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Discovery and Functional Analysis of a 4th Electron-Transferring Tryptophan Conserved Exclusively in Animal Cryptochromes and (6-4) Photolyases†

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A 4th electron transferring tryptophan in animal cryptochromes and (6-4) photolyases is discovered and functionally analyzed by transient absorption. It yields a much longer-lived flavin-tryptophan radical pair than the mere tryptophan triad in related flavoproteins, questioning the putative role of the primary light reaction of cryptochrome in animal magnetoreception.

DNA repair enzymes photolyases (PLs) and photoreceptors cryptochromes (Crys) form a superfamilly of light-harnessing flavoproteins occurring in all kingdoms of life.1 In spite of having vastly diverse functions, these proteins of 450 to 700 amino acids typically share a highly conserved domain of about 400 amino acids that harbours a non-covalently bound FAD cofactor. This domain contains also a chain of three tryptophan residues (Trp triad) connecting the FAD with the protein surface. This Trp chain is implied in photo-reduction of the FAD cofactor, converting fully oxidized FAD0 to semi-reduced FAD•− and/or FADH• radicals (putative signalling states of Crys) and FADH• to fully reduced FADH2 (required for DNA repair by PLs).1

Upon excitation by blue or near-UV light, FAD0 abstracts an electron from the nearest tryptophan residue in ~0.5 ps,2 yielding the FAD•− Trp•+ radical pair. This pair can either recombine or the charges can be stabilized by their separation through electron transfer (ET) from the 2nd and the 3rd Trp, leading to the FAD•− Trp•+ pair in ~100 ps.2 The solvent-exposed Trp•+ radical deprotonates within a few hundreds of nanoseconds.3 The resulting neutral Trp3 is eventually protonated and can then be further photo-reduced to FADH• via the same Trp triad.3a

A role of Cry in the magnetic sensing by migratory animals was suggested5 based on the discovery of Cry in animal eyes and its ability to form radical pairs. An external magnetic field is expected to affect singlet-triplet mixing in the relatively long-lived pair FAD•− Trp•+H•. Subsequent reactions stabilizing FAD•− (deprotonation of Trp•+H• and reduction of Trp•+H•) compete with spin-selective recombination of the radical pair to the FAD0 ground state (possible only from singlet pairs). Hence, the yield of long-lived FAD•− and/or FADH• could be modulated by an external magnetic field.5 The magnetic sensitivities of the forward light reactions of Arabidopsis Co, and E. coli PL with oxidized FAD have been tested in vitro,6 but only small effects and only at high magnetic field intensities (1000x the geomagnetic field) were observed, qualitatively in line with theoretical simulations for these systems.7 More
significant effects of weak (close-to-terrestrial) magnetic fields were observed in a model chemical magnetoreceptor.10

Our present structure and sequence analysis (Figures 1, 2, S1 and S2) revealed that unlike the tested plant Cry and bacterial PL, animal Cry (the actual putative magnetoreceptors) and animal (6-4) PLs (that specifically repair the so called (6-4) photoprotein in DNA)1e, 1f feature a unique chain containing a fourth tryptophan beyond the conserved triad, forming a tryptophan tetrad. This discovery motivated us to verify whether the fourth Trp is involved in photoinduced ET to FAD∞ in these proteins and whether they might have radical pair features different from those of systems with a mere triad of tryptophans and be more prone to effects of weak magnetic fields.

We have performed transient absorption spectroscopic measurements on the Xenopus laevis (6-4) photolyase (Xl(6-4)PL), which exhibits a high degree of homology with insect and vertebrate cryptochromes (Figures 2 and S2). Figure 3 shows transient absorption signals at the three most significant wavelengths: 376 nm (close to the maximum of FAD∞), 448 nm (maximum of FAD∞) and 562 nm (maximum of TrpH+); for other wavelengths, see Figure S3. The wild-type (WT) and a mutant protein, in which the 4th Trp (W370F), exhibited much longer much longer decay with a time constant τ~200 µs; the rest decayed nearly completely with τ~10 µs.

