Enzyme Mechanism

DOI: 10.1002/anie.201203476

Single Amino Acid Substitution Reveals Latent Photolyase Activity in *Arabidopsis* cry1**

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Cryptochromes are flavoprotein receptors found throughout the biological kingdom. In vertebrates, cryptochromes function in the circadian clock, are linked to human cancers, and have been proposed as magnetoreceptors in migratory birds. All cryptochromes are characterized by their striking structural similarity to light-activated DNA-repair enzymes, photolyases, despite their widespread occurrence and different signaling roles.[1–5] Like photolyases, cryptochromes bind a light-absorbing flavin cofactor (FAD) in a hydrophobic pocket and undergo intraprotein electron transfer and photoreduction in response to light.[6,7] However, unlike photolyases, cryptochromes have known signaling roles in plants and animals and do not repair DNA. The nature of the distinguishing characteristics required for signaling has remained elusive. It has recently been shown that animal and plant cryptochromes accumulate oxidized (OX) flavin in the dark and form the semi-reduced radical form (SR) upon illumination, whereas photolyases under the same conditions accumulate fully reduced anionic flavin (RED) in the dark, which is required for DNA repair.[8–10] It has also been shown that the SR form of plant and insect cryptochromes is correlated with biological activity.[8,9,11] Although the functional significance of the flavin oxidation state is still under discussion,[12] a critical difference between cryptochromes with known signaling roles and photolyases that repair DNA is the oxidation state of bound flavin in vivo.

Herein, we explore how the flavin redox state may provide a clue as to how plant and animal cryptochromes evolved from ancestral photolyases. Mechanistically, protonation of flavin may result from a conserved amino acid at position 396 of *Arabidopsis* cry1 (Atcry1), which is a negatively charged aspartic acid (D) residue in all plant cryptochromes, whereas in *E. coli* and other photolyases this is a neutral asparagine (N) or positively charged lysine (K) residue.[13] In cryptochromes, the D residue at this position has been suggested as a possible proton donor for flavin upon illumination[14,15] and may explain the difference in redox potentials and hence, the favored flavin redox states. To demonstrate that redox state may indeed be a defining distinction between cryptochromes and photolyases, the mutation D396N was introduced into Atcry1 and the purified recombinant protein isolated from a baculovirus expression system (Figure 1).

The purified D396N mutant protein binds OX flavin as is the case for wild-type protein (Figure 1a, panels 1,2; before illumination t_0 = dark). The absorption spectra remain unchanged by the mutation of D to N (Figure 1a, panels 1,2), consistent with D396 being protonated in the dark.[15] Upon illumination under aerobic conditions, in the presence of a mild reducing agent (10 mM β-mercaptoethanol (BME)), transition to the SR ($FADH^{\circ}$) form of flavin was detected by increased absorbance between 500–600 nm. With further illumination, significant formation of the fully reduced $FADH^{-}$ (RED) redox form can be seen because of a continuing decrease in absorbance at 450 nm, without an increase at 500–600 nm (Figure 1a, panels 1,3). Under these same illumination conditions, wild-type protein was only slightly reduced, failed to accumulate the RED form, and also accumulated far less of the SR form (Figure 1a, panels 2,4). Thus, illumination of the mutant protein D396N results in formation of the RED flavin, useful for DNA repair, rather than the SR flavin, which is correlated with cryptochrome activity.[8,9]

Another striking difference reported between cryptochromes and photolyases is the marked stability of the RED flavin in photolyases as compared to cryptochromes, where transition to OX occurs rapidly upon the return to darkness after illumination.[8–10,14] To determine whether the stability of the RED form is enhanced in the D396N mutant protein, Atcry1 was reduced in the presence of a strong reducing agent (10 mM dithiothreitol (DTT)) to drive SR accumulation under aerobic conditions. Samples were returned to darkness and spectra taken at intervals; the increase in absorbance at

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[**] This work was supported by grants from Human Frontiers RGP0045/2008-C and the National Science Foundation 33701 to M. Ahmad and the Deutsche Forschungsgemeinschaft FOR 526 to R. Bittl (Bi464-8), J. Heberle, and T. Kottke. We thank M. Narayanan and R. Stanley for assistance and helpful discussions in photoreactivation assays, P. Mueller for help with manuscript preparation, and members of the UR5 plant science laboratory at Université de Paris for assistance with experimental work and facilities.

Supporting information for this article (experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201203476.

