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Energetics of Radical Transfer in DNA Photolyase

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Abstract: Charge separation and radical transfer in DNA photolyase from *Escherichia coli* is investigated by computing electrostatic free energies from a solution of the Poisson−Boltzmann equation. For the initial charge separation 450 meV are available. According to recent experiments [Aubert et al. *Nature* **2000**, *405*, 586−590] the flavin receives an electron from the proximal tryptophan W382, which consequently forms a cationic radical $WH^{\bullet+}382$. The radical state is subsequently transferred along the triad W382−W359−W306 of conserved tryptophans. The radical transfer to the intermediate tryptophan W359 is nearly isoenergetic (58 meV uphill); the radical transfer from the intermediate W359 to the distal W306 is 200 meV downhill in energy, funneling and stabilizing the radical state at W306. The resulting cationic radical $WH^{\bullet+}306$ is further stabilized by deprotonation, yielding the neutral radical $W^{\bullet}306$, which is 214 meV below $WH^{\bullet+}306$. The time scale of the charge recombination process yielding back the resting enzyme with $FADH^{\bullet}$ is governed by reprotonation of W306, with a calculated lifetime of 1.2 ms that correlates well with the measured lifetime of 17 ms. In photolyase from *Anacystis nidulans* the radical state is partially transferred to a tyrosine [Aubert et al. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5423−5427]. In photolyase from *Escherichia coli*, there is a tyrosine (Y464) close to the distal tryptophan W306 that could play this role. We show that this tyrosine cannot be involved in radical transfer, because the electron transfer from tyrosine to W306 is much too endergonic (750 meV) and a direct hydrogen transfer is likely too slow. Coupling of specific charge states of the tryptophan triad with protonation patterns of titratable residues of photolyase is small.

Introduction

Photolyase is a DNA repair protein, that reverses cyclobutane−pyrimidine dimer (CPD) defects that are generated by UV/vis light through photoinduced electron transfer (ET). This enzyme, found in many organisms from bacteria to eukaryotes, is a single-chain protein consisting of 471 residues. Although, photolyases were biochemically and functionally characterized more than 10 years ago,[1,2] the reaction catalyzed by photolyase was revealed in atomic detail only after crystal structures from *Escherichia coli (E. coli)*[3] and from *Anacystis nidulans* (*A. nidulans*)[4] became available. Photolyase from *E. coli* contains two cofactors, flavin adenine dinucleotide (FAD) functioning as the redox catalyst, and 5,10-methenyltetrahydrofolate (MTHF), which acts as light-harvesting chromophore (Figure 1).

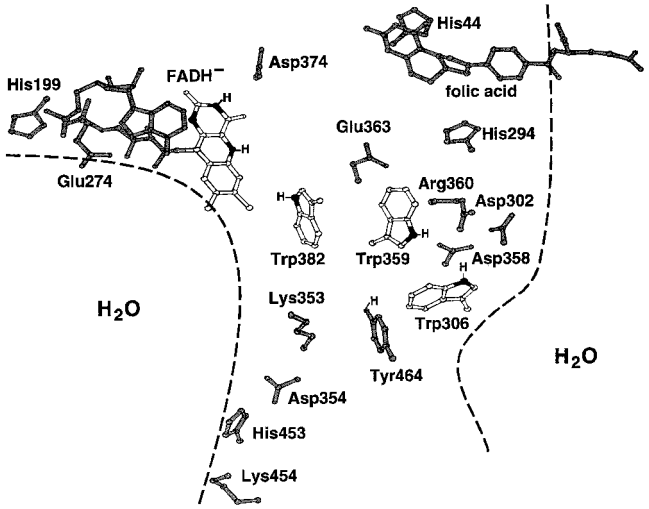

Figure 1. Structure of the cofactors FADH, folic acid, and the tryptophan triad W382−W359−W306 in DNA photolyase. The solvent boundary of photolyase is displayed by a dashed line. The hydrogen atoms relevant for deprotonation of the tryptophans and Tyr464 are explicitly indicated. Titratable residues that are close to the tryptophan triad and that vary their protonation state with a change in the charge state of the tryptophans are also shown. The figure was generated with MOLSCRIPT.[38]

In the resting state of the DNA photolyase from *E. coli*, flavin is typically in the semireduced radical state $FADH^{\bullet}$, which is

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catalytically inert. Illumination with UV/vis light converts the flavin to the reduced form $FADH^-$. In this photoactivation reaction, it was observed that an electronic excitation is transferred within 200 ps from MTHF to flavin with 60% efficiency. [4] In the excited state, the flavin ($FADH^{\bullet}$*) takes up an electron, resulting in a positively charged tryptophan W306[5] whose edge-to-edge distance to flavin is about 15 Å. The solvent-accessible W306 remains in the radical state[6] with a lifetime of about 10 ms, limited by back transfer.[7,8] Thus, it stabilizes the active state of the anionic flavin $FADH^-$. In photolyase from *A. nidulans*, the radical state resides to 40% on a tyrosine.[9]

Radical states of amino acids play a key role in a number of enzymatic reactions.[10] In these enzymes the radical state may be transferred over large distances as for instance in class I ribonucleotide reductase (RNR), where the transfer occurs from a tyrosyl radical to a cysteine over a distance of 35 Å.[11,12] In principle, the radical character of one molecular group can be transported to another group by ET or by hydrogen transfer. In the low dielectric medium of a protein, a hydrogen-transfer mechanism can be advantageous, since it saves the energy required for charge separation. Therefore, in RNR this radical transport mechanism is under discussion.[13,14] In photolyase, the ET process is photoactivated. Therefore, there is enough energy available to initialize the charge separation process. However, here also a hydrogen transfer mechanism may have some advantage. The transfer of a neutral hydrogen would require no charge reorganization energy as is needed for an ET, although in the low dielectric medium of a protein, the reorganization energy for an ET is small, and the barriers that a transferred hydrogen has to overcome may be large.

