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What Makes the Difference between a Cryptochrome and DNA Photolyase? A Spectroelectrochemical Comparison of the Flavin Redox Transitions

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Cryptochromes[1] and DNA photolyases[2] are flavoproteins of a highly homologous amino acid sequence and 3D structure that accomplish completely different tasks in the cell. While plant cryptochrome1 (CRY) functions as blue light photoreceptor that triggers various morphogenic reactions, CPD photolyases (PL) repair certain frequently occurring UV-induced DNA damages-cyclobutane pyrimidine dimers (CPD). Both enzymes share the active cofactor, a noncovalently bound flavin adenine dinucleotide, in characteristic bent U-configuration and the peculiarity that functioning is triggered by photoexcitation of the flavin moiety of this cofactor. For PL, a reaction mechanism has been suggested that involves electron transfer (ET) to the substrate from the excited-state of the flavin in its fully reduced (Red) state $FADH^-$ with subsequent electron return within a nanosecond.[3] For CRY, with the flavin initially in the oxidized (Ox) state FAD, it has previously been shown that photoexcitation of the flavin leads to formation of the semireduced (SR) neutral radical $FADH^•$ and an oxidized tyrosine that lives for several milliseconds.[4] This suggests an electron abstraction in analogy to PL photoactivation (restitution of $FADH^-$ from $FADH^•$),[5,6] but despite considerable efforts[7−9] none of the suggested signaling mechanisms (autophosphorylation, conformational change) up to now has been convincingly shown to be coupled to these primary events. Key parameters for the redox state of the flavin cofactor in the cell are the midpoint redox potentials E_1 and E_2 for the Ox⇔SR and SR⇔Red transitions, respectively. A link between CRY function and its cofactors' redox state has been suggested early on,[10] but no reliable determinations of midpoint potentials have been available. Here we report spectroelectrochemical titrations of cryptochrome1 from *Arabidopsis thaliana* (*A.t.*) and CPD photolyases from both *E. coli* (*E.c.*) and *Anacystis nidulans* (*A.n.*) that allow comparison of their redox transitions.

Redox titrations were carried out on a home-built potentiostat that imposes an adjustable potential upon the solution, via a three-electrode setup and suitably chosen soluble mediators, thus avoiding the potentially harmful[11] effects of dithionite application to flavoproteins. The potential E actually established in the solution was read out between the gold working electrode and the Ag/AgCl/3 M KCl reference electrode. For each E, the amount of flavin radical formed was determined by measuring an UV−vis spectrum of the sample (1 cm optical path). Between spectral measurements, the sample was kept in the dark to avoid photochemical reduction and the temperature was stabilized to 10 °C. Under these conditions, it was possible to complete a full reduction−oxidation−reduction cycle on a single sample without significant protein degradation. As none of the applied mediators nor the oxidized or reduced forms of the flavin do absorb above 570 nm, the sample absorbance at 600 nm can be taken as a direct measurement of the amount of $FADH^•$ which has a distinct absorption peak there (624 nm for PL). Details on the method can be found in ref 12 and in the Supporting Information (SI).

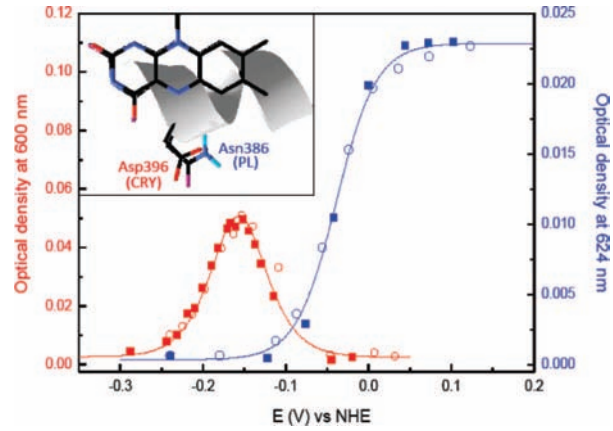

Figure 1. Detection of $FADH^•$ during reductive (■) and oxidative (○) titration of the FAD cofactor in CRY (35 μM, red) and PL (5 μM, blue). The *Y*-axes are scaled such that the top level of the PL titration curve corresponds to 100% $FADH^•$ for CRY as well. Inset: Overlay of the crystal structures around the flavin of PL from *A.n.* and CRY1 from *A.t.*

Redox titration of CRY (red symbols in Figure 1) with detection of $FADH^•$ at 600 nm yielded a bell-shaped curve peaking at −157 mV vs NHE. The peak absorption corresponds to 37% of the flavins being semireduced ($FADH^•$), as estimated using a ratio of 3.11 between the extinction coefficients of FAD at 444 nm and of $FADH^•$ at 600 nm (see SI). According to the Nernst equation applied to two one-electron transitions (Ox⇔SR ⇔Red) at 10 °C (see SI),

$$\frac{[\mathrm{SR}]}{[\mathrm{Ox}]+[\mathrm{SR}]+[\mathrm{Red}]}=\left\{1+\exp\left(\frac{E-E_1}{24\ \mathrm{mV}}\right)+\exp\left(\frac{E_2-E}{24\ \mathrm{mV}}\right)\right\}^{-1} \quad (1)$$

the peak population of SR is expected to be significantly lower than 100% for $E_1 - E_2 < 0.2$ V, as observed, for example, for the FAD domain of the human novel reductase1 ($E - E_2 = 85$ mV),[13] or the FAD domain of human NADPH-cytochrome P450 reductase ($E_1 - E_2 = 50$ mV).[14] Under such conditions, the height of the peak becomes a key to the correct determination of the midpoint potentials. For $E_1 = E_2$, this peak will be at 33% and for the inversed order ($E_1 < E_2$), as is the case with flavin in solution (cmpd FMN

