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Role of the Middle Residue in the Triple Tryptophan Electron Transfer Chain of DNA Photolyase: Ultrafast Spectroscopy of a Trp→Phe Mutant

Andras Lukacs,[†,‡] André P. M. Eker,[§] Martin Byrdin,[||] Sandrine Villette,[||] Jie Pan,[†,‡,⊥] Klaus Brettel,[||] and Marten H. Vos*[,†,‡]

CNRS UMR 7645, Laboratory for Optical Biosciences, Ecole Polytechnique, 91128 Palaiseau Cedex, France, INSERM U696, 91128 Palaiseau Cedex, France, Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University Medical Centre, PO Box 1738, 3000 DR Rotterdam, The Netherlands, and Service de Bioénergétique, CEA, and URA 2096 CNRS, CEA Saclay, 91191 Gif-sur-Yvette Cedex, France

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Photoreduction of the semi-reduced flavin adenine dinucleotide cofactor $FADH^{\bullet}$ in DNA photolyase from *Escherichia coli* into $FADH^{-}$ involves three tryptophan (W) residues that form a closely spaced electron-transfer chain $FADH^{\bullet}-W382-W359-W306$. To investigate this process, we have constructed a mutant photolyase in which W359 is replaced by phenylalanine (F). Monitoring its photoproducts by femtosecond spectroscopy, the excited-state $FADH^{\bullet *}$ was found to decay in ∼30 ps, similar as in wild type (WT) photolyase. In contrast to WT, however, in W359F mutant photolyase the ground-state $FADH^{\bullet}$ fully recovered virtually concomitantly with the decay of its excited state and, despite the presence of the primary electron donor W382, no measurable flavin reduction was observed at any time. Thus, W359F photolyase appears to behave like many other flavoproteins, where flavin excited states are quenched by very short-lived oxidation of aromatic residues. Our analysis indicates that both charge recombination of the primary charge separation state $FADH^{-}W382^{\bullet +}$ and (in WT) electron transfer from W359 to $W382^{\bullet +}$ occur with time constants <4 ps, considerably faster than the initial $W382 \rightarrow FADH^{\bullet *}$ electron-transfer step. Our results provide a first experimental indication that electron transfer between aromatic residues can take place on the time scale of $\sim 10^{-12}$ s.

Introduction

Directed channeling of electrons through proteins is an essential part of many biochemical processes. Electron transfer (ET) reactions often involve chains comprising redox active cofactors (heme, chlorin, flavin, metal cluster, etc.), intrinsic residues (essentially tryptophan and tyrosine), or both. In many cases, experimental access to intermediates in these chains is prohibited by difficulties to synchronize the reaction in all proteins in a sample. Exceptions are light-induced ET reactions, which can be triggered by short laser pulses and followed by absorption spectroscopy down to the femtosecond time scale.[1] One particularly interesting system is DNA photolyase[2,3] (PL). In this enzyme, two different ET reactions can be initiated by excitation of a flavin cofactor: photoreduction of the semi-reduced flavin adenine dinucleotide cofactor $FADH^{\bullet}$ ("photoactivation" of the isolated enzyme) and photooxidation of the fully reduced $FADH^{-}$ by pyrimidine dimer substrate ("photoreactivation"). Here, we focus on the former process, which involves a chain of three tryptophan residues, and provides a unique opportunity to study ET between identical amino acids.[4]

Photolyases possess a chain of three tryptophan residues that are well conserved, also in the related cryptochrome blue-light photoreceptor flavoproteins (Figure 1A). Strong evidence exists that, upon absorption of a photon, the neutral flavin radical is reduced via ET through the three tryptophans, followed by deprotonation of the surface-exposed $W306^{\bullet +}$ and reduction of the (neutral) W306 radical by external agents.[5−7] A generic scheme of the intraprotein ET steps is depicted in Figure 1B. Although the tryptophan radical intermediates do absorb in the visible range, they cannot a priori be distinguished by their absorption spectra, which complicates assessment of the intermediate reactions. Our main present knowledge, based on experiments with wild type (WT) and W382F mutant PL of *Escherichia coli*, concerns the initial and final steps of the chain. W306 is oxidized in less than 10 ns and deprotonates in ∼300 ns depending on buffer conditions.[6] The initial decay of the excited-state $FADH^{\bullet *}$ (by the competing processes **I** and $\mathbf{I}_b$) and formation of $FADH^{-}W^{\bullet +}$ takes ∼30 ps. The driving force for this reaction was roughly estimated at ∼0.5 eV;[6] with the very recent determination of the $(FADH^{-}/FADH^{\bullet})$ midpoint potential at 0 V,[8] this estimate is higher.[9] As upon replacement of W382 by phenylalanine, $FADH^{\bullet *}$ decays back to the neutral ground state virtually completely in only ∼80 ps (process $\mathbf{I}_b$ in Figure 1B),[7] the intrinsic time constant of initial ET to the flavin

