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Review

Intraprotein electron transfer and proton dynamics during photoactivation of DNA photolyase from *E. coli*: review and new insights from an "inverse" deuterium isotope effect

Martin Byrdin[a,*], Valérie Sartor[a,b], André P.M. Eker[c], Marten H. Vos[d], Corinne Aubert[a,e], Klaus Brettel[a,*], Paul Mathis[a]

[a] *Service de Bioénergétique, CEA, and URA 2096 CNRS, CEA Saclay, Bat. 532, F-91191 Gif-sur-Yvette Cedex, France*
[b] *Laboratoire de Chimie Organo-Minérale, Université Louis Pasteur, Institut Le Bel, 4 Rue Blaise Pascal, 67000 Strasbourg, France*
[c] *Department of Cell Biology and Genetics, MGC, Erasmus University Medical Centre, PO Box 1738, Rotterdam, 3000 DR, The Netherlands*
[d] *Laboratoire d'Optique et Biosciences, INSERM U451 - CNRS UMR7645, Ecole Polytechnique-ENSTA, 91128 Palaiseau Cedex, France*
[e] *Laboratoire de Bioénergétique et Ingénierie des Protéines - CNRS, 31 chemin J. Aiguier, 13402 Marseille Cedex 20, France*

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We dedicate this contribution to the memory of Jerry Babcock who inspired our research on biological electron transfer and amino acid radicals.

Abstract

We review our work on electron transfer and proton dynamics during photoactivation in DNA photolyase from *E. coli* and discuss a recent theoretical study on this issue. In addition, we present unpublished data on the charge recombination between the fully reduced $FADH^-$ and the neutral (deprotonated) radical of the solvent exposed tryptophan W306. We found a pronounced acceleration with decreasing pH and an inverse deuterium isotope effect ($k_H/k_D = 0.35$ at pL 6.5) and interpret it in a model of a fast protonation equilibrium for the W306 radical. Due to this fast equilibrium, two parallel recombination channels contribute differently at different pH values: one where reprotonation of the W306 radical is followed by electron transfer from $FADH^-$ (electron transfer time constant τ_{et} in the order of 10–50 μs), and one where electron transfer from $FADH^-$ ($\tau_{et} = 25$ ms) is followed by reprotonation of the W306 anion.

Keywords: Photolyase; Electron transfer; Proton transfer; Tryptophan radical; Deuterium isotope effect; Transient absorption spectroscopy

1. Introduction

DNA photolyase (see Refs. [1–3] for recent, more comprehensive reviews) is a soluble protein with a molecular mass of about 55,000 that is found in a variety of organisms ranging from bacteria to multicellular eucaryotes. It contains a redox active cofactor, flavin adenine dinucleotide (FAD), and an auxiliary antenna chromophore. In order to perform its biological role, the repair of UV-induced lesions in DNA by scission of covalent bonds between neighbouring pyrimidines, the enzyme must have the FAD cofactor in its fully reduced form, $FADH^-$. In this case, $FADH^-$ that has been excited by a photon in the near UV or blue spectral region can donate an electron to damaged DNA and initiate the repair reaction. The electron is thought to return afterwards, restoring the FAD cofactor to its (catalytically active) fully reduced state. In isolated photolyase, the FAD cofactor is typically found in the semi-reduced radical form $FADH^{\bullet}$. The fully reduced state can be obtained by an interesting mechanism, which will be our major concern here, the so-called photoactivation reaction. During photoactivation, excitation of $FADH^{\bullet}$ by a visible photon (up to 680 nm) leads to full reduction of the flavin by intraprotein electron transfer. The electron donor is a solvent exposed tryptophan (W306 in *E. coli* photolyase) [4] about 15 Å apart from the flavin. In between, the X-ray structure [5] shows a potential electron transfer pathway that consists of two more conserved tryptophans (W359 and W382). Reduction of the oxidised tryptophan W306 by external electron donors stabilises the fully reduced state of the flavin. When no external donor is available, electron

* Corresponding authors. Tel.: +33-16908-9869; fax: +33-16908-8717.
E-mail addresses: byrdin@dsvidf.cea.fr (M. Byrdin), brettel@dsvidf.cea.fr (K. Brettel).

