# Laser Flash Photolysis on an Intermediate in the Repair of Pyrimidine Dimers by DNA Photolyase

Sang-Tae Kim,† Martin Volk,‡ Gerhard Rousseau,‡
Paul F. Heelis,§ Aziz Sancar,*,† and
Maria-Elisabeth Michel-Beyerle‡

*Department of Biochemistry and Biophysics*
*University of North Carolina*
*Chapel Hill, North Carolina 27599*
*Institüt für Physikalische and Theoretische Chemie*
*TU Munchen, Garching, Germany*
*Faculty of Science, Health and Medical Studies*
*North East Wales Institute*
*Clwyd, United Kingdom*

*Received November 15, 1993*

Ultraviolet light (200–300 nm) induces adjacent pyrimidines in DNA to form cyclobutane-type dimers, which are mutagenic, carcinogenic, and lethal in a variety of organisms.[1] Photolyases repair damaged DNA by utilizing the photonic energy of near-UV or visible light (300–500 nm) to split the cyclobutane ring of the dimer.[2] All photolyases characterized so far appear to employ $FADH_2$ (or its anion) as the photoactive cofactor.[3] In the light-driven repair reaction, the excited singlet state of $FADH^-$ is quenched by the substrate, presumably because of single electron transfer (SET) from the excited flavin to the dimer.[4,5] In support of SET, a recent EPR study revealed that a paramagnetic species, most likely a reaction intermediate, was generated during photoenzymatic repair.[6]

Using picosecond flash photolysis and the deoxyuridine dinucleotide cyclobutane dimer (U◇U) as substrate, previously we detected a transient intermediate with $\lambda_{max}$ ~400 nm.[7] It was suggested that this transient represented an essential intermediate in the reaction pathway leading to dimer splitting. However, due to the lack of reference spectra, it was not possible to assign the transient to flavin or dimer radical. Similarly, since the lifetime of the 400-nm species could not be determined by picosecond flash photolysis, it was not possible to tell whether the paramagnetic species determined by EPR and the transient species identified by flash photolysis were the same intermediate or represented separate species along the reaction pathway. To resolve these questions, we have now conducted flash photolysis using U◇T and T◇T photodimers in a flash photolysis system which enabled us to measure the lifetime of the transient. We find that the transient species detected by flash photolysis arose from the substrate, has a lifetime of 0.5–2 ns, and is not the same species detected by the photoinduced EPR analysis.

*Escherichia coli* photolyase containing only the flavin cofactor was prepared as described previously.[8,9] The substrates, T◇T and U◇T, were prepared by acetone-photosensitized radiation of TpT and UpT at 313 nm.[10] The repair reaction mixture contained 0.2 mM photolyase in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 20% (v/v) glycerol and 1 mM substrate. The flavin chromophore of photolyase is oxidized to the blue neutral radical during purification.[2] Therefore, in all experiments the flavin was photoreduced to the $FADH^-$ form under a nitrogen-saturated atmosphere and mixed with substrate anaerobically.[6,7] Laser excitation at 355 nm was performed at 4 °C, and the absorption was monitored at 390–430 nm using an apparatus described elsewhere.[11]

Excitation of the enzyme alone yields a transient with lifetime of 1–2 ns which absorbs in the 400–800-nm region. This species has been characterized previously by both picosecond time-resolved emission and absorption measurements as the excited singlet state of reduced flavin,[4,6,7] and this excited state is quenched by substrate.[4b,7] In the presence of a 5-fold molar excess of U◇T, instead of a decrease by quenching, an approximately 2-fold increase at 400-nm absorption was observed 3 ns after the flash, indicative of a possible reaction intermediate which also absorbs in this region. Figure 1 shows the transient absorption spectrum in the 390–430-nm region obtained by moving the sample between each shot so that the beam hit a previously unirradiated part of the solution. A species with a $\lambda_{max}$ = 420 nm was resolved. In contrast, repetitive excitation of the same part of the sample with a frequency of 6 Hz resulted in a reduced transient signal. The signal obtained under the latter condition is quite featureless in the spectral region of 390–430 nm (Figure 1), consistent with the absorption spectrum of the excited singlet state of reduced flavin due to depletion of the substrate by repair.[7] Indeed, this signal is essentially identical to that observed without substrate. Thus we conclude that the species with $\lambda_{max}$ = 420 nm observed when the sample is moved after each shot is derived from the dimer splitting reaction.

The time course of the decay of the transient intermediate was monitored at 415 nm (Figure 2), again moving the sample between shots and repetitively hitting the same volume. Since the observed signal decayed with the instrumental time response of the spectrometer, the lifetime of the transient has to be less than 2 ns. On the other hand, this lifetime can be estimated to be more than 0.5 ns, since otherwise the instrumental resolution would not allow us to obtain the observed large signal. Thus, by deconvolution of the excitation pulse and transient signal, an estimate of 0.5–2 ns for the lifetime of the transient can be given with relative certainty.

