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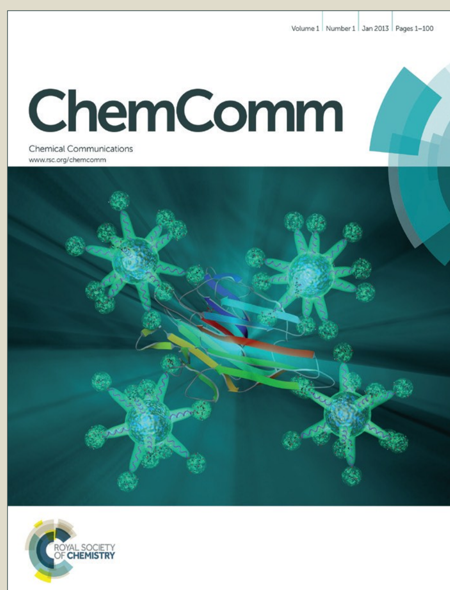

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Discovery and Functional Analysis of a 4th Electron-Transferring Tryptophan Conserved Exclusively in Animal Cryptochromes and (6-4) Photolyases†

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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 superfamily of light-harvesting 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 photoreduction of the FAD cofactor, converting fully oxidized FAD_{ox} to semi-reduced $FAD^{•-}$ and/or $FADH^{•}$ radicals (putative signalling states of Crys) and $FADH^{•}$ to fully reduced $FADH^{-}$ (required for DNA repair by PLs).[1]

Upon excitation by blue or near-UV light, FAD_{ox} abstracts an electron from the nearest tryptophan residue in ~0.5 ps,[2] yielding the $FAD^{•-}$ $Trp_1H^{•+}$ 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 $FAD^{•-}$ $Trp_3H^{•+}$ pair in ~100 ps.[2] The solvent-exposed $Trp_3H^{•+}$ radical deprotonates within a few hundreds of nanoseconds.[3] The resulting neutral $Trp_3^{•}$ radical may be reduced by extrinsic reductants preventing loss of $FAD^{•-}$ by recombination with $Trp_3^{•}$. In many members of the Cry/PL family, $FAD^{•-}$ is eventually protonated and can then be further

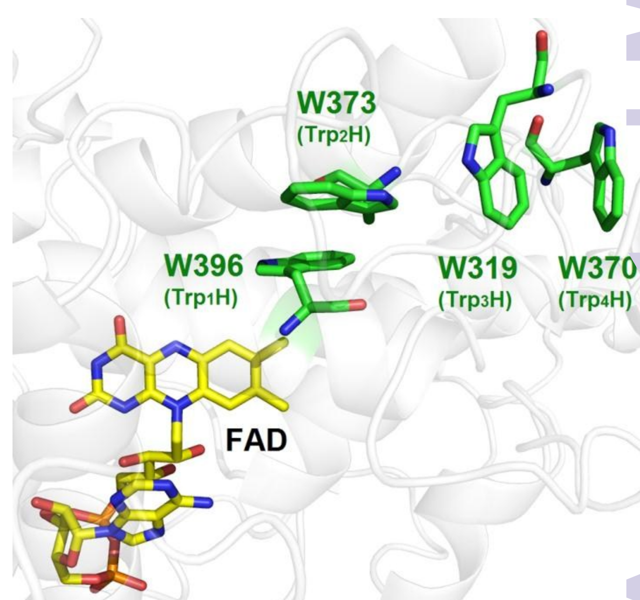

Fig. 1 Homology model of the *Xl*(6-4)PL structure. *Xl*(6-4)PL was aligned to the known structure of *Drosophila* (6-4) PL (structure 3CVU in RCSB PDB) using the SWISS-MODEL platform. FAD is highlighted in yellow, the tetrad of Trps involved in ET to photoexcited FAD in green.

photoreduced to $FADH^{-}$ via the same Trp triad.[3a]

A role of Cry in the magnetic sensing by migratory animals was suggested[4] 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 $FAD^{•-}$ $Trp_3H^{•+}$. Subsequent reactions stabilizing $FAD^{•-}$ (deprotonation of $Trp_3H^{•+}$ and reduction of $Trp_3^{•}$) compete with spin-selective recombination of the radical pair to the FAD_{ox} 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* Cry and *E. coli* PL with oxidized FAD have been tested *in vitro*,[6] but only small effects and only at high magnetic field intensities (1000× the geomagnetic field) were observed, qualitatively in line with theoretical simulations for these systems.[7] More