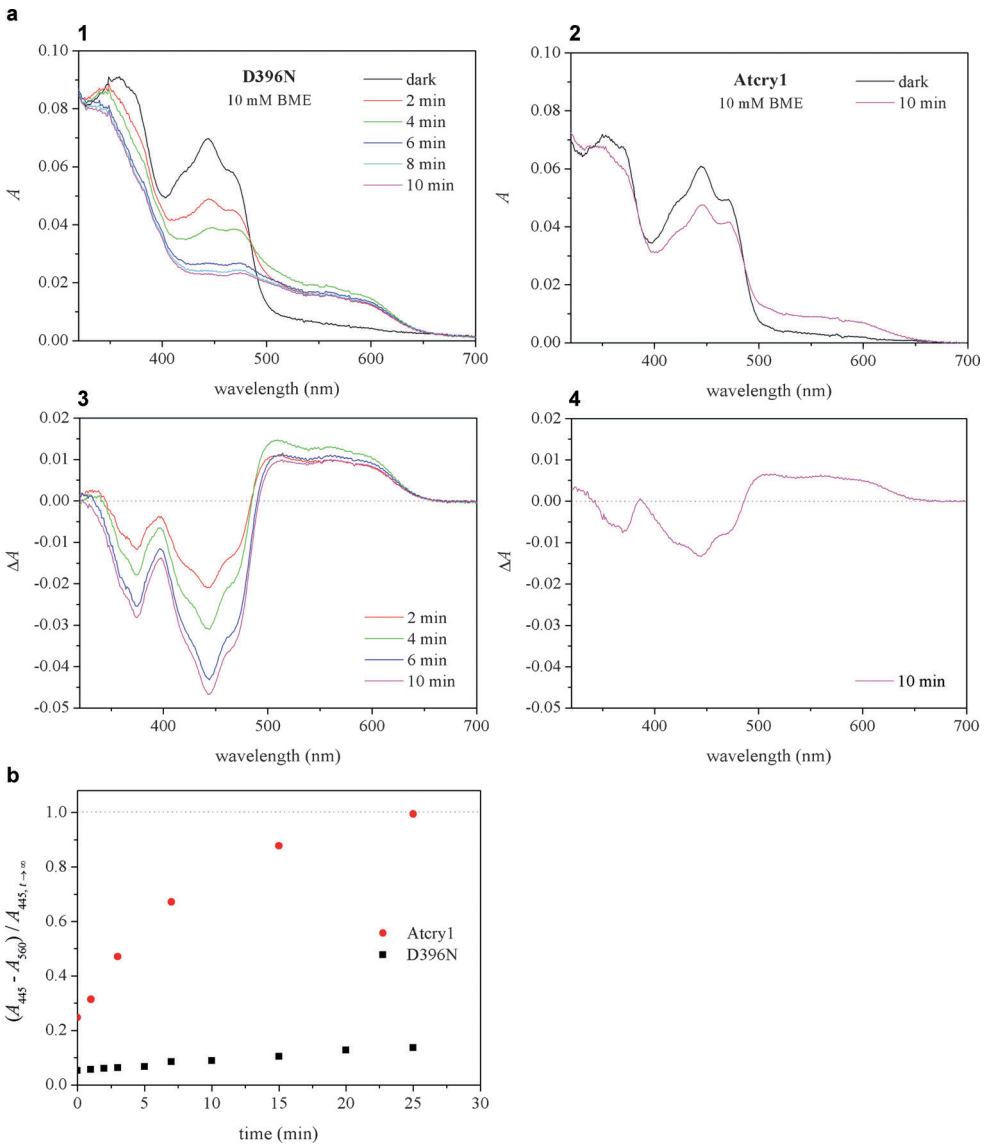

Figure 1. Photoreduction of D396N mutant protein. a) Panel 1 shows purified D396N protein binding a stoichiometric amount of flavin in 10 mM BME at 100 μmol m^{-2} sec^{-1} blue light, with absorption spectra taken before and after illumination for the indicated times. Wild-type Atcry1 (panel 2) was reduced under the same conditions. Below is the change in absorbance between the samples in the dark and the samples exposed to light for D396N (panel 3) and wild-type Atcry1 (panel 4). b) Wild-type Atcry1 and D396N mutant protein were photoreduced in 10 mM DTT and 10 mM BME, respectively, to achieve a comparable extent of photoreduction under aerobic conditions. Samples were returned to darkness and A_{445} (corresponding to the OX flavin peak) was recorded at the indicated times.

