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Excited-State Properties of *Escherichia coli* DNA Photolyase in the Picosecond to Millisecond Time Scale†

Paul F. Heelis,*,‡ Tadashi Okamura,§ and Aziz Sancar‖

Faculty of Science and Innovation, North East Wales Institute, Deeside, Clwyd CH5 4BR, United Kingdom, Laboratory of Physical Chemistry, Tokyo Zokei University, Hachioji, Tokyo 193, Japan, and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

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ABSTRACT: *Escherichia coli* DNA photolyase contains a stable flavin radical that is readily photoreduced in the presence of added electron donors. Picosecond, nanosecond, and conventional flash photolysis technique have been employed to investigate the events leading to photoreduction from 40 ps to tens of milliseconds following flash excitation. Direct light absorption by the flavin radical produces the first excited doublet state which undergoes rapid (within 100 ps) intersystem crossing to yield the lowest excited quartet ($n\pi^*$) state. In contrast, light absorption by the folate chromophore produces a new intermediate state via interaction of the folate excited singlet state with the ground-state flavin radical, leading to an enhanced yield of the excited radical doublet state and hence quartet state. Subsequent reaction of the excited quartet state involves hydrogen atom abstraction from a tryptophan residue. Secondary electron transfer from added electron donors occurs to the oxidized tryptophan radical with rate constants ranging from 10^4 (dithiothreitol) to 4×10^6 M^{-1} s^{-1} (*n*-propyl gallate). The low value of the latter rate compared to reduction of the tryptophan radical in lysozyme suggests that the reactive tryptophan is highly buried in photolyase. A redox potential diagram has been constructed for the ground and excited states involved. It is concluded that the one-electron reduction potential of the excited quartet state of the flavin radical must be at least 1.23 V more positive than the ground state, in agreement with the value of $\Delta E > 1.77$ V calculated from spectroscopic data.

DNA photolyase (EC 4.1.99.3) catalyzes the photochemical conversion of pyrimidine dimers in DNA into pyrimidines, thus reversing the effect of far-UV (200-300 nm) radiation. The enzyme from *Escherichia coli* contains the FAD blue neutral radical (Sancar & Sancar, 1984; Jorns et al., 1984) and a second chromophore that was recently identified as 5,10-methenyltetrahydrofolate (Johnson et al., 1988). Recent studies (Payne et al., 1987; Sancar et al., 1987b) have shown that in vitro photoreactivation proceeds via photoreduction of the flavin radical followed by dimer repair by the photoexcited reduced form. Hence, the mechanism of radical photoreduction is clearly important in understanding the in vitro photoreactivation mechanism.

We suggested previously (Heelis & Sancar, 1986; Heelis et al., 1987; Okamura et al., 1989) that excitation of the flavin radical (**1** → **2**, Figure 1) results in an intramolecular electron (or H atom) transfer from an unknown amino acid residue (DH) to the excited quartet state of the radical (**3** → **4**, Figure 1), to transiently form a reduced flavin-amino acid radical intermediate (**4**, Figure 1). A dark reversal of this electron-transfer process occurs (**4** → **1**, Figure 1) over 20 ms (Heelis et al., 1987) so that no net change in the enzyme is detected upon long-term irradiation. However, in the presence of external electron donors (RH, e.g., thiols, glycyltyrosine, or NADH) reduction of the oxidized amino acid radical occurs (**4** → **6**, Figure 1), resulting in permanent reduction (in the absence of O_2) of the flavin radical. The present study involves the use of both picosecond and nanosecond laser flash photolysis techniques together with conventional photolysis experiments to investigate in more detail the nature of the intermediates and the kinetics of the processes involved in radical photoreduction.

Experimental Procedures

DNA photolyase was prepared as described previously (Sancar et al., 1984, 1987a). The enzyme was in the "blue form", essentially free of oxidized FAD as shown by absorbance and fluorescence measurements. The stock solutions of the enzyme were stored at −20 or −80 °C in storage buffer containing 50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 10 mM DTT, 1 mM EDTA, and 50% glycerol. Experiments were carried out by diluting the stock solution from 2- to 60-fold into a buffer containing 50 mM Tris·HCl, pH 7.4, 100 mM NaCl, and other components as indicated. Enzyme concentrations were in the 10^{-5}-10^{-4} range with respect to the flavin

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* To whom correspondence should be addressed.
‡ North East Wales Institute.
§ Tokyo Zokei University.
‖ University of North Carolina.