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backtransfer (charge recombination) from $FADH^-$ to the oxidised tryptophan restores the semireduced form $FADH^{\bullet}$.

The photoactivation reaction of DNA photolyase from *E. coli* is particularly convenient for the study and deeper understanding of long-range intraprotein electron transfer, as:

(i) The reaction can be triggered by a short flash of light which allows highly resolved kinetic studies; the reversibility of the reaction in the absence of external electron donors allows improvement of the signal-to-noise ratio by signal accumulation on one sample.
(ii) The crystal structure of the protein is known to a resolution of 2.3 Å [5], which opens the possibility of structure-based simulations of electron transfer kinetics and energetics.
(iii) It is possible to directly modify the electron transfer chain components (tryptophans) by site-directed mutagenesis.

We used absorption spectroscopy in the picosecond to millisecond time domain after excitation of $FADH^{\bullet}$ by red laser flashes to study in detail the mechanism of electron transfer from tryptophan (TrpH; H denotes the N1 proton) to excited $FADH^{\bullet}$ in recombinant *E. coli* photolyase. Red flashes as applied in our work selectively excite the (semi-reduced) $FADH^{\bullet}$ radical and avoid possible ambiguities that arose in previous studies from near UV/blue flashes that also could excite the oxidised and fully reduced forms of FAD [6,7]. By pointing out these difficulties, Terry Babcock and his coworkers [7] contributed to the success of our work on photolyase. Major results [8–10] of our kinetic measurements are outlined below. Recent theoretical simulations of the energetics of electron and proton transfer reactions in *E. coli* photolyase [11] will serve us as a basis to discuss our kinetic results in a structure-based energetic context. Finally, we will present unpublished results, including an inverse deuterium isotope effect, that provide new insights into the coupling of electron transfer and proton uptake during charge recombination between $FADH^-$ and the tryptophanyl radical.

2. Review of our transient absorption studies of the photoactivation reaction [8–10]

To visualise the spectral evolution of *E. coli* photolyase during photoactivation, characteristic absorbance difference spectra at four different stages of the reaction are presented in Fig. 1. A reaction scheme that can account for our results is shown in Fig. 2.

The green line in Fig. 1 represents the earliest observed difference spectrum that we attribute to the formation of excited flavin radical (state II in Fig. 2). This spectrum decays in about 30 ps to the blue spectrum that did not change shape up to about 10 ns ($FADH^-...TrpH^{\bullet+}$, states III to V). Subsequently, the $TrpH^{\bullet+}$ radical deprotonates and the blue spectrum decays in about 300 ns to the red spectrum ($FADH^-...Trp^{\bullet}$, state VI). In the presence of external electron donors, the deprotonated $Trp^{\bullet}$ radical is eventually reduced, and $FADH^-$ can be observed for minutes (broken line). The horizontally blue hatched area represents the contribution of the protonated $TrpH^{\bullet+}$ radical, while the vertically red hatched area represents the contribution of the deprotonated $Trp^{\bullet}$ radical.

In the following we substantiate the evidence that lead us to set up the kinetic scheme depicted in Fig. 2. Ultrafast