445 nm (OX transition) was plotted as a function of time (Figure 1b). Parallel experiments were performed with the D396N mutant protein, reduced under milder conditions (10 mM BME). Once illumination stopped, the flavin rapidly reoxidized in wild-type Atcry1 (SR-OX transition) as measured by an increase in absorbance at 450 nm (Figure 1b). By contrast, the D396N mutant protein was reoxidized at a much slower rate, with a half-life at least tenfold longer than wild-type protein—full reoxidation was only found after overnight sample incubation. Therefore, D396N protein undergoes rapid photoreduction to its fully reduced form, which is stable for long periods of time under aerobic conditions, similar to photolyases.

Mechanistically, deprotonation of D396 has been proposed as the basis for these altered redox states.[14,15] To verify this prediction, further characterization involving FTIR spectroscopic analysis of the mutant protein in the absence of an external electron donor was performed. The mutant showed a similar change in absorbance upon illumination to that of the wild type,[15] with respect to the flavin state, but a difference in the negative band at 1735 cm^{-1} (Figure 2a, marked with arrow). This band has previously been assigned to deprotonation of a glutamic or aspartic acid in the wild-type Atcry1 and proposed to originate from D396 following illumination.[15] The absence of this band in the mutant indicates that in the wild-type protein deprotonation occurs at this residue, leaving a strong negative charge in the flavin binding pocket upon illumination. This negative charge so close to the flavin binding site could explain the slow rate of the SR to RED transition in cryptochrome, because reduction of flavin results in two negative charges in close proximity in the binding pocket, a highly unfavorable arrangement.

To confirm the in vivo relevance of studies performed with the purified proteins in vitro, EPR spectroscopy was used on living cells expressing baculovirus-encoded recombinant Atcry1 as previously described.[8,9,11] Insect-cell cultures adapted to the dark and expressing high levels of D396N mutant or wild-type Atcry1 (Supporting Information, Figure S3) were transferred to EPR tubes, irradiated with blue light at room temperature, and then shock-frozen in liquid nitrogen for analysis. Cells expressing wild-type Atcry1 and cells expressing mutant D396N protein at the same level were studied in parallel. Prior to illumination, the cell cultures showed EPR signals indistinguishable from that of uninfected control cell cultures

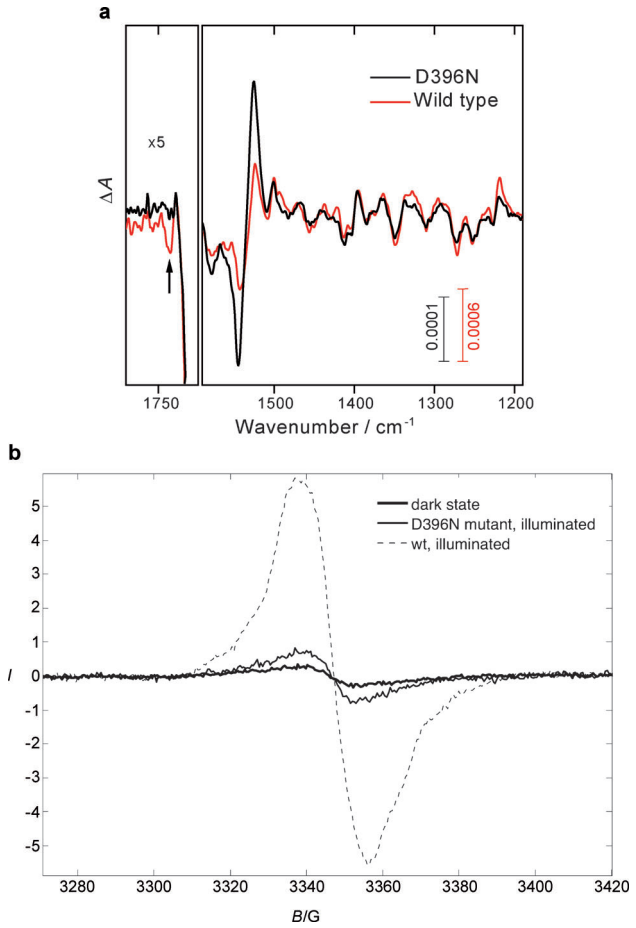

Figure 2. Detection of protonated radical species. a) The change in the FTIR absorbance spectra following exposure to light of the photoreduction of D396N mutant (this study) and wild-type Atcry1 (taken from [15]) in the absence of an external electron donor. Arrow = deprotonation of D396. b) EPR spectra of wild-type and D396N Atcry1 expressed in whole cells after illumination. Dark state = unilluminated control (not expressing protein); wt = expressing Atcry1.

