respectively) to the reduced HQ (2 h, broad 340–500 nm) peak. The 2-h Cr:EDTA trace is offset to aid the comparison. (The 550-nm peak is due to Cr:EDTA; Fig. S2.) Trypsin digestion at various time points after the addition of DT (*B*) and Cr:EDTA (*D*). O, oxidized; R, reduced HQ; ReOx, reoxidation of the sample for 1 h after chemical reduction; ReOx + light, light exposure of the reoxidized sample; S, ASQ.

TIM in the dark and the light but not to the same extent as C10 for dCRY (Fig. S7). Nonetheless, when CRY and JET are present, T10 shows similar light-induced degradation as WT TIM (Fig. 5*D*).

dCRY Small-Angle X-Ray Scattering. Small-angle X-ray scattering (SAXS) was used to measure the light response of dCRY by monitoring its overall size and shape in solution. Representative scattering profiles of dCRY before and after exposure to 440-nm blue laser light are consistent with conformational rearrangements within a monomeric species (Fig. 6*A*). The radius of gyration calculated from the Guinier analysis increased from 29.4 ± 0.2 Å to 30.1 ± 0.2 Å after light exposure, which is consistent with an overall extension of dCRY. Comparison of theoretical scattering profiles based on atomic coordinates for dCRY with the CTT docked and undocked revealed a small but reproducible decrease in scattering intensity in the mid-q region and an increase in intensity in the high-q region, as shown by the experimental data (Fig. 6 *A* and *B*). This trend was observed with any rearrangement that moved the CTT outside of its binding pocket.

Discussion

Light-induced conformational changes in CRY proteins have long been assumed to involve C-terminal regions that vary depending on CRY type (24, 26–28). Based on various in vitro and in vivo studies, two models of how light stimulates such conformational changes in dCRY have emerged (6, 12, 13, 15). According to model 1 (12, 15), dCRY has oxidized the FAD in the ground state and light reduces the cofactor to an ASQ, whose formation drives conformational changes to produce the signaling state (Fig. 6). In cells, these structural changes would lead to TIM binding and proteasome-mediated degradation of both dCRY and TIM. In vitro, the ASQ reoxidizes slowly in the dark, thereby reverting the conformational changes and completing the photocycle. In model 2 (6), cellular dCRY contains the ASQ in the ground state, and light exposure converts it to an excited state, ASQ*, which is the signaling state that differs in conformation. To be functionally relevant, the structural changes would persist longer than ASQ*. Thus, during in vitro recovery, changes to the protein conformation and cofactor electronic state will be kinetically uncoupled.

Evidence for model 1 derives from several observations (15): (*i*) In insect cell culture, overexpressed dCRY has oxidized flavin, which then becomes reduced to the ASQ upon exposure of blue light; (*ii*) dCRY photoreduction in cells is not dependent on the Trp triad; and (*iii*) light-induced degradation of dCRY in living flies has an action spectrum that matches that of oxidized flavin (15). Evidence for model 2 derives mainly from three experiments: (*i*) mutation of the dCRY Trp triad abolishes photoreduction in vitro, but light-induced degradation of dCRY in vivo is unaffected (13); (*ii*) the action spectrum for degradation of a luciferase-dCRY fusion protein in S2 cells matches well with ASQ absorption in the UV-A range (35); and (*iii*) DT reduces dCRY to the ASQ but does not promote conformational change at the CTT (6). Thus, these experiments indicate that ASQ formation is not sufficient to generate conformational changes in dCRY. However, dCRY purifies in the oxidized state. Thus, if model 2 were to hold