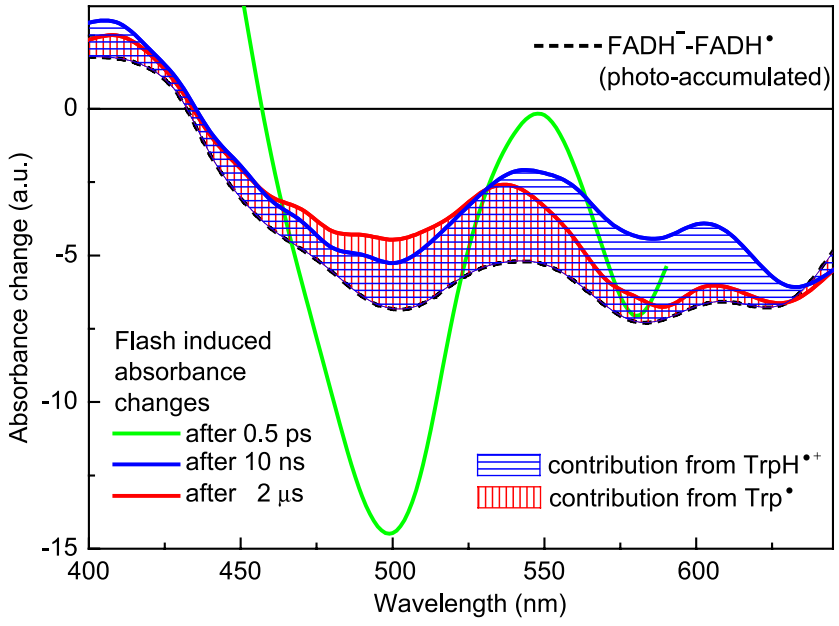

Fig. 1. Characteristic absorbance difference spectra occurring during photoactivation in *E. coli* photolyase. See text for details.

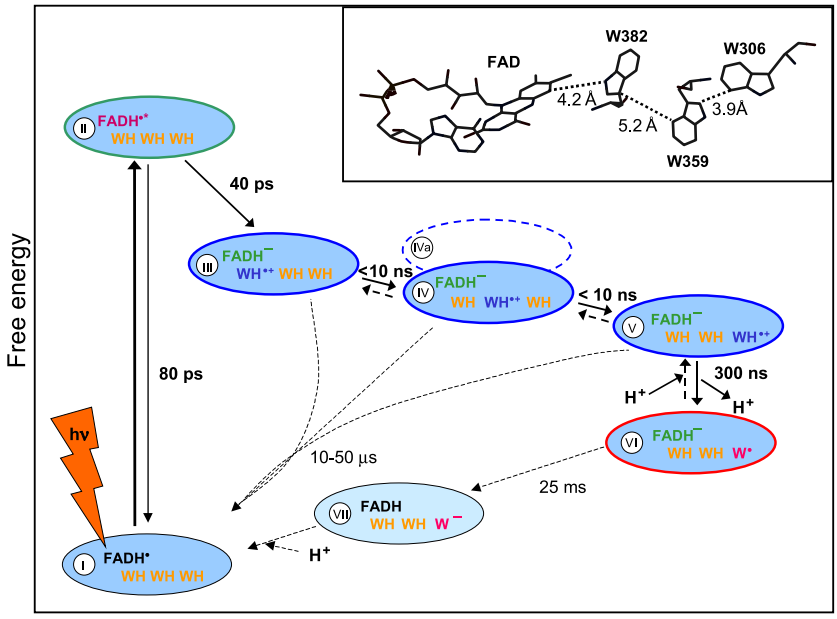

Fig. 2. Suggested reaction scheme for intraprotein electron transfer and proton dynamics during photoactivation in *E. coli* photolyase. Solid arrows: forward reactions, broken arrows: charge recombination. WH WH WH refers to tryptophans W382, W359 and W306. The free energy levels are not drawn to scale. Inset: Arrangement and shortest edge-to-edge distances between FAD, W382, W359 and W306 obtained from the X-ray structure of photolyase from *E. coli* [5]. Note the formal analogy of this scheme to charge separation and recombination in photosynthetic reaction centres [23].

pump-probe spectroscopy in the visible and near-infrared spectral regions revealed broad absorbance increases below 450 nm and between 650 and 800 nm (due to formation of the excited flavin radical $FADH^{\bullet}$*) and an absorbance decrease around 500 nm and around 580 nm (due to bleaching of $FADH^{\bullet}$) immediately after excitation (green spectrum in Fig. 1). The absorbance increase, attributed to excited state absorption of $FADH^{\bullet}$ [12], decayed nearly completely with $\tau \approx 30$ ps. Between 450 and 650 nm, the initial absorbance changes decayed with the same 30-ps lifetime to a resting spectrum which had the form of the blue spectrum in Fig. 1, i.e. corresponding to a state $FADH^{-}\ldots TrpH^{\bullet+}$. The shape of this spectrum did not change significantly between 200 ps and 10 ns. We concluded that electron transfer from a tryptophan to $FADH^{\bullet}$* occurred on a time scale of 30 ps. The observed electron transfer is several orders of magnitude faster than what was expected for a single step direct [13] or superexchange-mediated [14] electron transfer between the solvent accessible tryptophan residue W306 and the flavin ring (shortest edge-to-edge distance between aromatic/conjugated systems, 14.8 Å; note that the previously reported distance of 13.4 Å [8] had been measured between edges of the molecules instead). On the other hand, the distances in the chain FAD-W382-W359-W306 are short enough (≤ 5.2 Å) to allow for multistep electron transfer within a few picoseconds, provided that the energetics is favourable for this transfer (see below).

