Communication

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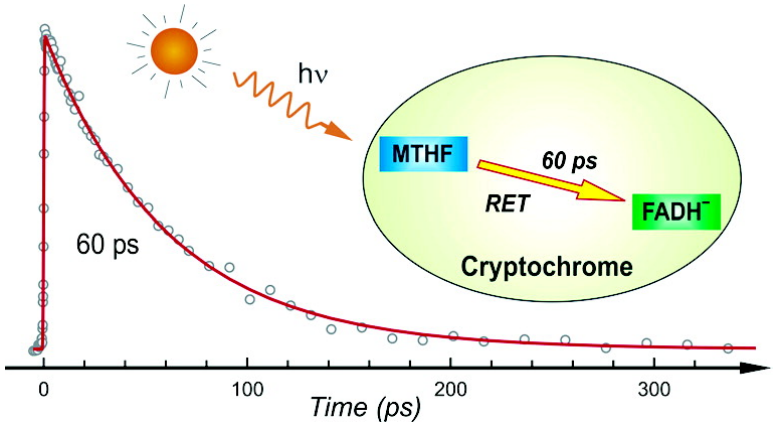

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Ultrafast Dynamics of Resonance Energy Transfer in Cryptochrome

Chaitanya Saxena,[¶] Haiyu Wang,[¶] I. Halil Kavakli,[‡] Aziz Sancar,[‡] and Dongping Zhong*[,¶]

Departments of Physics, Chemistry, and Biochemistry, OSU Biophysics, Chemical Physics, and Biochemistry Programs, 174 West 18th Avenue, The Ohio State University, Columbus, Ohio 43210, and Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Mary Ellen Johns Building, CB 7260, Chapel Hill, North Carolina 27599

Received December 29, 2004; E-mail: dongping@mps.ohio-state.edu

Cryptochrome, a recently discovered blue-light photoreceptor, synchronizes the circadian rhythm in animals and regulates growth and development in plants.[1−4] It is highly homologous to photolyase in sequence, a well-studied photoenzyme for repairing damaged DNA.[3] Both are flavoproteins containing two noncovalently bound chromophores. One chromophore is a flavin−adenine dinucleotide (FAD) as a key cofactor to carry out initial biological function upon photoexcitation, and the other is a pterin in the form of methenyltetrahydrofolate (MTHF) as a light-harvesting antenna to enhance biological efficiency. In vivo, it is believed that in both proteins the flavin is in the fully reduced $FADH^-$ form, but during purification, it is oxidized to FADH or FAD. The resonance energy transfer from MTHF to both FADH and $FADH^-$ in *Escherichia coli* photolyase has been well characterized.[5] However, the photophysics and photochemistry of the two chromophores in cryptochrome have not been studied so far for lack of purified cryptochrome containing both cofactors in significant quantities, and thus there are no data indicating interchromophore energy transfer in cryptochrome as has been found in photolyase. Recently, it was found that a cryptochrome purified from *Vibrio cholerae*, called VcCry1, contained both cofactors in near stoichiometric amount that makes ultrafast kinetic studies feasible.[6] In this communication, with femtosecond resolution, we report our first measurement of resonance energy-transfer dynamics from the photoantenna MTHF to the fully reduced active cofactor $FADH^-$ in *V. cholerae* cryptochrome, VcCry1.

VcCry1 is a monomeric protein with 466 amino acid residues, and its absorption and emission spectra are shown in Figure 1. In contrast to all cryptochromes purified so far that contain FAD in the two-electron oxidized form, in VcCry1, the flavin is mostly in $FADH^-$ form with a minor amount of oxidized FAD, which is unavoidable in the protein preparation.[6] The neutral radical FADH has absorption at longer than 500 nm, thus no FADH cofactor exists in our protein preparation. The fluorescence spectrum is dominated by the $MTHF^*$ emission with a $\lambda_{max} = 480$ nm, at longer wavelength overlapping with the emission from both FAD^* and $FADH^{-*}$. All experiments were done using a protein concentration of 21 μM in 50 mM Tris−HCl buffer at pH 7.4 with 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 50% (v/v) glycerol.[6] Femtosecond-resolved fluorescence up-conversion technique, as described elsewhere,[5] was used to obtain all fluorescence wavelength-dependent transients.