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                          Trp3                                                                   Trp4Trp2                            Trp1
EcCPD    295 HRPFIAWTDR VQWQSN-PAH LQAWQEGKTG YPIVDAAMRQ LNSTGWMHNR LRMITASFLV K-DLLIDWRE GERYFMSQLI DGDLAANNGG WQWAASTGTD 392
At64     317 KMKGNRICKQ IPWNED-HAM LAAWRDGKTG YPWIDAIMVQ LLKWGWMHHL ARHCVACFLT RGDLFIHWEQ GRDVFERLLI DSDWAINNGN WMWLSCSSFF 415
Dm64     318 RMLGNVYCMQ IPWQEH-PDH LEAWTHGRTG YPFIDAIMRQ LRQEGWIHHL ARHAVACFLT RGDLWISWEE GQRVFEQLLL DQDWALNAGN WMWLSASAFF 416
Xl64     307 KMEGNPVCVQ VDWDNN-KEH LEAWSEGRTG YPFIDAIMTQ LRTEGWIHHL ARHAVACFLT RGDLWISWEE GQKVFEELLL DADWSLNAGN WLWLSASAFF 405
AtCRY1   312 ERPLLGHLKF FPWAVD-ENY FKAWRQGRTG YPLVDAGMRE LWATGWLHDR IRVVVSSFFV K-VLQLPWRW GMKYFWDTLL DADLESDALG WQYITGTLPD 409
AtCRY2   307 EQSLLSHLRF FPWDAD-VDK FKAWRQGRTG YPLVDAGMRE LWATGWMHNR IRVIVSSFAV K-FLLLPWKW GMKYFWDTLL DADLECDILG WQYISGSIPD 406
XlCRYD   312 FFLRGLQDKD IPWKRD-PKL FDAWKEGRTG VPFVDANMRE LAMTGFMSNR GRQNVASFLT K-DLGIDWRM GAEWFEYLLV DYDVCSNYGN WLYSAGIGND 409
OtCPF1   339 FHLDGTAGRR ASWKRD-EKI LKAWKTGTTG YPLIDANMRE LAATGFMSNR GRQNVASWLA L-DAGIDWRH GADWFEHHLL DYDTASNWGN WCAAAGMTGG 436
PtCPF1   339 KMIDNPIARQ IPWDDD-PDL LLAWKMSKTG YPYIDAIMTQ LRETGWIHHL ARHSVACFLT RGDLWQSWED GATVFEEYLI DADWSINNFN WQWLSCTAHF 437
DmCRY1   330 RMEGNDICLS IPWAKPNENL LQSWRLGQTG FPLIDGAMRQ LLAEGWLHHT LRNTVATFLT RGGLWQSWEH GLQHFLKYLL DADWSVCAGN WMWVSSSAFE 429
DrCRY1a  308 KMEGNPICVQ IPWDKN-PEA LAKWAEGRTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWISWEE GMKVFEELLL DADWSVNAGS WMWLSCSSFF 406
XlCRY1   307 HMVGNPICLQ IEWYKN-EEQ LQKWREGKTG FPWIDAIMAQ LHEEGWIHHL ARHAVACFLT RGDLWISWEE GMKVFEELLL DADYSINAGN WMWLSASAFF 405
XlCRY2   312 QMEGNPICVQ IPWDKN-PKA LAKWTEGKTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWNSWEC GVKVFDELLL DADFSVNAGS WMWLSCSAFF 410
ErCRY1a  308 KMEGNPICVQ IPWDKN-PEA LAKWAEGRTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWISWEE GMKVFEELLL DADWSVNAGS WMWLSCSSFF 406
ErCRY1b  308 KMEGNPICVQ IPWDKN-PEA LAKWAEGRTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWISWEE GMKVFEELLL DADWSVNAGS WMWLSCSSFF 406
MmCRY1   308 KMEGNPICVQ IPWDKN-PEA LAKWAEGRTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWISWEE GMKVFEELLL DADWSINAGS WMWLSCSSFF 406
MmCRY2   326 RMEGNPICIQ IPWDRN-PEA LAKWAEGKTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWVSWES GVRVFDELLL DADFSVNAGS WMWLSCSAFF 424
HsCRY1   308 KMEGNPICVQ IPWDKN-PEA LAKWAEGRTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWISWEE GMKVFEELLL DADWSINAGS WMWLSCSSFF 406
HsCRY2   348 RMEGNPICIQ IPWDRN-PEA LAKWAEGKTG FPWIDAIMTQ LRQEGWIHHL ARHAVACFLT RGDLWVSWES GVRVFDELLL DADFSVNAGS WMWLSCSAFF 446
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Fig. 2 Partial sequence alignment of several representative Cry/PL proteins. The 4th tryptophan (red) is conserved exclusively in animal Crys, animal (6-4) photolyases, and in the dual-function protein *Pt*CPF1 (both photoreceptor and DNA repair enzyme)[8] found in the marine diatom *Phaeodactylum tricornutum*. Sequences of the protein studied here (*Xl*(6-4)PL) and of Cry1a found in the retina of magneto-sensitive European robin (*Erithacus rubecula*, *Er*)[9] are shown in boldface. *Ec* = *Escherichia coli*; *Ot* = *Ostreococcus tauri*; *At* = *Arabidopsis thaliana*; *Dm* = *Drosophila melanogaster*; *Dr* = *Danio rerio*; *Mm* = *Mus musculus*; *Hs* = *Homo sapiens*; CRYD = Cry *DASH*; CPD = cyclobutane pyrimidine dimer photolyase; See Figure S2 for more proteins and longer alignment.