To clarify these points the mechanism of photoactivation was recently studied for *E. coli* photolyase at pH = 7.4 by time-resolved absorption changes after selective excitation of $FADH^{\bullet}$ in the pico- to millisecond time regime.[15] It was found that after light excitation the tryptophanyl radical $WH^{\bullet+}306$ (H denotes the N1 proton of the indole ring as indicated in Figure 1.) forms in less than 10 ns, a time that is instrument limited. The cationic radical $WH^{\bullet+}306$ deprotonates to the neutral radical $W^{\bullet}306$ with a time constant of 300 ns. Finally, the radical $W^{\bullet}306$ relaxes exponentially with a time constant of 17 ms due to charge recombination. The recombination kinetics is strongly accelerated at lower pH. Hence, $W^{\bullet}306$ needs to be protonated before recombination. A radical transfer to the nearby tyrosine Y464 (Figure 1), which would be analogous to a tyrosine in the photolyase from *A. nidulans*, was not observed.

A closer inspection of the ET reaction shows that the excited-state $FADH^{\bullet}$* decays in about 30 ps, long before the formation of $WH^{\bullet+}306$. [15] The 15 Å distance between flavin and W306 is bridged by two other tryptophans, W382 and W359, which together with W306 form a triad of tryptophans, see Figure 1. Sequence comparisons of DNA photolyase from different species show that the triad of tryptophans is conserved. In photolyase from *A. nidulans* this triad comprises the chain of tryptophans W390−W367−W314, which are shifted in sequence number by 8 units as compared to the sequence of *E. coli* photolyase. In photolyase from *A. nidulans* tyrosine Y468 is closest to W314 at a distance of 8.6 Å. The next closest tyrosine is more than 13 Å away from W314. In photolyase from *E. coli* we find that tyrosine Y464 is closest to the corresponding tryptophan W306 at a distance of only 3.6 Å. Tyrosine Y464 in photolyase from *E. coli* is buried, whereas the corresponding tyrosine Y468 in photolyase from *A. nidulans* is on the surface. To answer the question why tyrosine Y464, although it is very close to W306, is not involved in the process of radical transfer for photolyase from *E. coli*, we consider the radical state of this tyrosine also in our investigations.

As suggested previously[3], the electron is first transferred from the nearest tryptophan W382 to flavin in about 30 ps. Then, the electron is transferred along the triad W382−W359−W306, generating the cationic radical $WH^{\bullet+}306$ in less than 10 ns.[15] Note that the formation of the tryptophan radical could not be resolved in time. Hence, the ET process may also proceed much faster than in 10 ns.[15] The deprotonation of $WH^{\bullet+}306$ is estimated to yield a decrease in free energy of about 200 meV at pH = 7.4 [15] assuming a $pK_a = 4$ for $WH^{\bullet+}$.[16,17] This energy decrease is essential to prevent a fast charge recombination and stabilizes the $FADH^-$ state. Computations with the pathway method also unraveled that the electron is transferred along the tryptophan triad.[18]

In this contribution, we further support and complete the model of radical transfer and stabilization in DNA photolyase by calculating the energetics of the redox and protonation states of FAD and the tryptophan residues. For this purpose, we evaluated the electrostatic energies of all relevant charge states of the titratable and redox-active groups in photolyase by solving the linear Poisson−Boltzmann equation and calculated the Boltzmann weighted probabilities for the different charge states of FAD and the tryptophan triad. With this method we recently contributed to the understanding of the ET and proton uptake events at the secondary quinone Q_B of bacterial photosynthetic reaction centers.[19−21]

Methods

Theoretical Background. The redox potentials of the FAD and the tryptophan residues in photolyase were determined by averaging over all protonation states of all other titratable groups. The probability $\langle \mathbf{x}_\mu \rangle$ of a variably charged group μ to be protonated or oxidized can be expressed by a thermodynamic average over all possible charge patterns,

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which for N variably charged groups of two different charge states are 2^N, yielding

$$\langle \mathbf{x}_\mu \rangle = \frac{1}{Z}\sum_{n=1}^{2^N} x_\mu^{(n)} \exp(-G^{(n)}/RT)$$

with

$$Z = \sum_{n=1}^{2^N} \exp(-G^{(n)}/RT) \quad (1)$$

where T is the absolute temperature and R the universal gas constant. The charge state of group μ for the charge pattern (n) is characterized by the integer $x_\mu^{(n)}$, which adopts the value 0 if group μ is unprotonated or reduced (negative charge state), or 1 if protonated or oxidized (positive charge state). The total free energy $G^{(n)}$ of the charge pattern (n) of the molecular system under consideration is given as

$$G^{(n)} = \sum_{\mu=1}^{N} [x_\mu^{(n)} \Delta G_\mu^{\text{intr}} + \sum_{\nu=1}^{N} x_\mu^{(n)} x_\nu^{(n)} W_{\mu\nu}] \quad (2)$$

The mutual interaction energies $W_{\mu\nu}$ of the titratable groups μ and ν refer to the charge pattern, where all other variably charged groups of the molecular system are unprotonated or reduced, respectively. Mutual interactions of variably charged groups in the unprotonated (reduced) state and interactions with background charges of nonvariably charged groups are accounted for by the intrinsic free energy $\Delta G_\mu^{\text{intr}}$. A detailed description of these energy terms and the way they are calculated by solving the linear Poisson−Boltzmann equation (LPBE) can be found in a recent review article.[22]

The free energy difference between the positive and negative charge states of the variably charged molecular group μ depending on pH value and redox potential of the solvent E_{sol} is defined in terms of the ratio of probabilities that this group is charged or uncharged according to

$$\Delta G_\mu(\text{pH},E_{\text{sol}}) = -RT \ln \frac{\langle \mathbf{x}_\mu \rangle}{1 - \langle \mathbf{x}_\mu \rangle} \quad (3)$$

With this free energy difference, the pK_a value of a titratable group μ in the protein environment can be written as

$$\text{p}K_{a,\mu}^{\text{protein}}(\text{pH},E_{\text{sol}}) = \text{pH} - \frac{\Delta G_\mu(\text{pH},E_{\text{sol}})}{RT \ln 10} \quad (4)$$

For a redox-active group μ, the standard redox potential of the group in the protein environment can be written as

$$\text{E}_\mu^{\text{protein}}(\text{pH},E_{\text{sol}}) = E_{\text{sol}} + \frac{\Delta G_\mu(\text{pH},E_{\text{sol}})}{F} \quad (5)$$

where F is the Faraday constant.