* Corresponding author, email marten.vos@polytechnique.edu; tel. +33169334777; FAX +33169333017.
† Ecole Polytechnique.
‡ INSERM.
§ Erasmus University Medical Centre.
|| Service de Bioénergétique, CEA, and URA 2096 CNRS.
⊥ Present address: Department of Chemistry, University of California, Davis, One Shields Avenue, Davis, California 95616.

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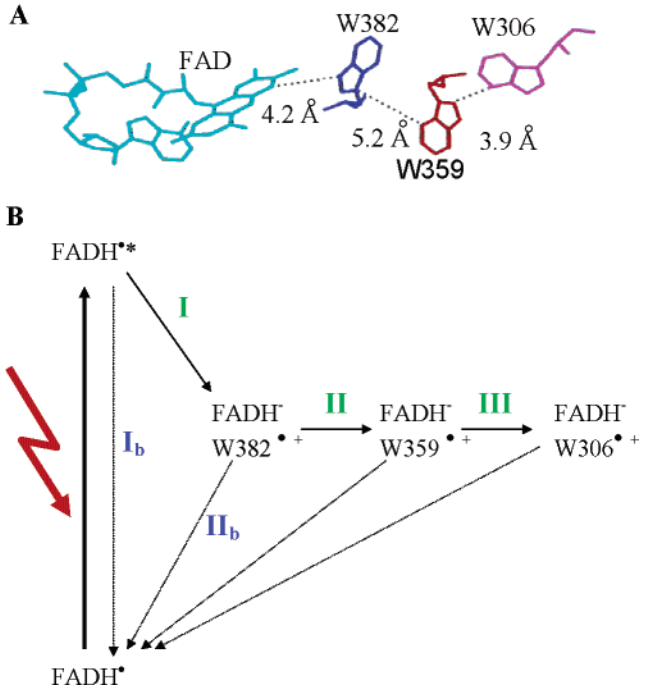

Figure 1. (A) Structural arrangement of FAD and the tryptophan chain in DNA photolyase from *E. coli* (from PDB file 1DNP,[34]). (B) Reaction scheme of the intraprotein charge-transfer processes during photoactivation. Further stabilization of the charge pair by release of a proton into the aqueous solvent around W306 and its eventual re-reduction by an external reductant are not included in the scheme. The reactions discussed in this paper are designed by roman numerals. The $FADH^{\bullet} \rightarrow FADH^{\bullet}*$ transition corresponds to ~2.0 eV. In the absence of experimental determinations, the three $FADH^{-}W^{\bullet+}$ states are drawn at the same energy level.

(process **I**) is ~45 ps, and the quantum yield (QY) of formation of the first product $FADH^{-}W382^{\bullet+}$ can be estimated at ~65%. It should be noted that precise determination of the QY of this reaction from the WT data is complicated by the strong contribution of excited-state absorption to the initial ($FADH^{\bullet}*$ *minus* $FADH^{\bullet}$) spectrum.[10] Recent single wavelength transient absorption experiments revealed a kinetic complexity in the WT kinetics that was interpreted in terms of two-exponential $FADH^{\bullet}*$ decay, with close-lying time constants, due to heterogeneous disposition of redox partners.[11,12]

Full spectral data unambiguously show that in WT PL, the product state after the ~30 ps phase(s) is $FADH^{-}W^{\bullet+}$,[13] and the involvement of W382 in reaching this state is obvious, but the tryptophan(s) on which the positive charge actually resides has not been identified. The distances between the redox partners are in principle all short enough (4−5 Å, Figure 1A) to allow ET rates as high as 10^{12} s^{-1}, depending on the energetic barriers and the details of the environments involved.[14,15] Therefore, the electron deficiency at $W382^{\bullet}$ could be transferred forward or equilibrated with W359 and/or W306 more rapidly than the initial ET step. From qualitative polarity arguments we have suggested a gradual decrease in free energy of the intermediates along the chain.[6] From a theoretical analysis of the energetics based on the static crystal structure in terms of the sequential scheme, as in Figure 1B, reaction **III** ($W359^{\bullet+} \rightarrow W306^{\bullet+}$) was also found to be downhill, but reaction **II** ($W382^{\bullet+} \rightarrow W359^{\bullet+}$) somewhat uphill.[16] Therefore, these authors suggested that the rate of the latter reaction is rate-limiting for W306 oxidation. This suggestion would imply that the population of the state $FADH^{-}W359^{\bullet+}$ cannot be buildup. In other theoretical work, W382 and W359 were not considered as real ET intermediates, but rather as part of the tunneling pathway between the redox partners $FADH^{\bullet}*$ and W306.[17] In terms of a sequential scheme (Figure 1B), back reactions are likely to compete with the stabilizing reactions, as the QY of $FADH^{-}$ formation on the microsecond time scale has been estimated at only ~10%.[18] To address these issues, here we study the early dynamics of engineered W359F PL from *E. coli* and compare it with the WT enzyme, using spectrally resolved femtosecond absorption spectroscopy.

