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Femtosecond Dynamics of Flavin Cofactor in DNA Photolyase: Radical Reduction, Local Solvation, and Charge Recombination

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We report here our femtosecond studies of the photoreduction dynamics of the neutral radical flavin (FADH) cofactor in *E. coli* photolyase, a process converting the inactive form to the biologically active one, a fully reduced deprotonated flavin $FADH^-$. The observed temporal absorption evolution revealed two initial electron-transfer reactions, occurring in 11 and 42 ps with the neighboring aromatic residues of W382 and F366, respectively. The new transient absorption, observed at 550 nm previously in photolyase, was found from the excited-state neutral radical and is probably caused by strong interactions with the adenine moiety through the flavin U-shaped configuration and the highly polar/charged surrounding residues. The solvation dynamics from the locally ordered water molecules in the active site was observed to occur in ∼2 ps. These ultrafast ordered-water motions are critical to stabilizing the photoreduction product $FADH^-$ instantaneously to prevent fast charge recombination. The back electron-transfer reaction was found to occur in ∼3 ns. This slow process, consistent with ultrafast stabilization of the catalytic cofactor, favors photoreduction in photolyase.

I. Introduction

Photolyase is an enzyme that uses light energy to repair UV-induced DNA damage.[1,2] The enzyme utilizes a fully reduced deprotonated flavin $FADH^-$ as a catalytic cofactor.[3] The excited cofactor $FADH^-$*, formed by absorption of one photon directly or through resonance energy transfer from a photoantenna molecule, donates one electron to the damaged DNA (thymine dimer), a key step for the repair function. However, the flavin cofactor in vitro is usually oxidized into the inactive neutral radical form FADH in most photolyases. This radical can be converted to the active form $FADH^-$ through electron transfer from neighboring aromatic residues by illumination with visible light, a process called photoreduction.

Two photoreduction processes involving a tryptophan triad and an α-helix in *E. coli* photolyase have been proposed according to the X-ray structure.[4] By site-specific mutagenesis, the ultimate electron donor was identified as the tryptophan residue 306, which is at the protein surface.[3,5] Ultrafast experiments[6,7] reported the observation of photoreduction along the tryptophan triad with an initial electron transfer in ∼26 ps. However, recent femtosecond-resolved studies[8] revealed two possible reduction pathways: one is along the tryptophan (W) triad but with the initial electron hop in ∼10 ps, and the other route starts with an initial electron separation in ∼40 ps through the neighboring phenylalanine (F) followed by either tunneling along an α-helix or hopping through the tryptophan triad again.

The X-ray structure[4] shows a unique U-shaped structure of the flavin cofactor at the active site (Figure 1), but the functional role of such an unusual configuration is still unknown. A recent report[9] showed an extra transient absorption band around 550 nm by comparison with the same species, but in a stretched shape, in glucose oxidase. Various speculations on such a new absorption were proposed, including the intramolecular charge-transfer interaction in flavin, facilitation of electron transfer in photoreduction, and stabilization of the reaction products. Molecular dynamics simulations[10] suggest that this unique shape mediates the electron transfer from the flavin to the dimer substrate through the adenine moiety.

The X-ray structure also reveals a favorable electrostatic environment[4] at the active site for the fully reduced $FADH^-$ cofactor (Figure 1). Several water molecules participate in the H-bonding network around the flavin, and fourteen amino acids, mostly polar and charged, tightly surround the cofactor. Through solvation processes, these strong electrostatic interactions must play an important role in the dynamics of the excited-state flavin cofactor and in the stabilization of the final products. The overall photoreduction quantum yield[11] is in the range 0.05−0.1, and therefore, charge recombination during a series of electron-transfer processes must be severe. The dynamics of these back electron-transfer reactions has not yet been characterized.

In this letter, we report our studies of the excited-state dynamics of the neutral flavin radical with femtosecond resolution, especially around the new additional absorption at 550 nm, and the local solvation dynamics at the active site. We also report the time scale of the charge recombination of the initial charge separation in photoreduction.

