Picosecond Laser Photolysis Studies on the Photorepair of Pyrimidine Dimers by DNA Photolyase. 1. Laser Photolysis of Photolyase-2-Deoxyuridine Dinucleotide Photodimer Complex

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Abstract: DNA photolyase splits the cyclobutane ring of pyrimidine dimers in DNA in a light-(350-500 nm) driven reaction and thus reverses the harmful effects of far-UV (200-300 nm). The *Escherichia coli* photolyase contains $FADH_2$ as the catalytic chromophore-cofactor. Using picosecond flash photolysis and the deoxyuridine dinucleotide photodimer, we have obtained the first experimental evidence for a radical intermediate in the reaction, indicating that photorepair is initiated by a photoinduced electron transfer between the pyrimidine dimer and the $FADH_2$ cofactor.

Irradiation of DNA with ultraviolet light causes the formation of cis, syn cyclobutane dimers between adjacent pyrimidine bases. It is well-known that these lesions are mutagenic and carcinogenic. A class of enzymes called DNA photolyase eliminates pyrimidine dimers from DNA and thus reverses the harmful effects of UV light. DNA photolyase binds to pyrimidine dimers independent of light but catalysis occurs in a light-dependent reaction.[1] *Escherichia coli* DNA photolyase is a 54-kDa protein and contains two chromophores: 1,5-dihydroflavinadenine dinucleotide ($FADH_2$)[2] and 5,10-methenyltetrahydropteroylpolyglutamate (folate).[3] The flavin in the purified enzyme exists as a neutral semiquinone radical ($FADH^0$),[4] which appears to be a purification artifact and is easily converted to the $FADH_2$ form by photoreduction.[2] Enzyme without the folate chromophore retains full catalytic activity[5] and therefore it has been proposed[6] that the folate functions only in harvesting light and not in the actual catalytic step.

The photophysical mechanism of this repair phenomenon is poorly understood even though photosensitized splitting of pyrimidine dimers has been studied in some detail in model systems.[7] In this study we have carried out picosecond laser photolysis on the folate-depleted *E. coli* DNA photolyase-deoxyuridine dinucleotide photodimer (U( )U) complex[8] in order to elucidate the dynamic mechanism of photoreactivation. Our results clearly show the formation of a radical intermediate, providing the first evidence for photoinduced electron transfer between the flavin and the photodimer.

Experimental Section

Materials. *E. coli* DNA photolyase was prepared as described previously.[9] The enzyme (greater than 95% pure) was in a buffer containing 5×10^{-2} M Tris-HCl, pH 7.5, 5×10^{-2} M NaCl, 10^{-3} M EDTA, 10^{-2} M dithiothreitol, and 50% glycerol. The U( )U photodimer was prepared by irradiation of uridine dinucleotide at 254 nm and purified by HPLC to >99% purity as described previously.[8] Before mixing with the enzyme solution, the U( )U photodimer or nondimerized uridine dinucleotide (UpU) was dissolved in a buffer containing 5×10^{-2} M Tris-HCl, pH 7.5, 5×10^{-2} M NaCl, and 10^{-3} M EDTA.

Flash Photolysis. Picosecond transient absorption spectra were measured by using a dye laser (Quantel PTL10) pumped by the second harmonic of a mode-locked Nd^{3+}:YAG laser (Quantel YG501C). The sample was excited by the second harmonic (340 nm, ca. 100 μJ) of the dye laser, while a picosecond continuum was generated by focusing the fundamental of the YAG laser into a cell containing a H_2O/D_2O mixture. The pulse width of the exciting source was 12 ps. This instrument (Osaka University) has been described elsewhere in detail.[10] Detected transient signals were transferred to a microcomputer. Thirty shots of data were accumulated to calculate transient absorbance. The enzyme sample was contained in a specially constructed anaerobic quartz cell of 1-cm optical path length. Deoxygenation was accomplished by directing a stream of pure nitrogen onto the enzyme solution. Prior to the laser photolysis, flavin photoreduction[1] and folate photodecomposition[5] were performed simultaneously by irradiation with filtered output ($\lambda > 350$ nm) of a high-pressure mercury lamp. This treatment reduces the catalytically inert $FADH^0$ to $FADH_2$[10] and selectively breaks down the folate cofactor to species that do not absorb at $\lambda > 300$ nm.[5,6] Therefore, in our flash photolysis experiments the contribution of the folate chromophore to the transient spectra was excluded. UpU and U( )U solutions, deoxygenated by purging with nitrogen, were introduced into the cell through the side arm with a microsyringe. Measurements were carried out at 293 K.