Materials and Methods

Mutagenesis, Protein Expression and Purification, and Sample Preparation. The PL W359F mutant was obtained from pKE,[19] which is pKK223-3 harboring the *E. coli* WT PL gene, by the Quick Change mutagenesis method (Stratagene) using CTGGTGAAAGATTTATTGATCGACT*TT*CGCGAAGGCGAGCG and its reverse complement as primers. The mutated gene was overexpressed in *E. coli* KY29,[20] a photoreactivation deficient strain in which the endogenous PL gene has been inactivated by replacement with a chloramphenicol resistance marker.[21]

After growth at 37°C in ampicillin- and chloramphenicol-containing medium, mutant PL was induced with isopropyl-beta-D-thiogalactopyranoside at 27 °C. Cells were destructed by sonication in the presence of 10 mM 2-mercaptoethanol. After centrifugation (twice) at 43 000 g for 75 min, PL was purified by chromatography on heparin-Sepharose CL-6B resin (Amersham Pharmacia) by elution with a 0.1−1.1 M NaCl gradient in 0.01 M potassium phosphate pH 7.0, 10 mM 2-mercaptoethanol, followed by chromatography on SP−Sepharose Fast Flow resin (Amersham Pharmacia) eluted with a 0.04−0.3 M NaCl gradient. To minimize oxidation of the FAD chromophore during purification, solutions were flushed with and kept under nitrogen. The final preparation was estimated to be >98% pure. *E. coli* WT[22] and W382F mutant[6] PL were obtained as described elsewhere. Approximately 300 μM PL was prepared as described[7] in 200 mM NaCl and 20 mM Tris, pH 7.4, in a 1-mm optical path length cell.

Spectroscopy. Steady-state spectra were obtained using a Kontron Uvikon UV−vis spectrometer. Multicolor pump−probe spectroscopy was performed with a 500-Hz setup based on a home-built Ti:sapphire oscillator and a 1-kHz regenerative amplifier (Spitfire, Spectra Physics), delivering 50-fs, 0.5-mJ pulses. Pump pulses centered at either 550 nm (for probing at λ>620 nm) or 620 nm (for probing at λ<580 nm), and with energy of ~300 nJ, were generated using a home-built, BBO (0.5 mm thick, θ=32°) based type I NOPA as described elsewhere.[23] These pulses exclusively excite the neutral radical flavin and not the UV-absorbing MTHF antenna also present in *E. coli* PL. White light probe pulses were generated by focusing part of the fundamental on a 1 mm thick CaF_2 window and separated in test and reference beams. These were, after passing through the sample, dispersed and detected shot-by-shot on a CCD camera (Roper Scientific, SPEC-10) configured as a dual-array detector. The pump and probe beams were set at magic angle (54.7°) using a half-wave plate and a wire-grid polarizer (ProFlux PPL04).

The sample was thermostated at below 10 °C to slow aggregation and renewed between subsequent pulse pairs by rotation perpendicular to the beams. One main challenge of this work is that the mutant is relatively instable and therefore, under the necessarily intense (due to the low extinction of flavin) pump beam conditions, working with a somewhat scattering sample is unavoidable. The full spectral detection allows filtering out of part of the corresponding baseline fluctuations.