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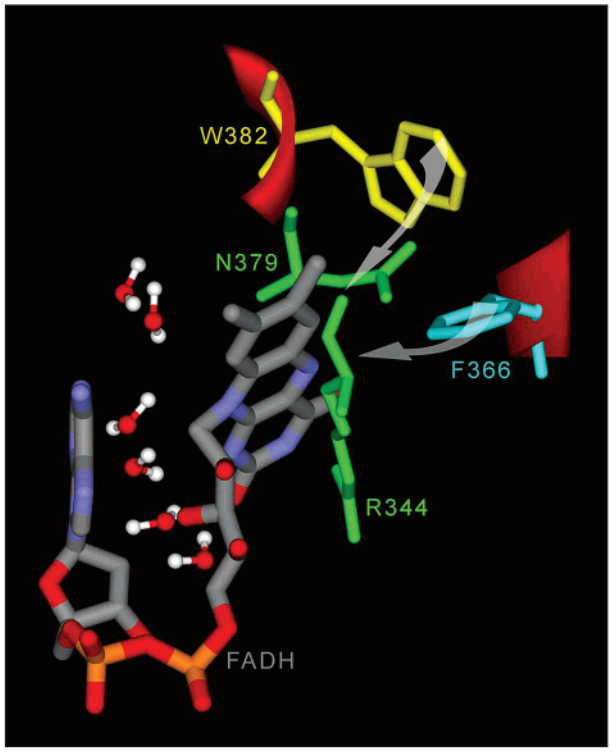

Figure 1. Configuration of the catalytic flavin cofactor from the X-ray crystal structure[4] of *E. coli* photolyase with six water molecules and four surrounding residues in less than 6 Å to the isoalloxazine ring of the cofactor. Two aromatic residues (W382 and F366) are involved in initial electron transfer in photoreduction. For clarity, only one polar (N378) and one charged (R344) residues out of fourteen surrounding ones are shown. Water molecules and highly polar/charged residues at the active site stabilize the photoreduction product, the active catalytic form $FADH^-$, as well as reaction intermediates during the repair process.

II. Experimental Section

All experimental measurements were carried out by a highly sensitive transient absorption method. The experimental setup has been described elsewhere.[8] Briefly, we used a pump wavelength of 620 nm which was generated from a doubling of the signal (1240 nm) from the output of an optical parametric amplifier (OPA) through a thin β-barium borate (BBO) crystal (0.2 mm). The probe wavelengths from 400 to 700 nm were generated by mixing of the idler or signal with the fundamental from a second OPA through another thin BBO crystal (0.2 mm). Both the pump and the probe pulses were compressed through a pair of prisms with double paths to reach a temporal resolution of 60 fs. The pump beam polarization was set at a magic angle (54.7°) directly with respect to the probe beam which was vertical. The sensitivity of the transient absorption method can reach $10^{-4}-10^{-5}$ of absorbance change.

DNA photolyase from *E. coli* was prepared as described previously.[12] Protein concentration of ~300 μM was used in a buffer containing 50 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 50% (v/v) glycerol. The absorption spectra with and without the photoantenna molecule (MTHF) are shown in Figure 2. This chromophore can be removed from the enzyme through a photodecomposition process.[11,13] After purification, the enzyme contains the oxidized neutral radical FADH with typical absorption longer than 500 nm (Figure 2). For all experiments, we used the enzyme with the MTHF. Because the pump wavelength was fixed at 620 nm where MTHF has no absorption, only the FADH radical was excited.

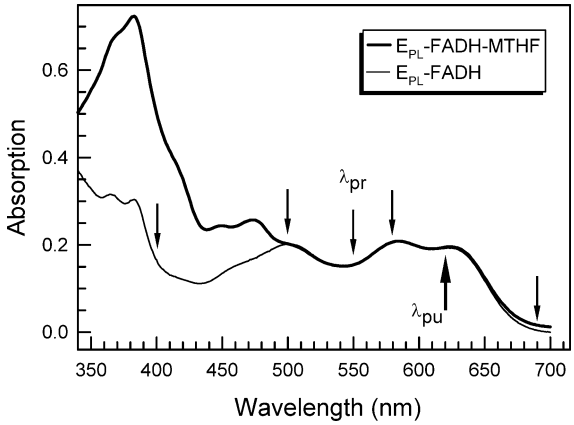

Figure 2. The absorption spectra of *E. coli* photolyase in the semi-oxidized neutral radical form with (thick dark line) and without (thin dark line) the photoantenna molecule (MTHF). The upward arrow indicates the excitation wavelength of 620 nm, and a series of downward arrows represent the probe wavelengths ranging from 400 to 700 nm.