In Figure 2, four typical femtosecond-resolved fluorescence transients are shown, covering the blue-side to the red-side emission. At 400 nm excitation, the fluorescence emission at 440 nm is purely from $MTHF^*$; both FAD^* and $FADH^{-*}$ emit at longer wavelengths with a peak at about 530 nm. The 440 nm transient can be best fitted by three exponential components: 10 ps decay with 14% of the total amplitude, 60 ps (58%), and 845 ps (28%). Protracted handling of the sample under aerobic conditions results in gradual oxidation of $FADH^-$ to FAD as revealed by absorption increase in the range of 450−500 nm (not shown). We repeated femtosecond-resolved fluorescence studies for the oxidized sample, and the obtained transient decayed with the same three time constants but with different amplitudes, as shown in the inset of Figure 2, with removal of the long (lifetime) decay components. The 10 ps component increased to 65%, and the 60 ps component decreased to ~10%. Thus, the 60 ps decay represents the resonance energy-transfer process from $MTHF^*$ to $FADH^-$, and the 10 ps component reflects the transfer dynamics from $MTHF^*$ to FAD. The 845 ps is, therefore, the lifetime of $MTHF^*$ in the protein because free MTHF that might be released from the protein is not stable at neutral pH and is converted to 10-formyltetrahydrolfolate that does not absorb at $\lambda > 300$ nm.[7] Using the obtained percentages for each chromophore and the known molar extinction coefficients of MTHF, $FADH^-$, and FAD,[3,8] the calculated absorption spectrum exactly reproduced the measured one in Figure 1.

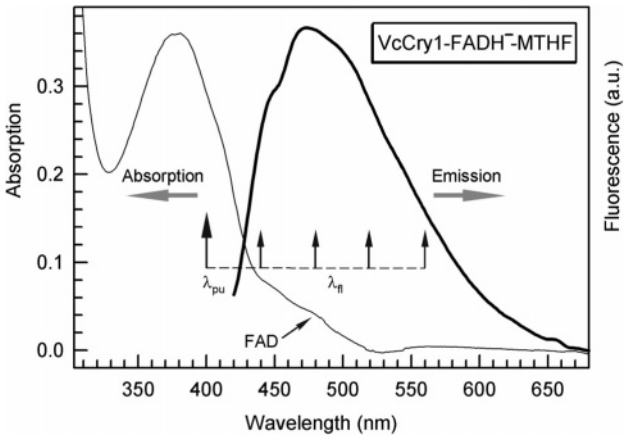

Figure 1. Absorption and emission spectra of *V. cholerae* cryptochrome. The absorption is dominated by the MTHF cofactor. The upward arrows indicate the excitation wavelength (400 nm) and the four gated fluorescence emissions. Note that a small fraction of FAD (14%) is also present as indicated by an arrow in the 450−500 nm region.

The fluorescence transients of VcCry1 gated at other three wavelengths of 480, 520, and 560 nm show a similar temporal behavior. All transients can be represented by the similar three decay components with 10 ps (14−16%), 60 ps (58−47%), and 845−1045 ps (28−37%). At the longer wavelengths, the $FADH^{-*}$ (and minor FAD^*) fluorescence emissions make some contributions. Overall, the observed ultrafast decay (10 and 60 ps) is independent of fluorescence wavelength detection. Thus, in a buffer with 50% (v/v) glycerol, the solvation process of $MTHF^*$ in the protein makes negligible contributions to the observed energy-transfer dynamics,

[¶] The Ohio State University.
[‡] University of North Carolina School of Medicine.

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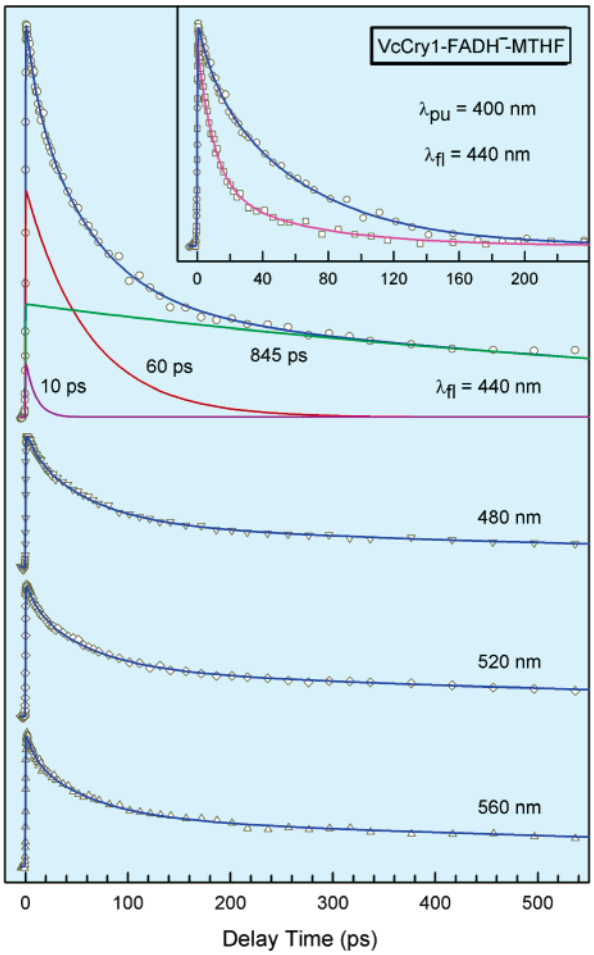