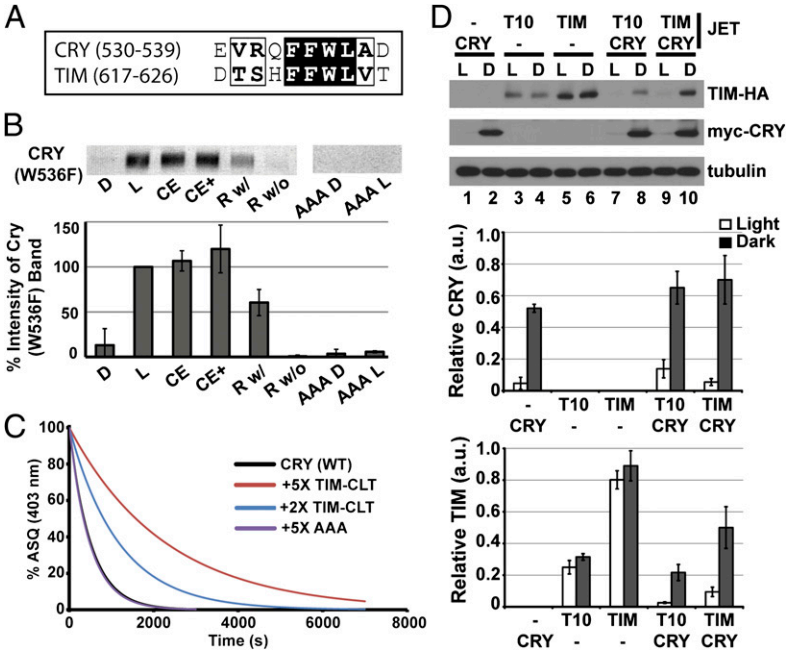

Fig. 5. Interaction of dCRY with the TIM-CTL. (*A*) dCRY CTT motif is conserved by TIM. (*B*) dCRY (W536F) was pulled down by a biotin-tagged peptide containing the TIM-CTL via streptavidin-conjugated agarose beads much more in the L than in the D and when dCRY is reduced. CE, dCRY treated with Cr:EDTA for 15 min; CE+, treatment for 2 h; R w/, dCRY 2 h recovery after light exposure with peptide; R w/o, dCRY 2 h recovery after light exposure without peptide; AAA, TIM-CTL with FFW mutated to AAA and incubated in either the D or the L. Quantification of dCRY band intensities from four independent experiments is shown. (*C*) ASQ recovery kinetics are slowed down by increasing concentrations of TIM-CTL but not by the AAA peptide. (*D*) Effect of TIM-CTL in dCRY-mediated TIM degradation. (*Upper*) Western blot analysis of TIM and dCRY expressed with JET in S2 cells under light or dark conditions. T10 represents TIM with the 10-residue CTL changed to poly-Ala; T10 and TIM both show dCRY-mediated degradation in the light. (*Middle* and *Lower*) Quantification of relative protein levels is shown below.

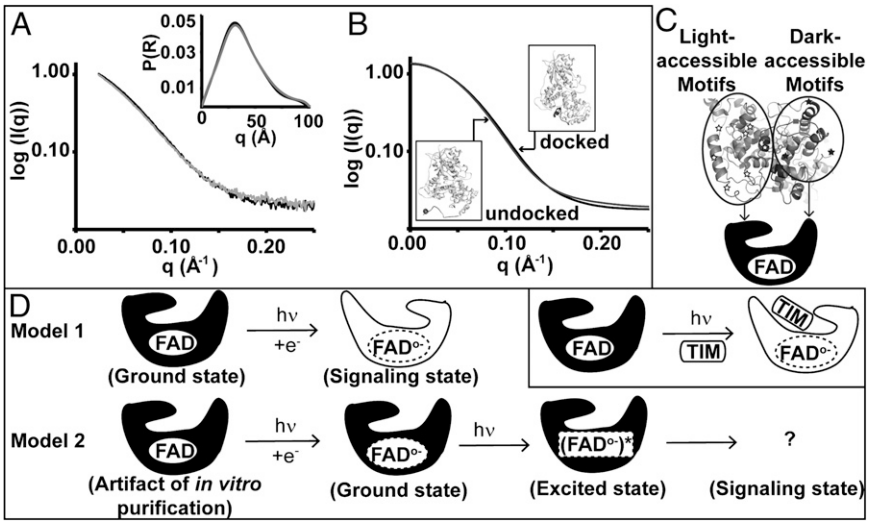

Fig. 6. SAXS analysis and the flip-flop switching model. (*A*) Comparison of experimental scattering profiles for dark-exposed (black) and light-exposed (gray) samples. (*Inset*) Pairwise distance distribution functions from the dark- and light-exposed experimental data. (*B*) Comparison of theoretical scattering profiles from the dark-state structure of dCRY (black) and a model with the CTT extended (gray). Insets show models. (*C*) Light-accessible proteolytic sites are confined to one side of the dCRY structure and light-protected sites to the other as represented in schematic below. (*D*) Two models of light-induced conformational changes with respect to the flavin electronic state. Data herein favor model 1 of FAD redox changes and (*Inset*) indicate a light-induced flip-flop conformational switch to gate dCRY–TIM interactions. e^-, electron; hν, photon.