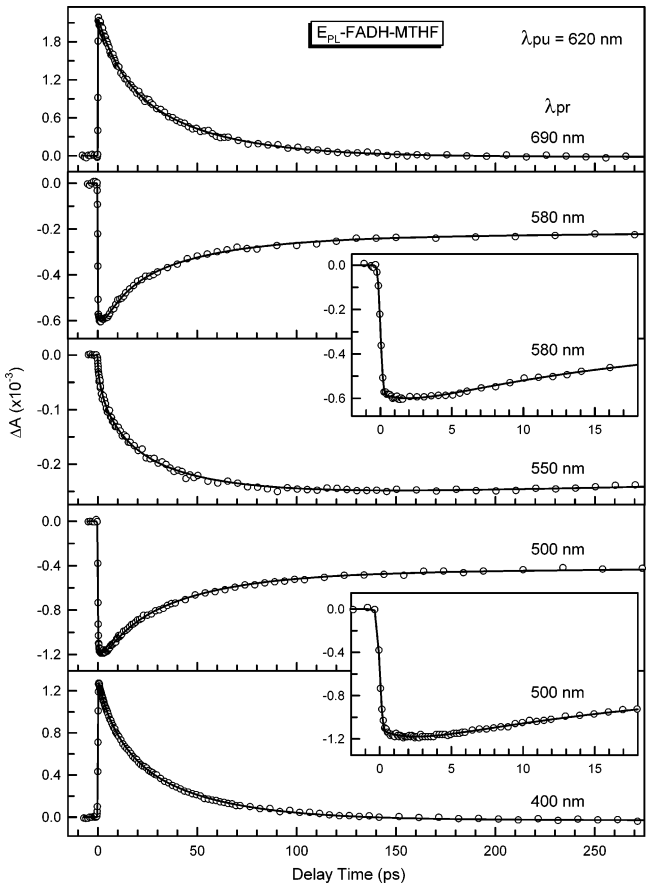

Figure 3. Femtosecond-resolved transient absorption measurements with excitation of 620 nm and probes of five different wavelengths at 690 nm (top), 580, 550, 500, and 400 nm (bottom). Only the FADH molecules were excited. The initial parts of the transients probed at 580 and 500 nm are shown in the inserts with a decay constant of about 2 ps. Note that the transient at 550 nm is very different from those probed at 580 and 500 nm.

All samples in a volume of 200 μL were kept in a rotating quartz cell to avoid heating and photobleaching.

III. Results and Discussion

A. Dynamics of the Excited-State Neutral Flavin Radical. Figure 3 shows five transients probed in the range 400−690 nm with excitation of 620 nm. All transients are a result of

TABLE 1: Time Constants (ps) and Relative Amplitudes Obtained from Fitting of Transients[a]

λ_{pr} (nm)	τ_1	a_1	τ_2	a_2	τ_3	a_3	a_3/a_2	τ_4	a_4
690			11	0.35	42	0.65	1.86		
580	2	0.15	11	−0.25	42	−0.39	1.56	3400	−0.36
550	2	0.15	11	0.34	42	0.51	1.50	3200	−1.11
500	2	0.16	11	−0.16	42	−0.48	3.00	3100	−0.36
400	2	0.06	11	0.36	42	0.58	1.61	3000	−0.02

[a] All transients were fitted by $\sum_{i=1}^{4} a_i e^{-t/\tau_i}$, and the positive amplitude represents decay dynamics, while the negative value means species formation (rise).

superposition of the negative ground-state bleaching and positive excited-state absorption. They were taken within 1.5 ns and can be fitted globally with four time constants: ~2, 11, and 42 ps, and ~3 ns. The time constants and the corresponding amplitudes are given in Table 1. Specifically, at 690 nm, we only observed a positive decay signal, and this signal is from the excited-state FADH*. The initial reactants (FADH and W/F) and the final electron-transfer products ($FADH^-$ and W^+/F^+), if the electron donor is tryptophan/phenylalanine, have no absorption at 690 nm ($FADH^-$ absorbs at $\lambda < 500$ nm[8]). The transient was best fitted by a double-exponential decay with two time constants of 11 ps (35%) and 42 ps (65%), respectively. The observed electron-transfer bifurcation is consistent with our findings at excitation of 580 nm,[8] where we also observed similar dynamics. The two decay processes represent two electron-transfer reactions (Figure 1): one is assigned to electron hop from W382 in 11 ps and the other to electron transfer from the nearby F366 in 42 ps. This bifurcation results from the different spatial configurations due to the dynamic motions of the cofactor and residues.