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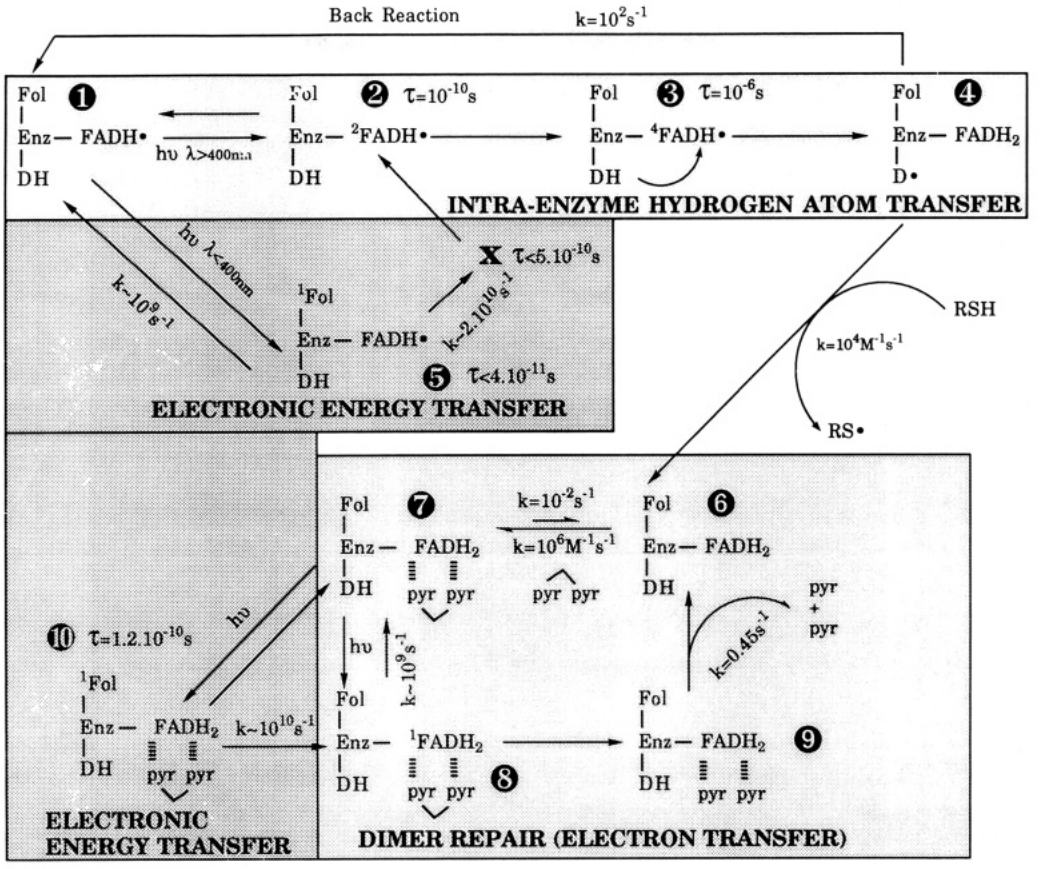