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 Crys (the actual putative magnetoreceptors) and animal (6-4) PLs (that specifically repair the so called (6-4) photoproduct in DNA)[1a, 11] 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_{ox} 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 $FAD^{\bullet-}$), 448 nm (maximum of FAD_{ox}) and 562 nm (maximum of $TrpH^{\bullet+}$); for other wavelengths, see Figure S3. The wild-type (WT) and a mutant protein, in which the 4th tryptophan was replaced by non-reducing phenylalanine (W370F), exhibited completely different kinetic behaviour, providing strong evidence that the 4th tryptophan does indeed participate in ET to FAD in the WT protein.

Initial signal amplitudes of both WT and W370F *Xl*(6-4)PL are very similar at all wavelengths; we attribute them to the formation of $FAD^{\bullet-}$ $Trp_4H^{\bullet+}$ and $FAD^{\bullet-}$ $Trp_3H^{\bullet+}$ radical pairs, respectively (Figures 3, 4 and S3), with a quantum yield of ~30% (see SI for details). The difference between the two proteins, however, becomes obvious already in the first few microseconds: in the WT protein, signals below 500 nm (essentially due to reduction of FAD_{ox} to $FAD^{\bullet-}$) remained virtually constant for at least 80 µs, indicating formation of a long-lived radical pair. At wavelengths > 515 nm, the signals decayed with a time constant $\tau \sim 2.5$ µs, which we attribute to deprotonation of $Trp_4H^{\bullet+}$ (see SI for discussion of this unusually slow deprotonation). In the W370F mutant, however, at all wavelengths, about 50% of the initial signal amplitudes decayed with $\tau \sim 200$ ns; the rest decayed nearly completely with $\tau \sim 10$ 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 $FAD^{\bullet-}Trp_3^{\bullet}$ pair at ~50% the yield of $FAD^{\bullet-}$ $Trp3H^{\bullet+}$ observed initially. We conclude that the 200 ns decay represents a competition between charge recombination in the $FAD^{\bullet-}$ $Trp3H^{\bullet+}$ pair and deprotonation of $Trp_3H^{\bullet+}$ (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 $FAD^{\bullet-}$ $Trp_3^{\bullet}$ 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 $TrpH^{\bullet+}$ and there were no detectable losses due to recombination on this time scale (Figures 3a and 4b). We hence conclude that recombination of the $FAD^{\bullet-}$ $Trp_4H^{\bullet+}$ radical pair must be at least 10× slower than $Trp_4H^{\bullet+}$ deprotonation, leading to a recombination time constant of >25 µs for $FAD^{\bullet-}$ $Trp_4H^{\bullet+}$.

The $FAD^{\bullet-}$ $Trp_4^{\bullet}$ 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 $FAD^{\bullet-}$ $Trp_3^{\bullet}$ 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 $FAD^{\bullet-}$ $Trp_4^{\bullet}$ (~40 ms) and a substantially slower protonation of $FAD^{\bullet-}$ (~200 ms). The overall mechanism of FAD_{ox} photoreduction in *Xl*(6-4)PL and the measured or estimated time constants are summarized in Scheme 1.