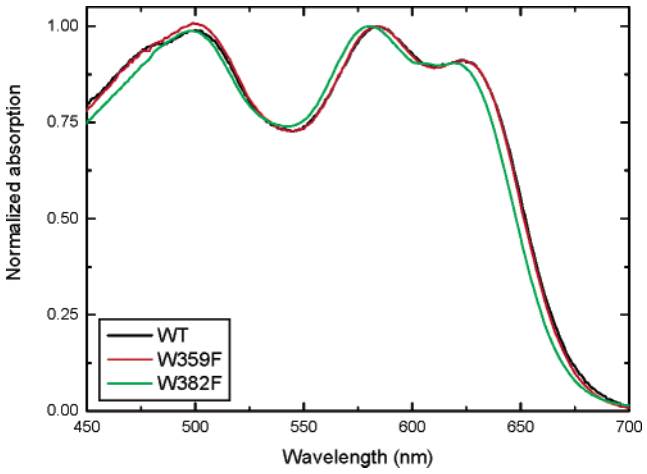

Figure 2. Steady-state absorption spectra of the neutral flavin radical FADH• in wild-type PL and W359F and W382F mutant PL.

Data Analysis. A singular value decomposition (SVD)[24] was performed on the measured $\Delta A(\lambda,t)$ matrix, and a component composed of baseline fluctuations was removed. Global fits to exponential decay processes were performed either using an SVD-based procedure[25] or using the SPLMOD algorithm[26] integrated in a MATLAB (MathWorks Inc.) application.[27]

Results and Discussion

Steady-State Absorption. WT photolyase is an unusual flavoprotein, in that the isolated protein contains a stable neutral flavin radical (FADH•)[2], evidenced by absorption maxima near 500, 580, and 630 nm (Figure 2). This is also the case for W359F mutant PL (Figure 2). Moreover, the W359F absorption spectrum is virtually identical to that of WT (in contrast to the slightly blue-shifted spectrum of W382F mutant PL, Figure 2). This indicates that also the electrostatic environment of the flavin, to which the absorption spectrum is sensitive,[28,29] is relatively unaffected by the mutation.

For W359F PL, from FADH• bleaching (20 mM dithiothreitol reductant, anaerobic), the yield of FADH• formation upon prolonged illumination at $\lambda > 550$ nm was estimated ~2% or less of that of WT PL.

Transient Absorption. The initial transient spectra of W359F mutant PL (Figure 3) are similar to those observed for WT[7] and can be ascribed to formation of the excited-state FADH•*. They contain contributions from the FADH• ground-state bleaching and FADH•* absorption, which is very sensitive to the flavin environment in flavoproteins.[10] In particular, a marked induced absorption feature roughly balances the ground state absorption around 560 nm. This feature is somewhat stronger in W359F mutant PL than in WT PL, possibly reflecting a slight alteration in the interaction of the isoalloxazine and adenine moieties of the FADH cofactor.[10]

In the monitored spectral region, the FADH• ground state and the potential products FADH• and $W^{\bullet+}$ have very distinct absorption spectra, so that formation of $FADH^{-}W^{\bullet+}$ is expected to give rise to spectral changes, as observed in WT after completion of the 30 ps kinetic phase[7] (Figure 3, inset). However, in W359F mutant PL, the transient spectra decay to zero in a monotonic way during the 150 ps time window, with well-defined isosbestic points. At all nonisosbestic wavelengths, the kinetics could be fit by a single-exponential decay with a time constant of 30 ps (Figure 4). The results of a global fit of the data matrix to a function of the form $\Delta A_0(\lambda) \exp(-t/\tau) + \Delta A_\infty(\lambda)$ are shown in Figure 4, inset. Allowing two-exponential decay did not significantly improve the fit. The lack of long-lived photoproduct is consistent with preliminary experiments on the millisecond time scale (not shown) that set an upper limit of 1% for the yield of $FADH^{-}$ formation with respect to WT.

Origin of Lack of $FADH^{-}$ Buildup in W359F. The scheme of Figure 1B would intuitively suggest that blocking reaction **II**, by the W359F mutation, still allows formation of the primary reaction product $FADH^{-}W382^{\bullet+}$. However, our results show this product is never populated to a sizable extent. Remarkably, the time constant of 30 ps for FADH•* decay is the same within experimental error as that reported previously for single-exponential fits of the kinetics in WT PL.[6,7] This similarity strongly suggests that the decay process is the same, predominantly via charge separation reaction **I**. We can rationalize our results on the mutant, where reaction **II** is blocked, by a high