FIGURE 1: Proposed reaction scheme for photoreduction of Enz-FADH• and repair by Enz-$FADH_2$. Absorption of light by the flavin radical (**1**) leads to formation of the excited doublet (**2**) followed by intersystem crossing to yield the quartet state (**3**). Subsequent intramolecular hydrogen atom transfer from an amino acid residue gives the "transient" reduced form (**4**). Electron transfer from added reducing agents (RH) reduces the D• radical to give the "permanent" reduced form (**6**). Binding of the enzyme to pyrimidine dimers (**6**) → (**7**) followed by light absorption (**7**) → (**8**) leads to dimer splitting (**8**) → (**9**) and subsequent dissociation (**9**) → (**6**). Absorption of light by the folate chromophore leads to electronic energy transfer to the flavin (**1**) → (**5**) → (**X**) → (**2**) or (**7**) → (**10**) → (**8**). τ is the actually observed lifetime of species. $k\sim$ denotes approximate calculation of the rate constant of a specific proces. $k=$ denotes measurement of the rate constant of a specific process. The τ value for the reduced flavin singlet **8** is that for the unbound chromophore. Sources for lifetimes and rate constants are as follows: $\tau 2 = 10^{-10}$ s (Okamura et al., 1989); $\tau 3 = 10^{-6}$ s (Heelis & Sancar, 1986); $\tau 5 < 4 \times 10^{-11}$ s (this work); $\tau X < 5 \times 10^{-10}$ s (this work); $\tau 10 = 1.2 \times 10^{-10}$ s (from fluorescence lifetime, unpublished observation). $k4,1 = 10^2$ s^{-1} (this work); $k5,1 = 10^9$ s^{-1} [calculated from observed lifetime of **5** and by assuming 95% energy transfer from **5** to **X** and hence 5% energy loss in going from **5** to **1**; see also Heelis et al. (1987)]; $k5,X \sim 2 \times 10^{10}$ s^{-1} (this work); $k4,6$ (RSH = dithiothreitol) $= 10^4$ M^{-1} s^{-1} (this work); $k6,7 = 10^6$ M^{-1} s^{-1} (Sancar et al., 1987a); $k7,6 = 10^{-2}$ s^{-1} (Sancar et al., 1987a); $k8,7 \sim 10^9$ s^{-1} (from lifetime of flavin singlet state absorbance, unpublished observation); $k10,8 \sim 10^{10}$ s^{-1} (calculated from lifetime of folate fluorescence and by assuming 90% energy transfer); $k9,6 = 0.45$ s^{-1} (Sancar et al., 1984; Sancar & Sancar, 1988).

radical as determined from the absorbance at 580 nm by using $\epsilon = 5000$ M^{-1} cm^{-1} (Payne et al., 1987). All experiments were carried out at 4 °C under aerobic conditions. Chemicals were purchased from either Sigma or British Drug House and were of the purest grade available.

The nanosecond laser flash photolysis system was based on a JK Lasers Systm 2000 Nd^{3+} YAG laser emitting pulses at 532 nm with energies in the 10–200-mJ range and a pulse duration of 20 ns and has been described in detail elsewhere (Heelis & Sancar, 1986). For measurements on a millisecond time scale, a DC-powered quartz–halogen lamp of constant intensity was used as the analyzing source. Picosecond transient absorption spectra were measured with a microcomputer-controlled mode-locked Na^{3+} YAG laser emitting pulses at 355 nm of 1-mJ energy and 22-ps duration as described elsewhere (Mashuhara et al., 1982). For all flash photolysis experiments, samples were contained in quartz cells of 1-cm optical path length. Appropriate filters were placed in the analyzing light path to reduce photolysis.

Conventional photolysis experiments were carried out with a Sunpak 4205G photographic flash unit on manual mode and will be termed camera flash experiments to distinguish them from the laser flash photolysis experiments. In all cases, the light output was passed through an Oriel OG515 cutoff filter to exclude wavelengths shorter than 520 nm to ensure absorption by the flavosemiquinone only.

Results

(*A*) *Nature of the Primary Excited States.* Recent studies using picosecond flash photolysis of folate-depleted photolyase (Okamura et al., 1989) have identified the primary excited state present 40 ps after excitation as the first excited doublet state of the radical (Enz-^{2}FADH•,[1] **2**, Figure 1). Intersystem crossing to the lowest excited quartet state (Enz-^{4}FADH•) occurs within 100 ps. In this work picosecond flash photolysis of photolyase containing the folate chromophore ($folate^+$) has been carried out. Figure 2 shows the transient difference spectrum observed 40 ps after laser excitation. For comparison the corresponding results with $folate^-$ enzyme are also shown

[1] Abbreviations: Enz-FADH•, enzyme-bound FAD neutral radical; Enz-$FADH_2$, enzyme-bound fully reduced FAD; Enz-^{2}FADH•, Enz-^{4}FADH•, the lowest excited doublet and quartet states, respectively, of the FAD neutral radical; DH, D•, the internal electron donor and its oxidized radical, respectively; TrpH, Trp•, $TrpH^{•+}$, tryptophan and its oxidized neutral and protonated radicals, respectively; Tyr, $Tyr^{•+}$, Cys, and $Cys^{•+}$, tyrosine, oxidized tyrosine radical, cysteine, and cysteine oxidized radical, respectively; $folate^+$, $folate^-$, enzyme with folate present and folate depleted, respectively; Fol, 5,10-methenyltetrahydrofolate.