Spectral analysis of the state reached after the 200 ns decay in the W370F mutant protein (difference spectrum at t = 3 µs in Figure 4c) indicates the presence of the FAD−Trp3− pair at ~50% of the yield of FAD−Trp3H+ observed initially. We conclude that the 200 ns decay represents a competition between recombination in the FAD−Trp3H+ pair and deprotonation of Trp3H+ (both processes contributing almost equally to the 200 ns phase, i.e., recombination and deprotonation have similar intrinsic time constants of ~400 ns) and that the ~10 µs decay represents recombination of the remaining FAD−Trp3− pairs. Note that while ~90% of all light-induced radical pairs were lost in the W370F mutant within the first 50 µs (Figures 3b and S3b), the terminal radical pair in the WT protein underwent only deprotonation of TrpH+ and there were no detectable losses due to recombination on this timescale (Figures 3a and 4b). We hence conclude that the recombination of the FAD−Trp3H+ radical pair must be at least 10x slower than Trp3H+ deprotonation, leading to a recombination time constant of >25 µs for FAD−Trp3H+.

The FAD−Trp3− radical pair formed in the WT protein is much longer-lived than 50 µs (Figure 3a). Measurements on a 0.4 s time scale yielded a life time of 35 ms (Figure S4), i.e., more than three orders of magnitude longer than for the FAD−Trp3− pair in the W370F mutant protein. Analysis of the data (see SI) suggests that the observed 35 ms decay results from a competition between recombination of the pair FAD−Trp3− (~40 ms) and a substantially slower protonation of FAD− (~200 ms). The overall mechanism of FAD∞ photoreduction Xl(6-4)PL and the measured or estimated time constants are summarized in Scheme 1.

Fig. 2 Partial sequence alignment of several representative Cry/PL proteins. The 4th tryptophan (red) is conserved exclusively in animal Cry, animal (6-4) photolases, and in the dual-function protein PSCP1 (both photoreceptor and DNA repair enzyme) found in the marine diatom Phaeodactylum tricornutum. Sequences of the protein studied here (Xl(6-4)PL) and of Cry/a found in the retina of magneto-sensitive European robin (Erithacus rubecula, Er) are shown in boldface. Ec = Escherichia coli; Ot = Ostracococcus tauri; Arabidopsis thaliana; Dr = Drosophila melanogaster; Dr = Danio rerio; Mm = Mus musculus; Hs = Homo sapiens; CRYD = Cry DASH; CPO = cyclobutane pyrimidine dimer photolyase; See Figure S2 for more proteins and longer alignment.
In conclusion, our comparison of WT and W370F mutant (6-4) photolyases from X. laevis provided strong evidence that tryptophan W370 functions as fourth and terminal electron donor to the photoexcited FAD cofactor in the WT protein, yielding radical pairs FAD$^\bullet$ – Trp$^\bullet$H$^\bullet$ and FAD$^\bullet$ – Trp$^\bullet$ that are much longer-lived than the corresponding pairs involving the third tryptophan observed in the W370F mutant protein. A longer lifetime of the terminal radical pair may be of advantage, as it gives more time to the extrinsic reducing agents to reduce the Trp$^\bullet$ radical and enhance the yield of long-lived FADH$^\bullet$, which is required for DNA repair.

As the fourth tryptophan is conserved in putatively magnetosensitive animal cryptochromes (Figure 2), it is likely that the radical pair features of Xl(6-4)PL apply also to those cryptochromes (see also discussion in the SI). A magnetic field effect on the outcome of a radical pair reaction requires that spin selective recombination (here to the singlet ground state of FAD) is fast enough to compete with spin relaxation, yielding an upper limit of 100 µs for the time constant of recombination in Crys.$^{14}$ The negligible recombination of the FAD$^\bullet$ – Trp$^\bullet$H$^\bullet$ pair and the lifetime of the FAD$^\bullet$ – Trp$^\bullet$ of tens of milliseconds observed here in a structural homologue of animal cryptochromes seem to be incompatible with the hypothesis that the primary light reaction in cryptochromes serves as the basis of animal magnetoreception. An alternative suggestion that FADH$^\bullet$O$_2$ formed during FADH$^\bullet$ reoxidation by O$_2$ may be the magnetosensitive radical pair in Crys$^{10b, 15}$ merits experimental verification.

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Notes and references