(Figure 2b), indicating that SR does not accumulate in the dark in vivo, consistent with prior reports.[8,9] By contrast, samples illuminated for 6 min with blue light prior to freezing showed a paramagnetic species that accumulates in both D396N and wild-type Atcry1 expressing cell cultures but not in the uninfected controls, indicating formation of the flavin SR state. However, the EPR signal strength from the D396N expressing cells was significantly lower than that of wild type Atcry1 expressing cells (Figure 2b), consistent with greatly reduced radical formation in the mutant D396N. These results are consistent with the in vitro measurements of reduced SR accumulation and efficient transition from SR to RED upon illumination of D396N (Figure 1a).

Taken together, the results from the optical and EPR spectroscopic analyses indicate that the D396N mutant cryptochrome has acquired one of the key characteristics of a photolyase, namely the stable accumulation of RED anionic flavin.

We next analyzed for the possibility of restoration of DNA repair activity in the D396N mutant. An in vitro assay for repair of cyclobutane pyrimidine TT dimers in ds (double-stranded) or ss (single-stranded) DNA was performed using a plasmid transformation assay (Figure 3a).[16] Plasmid DNA containing TT dimers cannot be efficiently replicated and therefore shows reduced transformation efficiency as compared to intact undamaged DNA (Figure 3a, t_0 versus + UV). Incubation of damaged plasmid substrates with wild-type Atcry1 in the presence of light caused no improvement in transformation efficiency under these conditions for either ssDNA or dsDNA substrates (Figure 3a). Mutant D396N likewise does not improve the efficiency of dsDNA repair. However, mutant D396N protein induced a significant increase in transformation efficiency of ssDNA plasmids, in marked contrast to the wild-type Atcry1 (Figure 3a). These data suggested that the D396N mutant protein has the ability to repair single-stranded DNA, similar to photolyases[13,16] and unlike wild type Atcry1.

To further define D396N repair activity, a modification of classical optical methods was utilized[16] using a ssDNA substrate (DNA pentamer (dT_5) containing a single TT dimer per molecule). Damaged dT_5 DNA substrate was incubated with Atcry1, D396N, or photolyase proteins and illuminated with photoreactivating light to induce repair (see Supporting Information). TT dimer repair results in increased UV absorbance (A_{260}) of the ssDNA substrate purified over an HPLC column (Figure 3b). In this assay, Atcry1 exhibited no significant increase in A_{260}, demonstrating little DNA repair activity after 120 minutes of illumination in photoreactivating light (Figure 3b). By comparison, *E. coli* photolyase induced a considerable increase in A_{260} as compared to t_0, consistent with a high degree of substrate repair. In contrast to wild-type Atcry1, the D396N protein also induced repair of ssDNA (Figure 3b). Repair by D396N was inefficient as compared to photolyase, likely because of an absence of many of the amino acid residues involved in substrate binding in photolyases[6,7] (see also Figure S4). By analogy the cryDASH cryptochromes, which carry positive surface charges for DNA binding[1,3] and have a photocycle similar to photolyases, exhibit similar ssDNA repair activity as compared to classic photolyases.[16] As a further qualitative test, DNA repair of a 12-mer ssDNA was assayed by restriction digest analysis (Figure S5), with consistent results.

In summary, the amino acid substitution D396N in Atcry1 favors stably reduced flavin formation as in photolyases and unlike in cryptochromes. Analogous substitutions reported for insect cryptochromes ApCRY1 (C402N) and AgCRY1 (C413N; where Cys occupies the homologous position to Asp in Atcry1) show increased efficiency in photoreduction and slowed reoxidation rates consistent with our results, although transition to the RED form was not reported.[17] Interestingly, the reverse substitution in *E. coli* photolyase (N378S) is reported to stabilize the OX state and to abolish DNA repair function, also consistent with our results.[17] Recently suggestions for alternative photocycles involved in insect cryptochrome function have been proposed to proceed by unknown photoreactions, however all of these mechanisms likewise require the formation and stable accumulation in vivo of the radical flavin form of cryptochrome.[18] Therefore, a common step on the pathway to cryptochrome photoreceptor evolu-