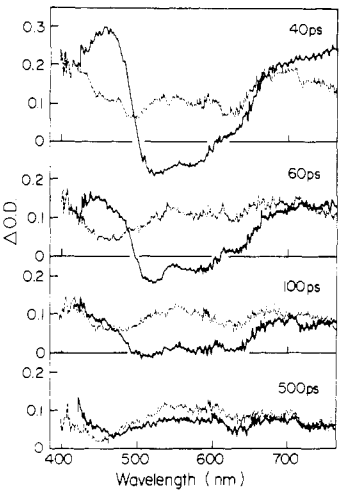

FIGURE 2: Picosecond flash-induced difference spectra for (—) folate$^+$ and (---) folate$^-$ enzymes. Delay times after the pulse are indicated in the figure. The enzyme concentration was 1.4×10^{-4} M, and the enzyme was 40% saturated with folate [see Johnson et al. (1988)].

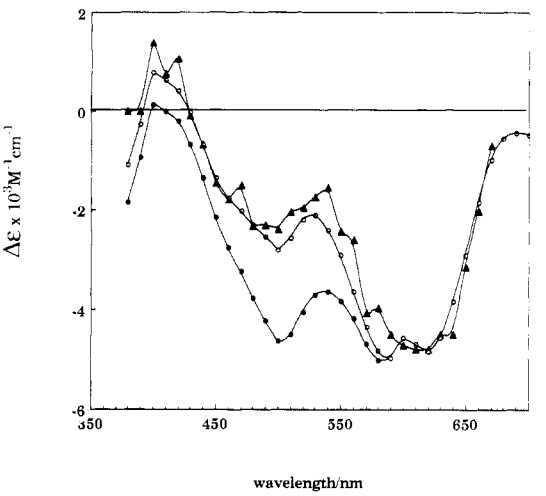

FIGURE 3: Flash-induced difference spectra for photolyase enzyme on an absolute extinction coefficient scale. (●) Determined 1 min after a single camera flash in the presence of 10 mM dithiothreitol. (○) Spectrum as for (●) with the absorption spectrum of the oxidized tryptophan radical (trp$^•$) added. (▲) Laser-induced transient spectrum determined 4 μs after excitation, normalized to (○) at 620 nm.

[from Okamura et al. (1989)]. As can be seen, the folate$^+$ enzyme yields strikingly different transient spectra from the folate$^-$ enzyme. The species present in folate$^+$ enzyme cannot be assigned simply to a folate excited state, e.g., the first excited singlet state (^{1}folate), since the substantial depletion from 500 to 600 nm shows the involvement of the flavin radical. Hence, it is suggested that the 40-ps species involves an interaction of unknown nature (species **X**, Figure 1) between ^{1}folate and Enz-FADH$^•$. Whatever the nature of the intermediate (**X**), it decays within 500 ps to yield a transient species previously assigned to the flavin radical quartet state (Enz-^{4}FADH$^•$), presumably via formation the excited doublet state.

(*B*) *Nature of the Primary Internal Electron Transfer.* Laser flash photolysis of photolyase using 532-nm excitation generates an initial transient spectrum (λ_{max} = 420 nm, data not shown) now assigned to that of the excited quartet state (Enz-^{4}FADH$^•$; Okamura et al., 1989) of the flavin radical (previously assigned to the excited doublet state, Enz-^{2}FADH$^•$). This excited state reacts within several microseconds to yield the transient reduced flavin (**4**, Figure 1, Enz-FADH$_2$; Heelis & Sancar, 1986), the transient difference spectrum of which is shown in Figure 3. This spectrum is similar to that previously reported (Heelis & Sancar, 1986) but is of considerably higher resolution due to the greater quantities of enzyme now available and the use of signal-averaging techniques. It was proposed previously (Heelis & Sancar, 1986; Heelis et al., 1987) that the electron source for reduction of the excited quartet state is an amino acid residue (DH, Figure 1).