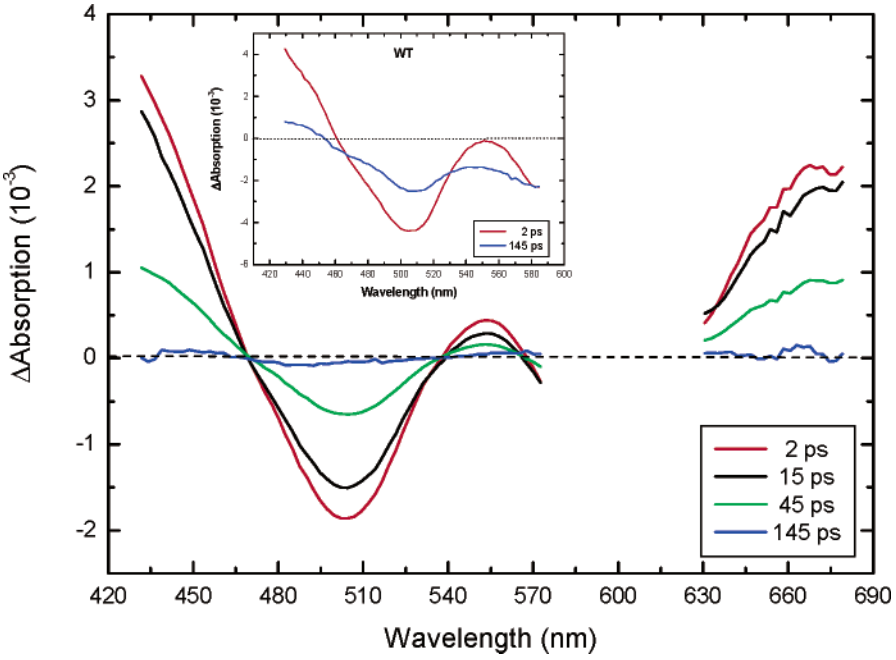

Figure 3. Transient absorption spectra at various delay times of W359F mutant PL. Inset: transient absorption spectra WT PL.

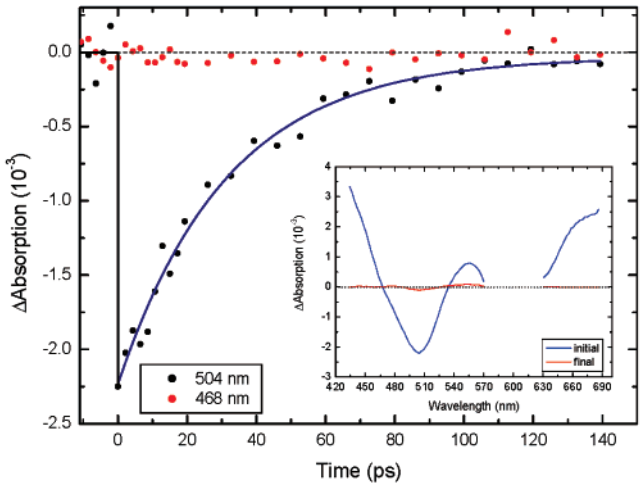

Figure 4. Kinetics of absorption change of W359F mutant PL at the main bleaching (504 nm) and at the 468 nm isosbestic point. Inset: Reconstructed spectra at time zero ($\Delta A_0(\lambda)$) and at infinity ($\Delta A_\infty(\lambda)$) from a global fit to a monoexponential decay $\Delta A(\lambda,t) = \Delta A_0(\lambda) \exp(-t/\tau) + \Delta A_\infty(\lambda)$.

rate of charge recombination $k_{\mathbf{IIb}}$. We estimate a lower limit for $k_{\mathbf{IIb}}$ as follows.

At the 468 nm isosbestic point, at any time the absorption change amounts to less than 5% of the maximum absorption change at 504 nm (Figure 3). Our previous analysis of the WT (FADH$^{\bullet}$* minus FADH$^{\bullet}$) spectrum indicates that the change at 504 nm is essentially due to bleaching of FADH$^{\bullet}$.[10] Taking into account that the (FADH−W$^{\bullet+}$ minus FADH$^{\bullet}$) extinction at 468 nm is ∼0.76 times that of FADH$^{\bullet}$ at 504 nm,[6] the maximum population of the intermediate state is ∼6.5% of the excited FADH$^{\bullet}$. Using the scheme of Figure 1B with $k_{\mathrm{II}} = 0$, and solving the respective coupled differential equation (with all population in FADH$^{\bullet}$* for $t = 0$), the FADH^{-}W382$^{\bullet+}$ population equals

$$\frac{k_{\mathrm{I}}}{k_{\mathrm{IIb}} - (k_{\mathrm{I}} + k_{\mathrm{Ib}})}\left(\exp(-(k_{\mathrm{I}} + k_{\mathrm{Ib}})t) - \exp(-k_{\mathrm{IIb}}t)\right)$$

With $k_{\mathrm{I}} = (45\ \mathrm{ps})^{-1}$ and $k_{\mathrm{Ib}} = (80\ \mathrm{ps})^{-1}$ (see Introduction), we find that the maximum of this function is <0.065 for $k_{\mathrm{IIb}} > (4\ \mathrm{ps})^{-1}$. This analysis thus suggests that the charge recombination $\mathbf{II}_{\mathrm{b}}$ (Figure 1B) occurs in <4 ps.