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Table 1. Midpoint Potentials in mV vs NHE as Derived from Nernst Fits to Data as Shown in Figure 1 or Taken from the Literature

flavin	E_1: Ox⇔SR	E_2: SR⇔Red
FMN in aq solution[a]	−313	−101
FAD in *A.t.* CRY1[b,c]	$-153_{+9}{}^{-6}$	$-161_{-9}{}^{+6}$
FAD in *A.n.* PL[b]		-39 ± 5
FAD in *E.c.* PL[b]		-48 ± 5
FAD in *E.c.* PL[d]		16 ± 6

[a] From ref 23, pH = 7.0. [b] This work, pH = 7.4. [c] Errors are due to an estimated incertainty of ±20% for the extinction coefficient of $FADH^{\bullet}$ (see SI). [d] From ref 15, pH = 7.0.

in Table 1), even less SR will be observed. The only published attempt[10] of reduction potential determination for CRY reported $E_1 > E_2$ based on a titration curve with a $FADH^{\bullet}$ peak population of only ∼18%, casting a doubt on the interpretation of that experiment. Our fit of the titration data in Figure 1 to eq 1 (see SI for the conversion of optical density to $FADH^{\bullet}$ population) yields nearly equal midpoint potentials for the two transitions as indicated in Table 1.

Titration curves strikingly different from that for CRY were obtained for photolyases from *A.n*. (blue symbols in Figure 1) and from *E.c*. (not shown; fit results presented in Table 1) in agreement with an earlier report.[15] During oxidative titrations, 100% $FADH^{\bullet}$ was formed and no trace of the transition to oxidized FAD was observed up to potentials as high as 0.4 V. As compared to CRY, the SR⇔Red transition in PL is up-shifted by about 120 mV. A further upshift of the SR⇔Red transition occurred upon binding of UV-damaged DNA, by 71/76 ± 7 mV (*A.n./E.c.*, see SI), in agreement with a previous report on PL from *E. coli*.[15] Interestingly, for cryptochrome1 from *C. reinhardtii*, upon binding of ATP, a slowed reoxidation of $FADH^{\bullet}$ has been observed and interpreted as selective stabilization of the semireduced form.[16]

The resolved crystal structures[17−19] for CRY and PL may serve as a basis for explaining these striking differences in redox behavior. The most common mechanisms of flavin redox potential tuning by the protein environment find little support by comparison of the structures: (i) No aromatic amino acids are found in π-stacking configuration with FAD, (ii) the H-bond pattern around the flavins is highly similar in CRY and PL, and (iii) flavin bending, although not detectable at the present structural resolutions, is expected to influence rather the SR⇔Red transition than the Ox⇔SR transition,[20] in contrast to our observations. We therefore suggest an alternative explanation on the basis of the (non)availability of an acceptor/donor for the flavin's N5 proton required to accomplish the Ox⇔SR transition. In the PL/CRY sequence alignment, the residue opposite to N5 that would serve this role is conserved: Asp in CRY (D396 in *A.t.*) and Asn in PL (N386/378 in *A.n./E.c.*). Asn is very hard to protonate ($pK_a < 0$), explaining that we could not oxidize SR flavin in PL. Asp in CRY might donate/accept the N5 proton, as suggested based on FTIR measurements.[7] For this role, D396 may either be normally protonated,[7] or be part of a larger proton transfer network and relay a proton to the flavin's N5 without being protonated on the average to a considerable extent. The more negative potential of the $FADH^{\bullet} \Leftrightarrow FADH^{-}$ transition in CRY compared to PL may be due to a negative charge on deprotonated D396 compared to neutral Asn in PL.

As PL requires fully reduced flavin ($FADH^{-}$) for photorepair of DNA, full oxidation to FAD is not necessary for biological function. For CRY, with a functional ET chain inherited from PL, it has been suggested[21] that FAD is the "dark state" that absorbs blue light to form the "signalling state" $FADH^{\bullet}$. The signaling state could be deactivated by green light to form fully reduced $FADH^{-}$ (followed by spontaneous dark oxidation). To make both transitions accessible by light in CRY, the flavin should be present in the fully oxidized state in the dark. With the midpoint potentials determined here, 90% of the flavins are expected to be fully oxidized, 10% semireduced, and <1% fully reduced at a typical potential of −100 mV[22] inside a plant cell. Blue light would increase the concentration of semireduced flavin to a level that might trigger downstream signaling by a yet unknown mechanism. We thus hypothesize that down-shift of both flavin redox transitions was essential in the evolution from PL to CRY. A particular role is likely to have been played by the replacement of the Asn residue opposite to the flavin's N5; interestingly, the N378S mutation in *E.coli* PL appears to stabilize the Ox state of the flavin.[9]

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Supporting Information Available: Experimental details for spectroelectrochemical redox titration. This material is available free of charge via the Internet at http://pubs.acs.org.

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