It follows that the transient difference spectrum observed 4 μs after the laser pulse may be a composite spectrum consisting of the Enz-FADH$^•$/E-FADH$_2$ difference spectrum plus the DH/D$^•$ difference spectrum. In contrast, for camera flash photoreduction experiments in the presence of electron donors (Heelis & Sancar, 1986) the difference spectrum is measured approximately 1 min afte excitation, by which time all radical species (i.e., D$^•$ or R$^•$) will have decayed. Hence, camera flash spectra should represent purely the Enz-FADH$^•$/Enz-FADH$_2$ difference spectrum. The latter spectrum can be calculated on an absolute scale (Figure 3, ●) by using $\Delta\epsilon$ = 5000 M^{-1} cm^{-1} at 580 nm (Payne et al., 1987). The spectra of the one-electron oxidized forms of many amino acids are available in the literature from pulse radiolysis experiments with one-electron oxidants such as $Br_2^{•-}$ (Adams et al., 1972). However, only the possible contribution to the spectrum from the oxidized radicals of tryptophan, tyrosine, or histidine need to be considered, as other amino acid radicals do not absorb significantly at $\lambda > 350$ nm. Hence, addition of the spectrum of the tryptophan (both neutral Trp$^•$ and cationic TrpH$^{•+}$, tyrosine, or histidine radicals to the camera flash difference spectrum was carried out. In only one case (Figure 3, ○) for the neutral electron deficient radical of tryptophan (Trp$^•$) was a reasonable agreement obtained with the spectrum actually observed following laser excitation (Figure 3, ▲), suggesting that the electron donor is a tryptophan at the active site of the enzyme.

The pH dependence of the efficiency of the primary electron transfer (Figure 1, **3** → **4**) reaction was detrmined in the pH range 6–10. However, the yield of Enz-FADH$_2$ (**4**, Figure 1) as determined from the ΔOD_{580} (t = 4 μs) was found to be essentially independent of the pH. These results further support the conclusion that TrpH is the electron donor by eliminating His and Cys as likely candidates.

(*C*) *Kinetics of the Secondary External Electron Transfer Reaction.* As was reported previously (Heelis et al., 1987), in the absence of added electron donors the primary reduction reaction is reversed over a period of tens of milliseconds (data not shown). The recovery process follows first-order kinetics with $K_{41} = 1\text{–}1.4 \times 10^2$ s^{-1}, the precise value varying slightly for each enzyme preparation. In all cases greater than 95% recovery of the initial depletion at 580 nm was observed.

In the presence of added electron donors, e.g., glycyltyrosine or thiols, it was previously shown that the initial recovery of the depletion at 580 nm was faster than in their absence (Heelis et al., 1987). However, the extent of recovery of the depletion was less in the presence of electron donors than in their absence, consistent with the prevention of the back reaction by reduction of D$^•$ (Figure 1, **4** → **6**). The bimolecular rate constant of the reduction of D$^•$ by added electron donors (RH = glycyltyrosine, cysteine, dithiothreitol, and *n*-propyl gallate) was studied by measuring the kinetics of the recovery of the depletion at 580 nm as a function of donor concentration. By use of eq 1, plots of k observed (k_{obs}) vs donor

$$k_{obs} = k_{41} + k_{46}[RH] \quad (1)$$

concentration were linear and yield the bimolecular rate constant (k_{46}) for repair of the D$^•$, radical species (Table I).

Table I: Photoreduction Parameters for Added Substrates

electron donor	k_{46}[a] (M^{-1} s^{-1})	$[RH]_{1/2}$[a] obsd (M)	$[RH]_{1/2}$ calcd from eq 3
n-propyl gallate	3.8×10^6	4×10^{-5}	3.4×10^{-5}
glycyl-L-tyrosine	5×10^4	2.5×10^{-3}	2.6×10^{-3}
L-cysteine	2.4×10^4	5.0×10^{-3}	5.4×10^{-3}
dithiothreitol	1.1×10^4	0.8×10^{-2}[b]	1.2×10^{-2}

[a] k_{46} = the bimolecular rate constant for repair of D•; $[RH]_{1/2}$ = concentration of substrate for 50% of maximum rate. [b] Due to precipitation of the enzyme at high concentrations of DTT, an accurate value of the saturating photoreduction rate and hence $[RH]_{1/2}$ could not be obtained.

The dependence of the extent of radical photoreduction on the electron-donor concentration was investigated by camera flash photoreduction experiments as previously described (Heelis & Sancar, 1986; Heelis et al., 1987).

For each electron donor, the extent of radical photoreduction following a single camera flash reaches a limiting value at high donor concentrations (data not shown). The concentrations of each electron donor needed to effect 50% of this maximum photoreduction rate will be termed $[RH]_{1/2}$ and are given in Table I. If the reaction pathway proposed is correct (Figure 1), then the value of $[RH]_{1/2}$ corresponds to 50% scavenging of D• by RH. Theoretical values of $[RH]_{1/2}$ can hence be calculated from eq 1 by using the relationship

for 50% scavenging of D•

$$k_{obs} = 2k_{41} \quad (2)$$

Combining eqs 1 and 2 gives

$$k_{41}/k_{46} = [RH]_{1/2} \quad (3)$$

Such theoretical values of $[RH]_{1/2}$ are listed in Table I. As can been seen, the good agreement between the observed and calculated values of $[RH]_{1/2}$ show that the proposed mechanism of radical photoreduction can adequately explain both qualitatively and quantitatively the observed photochemical properties.