Pulse Radiolysis. The spectrum of $FMNH_2^{\bullet}$ was determined by pulse radiolysis using the 8-14-MeV Vickers electron linear accelerator (Holt Radium Institute, Manchester) as previously described.[12] Doses of 1-10 Gy and pulse lengths of 5-50 ns were used. The spectrum of $FMNH_2^{\bullet-}$

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(1) (a) Sancar, A.; Sancar, G. B. *Annu. Rev. Biochem.* **1988**, *57*, 29. (b) Sancar, G. B.; Sancar, A. *Trends Biochem. Sci.* **1987**, *12*, 259.
(2) (a) Payne, G.; Heelis, P. F.; Rohrs, B. R.; Sancar, A. *Biochemistry* **1987**, *26*, 7121. (b) Jorns, M. S.; Baldwin, E. T.; Sancar, G. B.; Sancar, A. *J. Biol. Chem.* **1987**, *262*, 486.
(3) Johnson, J. L.; Hamm-Alvarez, S.; Payne, G.; Sancar, G. B.; Rajagopalan, K. V.; Sancar, A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2046.
(4) Jorns, M. S.; Sancar, G. B.; Sancar, A. *Biochemistry* **1984**, *23*, 2673.
(5) Heelis, P.; Payne, G.; Sancar, A. *Biochemistry* **1987**, *26*, 4634.
(6) Hamm-Alvarez, S.; Sancar, A.; Rajagopalan, K. V. *J. Biol. Chem.* **1989**, *264*, 9649.
(7) (a) Lamola, A. A. *Mol. Photochem.* **1972**, *4*, 107. (b) Roth, H. D.; Lamola, A. A. *J. Am. Chem. Soc.* **1972**, *94*, 1013. (c) Helene, C.; Charlier, M. *Photochem. Photobiol.* **1977**, *25*, 429. (d) Pac, C.; Kubo, J.; Majima, T.; Sakurai, T. *Photochem. Photobiol.* **1982**, *36*, 273. (e) Van Camp, J. R.; Young, T.; Hartman, R. F.; Rose, S. D. *Photochem. Photobiol.* **1987**, *45*, 365. (f) Rokita, S. E.; Walsh, C. T. *J. Am. Chem. Soc.* **1984**, *106*, 4589. (g) Jorns, M. S. *J. Am. Chem. Soc.* **1987**, *109*, 3133.
(8) It has been ascertained that *E. coli* DNA photolyase catalyzes the monomerization of this dimer. For reference, see: Witmer, M. R.; Altmann, E.; Young, H.; Begley, T. P.; Sancar, A. *J. Am. Chem. Soc.* **1989**, *111*, 9264.
(9) Sancar, A.; Smith, F. W.; Sancar, G. B. *J. Biol. Chem.* **1984**, *259*, 6028.
(10) Heelis, P. F.; Sancar, A. *Biochemistry* **1986**, *25*, 8163.
(11) Hirata, Y.; Ichikawa, M.; Mataga, M. *J. Phys. Chem.* **1990**, *94*, 3872.
(12) Heelis, P. F.; Parsons, B. J.; Phillips, G. O.; Swallow, A. J. *Int. J. Radiat. Biol.* **1989**, *55*, 557.