In principle, two other possible mechanisms may explain the lack of FADH^{-} buildup at any time: (i) mutation-induced structural modifications prohibit efficient ET so that only intrinsic FADH$^{\bullet}$* decay ($\mathbf{I}_{\mathrm{b}}$ in Figure 1B) occurs (in 30 ps), (ii) in WT PL, ET to FADH$^{\bullet}$* occurs via a super-exchange mechanism in which W382 is only a virtual intermediate. In the following, we will discuss these possibilities.

(i) It is conceivable that in W359F mutant PL, the relative positioning and/or the energetics of the primary reactants FADH$^{\bullet}$ and W382 is altered in such a way that ET can no longer compete with intrinsic decay of FADH$^{\bullet}$*. If this were the case, the striking agreement between the observed FADH$^{\bullet}$* decay rates of $\sim(30\ \mathrm{ps})^{-1}$ in W359F and WT[6] would be fortuitous, and this decay rate would reflect only direct decay to the ground state ($\mathbf{I}_{\mathrm{b}}$) in W359F mutant PL. This process would then be substantially faster in W359F PL than the value established for W382F PL (80 ps).[7] For WT PL, this rate cannot be directly established due to the competition with flavin reduction. However, a spectral deconvolution of the (FADH$^{\bullet}$* minus FADH$^{\bullet}$) spectrum using the 80 ps decay time yields amplitudes of the FADH$^{\bullet}$* absorption in the uncongested red spectral region that are quantitatively similar to other flavoproteins.[10] A rate of intrinsic FADH$^{\bullet}$* decay in WT deviating more than two-fold from $(80\ \mathrm{ps})^{-1}$ appears thus highly unlikely. Upon mutation of the relatively remote residue W359, the intrinsic decay rate of FADH$^{\bullet}$* is expected to be also close to $(80\ \mathrm{ps})^{-1}$, as the environment of the flavin is expected to be less modified than for mutation of the close-lying W382 (cf. also Figure 2). Altogether, although it cannot be fully excluded, the possibility of prohibition of ET from W382 to FADH$^{\bullet}$* due to the W359F mutation appears unlikely.

(ii) "Superexchange" tunneling of electrons via a virtual bridge state (here FADH^{-}W382$^{\bullet+}$) can be the dominating mechanism in systems where the energy level of the bridge state lies above that of the initial state (here FADH$^{\bullet}$*).[30] This is clearly not the case here, as FADH^{-}W382$^{\bullet+}$ lies well below the state FADH$^{\bullet}$*. Moreover, in a theoretical study reduction of FADH$^{\bullet}$* by W306 via the superexchange mechanism has been calculated to take ∼1 μs in the most favorable, activationless, case.[17] This is 4−5 orders of magnitude slower than the observed rate of FADH$^{\bullet}$* reduction in WT PL. Altogether, we exclude the possibility that in WT PL, FADH^{-}W382$^{\bullet+}$ only acts as a virtual state and was therefore not observed in W359F PL.

We thus favor fast FADH−W382$^{\bullet+}$ recombination to explain our data on W359F mutant PL. It is interesting to note that this mechanism makes the mutant to a certain extent behave like other, non-photoactive, flavoproteins: quenching of excited oxidized flavin by ET from closeby aromatic amino acids has been reported for a number of flavoproteins,[31−33] and has been proposed for excited semireduced flavin radical in flavodoxin.[10] In these systems the charge pair recombines within picoseconds and no effective flavin photoreduction occurs.

Implications for Flavin Photoreduction in WT Photolyase. WT PL differs from these systems in that a secondary ET reaction can efficiently compete with charge recombination so as to stabilize the reduced flavin species. Assuming that in WT PL charge recombination ($\mathbf{II}_{\mathrm{b}}$) also occurs in <4 ps, the forward reaction (**II**) must occur at least on the same order of magnitude (and over 3 orders of magnitude below the 10-ns upper limit previously established[6]). This suggests that in WT PL, the primary reaction product FADH^{-}W382$^{\bullet+}$ is also not populated significantly at any time, and that in the product that is actually built up by the ∼30-ps phase, the positive charge is rather located on W359 and/or W306. Indeed, as the state FADH^{-}W306$^{\bullet+}$ was suggested to lie ∼200 meV below FADH−W359$^{\bullet+}$,[16] stabilization of FADH^{-} may be greatly due to reaction **III**. We also note that a high rate for reaction **II**, as suggested by our analysis, may at first sight not be expected for the reaction being uphill, as suggested in ref 16. Experiments on PL modified at position 306 are planned to investigate these issues.