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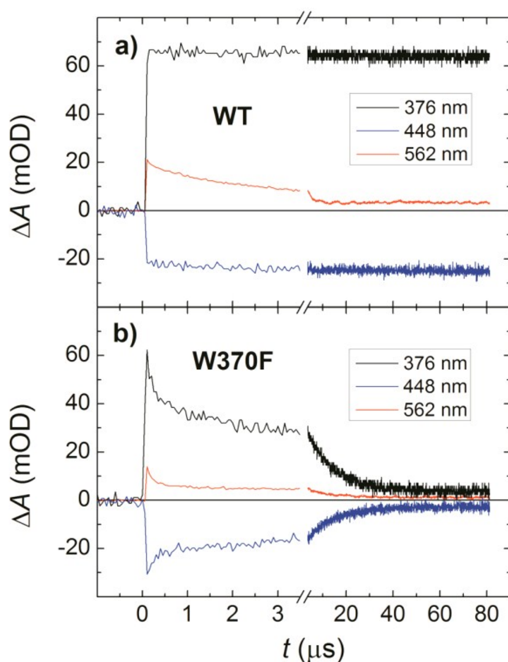

Fig. 3 Flash-induced absorption changes on ns and μs time scales for (a) WT *Xl*(6-4)PL and (b) its W370F mutant at three characteristic wavelengths. Samples were excited at 355 nm by a 100 ps pulse of E ~4.0 mJ.cm^{-2}. See Figure S3 for traces recorded at additional wavelengths.

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 $FAD^{\bullet-}$ $Trp_4H^{\bullet+}$ and $FAD^{\bullet-}$ $Trp_4^{\bullet}$ 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 $FADH^{-}$, 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.[12] The negligible recombination of the $FAD^{\bullet-}$ $Trp_4H^{\bullet+}$ pair and the lifetime of the $FAD^{\bullet-}$ $Trp_4^{\bullet}$ 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