0002-7863/91/1513-3143$02.50/0

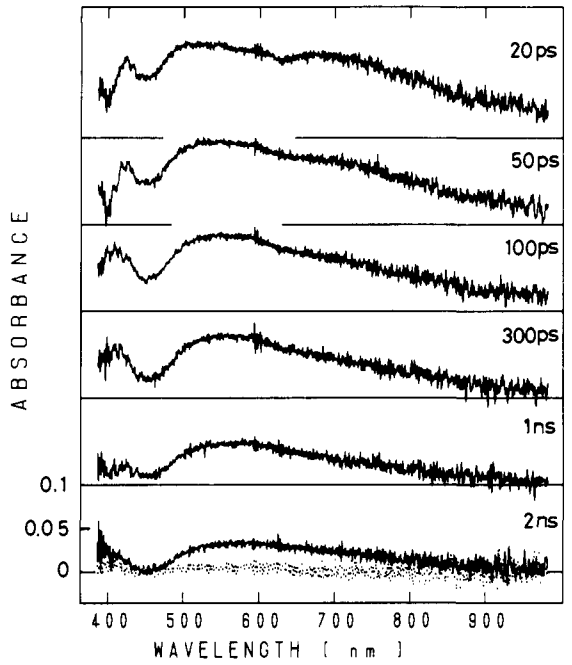


Figure 1. Transient absorption spectra of *E. coli* DNA photolyase containing $FADH_2$ (but no folate cofactor). Delay times after excitation are indicated in the figure. The dotted line shows a base line (signal without excitation). The heterogeneity of the spectrum at early times is likely to be due to an impurity in the enzyme with a short excited lifetime compared to that of enzyme-bound $FADH_2$.

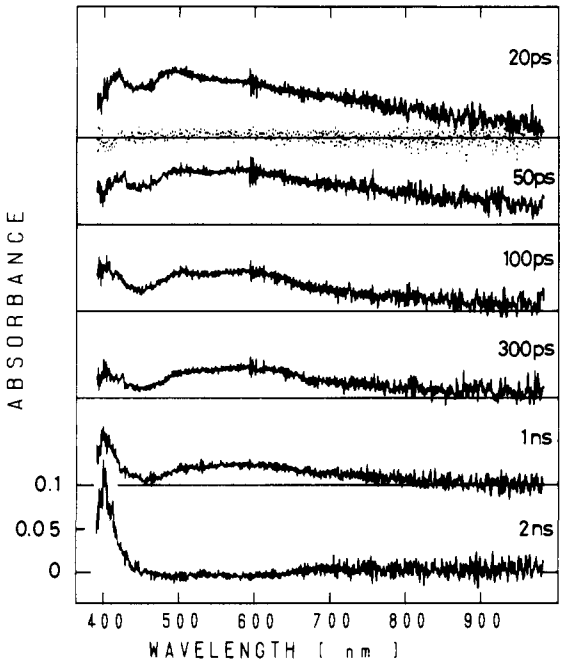


Figure 2. Transient absorption spectra of *E. coli* DNA photolyase containing $FADH_2$ (but no folate cofactor) plus 2′-deoxyuridine dinucleotide photodimer substrate system. Delay times are indicated in the figure. The dotted line shows a base line (signal without excitation). Solutions of the enzyme (1.9×10^{-4} M with respect to flavin) and U( )U) (2.1×10^{-2} M) were mixed at volume ratio of 5/1 (enzyme/U( )U). The spectra were measured under anaerobic conditions. The time courses of the excited state decay (500–700 nm) and photointermediate rise (400 nm) are approximately complementary. However, it is conceivable that there is another intermediate species with a low extinction coefficient between the flavin excited singlet and the photointermediate (radical) with a strong absorption at 400 nm.

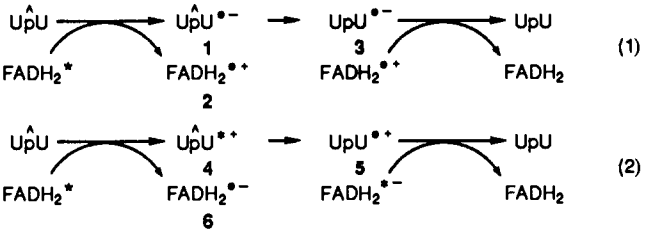
was obtained by the reaction of the hydrated electron directly with $FMNH_2$ (prepared by in situ anaerobic photoreduction by EDTA) in an N_2-saturated solution. The spectra so obtained were corrected for the contribution of the reaction of the hydroxyl radical with $FMNH_2$ by measurement of such spectra in a separate experiment using nitrous oxide saturated solutions, which yield only hydroxyl radicals during pulse radiolysis.

Results and Discussion

The transient absorption spectra of folate-depleted DNA photolyase after excitation with a 12-ps pulse of 340 nm are shown in Figure 1. The enzyme shows a characteristic absorption band in the visible region, which decays with a lifetime of 1.7 ns. This lifetime is comparable to the fluorescence lifetimes of reduced flavin in other flavoproteins;[13] furthermore, it has also been shown that $FADH_2$ in photolyase is fluorescent.[14] Thus, we assign the transient absorption to the first excited singlet state (S_1) of enzyme-bound $FADH_2$.