Discussion

The nature of the interaction of folate excited states with the flavin in its radical form is particularly important as we have previously shown that excitation of the folate chromophore leads to an enhanced yield of the flavin radical quartet state (Heelis et al., 1987; this species was previously assigned to the excited doublet state). This is most likely to occur via electronic energy transfer from the folate excited singlet state (**5**, Figure 1) to the flavin radical gound state doublet to yield the excited doublet state (**5** → **2**, Figure 1). The nature of the intermediate species **X** is obscure, but it may represent an encounter complex of the type Enz-^{2}FADH•...^{1}Fol.

The nature of the internal electron donor (DH) involved in the primary photoelectron transfer reaction (Figure 1, **3** → **4**) is of considerable importance. Previously, it was shown that the folate chromophore (the so-called "second chromophore") is not essential for the photoreduction reaction to proceed. Hence, an amino acid residue is implicated as the internal donor (DH). A redox potential diagram (Figure 4a) can be constructed from information in the literature for the one-electron reduction potentials of oxygen (Ilan et al., 1974), NAD• (Anderson, 1980), $Cys^{\bullet+}$ (Prutz et al., 1986), $FADH^\bullet/FADH_2$ (Anderson, 1983), $Tyr^{\bullet+}$, and Trp• (Butler et al., 1986). An attempt can be made to estimate the redox potentials of the relevant states involved in the radical form of photolyase (Figure 4B). The fully reduced enzyme (E-$FADH_2$) has been shown to react with oxygen to regenerate the radical form (Heelis & Sancar, 1986). Hence, the E-

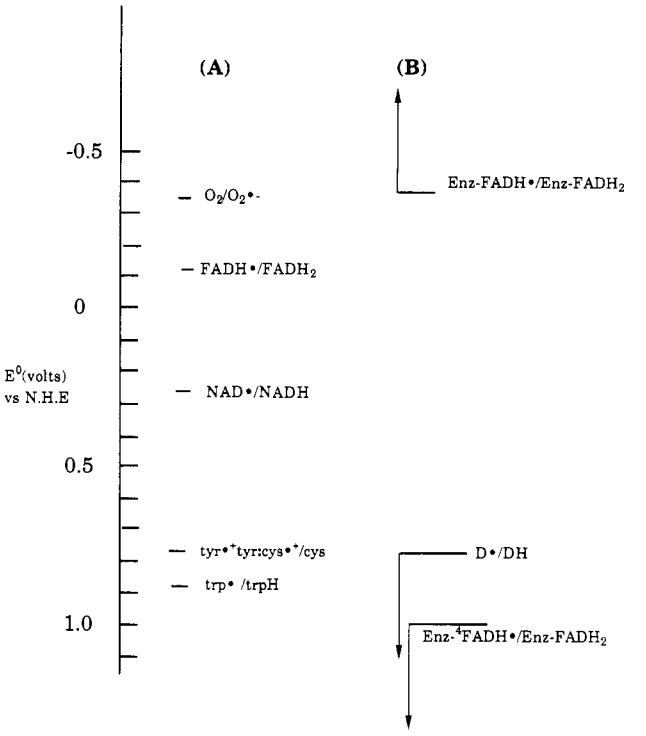

FIGURE 4: Redox potentials (vs NHE) of known (A) and postulated states (B) of photolyase. The arrows signify upper and lower limits, respectively; their magnitudes are not significant.

$FADH^\bullet$/E-$FADH_2$ potential lies below the $O_2/O_2^{\bullet-}$ potential of −0.33 V. Further, as pointed out previously (Heelis et al., 1987), the reduction potential (R•/RH) for an electron donor capable of reducing D• in the secondary reaction must lie below the D•/DH potential. From Figure 4A, the highest such potential of an effective electron donor is +0.75 (tyrosine or cysteine), thus providing a lower limit for the D•/DH reduction potential. In contrast, tryptophan (E_0 = +0.87 V) does not reduce the D• radical, suggesting the D•/DH potential lies between 0.75 and 0.87 V. Taking into account that the redox potential of an amino acid residue is quite possibly slightly different from that of the free amino acid, the donor DH can be identified as either a tryptophan, tyrosine, or cysteine residue. Cysteine can probably be eliminated as no pH dependence of the primary reaction was observed in the pH range 6–10. Since the pK_a of the thiol group of cysteine is 8.3, a pronounced increase in the efficiency of the primary reaction would be expected at pH >8.3 due to the greater electron-donating power of the thiolate anion as compared to the thiol group.