Note that electrostatic free energies, which are calculated by this machinery refer to the thermodynamic equilibrium of the considered molecular system. Thus, structural relaxation processes, which are slow on the time scale of the considered ET processes and may occur in proteins are not included. Nevertheless, all of the electronic polarizablility, which is instantaneous, and the conventional nuclear polarizability of molecular systems, that is, the reorientation of charged and polar groups, which occurs in the subpicosecond time scale is considered appropriately by using a dielectric constant of $\epsilon_p = 4$ inside the protein.

Calculating Protonation and Redox Patterns of Variably Charged Groups. In the present approach, charge patterns of molecular systems were determined by calculating the electrostatic energy differences of individual variably charged groups in the protein environment relative to suitable model systems, which provide the reference values. For titratable amino acid side chains, these model systems are the corresponding isolated amino acids in aqueous solution, where the basic and acidic groups of the backbone are acetylated and amidated, respectively. The electrostatic energies of the model systems were matched with the corresponding experimental pK_a values. For these computations, a dielectric constant of $\epsilon_p = 4$ inside and $\epsilon_s = 80$ outside of the considered molecular systems was used. For the protein the ionic strength 100 mM with an ion exclusion layer of 2 Å and a solvent probe radius of 1.4 Å were used. The redox potential of the solvent was set to $E_{\text{sol}} = 0$ mV.

The number of variably charged molecular groups in photolyase is 167. These are too many for a direct evaluation of the Boltzmann averaged sums (1). Therefore, a Monte Carlo (MC) titration method with Metropolis sampling was employed, for which we used our own program Karlsberg.[20] It is based on the same method as the MCTI program from Beroza et al.[24] but has additional features.[21] Karlsberg is freely available under the GNU public license from our web server (http://lie.chemie.fu-berlin.de/karlsberg/). We used the same protocol of MC moves as in previous applications.[20,21]

All residues known to be titratable: C- and N-terminus, Asp, Glu, Arg, Lys, Cys, Tyr and His were treated as such. In addition also the diphosphoric acid (DPA) as part of FAD, and the oxidized tryptophan ($WH^{\bullet+}$) and tyrosine ($YH^{\bullet+}$) were considered titratable. MHTF involves two acidic groups, which we treated as independent titratable groups. One group is equivalent to the side chain of glutamate, the other to the C-terminal end group. The tryptophan triad, the nearby tyrosine Y464, and the pair of states $FADH^{\bullet}/FADH^{-}$ from flavin were considered to be redox-active.

For most titratable groups, the corresponding pK_a values were listed by Ullmann and Knapp.[22] For tryptophan, we used the redox potential of 1.07 V as reference value in aqueous solution. This value was measured at pH values below 3, where the redox potential becomes pH-independent because tryptophan remains protonated.[16] For the oxidized tryptophan ($WH^{\bullet+}$), we took p$K_a = 4$.[16,17] From experimental data at low pH, the redox potential of tyrosine was estimated to be above 1.35 V.[16] We used the value of 1.376 V, derived by extrapolating experimental data to pH values below −2, where tyrosine remains protonated. The oxidized tyrosine ($YH^{\bullet+}$) has p$K_a = -2$.[16,26] FAD was considered to consist of two independent variably charged groups, DPA and flavin. In aqueous solution the redox potential of flavin referring to the pair $FADH^{\bullet}/FADH^{-}$ is not known with certainty, since the pK_a of the pair $FADH_2/FADH^{-}$ is with 6.7 close to the neutral pH = 7 where the measurement of the redox potential was performed.[27,28] At this pH, $FADH^{-}$ will be partially protonated and the measured (estimated) redox potential of $FADH^{\bullet}$ amounting to −167 meV[27] (−124 meV[28]) can only be considered as an upper limit. As an estimate we will assume the value −200 meV. We will discuss how the energetics of the radical transfer will change, if this value is varied. In agreement with experiments we did not consider a change of the protonation state of flavin.[15] For the DPA group of FAD no pK_a value was available. As estimate we took pK_a values from the corresponding free DPA[29] where the equilibrium between the pair of protonation states $(HO_3R^{(1)}P-O-PR^{(2)}O_3H)^0/(O_3R^{(1)}P-O-PR^{(2)}O_3H)^-$ and $(HO_3R^{(1)}P-O-PR^{(2)}O_3)^-/(O_3R^{(1)}P-O-PR^{(2)}O_3)^{-2}$ is described by p$K_a = 6$ and 8, respectively. There are two different states of DPA with charge −1, namely, $(O_3R^{(1)}P-O-PR^{(2)}O_3H)^-$ and $(HO_3R^{(1)}P-O-PR^{(2)}O_3)^-$, which are not equivalent due to the different substituents of DPA as part of FAD

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($R^{(1)}$, $R^{(2)}$) and the protein environment. The program MEAD[30] available to calculate the electrostatic energies of titratable groups in different protonation states cannot handle titratable groups with more than two protonation states. To find out whether the protonation equilibrium of DPA is inclined toward the protonation states DPA^{-}/DPA^{-2} or toward DPA^{0}/DPA^{-}, we considered both equilibria separately and described the two different protonation states of DPA^{-} in the same way as we did it for histidine.

Atomic Partial Charges. Using an appropriate set of charges is critical to obtain reliable values of electrostatic energies. A proper choice of charges is also a matter of experience. Most of the charges that we used in the present application were used successfully before to calculate redox potentials of cofactors in proteins.[19,21,31] Additional charges that were needed for computations of electrostatic energies in this application were computed with procedures that we used in such applications before.