The oxidation potential of tryptophan (W/W^+)[14] is ~+1.0 V vs normal hydrogen electrode (NHE), and the reduction potential of the flavin cofactor ($FADH/FADH^-$)[15] was estimated between −0.33 and −0.5 V vs NHE. Under excitation of 620 nm (2 eV, close to 0−0 transition), the net free energy change ($\Delta G°$) of the electron-transfer reaction from W382 to FADH is negative, between −0.67 and −0.5 V, and the reaction is feasible. The oxidation potential of phenylalanine is not known but is estimated to be less than +2 V vs NHE.[16] The net free energy change ($\Delta G°$) for electron transfer from F366 to FADH is around zero, and the reactivity highly depends on the local environment. Most of the surrounding residues around F366 are polar and charged, and four oxygen atoms of peptide bonds are in proximity of the F366 ring within 4.5 Å. All of these favor charge separation and stabilize the $F366^+$ cation. The electron-transfer reaction between the excited, fully oxidized flavin FAD* and phenylalanine in flavodoxin has been recently reported to occur in 18 ps.[17] Another possibility is that the reaction of FADH* with phenylalanine (F366) follows Mulliken's charge-transfer mechanism[18] as observed in the benzene−iodine complex.[19] Because of the van der Waals contact and the strong orbital overlap between the donor and acceptor, there might exist a charge-transfer excited state. The charge-transfer potential surface can cross with the excited local-state one;[19] thus, excitation of a local state (FADH*) finally results in a charge separation. The distance between FADH and F366 is 4 Å, also favoring a charge-transfer reaction. Thus, we believe that the electron-transfer reaction between FADH* with F366 is feasible in *E. coli* photolyase, which is also supported by recent theoretical calculations.[20] The electron-transfer processes can be written as follows:

$$\text{FADH}^* + \text{W382/F366} \xrightarrow{k_1} \text{FADH}^- + \text{W382}^+/\text{F366}^+$$

$$\xrightarrow{k_2} \text{FADH} + \text{W382/F366} \quad (3.1)$$

$$\xrightarrow{k_3} \text{FADH}^- + \text{W382/F366} \quad (3.2)$$

The second step (k_2) of eq 3.1 is the charge recombination, which is unfavorable to photoreduction, and the process (k_3) of eq 3.2 represents the photoreduction pathway that the electron hops along on the tryptophan triad and/or tunnels through the α-15 helix. Here, we determined the k_1 rate for transfer from W382, $(11 \text{ ps})^{-1}$, and F366, $(42 \text{ ps})^{-1}$.

With the 580-nm probe, we observed an initial negative bleaching/decay signal and then a formation/recovery component. Because the probe is at a shorter wavelength than the pump, the stimulated emission process, although it gives a negative signal in our observation, cannot occur because of energetics. The bleaching signal is due to the depletion of the ground-state population of FADH by pump excitation at 620 nm. The ultrafast positive decay component (~2 ps, Table 1) reflects the dynamics of the excited-state FADH*. As discussed in section B, the 2-ps decay manifests the local solvation process. The observed rise signal of 11 and 42 ps represents the formation of $W382^+$ and $F366^+$, respectively, consistent with the observed dynamics at the 690-nm probe. The observation is also supported by the fact that the $FADH^-$ has no absorption at 580 nm, and both cations have absorption at this wavelength, particularly with a characteristic absorption band around 580 nm for the tryptophan cation ($W382^+$).[5,6] The observed ~3-ns rise component is attributed to the recovery signal of FADH (k_2) through charge recombination. Because k_2 is much smaller than k_1, the recovery dynamics is apparently dominated by the k_2 process.