in an in vitro test, light must photoreduce the protein and generate ASQ*, and these two processes are not easily separated.

Here, we show that (*i*) photoreduction to the ASQ induces conformational changes in dCRY as monitored by protease sensitivity, peptide binding, and SAXS; (*ii*) these conformational changes involve (but are not limited to) the CTT, which is structurally positioned to respond to changes in the FAD redox state and known to be functionally important in gating interactions with TIM (6, 24, 26–28); (*iii*) conformational changes at the CTT are reversible and kinetically coupled to the extent of FAD reduction and rate of FAD reoxidation; (*iv*) chemical reduction of the FAD to the ASQ produces the same conformational changes as photoreduction; (*v*) further chemical reduction to the HQ produces similar changes in structure as reduction to the ASQ; (*vi*) dCRY cannot be photoreduced to the HQ; (*vii*) global changes in dCRY structure on light exposure do not involve changes in oligomeric state and are best modeled by repositioning of the CTT; and (*viii*) reduced dCRY binds a region of TIM that resembles the CTT. Taken together, these data support model 1 as the best description for the dCRY photocycle.

The correspondence between rates of CTT proteolytic protection and FAD reoxidation strongly indicates that these two processes are coupled. Interestingly, the $t_{1/2}$ = 14 min of ASQ reoxidation in aerobic conditions agrees well with the $t_{1/2}$ = 15 min of the CRY signaling state in vivo and decay of the CRY–JET interaction (6). The ability of chemical reductants to induce the same conformational changes in dCRY as light indicates that a reduced flavin (ASQ) drives these changes, no matter how it is produced. This also raises the possibility that dCRY signaling could be triggered by redox processes that form (or deplete) the ASQ in the absence of light. The Trp triad that reductively quenches the flavin in vitro is not required for dCRY photoreduction in cells (13). However, other physiological electron donors appear to be capable of mediating photoreduction in the absence of these residues (15). Such a change in reductant may be related to the observation that the dCRY W342F mutant is less sensitive to photodegradation in cells (13, 36), although other explanations have also been offered (13). Similarly, the Trp triad is not needed for *Arabidopsis* CRY to mediate light signaling in plants (37), although, again, other endogenous reductants could participate in CRY photochemistry.

Previous proteolytic protection assays also observed DT-induced ASQ formation in dCRY but found no associated conformational changes at the CTT (6). The discrepancy may derive from differences in the experimental conditions of reduction and digestion. We found that in a thoroughly degassed 13 μL of a 200 μM solution of CRY, 3.7 mM DT prevents reoxidation of the FAD for 2 h, whereas DT concentrations of less than ∼1 mM will reduce the FAD to the ASQ but will not protect against reoxidation after a few minutes. Less than complete anaerobic conditions during proteolytic digestion can also lead to dark-state reversion. To confirm the reactivity we observed with DT, we used another reducing agent, the Cr:EDTA complex, and also found good correspondence with ASQ formation and light-induced dCRY conformational changes. Previous studies (6) used a higher DT-to-protein ratio compared with our experiment. We find that trypsin digestion in the presence of DT is less clean than with Cr:EDTA and that excess DT produces more irreversible changes in the protein (Fig. 4 *B* and *D* and Fig. S3). Thus, we have higher confidence in the Cr:EDTA results.