Direct experimental proof for the involvement of W382 in multistep electron transfer to $FADH^{\bullet}$* was recently provided by a study [10] of a mutant where W382 had been replaced by redox inert phenylalanine. In this mutant, electron transfer to $FADH^{\bullet}$* was drastically impaired. $FADH^{\bullet}$* decayed with an intrinsic lifetime of 80 ps, which allowed to calculate the intrinsic time constant of the first electron transfer step in the wild-type system as 40 ps. Tiny absorbance changes observed on a millisecond time scale in the W382F mutant were interpreted as indicating low yield electron transfer from W359 to $FADH^{\bullet}$* in ~25 ns over a distance of 10 Å.

Electron transfer between identical species such as the three tryptophan residues does not give rise to absorbance changes and, hence, cannot be monitored directly by transient absorption spectroscopy. We nonetheless estimated an upper limit of 10 ns for the electron transfer steps between the tryptophan residues. This limit was derived from the onset of the next reaction, the deprotonation of the $TrpH^{\bullet+}$ radical, which we monitored with nanosecond time resolution.

Throughout the 300–645-nm range studied in Ref. [8], the instrument-limited (< 10 ns) initial absorbance change due to formation of the state $FADH^{-}\ldots TrpH^{\bullet+}$ (blue line in Fig. 1) was followed by a second kinetic phase with $\tau \approx 300$ ns. The spectrum of the amplitude of the 300 ns phase (difference between blue line and red line in Fig. 1) agreed well with the difference between the solution spectra of $TrpH^{\bullet+}$ and $Trp^{\bullet}$ [15], strongly indicating that the 300-ns phase reflected the deprotonation of $TrpH^{\bullet+}$. This conclusion was supported by the "normal" kinetic isotope effect (see Ref. [16] for a comprehensive treatment) that we observed upon exchange of H_2O for D_2O: $k_H/k_D = 2.2$. To our knowledge, this is the first example where the deproto-

nation of an amino acid radical has been monitored in real time. We therefore further characterised the deprotonation of the tryptophanyl radical and showed that increasing the glycerol concentration in the solvent caused a pronounced decrease in the rate of $TrpH^{\bullet+}$ deprotonation, suggesting that the proton from $TrpH^{\bullet+}$ is released to the solvent rather than being transferred to a basic residue in the protein. Experiments varying pH and buffer concentration showed that at buffer concentration below about 20 mM, the dominating proton acceptor was water rather than buffer molecules. In this case, the deprotonation rate was independent of pH within the range from pH 5.4 to 8.6.

The final deprotonated state of the tryptophanyl radical was confirmed by absorption changes accompanying the recombination between $FADH^-$ and the tryptophanyl radical in the absence of an external electron donor on a millisecond time scale. Throughout the 400 to 645 nm spectral range studied, we observed absorbance changes relaxing exponentially due to charge recombination with a time constant $\tau = 17$ ms (at pH 7.4). The spectrum of these absorbance changes was identical to the red line in Fig. 1, i.e. consistent with a contribution from $Trp^{\bullet}$ but not from $TrpH^{\bullet+}$. The presence of $Trp^{\bullet}$ is also supported by the acidity of $TrpH^{\bullet+}$ which has a $pK_a \approx 4$ in water [15,17]. Independent evidence for the deprotonated form of the tryptophanyl radical came from our observation that the recombination reaction accelerated strongly upon lowering of the pH, indicating that recombination involves $Trp^{\bullet}$ reprotonation. Possible pathways for the recombination reactions are discussed in more detail below.