The atomic partial charges of the amino acids including the charged and neutral states of the titratable residues were taken from the CHARMM22 parameter set.[32] But, for some of the titratable residues (Arg, Lys, Cys, Tyr) charges are only available in standard protonation. For these residues, we took atomic partial charges as calculated and used before in ref 33. The charge distributions of FAD (including the DPA group) and MTHF (including the two acidic groups) were calculated with the Hartree−Fock method from GAUSSIAN98[34] using the 6-31G* basis set. The atomic partial charges were obtained from the electronic wave functions by adjusting the electrostatic potential in the neighborhood of the molecules with the Merz−Kollman approach.[35] The atomic partial charges of FAD and MTHF and of all titratable residues, whose charges were not available yet, are given in Table S1 of the Supporting Information.

Preparing the Coordinates of Photolyase and Solving the LPBE. DNA photolyase from *E. coli* possesses 471 residues, and two cofactors one FAD and one MTHF. Atomic coordinates are available for both molecules (chain A and B) in the unit cell of the photolyase crystals (PDB code 1dnp). Root-mean-square deviations between non-hydrogen atoms of chain A and B from the crystal structure of photolyase are for all atoms 0.33 Å and for the atoms of the functionally relevant molecular groups of photolyase as displayed in Figure 1, 0.03 Å only. Hence, we considered the coordinates of chain A only and can safely assume that our conclusions will be valid for chain B as well. Atomic coordinates of the two last residues at the C-terminus are not available. Since these are far away from the flavin of FAD and the tryptophan triad, we can safely ignore them. All crystal waters were removed, since the positions of their hydrogen atoms are particularly uncertain. Their influence is accounted for by cavities of high dielectric medium that are formed in their absence. A more thorough discussion of this point is given in ref 19. We consider a total of 3855 non-hydrogen atoms of photolyase. An all atom representation of DNA photolyase (total number of atoms 7589) was obtained by adding all hydrogen atoms with the HBUILD command of CHARMM22.[32,36] Subsequently, the hydrogen atom positions were energetically minimized with the CHARMM22 force field keeping all other atoms fixed.

To compute the electrostatic energies, we solved the LPBE for DNA photolyase with a three-step grid focusing procedure starting from a 162 Å cube lattice with a grid spacing of 2.0 Å, followed by a 101 Å cube with 1.0 Å grid spacing and finally a 22.75 Å cube with a grid spacing of 0.25 Å. The two lattices of lower resolution were centered with respect to the geometric center of photolyase. The lattices with highest resolution were centered at the corresponding variably charged groups. More technical details on the computation of the electrostatic energies $G^{(n)}$, eq 2, for the different charge patterns (n) of a molecular system by solving the LPBE are given elsewhere.[22]

Results and Discussion

Protonation Pattern of Titratable Groups in Photolyase, except Tryptophans of the Triad. Except for histidines, which are mostly neutral, most of the titratable groups in DNA photolyase are at pH = 7.4 in the standard protonation state, that is, the basic residues are charged positive and the acidic residues are charged negative. Folic acid is situated on the surface of photolyase opposite to flavin. Its two acidic groups are fully exposed to the solvent and are therefore found to be both unprotonated. At pH = 7, the DPA group is singly protonated in aqueous solution but was found fully deprotonated in DNA photolyase. The stabilization of the two unit negative charges at the DPA group in photolyase is accomplished by optimally orienting the polar groups in the neighborhood. These are the backbone NH groups of Trp271, Ser235, Ser238, Leu237, Arg236 and the side chain OH groups of Ser235, Tyr222, and Thr234, which form hydrogen bonds with the phosphoric oxygens. The titratable residues in nonstandard partial protonation state that varies with the charge state of the tryptophan triad are Glu274, Asp354, Asp374, Lys454, N-terminus, and four histidines (H44, H199, H294, H453). Their protonation patterns at pH = 7.4 are listed in Table 1. From these residues, only Glu274 and Asp374 are not too far from FADH, but none of these residues is close to the tryptophan triad (Figure 1). The acidic group of Glu274 is on the surface of photolyase but is nevertheless partially protonated. This is due to the double negative charge of the DPA group in the neighborhood of Glu274. Asp354 varies the most with a change in the charge state of the tryptophan triad, although it is with an edge-to-edge distance of 10.9 Å to W382 and 10.7 Å to W306 not very close to any of the three tryptophans (Figure 1). Only four of the 15 histidines in photolyase (displayed in Figure 1) vary their protonation pattern with a change in the charge state of the tryptophan triad. Only one of them, His294 is close to the intermediate tryptophan W359 (6.1 Å edge-to-edge distance). The fact that none of the titratable residues couples strongly with the different charge states of the tryptophan triad accounts for a low reorganization energy of the ET processes between the tryptophan triad. The calculated total charge of photolyase (see Figure 3) and the sum of charges from the tryptophan triad and the nine residues displayed in Table 1 (last two columns) agree well for the different charge states of

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Table 1. Nonstandard Protonation Probability of Residues in DNA Photolyase[a]