Our results constitute a first experimental indication for ET between aromatic residues occurring on the order of 10^{-12} s. The finding of both fast recombination and fast stabilizing ET reactions can, in principle, be rationalized by the distances between all reactants. Using the crystallographic edge-to-edge distances[34] (Figure 1A), the simplest form of the semiempirical distance rule of Dutton and co-workers[35] used to calculate the electronic coupling constants gives "optimal" (activationless) time constants of 0.3, 1.3, and 0.2 ps, respectively for reactions **I**, **II**, and **III**. As especially at such short distances, the approximation of the inter-reactant space by a dielectric continuum made in this rule may be considered rough, a pathway-type analysis of the individual steps would be of interest. In the framework of Marcus theory,[36] the high ET rates

deduced for reactions **I**, $\mathbf{II}_b$, and **II** would indicate that the reorganization energy λ does not deviate too much from the driving force. Considering the approximate energetics displayed in Figure 1B, one would therefore expect λ to be relatively high for the reaction **I** and $\mathbf{II}_b$ involving a flavin redox change, and low for reaction **II** involving only tryptophans. This reasoning is in general agreement with the facts that the flavin is partially accessible by the aqueous phase and that W382 and W359 are buried in the protein.[34] In this context it is of interest to note that a short-distance ET step between protein-buried reactants has been shown recently to occur with λ as low as <200 meV.[37] However, reactions as fast as $\sim 10^{12}$ s^{-1} likely involve nonthermal near-adiabatically coupled nuclear dynamics, and we therefore refrain from a more detailed analysis in terms of nonadiabatic thermalized ET.[15,36]

Finally, whereas the conserved triple tryptophan chain in PL provides a well-defined pathway to channel electrons from the aqueous environment to the catalytic flavin cofactor, suggestions have been made that other residues, and in particular phenylalanine, can be used to replace or bypass this channel.[2,11] Our results on W359F mutant PL show that the middle tryptophan in this chain, W359, is required to obtain a sizable flavin reduction at any time after excitation. Together with our previous work on W382 mutant PL,[7] this finding highlights the role of tryptophan and unambiguously shows that it cannot be taken over by phenylalanine. These findings are in general agreement with a theoretical analysis of the PL structure,[17] that predicted substantial decrease in the electronic coupling upon replacement of either W382 or W359 by phenylalanine.

Conclusions

In conclusion, we have shown that the secondary electron donor W359 is required for long-lived $FADH^\bullet$ photoreduction in photolyase. Our analysis further indicates that the initial $W382 \rightarrow FADH^{\bullet *}$ ET step is rate limiting for oxidation of at least W359, and possibly even W306.