3. Energetics of electron transfer through the triple-tryptophan chain

The initial electron transfer step, from W382 to $FADH^{\bullet}$*, proceeds downhill with a driving force in the order of about 0.5 eV [8]. The next two electron transfer steps (between the three tryptophan residues) may be, on the first glance, assumed to be isoenergetic. However, different polarities of the tryptophan environments could give rise to different reduction potentials for the $TrpH^{\bullet+}$/TrpH couple (it is established that this potential is higher in a hydrophobic protein environment than in water [17]). From an inspection of the crystal structure, we suggested qualitatively that the polarities increase and hence the potentials decrease in the order W382, W359, W306, favouring radical localisation on W306 already prior to its deprotonation. The proton release from $TrpH^{\bullet+}_{306}$ to the aqueous medium in about 300 ns is driven by a substantial decrease in free energy (about 0.2 eV at pH 7.4, assuming a pK_a of 4 for $TrpH^{\bullet+}$ [15,17]). This energetic stabilisation is essential to trap the oxidising radical on W306, which is accessible to external reductants and sufficiently far apart from $FADH^-$ to prevent a fast direct recombination. Moreover, the deprotonation creates a free energy barrier in a potential recombination pathway via repopulation of $TrpH^{\bullet+}_{306}$ and reverse electron transfer in the chain FAD-W382-W359-W306.

The energetics of electron transfer through the tryptophan chain has recently been investigated theoretically by Popovic et al. [11]. They computed the electrostatic free energies of the redox active groups from a solution of the linear Poisson–Boltzmann equation for the whole protein and its aqueous surroundings. Electron transfer from $TrpH_{359}$ to $TrpH^{\bullet+}_{382}$ was found to be slightly uphill ($\Delta G^\circ = 58$ meV; indicated by state IVa in Fig. 2), and electron transfer from $TrpH_{306}$ to $TrpH^{\bullet+}_{359}$ was found to be clearly downhill ($\Delta G^\circ = -200$ meV). The deprotonation free energy of $TrpH^{\bullet+}_{306}$ was calculated to be -214 meV at pH 7.4, i.e. very close to the solution value. For the uphill electron transfer from $TrpH_{359}$ to $TrpH^{\bullet+}_{382}$, Popovic et al. [11] estimated a rate of $(5\ \text{ns})^{-1}$, so there is no contradiction between the calculated energetics and our experimental results (overall electron transfer in less than 10 ns). Nevertheless, we would like to point out that straightforward application of the free energies calculated in Ref. [11] to the fast photoreactions in photolyase may be problematic, as the electrostatic interaction energies were calculated for protonation patterns of the protein in thermodynamic equilibrium. It is unlikely that all titrable groups reach protonation equilibrium on the picosecond to nanosecond time scale of electron transfer. For comparison, it might be instructive to calculate the free energy levels of the states III, IV and V with the protonation pattern fixed to its equilibrium state prior to excitation.

4. New results on proton coupled electron transfer during charge recombination

Charge recombination between $FADH^-$ and the terminal tryptophan radical accelerates strongly with decreasing pH [8]. We qualitatively attributed this effect to the reprotonation of $Trp^{\bullet}_{306}$ assumed to be required for recombination, but we had not established the mechanism of coupling between electron transfer and protonation. Popović et al. [11] suggested that the overall rate of charge recombination is dominated by the proton uptake. Their structure based calculation (see above) yielded a proton uptake rate of $(1.2\ \text{ms})^{-1}$ for $Trp^{\bullet}_{306}$ at pH 7.4; this rate was considered to compare favourably with the recombination rate of $(17)\ \text{ms}^{-1}$ observed by us at pH 7.4.

We have now investigated the charge recombination reaction in more detail. In a first experiment, we added 50% (v/v) glycerol to a sample at pH 6.6 in order to increase viscosity and hence slow proton uptake from the solution. Surprisingly, charge recombination as monitored at 580 nm (see Ref. [18] for experimental details) did not slow down. This observation indicates that the overall reaction was not kinetically limited by reprotonation of the terminal tryptophan radical. Alternatively, if the rate constant k_1 of electron transfer from $FADH^-$ to $TrpH^{\bullet+}_{306}$ is slower than deproto-