Transient absorption spectra of DNA photolyase complexed to U( )U are shown in Figure 2. When these spectra are compared with those in Figure 1 several changes are evident. First, the broad band appearing in the 500–900-nm region decays faster than it does in the absence of substrate. This is consistent with a previous report[14a] showing fluorescence quenching of photolyase by a thymine dimer containing substrate. The most interesting change we observe, however, is that a strong new band appears at around 400 nm with increasing delay time. It should be noticed that at 2 ns this is the only absorption band in the spectrum. In contrast, measurement of the picosecond transient absorption spectra of DNA photolyase in the presence of UpU demonstrated that the dinucleotide had no effect on the transient spectra (data not shown). Thus, we conclude that the 400-nm species is a reaction intermediate in photorepair.

Mainly two mechanisms have been proposed for the photorepair process.[7,8] One of these involves initial electron transfer from an excited photosensitizer to the pyrimidine dimer to generate the cation radical of the sensitizer and the anion radical of the photodimer followed by splitting of the dimer into monomers. This proposal is based on model photosensitized cleavage reactions with indole and its derivatives.[7c,e] The second mechanism, based on model photosensitized cleavage reactions with quinones,[7a,b] involves initial electron transfer from the photodimer to the excited photosensitizer to yield the radical anion of the sensitizer and the radical cation of the dimer, which then similarly yields monomers. Application of these two mechanisms to our system may be represented as follows:



No experimental evidence exists to differentiate between these two mechanisms. A recent study[8] investigating the secondary deuterium isotope effect on the reaction gave results that were compatible with either mechanism. Now it should be possible to differentiate between the two mechanisms by analyzing the transient absorption spectra for absorption(s) that can be attributed to ion radicals of the sensitizer (**2** and **6**) or those of the substrate (**1**, **3**, **4**, and **5**).

Our results show the buildup of a transient band at 400 nm, which at this stage cannot be assigned unambiguously to a particular ion radical for the lack of a complete set of reference spectra. The following arguments could be proposed in favor of mechanism 2. First, our transient spectra failed to reveal an absorption band attributable to $FADH_2^{\bullet+}$ (**2**). It is known that

(13) (a) Ghisla, S.; Massey, V.; Lhoste, J.; Mayhew, S. G. *Biochemistry* **1974**, *13*, 589. (b) Visser, A. J. W. G.; Ghisla, S.; Massey, V.; Muller, F.; Veeger, C. *Eur. J. Biochem.* **1979**, *101*, 13. (c) Bastiaens, P. I. H.; Bonants, P. J. M.; Mueller, F.; Visser, A. J. W. G. *Biochemistry* **1989**, *28*, 8416.

(14) (a) Jordan, S. P.; Jorns, M. S. *Biochemistry* **1988**, *27*, 8015. (b) Li, Y. F.; Sancar, A. *Biochemistry* **1990**, *29*, 5698.

the cation radical of reduced flavin shows a strong absorption band near 500 nm; e.g., cation radicals of reduced lumiflavin ($LFH_2^{\bullet+}$) and of reduced riboflavin ($RFH_2^{\bullet+}$) have absorption maxima at 488 nm (ϵ = 7200) and ca. 483 nm (ϵ = 13 000),[15,16] respectively. If mechanism 1 were operative in our system we would observe this absorption band together with the absorption assigned to transient species produced from the substrate such as **1** and **3** (provided these species absorb at $\lambda > 400$ nm). Second, recent pulse radiolysis studies[17] indicate that $FADH_2^{\bullet-}$ has an absorption peak at ca. 400 nm with no significant absorption at longer wavelengths, suggesting that the 400-nm peak observed in our spectra at $\tau > 1$ ns may be the cofactor radical, consistent with mechanism 2. Finally, the fact that in aqueous solutions dimer radical anions split very inefficiently in all model systems used ($\phi \sim 5 \times 10^{-2}$)[7a,e,g] while dimer radical cations split with high quantum yield (ϕ = 0.5–1.0)[7a,b,d] and that photoenzymatic repair with $FADH_2$ occurs with a quantum yield of 0.7[18] might be taken as evidence for mechanism 2.