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References and Notes

(1) Vos, M. H.; Martin, J.-L. *Biochim. Biophys. Acta* **1999**, *1411*, 1.
(2) Sancar, A. *Chem. Rev.* **2003**, *103*, 2203.
(3) Weber, S. *Biochim. Biophys. Acta* **2005**, *1707*, 1.
(4) Byrdin, M.; Sartor, V.; Eker, A. P. M.; Vos, M. H.; Aubert, C.; Mathis, P.; Brettel, K. *Biochim. Biophys. Acta* **2004**, *1655*, 64.
(5) Li, Y. F.; Heelis, P. F.; Sancar, A. *Biochemistry* **1991**, *30*, 6322.
(6) Aubert, C.; Vos, M. H.; Mathis, P.; Eker, A. P. M.; Brettel, K. *Nature* **2000**, *405*, 586.
(7) Byrdin, M.; Eker, A. P. M.; Vos, M. H.; Brettel, K. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8676.
(8) Gindt, Y. M.; Schelvis, J. P. M.; Thoren, K. L.; Huang, T. H. *J. Am. Chem. Soc.* **2005**, *127*, 10472.
(9) For the midpoint potentials, taking 0.0 V for $FADH^\bullet/FADH^-$ [8] and assuming 1.1 V for $W^{\bullet +}/W$ buried in a protein (Tommos, C.; Skalicky, J. J.; Pilloud, D. L.; Wand, A. J.; Dutton, P. L. *Biochemistry* **1999**, *38*, 9495), we estimate that $FADH-W^{\bullet +}$ lies roughly 1.1 eV above the ground state. Taking $FADH^{\bullet *}$ 2.0 eV above $FADH^\bullet$ (corresponding to the energy of a 630 nm photon), the driving force would be $\sim$0.9 eV.
(10) Pan, J.; Byrdin, M.; Aubert, C.; Eker, A. P. M.; Brettel, K.; Vos, M. H. *J. Phys. Chem. B* **2004**, *108*, 10160.
(11) Wang, H.; Saxena, C.; Quan, D.; Sancar, A.; Zhong, D. *J. Phys. Chem. B* **2005**, *109*, 1329.
(12) Saxena, C.; Sancar, A.; Zhong, D. *J. Phys. Chem. B* **2004**, *108*, 18026.
(13) Byrdin, M.; Brettel, K.; Aubert, C.; Eker, A.; Vos, M. H. Ultrafast Spectroscopy of the Excited Flavin Radical and its primary photoproduct in DNA Photolyase from *E. coli*; in *Flavins and Flavoproteins 2002*; Chapman. S.; Perham, R., Scrutton, N., Eds.; Rudolf Weber: Berlin, 2002; p 701.
(14) Gray, H. B.; Winkler, J. R. *Q. Rev. Biophys.* **2003**, *35*, 341.
(15) Page, C. C.; Moser, C. C.; Chen, X.; Dutton, P. L. *Nature* **1999**, *402*, 47.
(16) Popovic, D. M.; Zmiric, A.; Zaric, S. D.; Knapp, E.-W. *J. Am. Chem. Soc.* **2002**, *124*, 3775.
(17) Cheung, M. S.; Daizadeh, I.; Stuchebrukhov, A. A.; Heelis, P. F. *Biophys. J.* **1999**, *76*, 1241.
(18) Heelis, P. F.; Sancar, A. *Biochemistry* **1986**, *25*, 8163.
(19) Takao, M.; Oikawa, A.; Eker, A. P. M.; Yasui, A. *Photochem. Photobiol.* **1989**, *50*, 633.
(20) Nakajima, S.; Sugiyama, M.; Iwai, S.; Hitomi, K.; Otoshi, E.; Kim, S.; Jiang, C. Z.; Todo, T.; Britt, A. B.; Yamamoto, K. *Nucl. Acids Res.* **1998**, *26*, 638.
(21) Akasaka, S.; Yamamoto, K. *Mutat. Res.* **1991**, *254*, 27.
(22) Eker, A. P. M.; Yajima, H.; Yasui, A. *Photochem. Photobiol.* **1994**, *60*, 125−133.
(23) Cerullo, G.; Silvestri, S. D. *Rev. Sci. Instrum.* **2003**, *74*, 1.
(24) Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes*; Cambridge University Press: New York, 1989.
(25) Liebl, U.; Lambry, J.-C.; Leibl, W.; Breton, J.; Martin, J.-L.; Vos, M. H. *Biochemistry* **1996**, *35*, 9925.
(26) *Regularization Techniques for Inverse Problems in Molecular Biology*; Provencher, S. W., Vogel, R. H., Eds.; Birkhauser: Boston, 1983; Vol. 2, p 304.
(27) Morgan, J. E.; Verkhovsky, M. I.; Puustinen, A.; Wikström, M. *Biochemistry* **1995**, *34*, 15633.
(28) MacFarlane, A. W., IV; Stanley, R. J. *Biochemistry* **2001**, *40*, 15203.
(29) Schelvis, J. P. M.; Ramsey, M.; Sokolova, O.; Tavares, C.; Cecala, C.; Connell, K.; Wagner, S.; Gindt, Y. M. *J. Phys. Chem. B* **2003**, *107*, 12352.
(30) Bixon, M.; Jortner, J. *J. Chem. Phys.* **1997**, *107*, 5154.
(31) Mataga, N.; Chosrowjan, H.; Shibata, Y.; Tanaka, F.; Nishina, Y.; Shiga, K. *J. Phys. Chem. B* **2000**, *104*, 10667.
(32) Mataga, N.; Chosrowjan, H.; Taniguchi, S.; Tanaka, F.; Kido, N.; Kitamura, M. *J. Phys. Chem. B* **2002**, *106*, 8917.
(33) Zhong, D.; Zewail, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11867.
(34) Park, H.-W.; Kim, S.-T.; Sancar, A.; Deisenhofer, J. *Science* **1995**, *268*, 1866.
(35) Moser, C. C.; Keske, J. M.; Warncke, K.; Farid, R. S.; Dutton, P. L. *Nature* **1992**, *355*, 796
(36) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.
(37) Jasaitis, A.; Rappaport, F.; Pilet, E.; Liebl, U.; Vos, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10882.