nation of $TrpH^{\bullet+}_{306}$ (measured rate (300 ns)$^{-1}$, see above), the deprotonation/reprotonation of this radical can be treated as a fast pre-equilibrium. The overall recombination rate k_{rec} is then approximately given by weighting k_1 with the relative population of $TrpH^{\bullet+}_{306}$: $k_{rec} = \frac{[TrpH^{\bullet+}_{306}]}{[TrpH^{\bullet+}_{306}]+[Trp_{306}]} k_1 = \frac{[H^+]}{K_a+[H^+]} k_1$ *with* $K_a = \frac{[Trp_{306}][H^+]}{[TrpH^{\bullet+}_{306}]}$. Hence, at a given pH, k_{rec} depends on the equilibrium constant $K_a = 10^{-pKa}$ rather than only on the reprotonation rate, in line with the glycerol result.

In a second step, we measured k_{rec} at various pL values (L = H or D) with H_2O (Fig. 3, black squares) and D_2O (red circles). The data confirm the acceleration of the recombination with decreasing pH as reported previously [8], but two unexpected points attracted our attention: (1) At pL < 7.5, exchange of H_2O for D_2O significantly accelerated the recombination (about 3 times at pL 6.5). This is in contrast to the "normal" deuterium isotope effect, where deuteration slows down reactions involving transfer of a hydrogen nucleus [16,19]. (2) The recombination became nearly pL independent above pH 8 or pD 8.5.

Observation (1) might be explained by the fact that many weak acids show an increase in pK_a in D_2O versus H_2O [19]. Within the pre-equilibrium model presented above, an increase of the pK_a (decrease of K_a) of the tryptophanyl radical in D_2O compared to H_2O would shift the equilibrium towards the protonated state and thus give rise to the observed "inverse" deuterium isotope effect.

Observation (2) suggests the existence of a second, pL-independent, recombination channel. An obvious pathway would be electron transfer from $FADH^-$ to $Trp^{\bullet}_{306}$ followed by reprotonation of Trp_{306}^- (reactions VI → VII → I in Fig. 2; note that reprotonation of Trp^- is not expected to give rise to absorbance changes in the visible and could therefore not be observed in our experiments).

We hence tried to describe the experimental data by the kinetic scheme shown in the inset of Fig. 3 (the numbering of the states refers to Fig. 2). Assuming a fast equilibrium between states V and VI, the decay of this quasi-equilibrium state is given by a population-weighted sum of the two rates k_1 (recombination via $TrpH^{\bullet+}_{306}$) and k_2 (recombination via $Trp^{\bullet}_{306}$):

$$k_{rec} = \frac{[H^+]}{K_a + [H^+]} k_1 + \frac{K_a}{K_a + [H^+]} k_2 \qquad (1)$$

Substituting $K_a = 10^{-pKa}$ and $[H^+] = 10^{-pH}$, we obtain

$$k_{rec} = (1 + 10^{pH-pK_a})^{-1} k_1 + (1 + 10^{-(pH-pK_a)})^{-1} k_2 \qquad (2)$$

According to these equations, at low pH, the first term dominates (k_{rec} approaches k_1 for $pH \ll pK_a$) while at high pH the second one dominates (k_{rec} approaches k_2 for $pH \gg pK_a$). At intermediate pH values, both terms contribute depending on pH and on the values of k_1 and k_2. This behaviour reflects essential features of our equilibrium model: At high pH, k_{rec} approaches k_2 as the recombination channel via $TrpH^{\bullet+}_{306}$ becomes negligible; with decreasing pH, the recombination accelerates (if, as in our case, $k_1 > k_2$) due to an increasing contribution of the recombination via $TrpH^{\bullet+}_{306}$. The limiting case $pH \ll pK_a$ where the recombi-