charge state of photolyase	Glu 274	Asp 354	Asp 374	Lys 454	N-term	His44			His199			His294			His453			reduced sum of protons[b]	total sum of protons[c]
						double	δ	ϵ	double	δ	ϵ	double	δ	ϵ	double	δ	ϵ		
$FADH^-WH^{\bullet}+WHWH$	0.385	0.626	0.016	0.701	0.277	0.807	0.003	0.189	0.339	0.239	0.422	0.004	0.555	0.441	0.045	0.772	0.182	0.196	0.23
$FADH^-WHWH^{\bullet+}$ WH	0.504	0.771	0.033	0.677	0.282	0.838	0.003	0.160	0.370	0.242	0.387	0.001	0.419	0.580	0.033	0.754	0.213	0.505	0.51
$FADH^-WHWH\ WH^{\bullet+}$	0.565	0.777	0.057	0.684	0.280	0.851	0.002	0.147	0.375	0.238	0.387	0.009	0.663	0.328	0.030	0.762	0.208	0.624	0.66
$FADH^-WH\ WH\ W^{\bullet}$	0.595	0.894	0.059	0.676	0.271	0.861	0.002	0.137	0.373	0.240	0.387	0.014	0.692	0.294	0.022	0.764	0.213	−0.239	−0.22
$FADH^-WHW^{\bullet}$ WH	0.586	0.916	0.062	0.678	0.273	0.849	0.002	0.149	0.368	0.252	0.380	0.012	0.660	0.328	0.015	0.771	0.215	−0.245	−0.20
$FADH^-W^{\bullet}$ WH WH	0.617	0.908	0.063	0.678	0.275	0.969	0.002	0.129	0.382	0.376	0.242	0.012	0.688	0.300	0.018	0.768	0.215	−0.082	−0.16
$FADH^{\bullet}WHWH$ WH	0.221	0.869	0.000	0.679	0.275	0.671	0.004	0.325	0.258	0.260	0.482	0.009	0.645	0.346	0.022	0.762	0.216	0.000	0.00

[a] With the exception of histidines, we listed all titratable residues of photolyase, which deviate by at least 0.05 unit charges from the standard protonation state at pH = 7.4. From the 15 histidines we listed only those four whose protonation probabilities vary by more than 0.05. The small deviations between the total sum and the reduced sum of protons demonstrates that the main contribution to changes in the total charge of photolyase is due to these nine residues. [b] Sum of protonation probabilities of the nine listed titratable groups including the protons in the tryptophan triad relative to the sum of the ground-state $FADH^{\bullet}$ WH WH WH probabilities, which is $\sum p_0 = 3.004$. [c] Sum of all protons in photolyase relative to the sum of protons in the ground-state $FADH^{\bullet}$ WH WH WH.

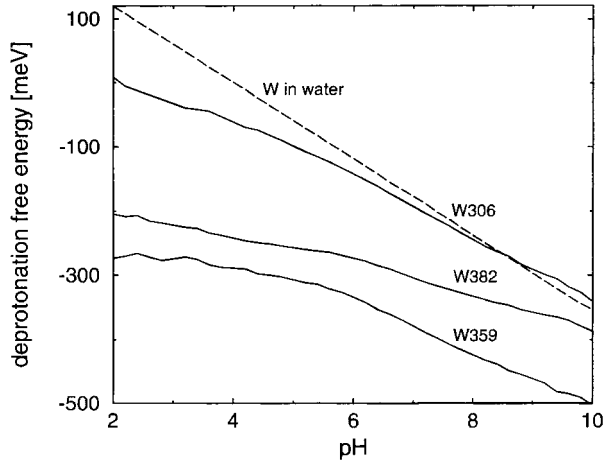

Figure 2. pH dependence of the deprotonation free energy of the tryptophans from the triad evaluated by calculating the electrostatic free energies from the solution of the LPBE. The dashed line corresponds to the pH dependence in pure water. Flavin is kept in the reduced state $FADH^-$ as it corresponds to the activated photolyase. For more details see text.

the tryptophan triad, indicating that these are the most relevant residues responsible for changes of the total charge in photolyase.

Protonation of Tryptophans from the Triad. In aqueous solution, an oxidized (positively charged) tryptophan ($WH^{\bullet+}$) deprotonates with $pK_a = 4$ to yield the neutral charge state $W^{\bullet}$.[16,17] Since W306 is partially solvent exposed, it is not so surprising that it behaves similarly as in solution. The calculated pK_a values are 2 for W306 and −11 and −14 for the buried tryptophans W359 and W382, respectively. The complete pH dependence of the deprotonation free energy of the tryptophan cation radicals from the triad is exhibited in Figure 2. The pH dependence of the deprotonation free energy is approximately linear for W306 and close to the behavior of a fictitious isolated (noninteracting) tryptophan cation radical in solution (dashed line in Figure 2). The deprotonation free energy of $WH^{\bullet+}382$ and $WH^{\bullet+}359$ is considerably lower than that of the solvent exposed $WH^{\bullet+}306$. Hence, the two other tryptophans prefer even more than W306 to be in the neutral charge state $W^{\bullet}$. This behavior is to be expected, since bare charges can be accommodated more easily in a medium of high dielectric constant than in a medium of low dielectric constant like a protein.

The difference in protonation energy of the tryptophans from the triad is also reflected in the variation of the total charge of photolyase. The formal total charge is computed by using the standard protonation state of all titratable residues and assuming that histidines are neutral. Considering that folic acid possesses a total charge of −1 (two negatively charged carboxylates and a unit positive charge on N6) and FAD in the catalytic active state ($FADH^-$) a total charge of −3 (due to DPA and flavin), the formal total charge of photolyase is −6 in the resting state $FADH^{\bullet}-WH-WH-WH-YH$ and in the four possible charge separated states $FADH^--WH^{\bullet+}-WH-WH-YH$, $FADH^--WH-WH^{\bullet+}-WH-YH$, $FADH^--WH-WH-WH^{\bullet+}-YH$, and $FADH^--WH-WH-WH-YH^{\bullet+}$ (Figure 3). Consequently, the formal total charge is −7 if one of the tryptophans from the triad or the tyrosine becomes deprotonated. The total charge of photolyase computed from the distribution of protonation pattern is close to −5 if all tryptophans from the triad and the tyrosine Y464 are protonated. The calculated total charge diminishes by a fraction of a unit charge, if one of the buried tryptophans or the tyrosine becomes deprotonated (W382: −0.39; W359: −0.71; Y464: −0.54), whereas it decreases by almost a unit charge (−0.88) if the solvent-accessible W306 is deprotonated (see Figure 3). Hence, the protein tries in part to compensate for the decrease in charge if a buried tryptophan becomes deprotonated by an increase in protonation at a number of other residues. This charge compensation is not necessary for the solvent exposed tryptophan W306.