With the 550-nm probe, although the signal was very weak, we clearly observed three decay components (2, 11, and 42 ps), *but not a formation signal*, and a recovery transient (~3 ns). The time scales are the same as we observed using the probe of 690 and 580 nm, and hence, the decay signal has to represent the FADH* dynamics. Therefore, the additional positive absorption at 550 nm is directly from the excited-state FADH*, which at this probe wavelength dominates over the absorption of $W382^+$ and $F366^+$. The ~3-ns rise signal is again from the recovery of the FADH. In comparison with the absorption of the FADH* in glucose oxidase,[9] the new absorption at 550 nm in photolyase must be due to the strong interactions of the excited-state FADH* with the adenine moiety through the unusual U-shaped configuration and with the highly polar surrounding environment.

When the probe wavelength was tuned to 500 nm, the results were similar to those observed at 580-nm excitation in our early studies.[8] Previously, we fitted the formation component dominantly with 42 ps, neglecting the initial 2-ps decay signal. The overall transient profile is similar to that with the probe of 580 nm. Here, we fitted one decay (2 ps) and two rise components (11 and 42 ps) with a dominant contribution from the 42-ps signal (Table 1). The 42-ps signal is from the $F366^+$ formation and is also based on the peak absorption of the benzene cation around 500 nm.[21] Although the deprotonated tryptophan neutral radical has an absorption peak around 500 nm,[5,6] it seems unlikely that deprotonation of the tryptophan cation occurs within 40 ps, and a recent study showed that the process takes about 300 ns in photolyase.[22]

With the 400-nm probe, the obtained signal is similar to that at excitation of 580 nm, as well as to those probed at 690 nm with both 580-nm and 620-nm excitations.[8] The excited-state

FADH* decay dominates the overall signal with 11-ps and 42-ps time constants (Table 1). Thus, at 400 nm, the excited-state absorption of FADH* is dominant.

It is now possible to understand the initial photoreduction dynamics by following the absorption evolution of all species involved in eqs 3.1 and 3.2. For the excited neutral radical FADH*, there are two strong absorption bands around 400 and 700 nm with an immediate absorption around 550 nm. The tryptophan cation ($W382^+$) has an absorption peak around 580 nm, and the phenylalanine cation ($F366^+$) probably peaks around 500 nm. Given the distances of FADH with W382 (4.2 Å) and with F366 (4 Å) and their redox potentials, the forward electron-transfer dynamics (11 and 42 ps) are ultrafast but reasonable, compared with the observed electron-transfer processes in other flavin proteins.[17,23,24] However, the back electron transfer observed here (~3 ns) seems slow, which favors flavin photoreduction. Therefore, the unusual U-shaped configuration of the flavin cofactor and the highly polar environment at the active site may not facilitate the forward electron transfer in photoreduction, but probably stabilize the photoreduction product $FADH^-$ by slowing the rate of back electron transfer.

B. Solvation Dynamics at the Active Site. The flavin cofactor is accessible to the flat enzyme surface through a hole in the middle of the α-helical domain, and water molecules can easily diffuse in and out of the hole. The X-ray structure[4] does show several water molecules involved in the H-bonding network around the cofactor in the active site. Fourteen amino acid residues are also bound to the cofactor, and most of them are polar and charged. The excited-state flavin FADH* can be easily solvated by such an environment through strong electrostatic interactions.

The ~2-ps decay components appear in most transients (see Table 1 and inserts in Figure 3) and must reflect solvation processes of the excited-state FADH*. Ultrafast hydration dynamics on protein surfaces or in shallow clefts have recently been observed to follow a double-exponential decay, for most cases, in several and tens of picoseconds using an intrinsic tryptophan as a local optical probe.[25−29] The observed electron-transfer dynamics in ~10 and ~40 ps are longer than the initial solvation process occurring in several picoseconds, resulting in the observation of the 2-ps solvation component in most transients. The second longer solvation process, especially in the active site, is probably much slower than the observed electron-transfer reactions (11 and 42 ps). Thus, the excited-state population is mainly quenched by the reactions before reaching the final relaxed state.

The observation of ultrafast solvation dynamics at the active site in the enzyme is significant because solvation processes, especially water motions, are believed to play an important functional role in enzymatic reactions.[30] Ultrafast water motions are crucial to protein conformational flexibility, which is essential for enzymatic activity. The catalytic process is mediated by ordered water molecules located at the molecular distance scale (Figure 1). To maintain the order and selectivity of water molecules in function, the observed picosecond time scale is ideal for ordered water motions, for the case studied here, to stabilize the biologically active cofactor $FADH^-$ as well as the reaction intermediates during the DNA-repair process. These processes are faster than the local protein motions to allow the protein to optimize an ideal configuration immediately for efficient biological function.