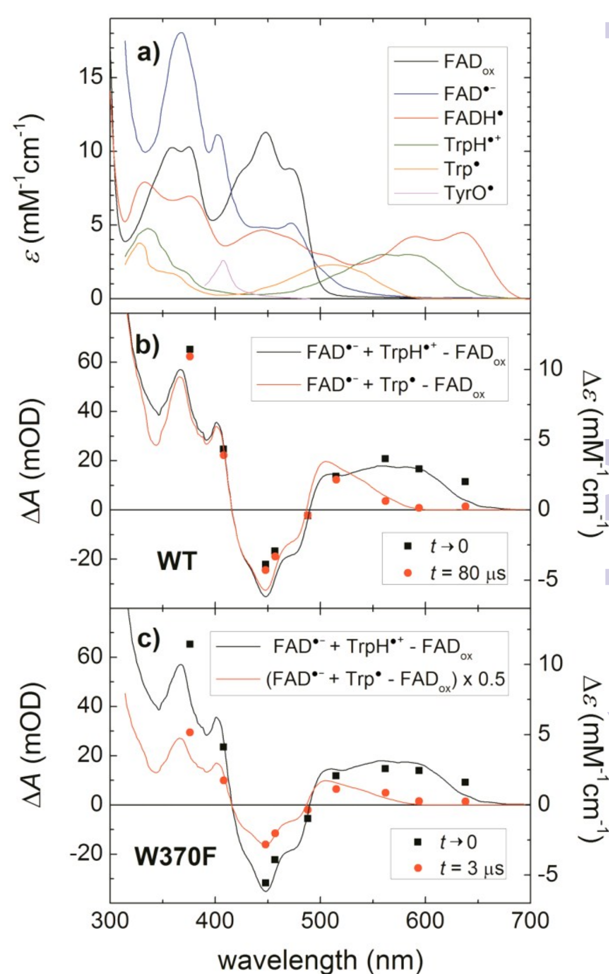

Fig. 4 Spectral analysis of transient absorption kinetics. (a) Absorption spectra of species susceptible to contribute to the photoreactions of *Xl*(6-4)PL upon FAD excitation. The FAD_{ox} spectrum was measured in *Xl*(6-4)PL and scaled to ε (at λ_{max}) = 11 300 M^{-1}cm^{-1}.[13] The $FADH^{\bullet}$ spectrum was constructed as described previously[3b] using the *Xl*(6-4)PL FAD_{ox} spectrum and that of a mixture of FAD_{ox} and $FADH^{\bullet}$ in the same sample (obtained by partial photoreduction). The $FAD^{\bullet-}$ spectrum (from an insect cryptochrome) and spectra of Trp and Tyr radicals are adopted from the literature.[14] (b) Superposition of observed (symbols) signal amplitudes (ΔA) for WT *Xl*(6-4)PL at $t \to 0$ (extrapolation, see SI) and at t = 80 μs with expected (lines) difference spectra for the formation of the radical pair states $FAD^{\bullet-}$ $TrpH^{\bullet+}$ and $FAD^{\bullet-}$ $Trp^{\bullet}$ constructed from the spectra in panel (a). (c) Same as (b), but for the W370F mutant protein and with data taken at 3 μs. The expected difference spectrum for formation of $FAD^{\bullet-}$ $Trp^{\bullet}$ was downscaled by a factor 0.5 to account for the loss of radical pairs in the first 3 μs (see Figure 3b).

suggestion that $FADH^{\bullet}$ $O_2^{\bullet-}$ formed during $FADH^{-}$ reoxidation by O_2 may be the magnetosensitive radical pair in Cry[10-15] merits experimental verification.

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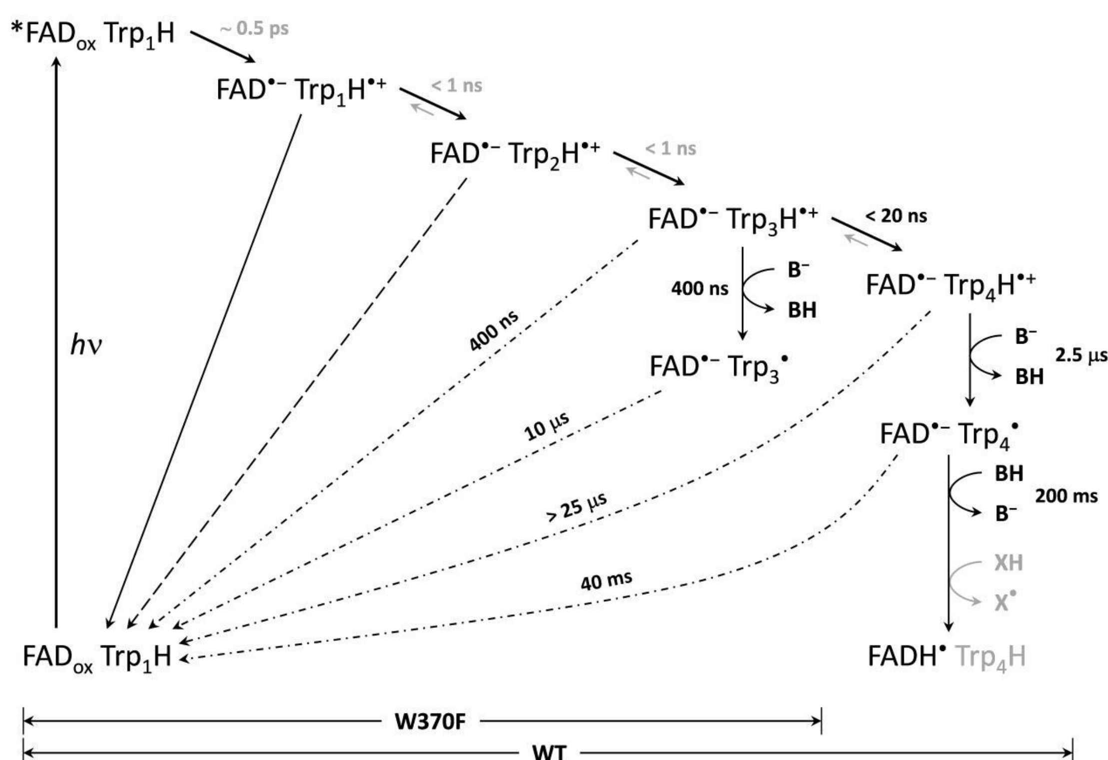

Scheme 1 Reaction scheme of FAD$_{ox}$ photoreduction in *Xl*(6-4)PL. Time constants in grey were taken from the literature on other CPF proteins[2]. Time constants in black were obtained in the present study in 50 mM Tris buffer of pH ~8.3 at 10°C. The rightmost reactions do not occur in the W370F mutant protein because phenylalanine at the position of the 4th tryptophan cannot be oxidized by Trp$_3$H$^{•+}$.