Redox Potential of Flavin and of Tryptophans from the Triad. The redox potential for the redox pair $FADH^{\bullet}/FADH^-$ of flavin is not known with certainty. We estimated it to be −200 meV. The resulting calculated redox potential of −370 meV in photolyase is consistent with a measurement estimating that the redox potential is between −330 and −500 meV.[37] Oxidized tryptophan $WH^{\bullet+}$ possesses a $pK_a = 4$.[16,17] Thus, above pH = 4 the oxidation reaction of tryptophan in water is inevitably coupled to a deprotonation reaction, which complicates the interpretation of measurements on the redox potential of tryptophan in water. We simulated this pH dependence of the redox potential of tryptophan in water considering the three states, $WH^{\bullet+}$, WH, and $W^{\bullet}$ involved in the redox reaction by using a technique analogous to one employed for histidine. We used 1.07 V for the redox potential, which is the pH independent value below pH = 3.[16,25] The aqueous solution was modeled by a dielectric continuum of $\epsilon = 80$. The resulting pH dependence (solid line in Figure 4) from the computation of electrostatic energies is very similar to the measured data.[16,26]

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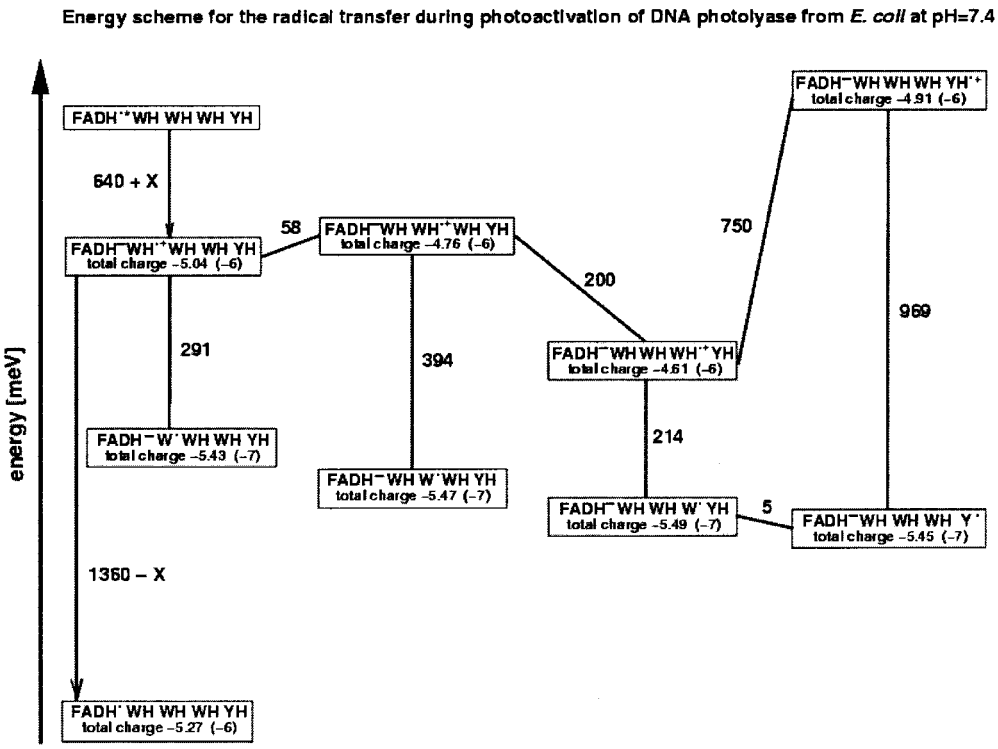

Figure 3. Reaction and energy scheme of the redox and protonation states of FAD, the tryptophan triad, and the tyrosine. FADH is photoactivated $FADH^{\bullet} \rightarrow FADH^{\bullet *}$, the charge separation occurs $FADH^{\bullet *}WH \rightarrow FADH^{-}WH^{\bullet +}$. We estimated the unknown redox potential of $FADH^{\bullet}$ to be $X = -200$ meV as explained in text. This value is shifting the energy of the charge-separated state $FADH^{-}WH^{\bullet +}$ as indicated in the figure. Starting from the state $FADH^{-}WH^{\bullet +}$, the radical character is transferred within the tryptophan triad to the distal tryptophan W306. By deprotonation of tryptophan $WH^{\bullet +} \rightarrow W^{\bullet}$ a neutral tryptophan radical is formed. Finally, the radical may be transferred to Tyr464. However, in contrast to photolyase from *A. nidulans* this was not observed for photolyase from *E. coli*. Solid lines connecting different states denote changes in electronic state or protonation state (vertical lines) or denote ET processes (more horizontal lines). The numbers at these lines provide the difference in electrostatic free energies in units of meV between the pairs of states that are connected by the lines. Values of total charge and formal charge (in parentheses) are given in the boxes.

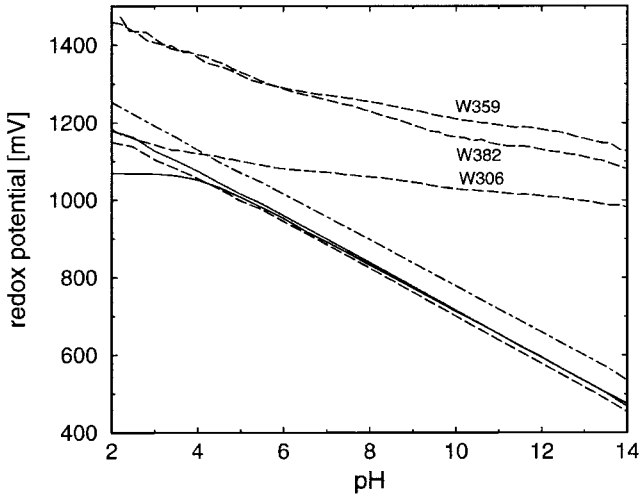

Figure 4. pH dependence of the redox potentials of the three tryptophans in photolyase and in water. (Upper three curves (dashed lines)) pH dependence of the redox potential of the three tryptophans from the triad remaining in the protonated state, i.e., considering the redox pair $WH/WH^{\bullet +}$. (Solid line) pH dependence of tryptophan in water calculated by using a dielectric medium of $\epsilon = 80$ and allowing the tryptophan to deprotonate at high pH as it occurs in aqueous solution. The three curves parallel to the solid line exhibit the pH dependence of the redox potential of the tryptophans from the triad in photolyase by allowing them to deprotonate in the oxidized state. (Dashed line) W306; (dotted line) W359; (dashed−dotted line) W382.