C. Dynamics of Back Electron Transfer. Charge recombination is critical to photoreduction, and the overall quantum efficiency of photoreduction in *E. coli* photolyase is only about 0.05−0.1.[11] All experiments shown in Figure 3 were done in the presence of oxygen, and oxygen has been shown[8] to oxidize $FADH^-$ to the neutral radical FADH in less than 2 ms through the reduction of O_2 to O_2^-. Figure 4 shows two transients which were taken in the presence and absence of oxygen. Both transients are identical and have the same dynamics. Thus, the observed ~3 ns is the time of the first charge-recombination process (k_2) in a series of electron-transfer reactions in photoreduction. The second step of photoreduction (k_3) must also occur on the similar time scale of several nanoseconds, because if k_3 were much larger than k_2, we would not observe considerable recovery of FADH, and if k_3 were much smaller than k_2, we would not have detectable flavin photoreduction. The oxidation of $FADH^-$ by oxygen takes longer than ~3 ns, and it is probably in the microsecond range.

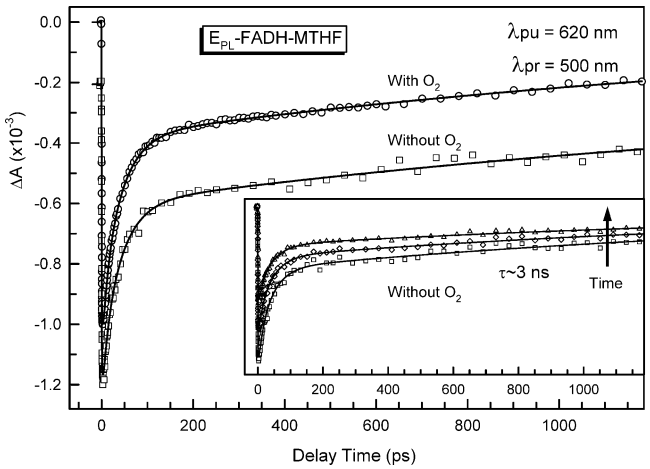

Figure 4. Femtosecond-resolved absorption transients probed at 500 nm with and without O_2. Two transients are identical, and the shift of the transient in absorbance change without O_2 is just for clarity. In the insert, the transients with three different data-collecting time intervals (1, 2, and 3 h) under anaerobic conditions are shown. After a 3-h period, the signal only dropped 38% in total; see text. The recovery of the long component signal takes ~3 ns.

In the insert of Figure 4, we also show the transients taken under anaerobic conditions (without O_2) for three time intervals. After a period of time, $FADH^-$ molecules accumulated in the sample, but they have no absorption at an excitation of 620 nm. Thus, the total signal probed at 500 nm decreased. As shown in Figure 4, after 3 h of laser irradiation, the signal only dropped 38% in total. Therefore, the overall charge-recombination reactions are severe.

IV. Conclusion

In this contribution, we report our studies of the excited-state reduction dynamics of the neutral radical flavin cofactor in *E. coli* photolyase with femtosecond resolution. The observed temporal absorption evolution from 400 to 700 nm revealed two initial electron-transfer reactions in 11 and 42 ps through the neighboring aromatic residues of W382 and F366, respectively. The new additional absorption observed at 550 nm previously[9] was found from the excited-state neutral radical, probably formed through strong intramolecular interactions with the adenine moiety by the flavin U-shaped configuration and intermolecular interactions with the highly polar/charged residues at the active site.

Ultrafast solvation dynamics was observed to occur in about 2 ps at the active site through several local ordered-water motions. The electron-transfer reactions (11 and 42 ps) quenched the excited state and thus the longer solvation processes, which

typically occur in tens to hundreds of picoseconds,[25−29] were not observed. The observed ultrafast local ordered-water motions are critical to stabilizing the reaction product ($FADH^-$) to prevent fast charge recombination. The back electron transfer was determined to be in ~3 ns, and this time scale is significantly longer than the expected value but consistent with ultrafast stabilization of the reaction products and favoring the photoreduction. Studies on various mutants using site-directed mutagenesis are on the way to determine forward and backward electron-transfer time scales, unravel photoreduction pathways, and finally elucidate the nature of electron-transfer mechanisms.

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