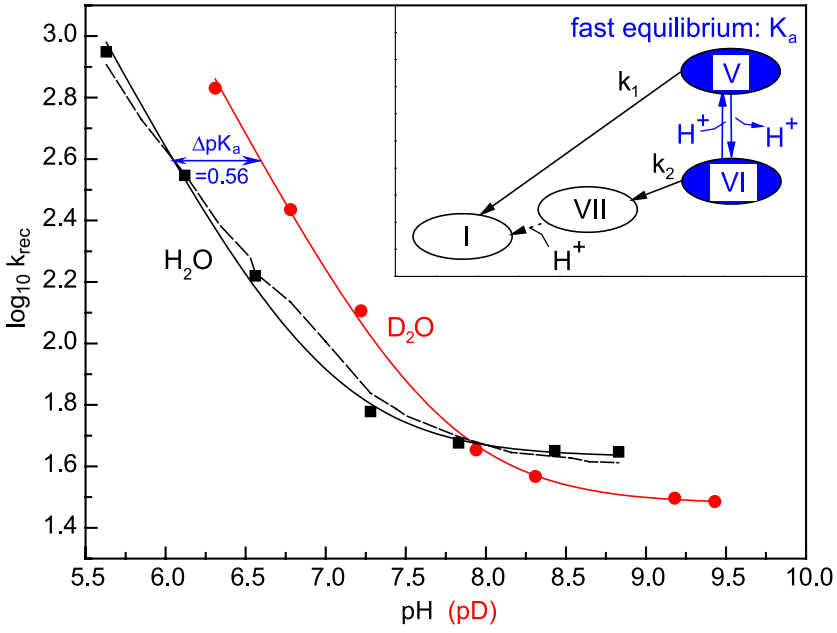

Fig. 3. Deuterium isotope effect and pH dependence of the charge recombination kinetics in *E. coli* photolyase. Recombination rate constants k_{rec} (in s^{-1}) were obtained from monoexponential fits to flash-induced transient absorption changes at 580 nm that were measured at 10 °C, as described [18]. "D_2O" samples were prepared by buffer exchange [24]. pD values were obtained from the reading of a glass electrode by adding 0.4 [19]. Solid lines represent fits using Eq. (2) based on the fast equilibrium model shown in the inset (roman state numbering as in Fig. 2). For fit parameters, see Table 1. The dashed line represents a fit based on the (pH-dependent) deprotonation free energies as calculated in Ref. [11] for $TrpH^{\bullet+}_{306}$ in *E. coli* photolyase (see text).

nation rate approaches k_1 could not be reached in our experiments due to protein degradation at pH < 5.5.

We applied the ORIGIN Advanced fitting tool to fit Eq. (2) to the data in Fig. 3 (solid lines in Fig. 3; see Table 1 for best fit parameters pK_a, k_1 and k_2). While k_2 could be determined reliably, the other two parameters pK_a and k_1 turned out to be interdependent, i.e. several of their combinations resulted in fit curves of equal quality. Therefore, we fixed pK_a for the H_2O data to three different values around 4, the value reported for the tryptophanyl radical in water [15,17]. The best fit curves for pK_a = 3.8 and pK_a = 4.2 were virtually identical to that for pK_a = 4.0 (shown in Fig. 3). The k_1 values differed considerably between the three fits (Table 1), obviously compensating for the differences in pK_a. For the fit of the D_2O data, pK_a was a free running parameter, but k_1 was fixed to the value obtained from the fit to the H_2O data, as we assumed that there is no deuterium isotope effect on the pure electron transfer process described by k_1.

The fit results reveal that the inverse isotope effect on the recombination rate below pL 7.5 can be explained by a pK_a shift of the terminal tryptophanyl radical. The $\Delta pK_a = pK_a(D_2O) - pK_a(H_2O) = 0.56$ which is invariably obtained for all three pK_a values tested (compare Table 1) reflects the horizontal displacement of the D_2O curve in Fig. 3 with respect to the H_2O curve. This value compares favourably with the 0.3–0.7 units typically found for weak acids [19]. The intrinsic rate k_1 of electron transfer from $FADH^-$ to $TrpH^{\bullet+}_{306}$ was found in the order of $(10\ \mu s)^{-1}$ to $(50\ \mu s)^{-1}$. Although the fit result for k_1 depends on the choice of pK_a for the tryptophanyl radical (see Table 1), this electron transfer rate is certainly much slower than the directly measured deprotonation rate of $TrpH^{\bullet+}_{306}$ $(300\ ns)^{-1}$, see above. Thus, the condition for the fast pre-equilibrium model is fulfilled. It is not clear at present whether electron transfer from $FADH^-$ to $TrpH^{\bullet+}_{306}$ is direct (single step) over the distance of 14.8 Å, or indirect (in short steps via one or two of the intermediate tryptophans). Single-step electron transfer over 14.8 Å might well be as fast as 10–50 μs, as the "Moser–Dutton ruler" [20] allows for rates up to ~1 μs^{-1} (for the optimal condition of a driving force equal to the reorganisation energy). Hence, we cannot exclude direct charge recombination. Direct recombination might also explain our finding [10] that the recombination rate in the W382F mutant was quite similar to that found in wild type.