The pH dependence of the redox potentials of the tryptophans from the triad in photolyase corresponding to the redox pair $WH^{\bullet +}/WH$ is much weaker than for the redox pair $WH/W^{\bullet}$ involving the abstraction of a whole hydrogen atom. That is in particular the case for the solvent exposed tryptophan W306, where the coupling of the ET with changes of protonation pattern is screened most efficiently (dashed lines in upper part of Figure 4). Considering the redox pair $WH/W^{\bullet}$ involving a coupled electron−proton transfer also for the tryptophans in photolyase the pH dependence of the redox potential becomes very similar to the one of tryptophan in aqueous solution (Figure 4). At low pH the values of the redox potentials from the redox pair $WH^{\bullet +}/WH$ and $WH/W^{\bullet}$ merge, since deprotonation is not possible. This merging is observed at pH = 2 for the solvent exposed tryptophan W306, but occurs at lower pH for the buried tryptophans whose pK_a is shifted to smaller values.

Energetics of Charge Separation and Radical Transfer Involving Flavin and the Tryptophan Triad. The reaction scheme of the charge separation and radical transfer in DNA photolyase is displayed in Figure 3. The electronic excited state in photolyase $FADH^{\bullet *}$ is estimated to be 2 eV above the ground-state $FADH^{\bullet}$.[15] Assuming that the redox potential of flavin for the redox pair $FADH^{\bullet}/FADH^{-}$ in solution is $X = -200$ meV the energy of the initial charge separated state $FADH^{-}$ $WH^{\bullet +}$ with the proximal tryptophan W382 is calculated to be 1.560 eV above the ground state energy. Hence, there are 0.440 eV available for the charge separation process. In the next step, an electron is transferred from the intermediate tryptophan W359 to the proximal W382 with a slightly positive reaction energy of +58 meV. The ET from the distal tryptophan W306 to W359 is calculated to be downhill in energy by −200 meV. Finally, the deprotonation of the distal tryptophan occurs at pH = 7.4 with a reaction energy that is calculated to be −214 meV in

agreement with an estimate that assumes W306 to behave as in solution.[15] Since this tryptophan is solvent-exposed, the proton can directly be dumped into the solvent.

Deprotonation of the Tryptophans from the Triad. The two buried tryptophans may also become deprotonated, although there is no direct pathway for the proton to reach the solvent. Thus, a deprotonation of the buried tryptophans will be very slow compared to the ET, which is faster than 10 ns. However, there could be a hydrogen atom transfer or proton transfer from the intermediate W359 to the distal W306 when the latter is deprotonated and subsequently from the proximal W382 to the intermediate W359. The corresponding edge-to-edge distances between the tryptophans are 3.9 and 5.2 Å, respectively. Hydrogen or proton transfer between W359 and the distal W306 may be possible, since the positions where the hydrogen atom binds are only 5.1 Å apart. The transfer may be supported by two crystal waters, each one forming a hydrogen bond with the NH group of the indole ring of one of the tryptophans and the Asp358 that is situated between the two tryptophans (Figure 1). One acidic oxygen is close to the hydrogen of W359 (3.6 Å). The other oxygen is engaged in a salt bridge with Arg360. The salt bridge may become weaker after a proton transfer from W359 to Asp358 and allow the acidic group of Asp358 to reorient and contact the empty proton binding site at the indole ring of the distal tryptophan W306. For the pair W382 and W359 the indole rings are oriented such that the corresponding hydrogen atom positions are pointing away from each other (see Figure 1), and no transfer pathway of the hydrogen could be recognized by inspection of the crystal structure. The same is the case for the hydrogen transfer between the pair Y464 and W306. Calculating the energetics of the deprotonated radical states of the tryptophan triad, we found that the energy difference is 20 meV between WH359−W$^{\bullet}$306 and W$^{\bullet}$359−WH306 and 45 meV between WH382−W$^{\bullet}$359 and W$^{\bullet}$382−WH359 (see Figure 3). Hence, a hydrogen atom transfer or a coupled proton transfer and ET, where the neutral radical state moves from W306 via W359 to W382 is energetically easily possible, but kinetically probably very slow.

Estimating the ET Rates of the Forward and Backward Reaction. The ET of the forward reaction was measured to be faster than 10 ns.[15] The overall rate of the forward ET process is probably dominated by the weakly endergonic ET from W359 to W382. An empirical expression to estimate the rate for endergonic ET processes in proteins at $T = 300$ K is[14]

$$\log_{10}(k_{T=300K}^{\text{endergonicET}}) = 13.0 - 0.6(D - 3.6) - 3.1(-|\Delta G| + \lambda)^2/\lambda - |\Delta G|/0.06 \quad (6)$$

A corresponding rate expression applicable for exergonic ET processes is obtained by omitting the last term in expression 6.[14] With $\Delta G = 0.058$ eV, an edge-to-edge distance between the tryptophans W382 and W359 of $D = 5.2$ Å, and the generic value $\lambda = 1.0$ eV for the reorganization energy of ET processes in proteins, [14] we obtain $k_{\text{forward}}^{\text{endergonic ET}} = 2.1\ 10^8 \text{s}^{-1}$ for the endergonic ET from W359 to W382. This value of the rate constant of the forward ET process is large enough to be consistent with the fact that the tryptophanyl radical WH$^{\bullet+}$306 appears in less than 10 ns. Using the parameter values $\Delta G = -0.2$ eV, $\lambda = 1.0$ eV and $D = 3.9$ Å, the rate of the exergonic ET from W306 to W359 is estimated to be $k_{\text{forward}}^{\text{exergonic ET}} = 6.85\ 10^{10}\ \text{s}^{-1}$. The reverse ET process leading to charge recombination is dominated by the endergonic ET process WH359−WH$^{\bullet+}$306 → WH$^{\bullet+}$359−WH306. According to eq 6, its rate is estimated to be $k_{\text{back}}^{\text{endergonic ET}} = 3.2\ 10^7\ \text{s}^{-1}$. Hence, the cationic tryptophanyl radical is most of the time localized at W306, which facilitates deprotonation of W306 to stabilize the active charge state FADH$^-$ in photolyase.