Figure 2. Femtosecond-resolved fluorescence transients of the MTHF* decay probed from the blue-side to red-side emission at 400 nm excitation. All four transients show a three exponential decay of 10, 60, and ~845 ps. In the inset, the signal with a faster decay (□) represents the protein sample with FAD as the dominant flavin cofactor; see text.

as observed in *E. coli* photolyase.[5,9] Thus, the chromophore MTHF is buried in the protein, and the solvation process, if any, would be much longer than the observed energy-transfer dynamics (10 and 60 ps).

We also measured the anisotropy dynamics of MTHF* at 440 nm emission, and the obtained anisotropy was constant with a value of ~0.32 within 1.5 ns (not shown). The result revealed a rigid local structure around the MTHF, indicating that the chromophore is tightly bound to the protein with minimal local motions, also consistent with the absence of ultrafast solvation processes. The recently solved X-ray structure of the photolyase homology (PHR) domain of *A. thaliana* cryptochrome[10] and of *Synechocystis* cryptochrome[11] revealed flavin binding sites very similar to that of photolyase.[12] Thus, the local motion of the $FADH^-$ is highly restricted in this family of proteins. Therefore, the relative orientations of the energy-transfer pairs, MTHF with FAD/$FADH^-$, are well aligned and the orientation factor (κ^2) is well defined, as we recently observed in *E. coli* photolyase.[5] The observed long lifetime component in Figure 2 can be ascribed to MTHF in the absence of flavin since in a fraction of VcCry1 the flavin is lost during purification.[6] According to Förster resonance energy-transfer formulation, the ratio of two energy-transfer rates from MTHF* to $FADH^-$ and to FAD is $k_{FADH^-}/k_{FAD} = (J_{FADH^-}\kappa^2_{FADH^-})/(J_{FAD}\kappa^2_{FAD})$. J is the spectral overlap integral and was calculated to be 0.34×10^{-14} $cm^3 \cdot M^{-1}$ for the MTHF*/$FADH^-$ pair[7] and 3.48×10^{-14} $cm^3 \cdot M^{-1}$ for MTHF*/FAD. From our measurements, the ratio of the energy-transfer rates of MTHF* to $FADH^-$ and FAD is 1/6. Thus, we obtained a ratio of 1.7 for the orientation factors for the two energy-transfer pairs ($\kappa^2_{FADH^-}$ to κ^2_{FAD}). This result indicates a local structural change from the charged ($FADH^-$) to the polar (FAD) flavin redox state due to the strong electrostatic interactions with the surrounding residues and water molecules at the binding site.[10] Similar results have also been observed in photolyase containing $FADH^-$ and FADH, respectively.[5]

Both cryptochrome and photolyase have similar flavin binding sites with a unique hole configuration.[10−12] Structure of photolyase containing both cofactors is available. However, the available cryptochrome structures were obtained with proteins devoid of MTHF,[10] and hence the relative configuration of the two cofactors in cryptochromes is not known. The results reported here suggest different binding interactions and local structures of MTHF in the two proteins, at least with respect to VcCry1. The MTHF* lifetime of 845 ps in VcCry1 cryptochrome is more than 2 times longer than that in *E. coli* photolyase (354 ps),[7] indicating a more hydrophobic and rigid environment. The energy-transfer process of 60 ps is more than 4 times faster than that in photolyase (292 ps),[5] suggesting a shorter distance or a highly favorable orientation of the two chromophores. The ultrafast energy-transfer dynamics in cryptochrome reported here (60 ps) must enhance its functional efficiency, in this case, to facilitate formation of the initial signaling state. Importantly, our results for the first time show energy transfer from MTHF to flavin in cryptochrome, suggesting some mechanistic similarities between photolyase that repairs damaged DNA and cryptochrome that mediates blue-light signaling.

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Supporting Information Available: Complete ref 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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