However, mechanism 1 cannot be ruled out on the basis of these considerations. The following arguments could be made in favor of mechanism 1. First, it is conceivable that the reduced flavin in photolyase is an anion (i.e., $FADH^-$) as is often found in flavoenzymes.[19] It is well-known[19b] that the absorption spectra of reduced flavins and flavoenzymes are influenced by the degree of planarity. Neutral reduced flavins (free or enzyme bound) possess maximum planarity and typically exhibit $\lambda_{max} \sim 390$ nm (ϵ = 2000–3000). Ionization at N1 ($pK_a \sim 6.6$ for free flavin) promotes flexing along the N5–N10 axis, resulting in a blue shift to 350 ± 10 nm (ϵ = 5000–6000). In this regard it is interesting to note that the reduced flavin in photolyase shows λ_{max} at 366 nm (ϵ = 5700)[5,18b] and therefore the anionic form may be prevalent. Then electron loss by this species would be expected to produce the neutral one-electron-reduced radical $FADH^0$. This species has a broad, not well-defined absorption over 500–700 nm and hence might be obscured by the very similar absorption of excited singlet $FADH_2$. That no absorption at $\lambda > 450$ nm is observed after 2 ns implies that $FADH^-$ is reformed by back electron transfer within this time scale. Another alternative is that electron donation by excited singlet $FADH^-$ followed by proton loss would yield $FAD^{\bullet-}$. The $FAD^{\bullet-}/FADH^-$ difference spectrum reported by Surdhar and Armstrong[20] shows a peak at ca. 380 nm, consistent with the proposed mechanism. Second, preliminary measurements of the redox potentials of the species involved (U( )U and excited singlet $FADH_2$) as well as previous work with model systems[7a,d] indicate that the reduction potential of excited singlet $FADH_2$ is too negative by at least 1.5 V to abstract an electron from the uracil photodimer. (The reduction potential of enzyme-bound $FADH_2$ is estimated to be -2.8 V $< E^\circ < -1.9$ V since free $FMNH_2$ reacts with $e^-(aq)$ but is unreactive toward $CO_2^{\bullet-}$.) In contrast, electron donation by excited singlet $FADH_2$ to U( )U is thermodynamically feasible. Finally, the recent observation by Kim et al.[21] that in solvents of low polarity the dimer radical anion is split with a quantum yield of 0.41 suggests that dimer anions are not intrinsically resistant to splitting and thus a dimer anion mechanism is a likely possibility for photolyase, which appears to have a nonpolar active site.[22]

In conclusion, our data clearly show that photolyase repairs pyrimidine dimers by photoinduced electron transfer through a radical intermediate that does not include $FADH_2^{\bullet+}$. Experiments are under way to determine unambiguously the direction of electron transfer between enzyme and substrate.

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Registry No. $FADH_2$, 1910-41-4.

(15) Dudley, K. H.; et al. *Helv. Chim. Acta* **1964**, *47*, 1354.
(16) These values are estimated from a spectrum shown in: Land, E. J.; Swallow, A. J. *Biochemistry* **1969**, *8*, 2117.
(17) Heelis, P. F.; Parsons, B. J., unpublished observation. These pulse radiolysis experiments show that $FMNH_2^{\bullet-}$ clearly has a peak at ~400 nm of $\epsilon \sim 2000$ M^{-1} cm^{-1} and very little absorption elsewhere. Actual data need further refinement for a more quantitative statement regarding the absorption spectrum of this radical.
(18) (a) Sancar, G. B.; Jorns, M. S.; Payne, G.; Fluke, D. J.; Rupert, C. S.; Sancar, A. *J. Biol. Chem.* **1987**, *262*, 492. (b) Payne, G.; Sancar, A. *Biochemistry* **1990**, *29*, 7715.
(19) (a) Van Schagen, C. G.; Müller, F. *Eur. J. Biochem.* **1981**, *120*, 33. (b) Ghisla, S.; Massey, V.; Lhoste, J.-M.; Mayhew, S. G. *Biochemistry* **1974**, *13*, 589.
(20) Surdhar, P. S.; Armstrong, D. A. *J. Phys. Chem.* **1985**, *89*, 5514.
(21) Kim, S.-T.; Hartman, R. F.; Rose, S. D. *Photochem. Photobiol.* **1990**, *52*, 789.
(22) Payne, G.; Wills, M.; Walsh, C.; Sancar, A. *Biochemistry* **1990**, *29*, 5707.