Estimating the Rate of De- and Reprotonation of Trp306. The measured deprotonation rate of WH$^{\bullet+}$306 at pH = 7.4 is $k_{\text{forward}}^{\text{PT}} = 3.3\ 10^6\ \text{s}^{-1}$.[15] Assuming that the reprotonation process of W$^{\bullet}$306 is governed by the same activation energy the corresponding rate is estimated to be $k_{\text{back}}^{\text{PT}} = 3.3\ 10^6\ \text{s}^{-1} * \exp(-0.214\ \text{eV}/k_B T) = 840\ \text{s}^{-1}$. Compared to the forward reaction of deprotonation the back reaction may be kinetically slowed, since the proton from the partially solvent exposed W306 can easily be dumped in the solvent but may not be so readily available as is the proton for the deprotonation process. The electron back-transfer is much faster than the proton uptake such that the overall rate of charge recombination is dominated by the proton uptake W$^{\bullet}$306 + H$^+$ → WH$^{\bullet+}$306. This result is also supported by the finding that the back-transfer is pH-dependent.[15] The calculated rate of proton uptake $k_{\text{back}}^{\text{PT}} = 840\ \text{s}^{-1}$ corresponds to a lifetime of about 1.2 ms for the neutral radical state FADH$^-$ WH WH W$^{\bullet}$, that compares favorably with the measured lifetime of 17 ms for the charge separated state at pH = 7.[15]

Possible Role of Tyrosine Y464 for Stabilizing the Radical State. In contrast to photolyase from *E. coli*, it was found that in photolyase from *A. nidulans* 60% the radical state resides at a tryptophan and 40% at a tyrosine.[9] Tryptophan W306 in photolyase from *E. coli*, which stabilizes the radical FADH$^-$, corresponds to W314 in photolyase from *A. nidulans*. The tyrosine closest to this tryptophan is in photolyase from *A. nidulans* Y468 at a distance of 8.6 Å, whereas Y464 is just 3.6 Å away from W306 in photolyase from *E. coli*. To clarify the question why in photolyase from *E. coli* the radical transfer stops at tryptophane W306, we also computed the energetics of a possible radical transfer from W306 to Y464. The corresponding ET from YH464 to WH$^{\bullet+}$306 is strongly endergonic by 750 meV, since the resulting positively charged tyrosine YH464$^{\bullet+}$ is in contrast to the solvent-exposed W306 buried in the low dielectric medium of the protein. For the same reason the deprotonation of the charged YH464$^{\bullet+}$ yielding the charge neutral state Y464$^{\bullet}$ is strongly exergonic by 969 meV, such that the charge neutral radical states W$^{\bullet}$306-YH and WH306-Y$^{\bullet}$ are nearly isoenergetic (5 meV difference). Using eq 6 with the generic value of $\lambda = 1.0$ eV for reorganization energy and the distance $D = 3.6$ Å, the endergonic ET rate is estimated to be only 2 s^{-1}, which is an order of magnitude smaller than the rate of charge recombination. Furthermore, the lifetime of the cationic radical state YH$^{\bullet+}$464 is limited by electron back-transfer from WH306 to about 10^{-13} s. On this short time scale a proton transfer yielding a deprotonated Y464 becomes highly unlikely and since this tyrosine is buried a suitable nearby proton acceptor group would be needed to make the deprotonation process possible. Also a direct hydrogen transfer is likely to be too slow to compete with charge recombination process, since the pair Y464 and W306 is oriented such that the corresponding hydrogen atom positions are pointing away from each other (see Figure 1), and no transfer

pathway of the hydrogen could be recognized by inspection of the crystal structure. In photolyase from *A. nidulans* the tyrosine Y468 closest to the corresponding tryptophan W314 is solvent-exposed. As a consequence, we expect that the cationic radical state $YH^{\bullet+}468$ from *A. nidulans* has a much lower energy than has $YH^{\bullet+}464$ from *E. coli* such that the lifetime of the cationic radical state of Y468 is long enough that the neutral radical state can be reached.

Summary

The present computations are consistent with all experimental findings of radical transfer within the tryptophan triad and radical stabilization at the distal tryptophan W306.[15] They provide further insight, yielding energies for all relevant states and rates for the most relevant processes. The more detailed picture of charge separation and radical transfer from these computations yields an energy of about 450 meV available for the charge separation process and shows that, while the first ET from W359 to W382 is nearly isoenergetic, the ET from W306 to W359 is downhill in energy by 200 meV, which stabilizes the radical state at the distal tryptophan W306. The radical state $WH^{\bullet+}306$ is stabilized further by 214 meV in an exergonic deprotonation reaction that leads to the neutral radical state $W^{\bullet}306$. The generic value of the reorganization energy of ET processes in proteins[14] of $\lambda = 1.0$ eV that we used, yielded consistent values for the ET rates. The energy of the cationic radical state of WH306 is lower than the one of W382 and W359, presumably since W306 is solvent exposed and can therefore better accommodate its positive charge, while the energies of the neutral radical state of the tryptophans differ by no more than 65 meV. The rate of back reaction is determined by a slow reprotonation of W306. The calculated rate of charge recombination agrees within an order of magnitude with the measured rate of about 60 s^{-1}.[15]

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Supporting Information Available: Table S1, atomic partial charges of folic acid, FAD, tryptophan, tyrosine in the different protonation and redox states. This material is available free of charge via the Internet at http://pubs.acs.org.

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