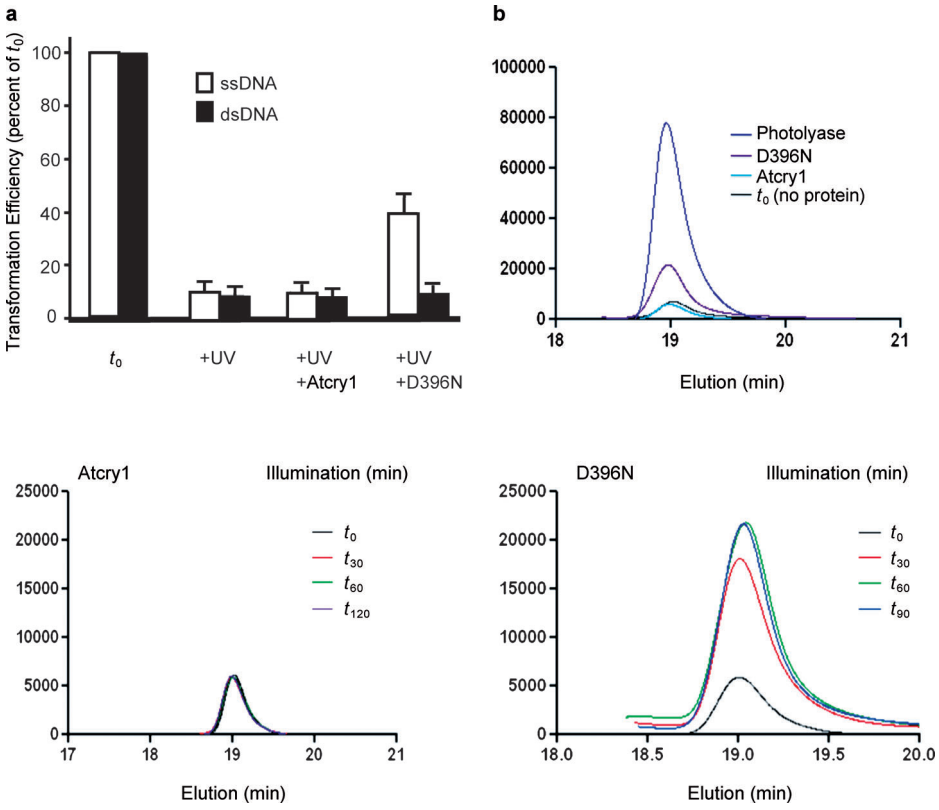

Figure 3. DNA repair activity of D396N. a) D396N and Atcry1 proteins were incubated with DNA plasmids in vitro and illuminated for 30 min with UV light essentially as described.[16] The y axis represents the number of colonies formed per transformation as a percentage of the transformation efficiency of an undamaged substrate, t_0. Error bars show the standard deviation (SD) from three trials. b) TT dimer substrate in vitro repair assay. Upper panel) Purified substrate DNA (dT_5 containing TT dimer) was incubated together with D396N, wild-type Atcry1, or *E. coli* photolyase proteins under repair conditions (see Supporting Information). Samples were separated using HPLC and the eluted DNA peak corresponded to repaired substrate. Lower panels) Time course of repair by Atcry1 and D396N after the indicated times of illumination.

tion from photolyases in both plants and animals appears to have been transition to a more oxidized redox form than for photolyases.

Unlike in the repair enzyme photolyase, which undergoes rapid electron transfer within microseconds subsequent to illumination,[6,7] a photoreceptor needs to cycle between active and inactive forms on a time scale sufficiently long to initiate downstream signaling events. We therefore propose the following model for the functional evolution of cryptochromes: a light induced redox transition from OX to SR flavin represents an almost ideal biological switch for light signaling. The reaction is slow, leads to a relatively stable intermediate (SR), and moreover reverses spontaneously in the dark, occurring on the order of minutes for both forward (OX to SR) and reverse (SR to OX) transitions (Figure S1). It can be assumed that OX and SR forms of cryptochrome have differing affinity for their signaling partners to develop a viable molecular switch. In fact, several independent studies have proposed precisely such a mechanism for cryptochrome, suggesting the signaling state may undergo a conformational change upon illumination, leading to an interaction with the substrate.[19–22] Given that plant and animal cryptochromes evolved independently from at least two different photolyase ancestors, the simplicity of evolutionary transitions from photolyase to a signaling protein[1–3,9] can be expected.

Received: May 5, 2012
Published online: August 13, 2012

Keywords: biophysics · cryptochromes · DNA damage · flavin reduction · photochemistry

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