Table 1
Parameters resulting from fits of Eq. (2) to data in Fig. 3

H_2O-buffer			D_2O-buffer			$\Delta pK_a = pK_a(D_2O) - pK_a(H_2O)$
pK_a (fixed)[a]	$1/k_1$ (μs)	$1/k_2$ (ms)	$1/k_1$ (fixed) (μs)[b]	$1/k_2$ (ms)	pK_a	
3.8	15.9	23.4	15.9	33.2	4.36	0.56
4.0	25.1	23.4	25.1	33.2	4.56	0.56
4.2	39.5	23.4	39.5	33.2	4.76	0.56

[a] Different fixed values were tested to account for the uncertainty in the pK_a value of $TrpH^{\bullet+}_{306}$.

[b] k_1 was fixed to the values obtained by the H_2O fit; as for this pure electron transfer, no deuterium isotope effect is expected.

According to our fit results, electron transfer from $FADH^-$ to $Trp^{\bullet}_{306}$ (k_2) would be three orders of magnitude slower than electron transfer from $FADH^-$ to $TrpH^{\bullet+}_{306}$ (k_1). If both reactions represented direct electron transfer, the different rates might be due to different driving forces (see Fig. 2). Alternatively, if the other two tryptophans were engaged as intermediates, electron transfer from $FADH^-$ to $TrpH^{\bullet+}_{306}$ would be a (fast) three-step pure electron transfer, while electron transfer from $FADH^-$ to $Trp^{\bullet}_{306}$ would have to involve (slow) hydrogen atom transfer between tryptophans.

Our data at high pH (limiting case where $k_{rec} \approx k_2$) show that, in line with the fit results for k_2, the k_2 recombination pathway is slower in D_2O than in H_2O, i.e. they indicate a weak normal deuterium isotope effect ($k_H/k_D = 1.4$) for electron transfer from $FADH^-$ to $Trp^{\bullet}_{306}$. We have no straightforward explanation for this effect, unless hydrogen atom transfer between tryptophans is to some extent involved in this recombination pathway (see above).

Strictly speaking, in a protein, K_a values are not necessarily pH-independent, as the equilibrium protonation state of any titrable group becomes pH-dependent via electrostatic interactions with surrounding titrable amino acids. Popovic et al. [11] calculated this dependence for $Trp^{\bullet}_{306}$. Using the pH-dependent deprotonation free energies ΔG^0_a from Fig. 2 in Ref. [11] and substituting $K_a = [H^+]e^{-\Delta G^0_a/k_B T}$ in our Eq. (1), we obtained a good fit for the H_2O data with $k_1 = (8.5\ \mu s)^{-1}$ and $k_2 = (25.5\ ms)^{-1}$ (dashed line in Fig. 3).

In conclusion, the recombination between $FADH^-$ and $Trp^{\bullet}_{306}$ appears to be governed by a fast protonation/deprotonation equilibrium and subsequent slower electron transfer steps rather than being limited by slow reprotonation of $Trp^{\bullet}_{306}$.

5. Concluding remark

Our studies on photoactivation of *E. coli* photolyase provide evidence for a fast multistep electron transfer to excited $FADH^{\bullet}$ through a chain of three tryptophan residues. Deprotonation of the terminal $TrpH^{\bullet+}_{306}$ was kinetically resolved and turned out to be several orders of magnitude slower than the preceding electron transfer. As a consequence, charged amino acids should exist, at least transiently, within the protein, despite the energetic cost of charges inside the low-dielectricity medium. This observation may be relevant for the understanding of long-range radical transfer through amino acids in proteins, as, e.g. in ribonucleotide reductase [13,21]. Recent experimental findings indicate that an electron transfer chain similar to that in *E. coli* photolyase is also functional in cryptochrome blue light photoreceptors [22].

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