Notes and references

1 (a) A. Sancar, *Chemical Reviews*, 2003, **103**, 2203; (b) I. Chaves, R. Pokorny, M. Byrdin, N. Hoang, T. Ritz, K. Brettel, L.-O. Essen, G. T. J. van der Horst, A. Batschauer and M. Ahmad, in *Annu. Rev. Plant Biol.*, 2011, **62**, 335.

2 (a) J. Brazard, A. Usman, F. Lacombat, C. Ley, M. M. Martin, P. Plaza, L. Mony, M. Heijde, G. Zabulon and C. Bowler, *J. Am. Chem. Soc.*, 2010, **132**, 4935; (b) D. Immeln, A. Weigel, T. Kottke and J. L. Perez Lustres, *J. Am. Chem. Soc.*, 2012, **134**, 12536; (c) Z. Liu, C. Tan, X. Guo, J. Li, L. Wang, A. Sancar and D. Zhong, *Proc. Natl. Acad. Sci. USA*, 2013, **110**, 12966.

3 (a) C. Aubert, M. H. Vos, P. Mathis, A. P. M. Eker and K. Brettel, *Nature*, 2000, **405**, 586; (b) P. Müller, J.-P. Bouly, K. Hitomi, V. Balland, E. D. Getzoff, T. Ritz and K. Brettel, *Sci. Rep.*, 2014, **4**, 5175.

4 T. Ritz, S. Adem and K. Schulten, *Biophys. J.*, 2000, **78**, 707.

5 I. A. Solov'yov, D. E. Chandler and K. Schulten, *Biophys. J.*, 2007, **92**, 2711.

6 (a) K. B. Henbest, K. Maeda, P. J. Hore, M. Joshi, A. Bacher, R. Bittl, S. Weber, C. R. Timmel and E. Schleicher, *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 14395; (b) K. Maeda, A. J. Robinson, K. B. Henbest, H. J. Hogben, T. Biskup, M. Ahmad, E. Schleicher, S. Weber, C. R. Timmel and P. J. Hore, *Proc. Natl. Acad. Sci. USA*, 2012, **109**, 4774.

7 E. W. Evans, C. A. Dodson, K. Maeda, T. Biskup, C. J. Wedge and C. R. Timmel, *Interface Focus*, 2013, **3**, 20130037.

8 S. Coesel, M. Mangogna, T. Ishikawa, M. Heijde, A. Rogato, G. Finazzi, T. Todo, C. Bowler and A. Falciatore, *EMBO Rep.*, 2009, **10**, 655.

9 C. Nießner, S. Denzau, J. C. Gross, L. Peichl, H.-J. Bischof, G. Fleissner, W. Wiltschko and R. Wiltschko, *PLoS ONE*, 2011, **6**, e20091.

10 (a) K. Maeda, K. B. Henbest, F. Cintolesi, I. Kuprov, C. T. Rodgers, P. A. Liddell, D. Gust, C. R. Timmel and P. J. Hore, *Nature*, 2008, **453**, 387; (b) K. Maeda, C. J. Wedge, J. G. Storey, K. B. Henbest, P. A. Liddell, G. Kodis, D. Gust, P. J. Hore and C. R. Timmel, *Chem. Commun.*, 2011, **47**, 6563.

11 J. Yamamoto, R. Martin, S. Iwai, P. Plaza and K. Brettel, *Angew. Chem. Int. Ed.*, 2013, **52**, 7432.

12 O. Efimova and P. J. Hore, *Biophys. J.*, 2008, **94**, 1565.

13 P. Macheroux, *Meth. Mol. Biol.*, 1999, **131**, 1.

14 (a) S. Solar, N. Getoff, P. S. Surdhar, D. A. Armstrong and A. Singh, *J. Phys. Chem.*, 1991, **95**, 3639; (b) B. Giese, M. Wang, J. Gao, M. Stoltz, P. Müller and M. Graber, *J. Org. Chem.*, 2009, **74**, 3621; (c) B. Liu, H. Liu, D. Zhong and C. Lin, *Curr. Opin. Plant Biol.*, 2010, **13**, 578.

15 P. Müller and M. Ahmad, *J. Biol. Chem.*, 2011, **286**, 21033.

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