The proteolytic accessibility of dCRY in light reported herein largely agrees with previous work (6), although additional light-sensitive sites were identified, as well as some that undergo protection during light activation. Light-sensitive positions K503 (band II), R532 (band VII), and R430 (band XI) were identified as before; however, in addition, R494 (band II), R354 (band VII), and R368 (band VII) were found to be preferentially light-accessible. Increased sensitivity of R494 at the N terminus of the helix that connects to the CTT indicates rearrangement of up to 50 C-terminal residues that then couple to increased access of R430 in the C-terminal lid. Proteolytic sites remote from the CCM (e.g., R354, R368) indicate that the light-induced conformational changes involve a global change in dCRY structure. In contrast to the light-sensitive sites, R247, which is near the phosphate-binding loop, and R315, which is near the protrusion motif, are preferentially cleaved in the dark. R315 resides in a helix adjacent to the FAD, and the neighboring residue, W314, is only 4.7 Å from the FAD adenosine moiety. Thus, this region may respond directly to changes in flavin redox state. Dark accessibility of R179 also reflects global changes, because this residue resides in a solvent-exposed loop connecting the α/β-domain and α-helical domain of dCRY. Protection of dark-accessible sites in the light demonstrates that the protein is not simply destabilized in the light and that cleavage of the CTT does not merely unfold the protein to generate sensitivity at the more remote positions. Furthermore, not all sites undergo changes during dark/light conversion (e.g., K289/R298, R381). Mapping the proteolysis data onto the structure of dCRY (Fig. 6*C*) indicates that light-accessible sites are confined to one side of the FAD pocket, whereas light-protected sites localize to the opposite side. This suggests a conformational "flip-flop" mechanism, where ASQ formation displaces structural elements of the CCM and adjacent flexible regions then relax against the PHD and become relatively sequestered (Fig. 6*D*). The protected regions (e.g., protrusion motif, phosphate-binding loop, W314-containing helix) may reflect

reduced dynamics associated with formation of binding interfaces for downstream targets such as JET and TIM.

The CTT has been strongly implicated as an autoinhibitory motif for gating CRY engagement with TIM; as such, its release from the flavin-active center provides a reasonable model to explain this behavior. It is striking that TIM conserves a sequence so similar to the CRY CTT (TIM-CTL). A peptide composed of this motif interacts with dCRY much more strongly in the light (ASQ state) than in the dark, and binding depends on its FFW motif. Furthermore, the TIM-CTL likely binds in the pocket vacated by the CTT because it protects the ASQ from oxidation. In the dark state, the CTT blocks the flavin pocket and prevents the TIM-CTL from binding in the same position. In the ASQ state, the CTT is released, exposing the pocket and providing access to the TIM-CTL. However, in a cellular assay, mutation of the TIM-CTL does not prevent TIM-mediated degradation by dCRY and JET. Although this may indicate that dCRY does not bind TIM through the TIM-CTL in vivo, other TIM regions may be sufficient to mediate dCRY/JET recognition in this assay. Additionally, TIM destabilization resulting from the T10 substitution could act as a gain of function that facilitates dCRY-dependent TIM degradation by an unknown mechanism. The stabilization of both TIM and dCRY in cell culture when the TIM-CTL is changed to the CTT sequence suggests that in addition to gating the TIM interaction, the CTT may further facilitate dCRY:TIM complex formation.

In conclusion, light reduces dCRY to the ASQ, which is sufficient to induce conformational changes in dCRY. These changes involve a complex rearrangement of the CTT and surrounding regions that exposes the flavin pocket to bind peptides and react with oxidants. The recent structure of mammalian CRY2 demonstrates how the analogous flavin pocket in CRY2 recognizes the SCFFBXL3 ubiquitin ligase (38). Activation of CRY by flavin reduction has implications for signaling mechanisms beyond light-setting of the circadian clock. In insects, dCRY has recently been implicated in nonphotosensory signaling (32, 39), and in mammals, it is unlikely that CRY has any photosensory role (5, 40, 41). The ability to trigger CRY by flavin reduction, whether by light or cellular reductants, may explain the conservation of the flavin cofactor and involvement of CRYs in diverse signaling networks.

Materials and Methods

Materials and methods are reported in *SI Materials and Methods*. Described therein are protocols followed for dCRY reduction, spectroscopy, proteolysis analysis, and cell assays. Mass-spectrometry and SAXS experiments are also detailed.

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