The correction of the camera flash induced difference spectra for various amino acid cation radicals (Figure 3) strongly suggested that the neutral tryptophan radical was present. Hence, it is concluded that a tryptophan residue located close to the flavin radical site is the internal electron donor. Further, since the neutral tryptophan radical and not the cation form is present, it would appear that hydrogen atom abstraction from tryptophan occurs. The alternative process of electron abstraction followed by protonation is unlikely to occur in the short time scale involved. As a consequence of the redox potential scale in Figure 4, the reduction potential of the excited quartet state must lie above the Trp•/Trp reduction potential, i.e., >0.9 V. This can be compared to the ground-state doublet redution potential of <−0.33 V, implying an increase of >1.23 V upon excitation. It is to be expected that reduction of the excited state would be more favorable than the ground state as electron addition to the former occurs

to a lower lying molecular orbital than the latter, making reduction more exoenergic. In fact, the increase in reduction potential of an excited state relative to ground state can be calculated from the energy of the excited state. For the flavin radical of photolyase, the lack of any vibrational fine structure in the absorption spectrum precludes an accurate assessment of the position of the $0 \rightarrow 0$ vibronic band within the lowest electronic transition. However, the energy of E-^{2}FADH$^\bullet$ cannot be less than 170 kJ mol^{-1}, which corresponds to the red edge of the absorption spectrum (700 nm). Hence, a change in the reduction potential of E-^{2}FADH$^\bullet$ compared to the ground state of $>+1.77$ V can be calculated. While the increase in reduction potential of the quartet state (E-^{4}FADH$^\bullet$) would be slightly less than the doublet (due to the doublet - quartet splitting energy), this is still consistent with the minimum increase of +1.23 V for the proposed reaction scheme to be feasible.

The repair of oxidized tryptophan radicals by *n*-propyl gallate has previously been reported (Hoey & Butler, 1984). Bimolecular rate constants of 3.8×10^8 and 1.2×10^8 m^{-1} s^{-1} have been determined for repair of the oxidized free tryptophan and oxidized tryptophan residues of lysozyme, respectively. The much lower repair efficiency for *n*-propyl gallate determined in this work (3.8×10^6 M^{-1} s^{-1}) suggests that the active tryptophan residue of photolyase is more shielded from the solvent than the tryptophan residues of lysozyme.

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Active Site of *Escherichia coli* DNA Photolyase: Mutations at Trp277 Alter the Selectivity of the Enzyme without Affecting the Quantum Yield of Photorepair†

Ywan Feng Li and Aziz Sancar*

Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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ABSTRACT: *Escherichia coli* DNA photolyase repairs pyrimidine dimers by a photoinduced electron-transfer reaction. The enzyme binds to UV-damaged DNA independent of light (the dark reaction) and upon absorbing a 300–500-nm photon breaks the cyclobutane ring of the dimer (the light reaction) and thus restores the DNA. No structural information on the enzyme is available at present. However, comparison of the sequences of photolyases from five different organisms has identified highly conserved regions of homology. These regions are presumably involved in chromophore (flavin and folate) and substrate binding or catalysis. Trp277 (W277) in *E. coli* photolyase is conserved in all photolyases sequenced to date. We replaced this residue with Arg, Glu, Gln, His, and Phe by site-specific mutagenesis. Properties of the mutant proteins indicate that W277 is involved in binding to DNA but not in chromophore binding or catalysis. Of particular significance is the finding that compared to wild type W277R and W277E mutants have about 300- and 1000-fold lower affinity, respectively, for substrate but were indistinguishable from wild-type enzyme in their photochemical and photocatalytic properties.

Mechanisms and rate of electron transfer in biological systems are of much current interest (Closs & Miller, 1988). Of the various biological electron-transfer systems, those that involve photoinduced electron transfer are of particular significance because the transfer reactions are easily controlled by exposing the system to light flashes of desired intensity and duration. The most widely known and best characterized

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