In contrast to the results obtained with the U◇T substrate, excitation of the enzyme in the presence of 5-fold molar excess of T◇T did not yield a transient which absorbs at $\lambda > 400$ nm.

* Author to whom correspondence should be addressed. Telephone: 919-962-0115. Fax: 919-966-2852.
† University of North Carolina.
‡ TU Munchen.
§ North East Wales University.

(1) Wang, S. Y. In *Photochemistry and Photobiology of Nucleic Acids*; Academic Press: New York, 1976; Vols. I and II.
(2) (a) Sancar, A. Photolyase. In *Advances in Electron Transfer Chemistry Chemistry*; Mariano, P. E., Ed.; JAI Press: London, 1992; Vol. 2, pp 215–272. (b) Heelis, P. F.; Kim, S. T.; Okamura, T., Sancar, A. *J. Photochem. Photobiol. B: Biol.* **1993**, *17*, 219. (c) Kim, S. T.; Sancar, A. *Photochem. Photobiol.* **1993**, *57*, 895.
(3) (a) Sancar, G. B.; Smith, F. W.; Heelis, P. F. *J. Biol. Chem.* **1987**, *262*, 15457. (b) Kiener, A.; Husain, I.; Sancar, A.; Walsh, C. *J. Biol. Chem.* **1989**, *264*, 13880. (c) Eker, A. P. M.; Kooiman, P.; Hessels, J. K. C.; Yasui, A. *J. Biol. Chem.* **1990**, *265*, 8009. (d) Payne, G.; Sancar, A. *Biochemistry* **1990**, *29*, 7715. (e) Malhotra, K.; Kim, S. T.; Walsh, C. T.; Sancar, A. *J. Biol. Chem.* **1992**, *267*, 15406.
(4) (a) Jorns, M. A.; Wang, B.; Jordan, S. P.; Chanderkar, L. P. *Biochemistry* **1990**, *29*, 552. (b) Kim, S. T.; Heelis, P. F.; Okamura, T.; Hirata, Y.; Mataga, N.; Sancar, A. *Biochemistry* **1991**, *30*, 11262.
(5) (a) Roth, H. D.; Lamola, A. A. *J. Am. Chem. Soc.* **1972**, *94*, 1013. (b) Kemmink, J.; Eker, A. P. M.; Kaptein, R. *Photochem. Photobiol.* **1986**, *44*, 137. (c) Young, T.; Kim, S. T.; Van Camp, J. R.; Hartman, R. F.; Rose, S. D. *Photochem. Photobiol.* **1988**, *48*, 635. (d) Young, T.; Nieman, R.; Rose, S. D. *Photochem. Photobiol.* **1990**, *52*, 661. (e) Yeh, S. R.; Falvey, D. E. *J. Am. Chem. Soc.* **1991**, *113*, 8557. (f) Heelis, P. F.; Hartman, R. F.; Rose, S. D. *Photochem. Photobiol.* **1993**, *57*, 1053.
(6) Kim, S. T.; Sancar, A.; Essenmacher, C.; Babcock, G. T. *J. Am. Chem. Soc.* **1992**, *114*, 4442.
(7) Okamura, T.; Sancar, A.; Heelis, P. F.; Begley, T. P.; Hirata, Y.; Mataga, N. *J. Am. Chem. Soc.* **1991**, *113*, 3143.
(8) Sancar, A.; Smith, F. W.; Sancar, G. B. *J. Biol. Chem.* **1984**, *259*, 6028.
(9) Heelis, P. F.; Payne, G.; Sancar, A. *Biochemistry* **1987**, *26*, 4634.
(10) (a) Taylor, J.-S., Brockie, I. R.; O'Day, C. L. *J. Am. Chem. Soc.* **1987**, *109*, 6735. (b) Kim, S. T.; Sancar, A. *Biochemistry* **1991**, *30*, 8623. (c) Nadji, S.; Taylor, J.-S. *Tetrahedron* **1991**, *47*, 2579.
(11) (a) Volk, M.; Aumeier, G.; Haberle, T.; Ogrodnik, A.; Feick, R.; Michel-Beyerle, M. E. *Biochim. Biophys. Acta* **1992**, *1102*, 253. (b) The sample was excited at 355 nm with a Nd:YAG laser pulse (pulse width 4 ns, excitation intensity approximately 100 $\mu J/mm^2$, and instrumental response time approximately 4.5 ns) and probed with a $N_2$ laser-pumped dye laser (pulse width 2 ns). The delay between excitation and probing could be adjusted electronically between 1 ns and 10 ms.

0002-7863/94/1516-3115$04.50/0 

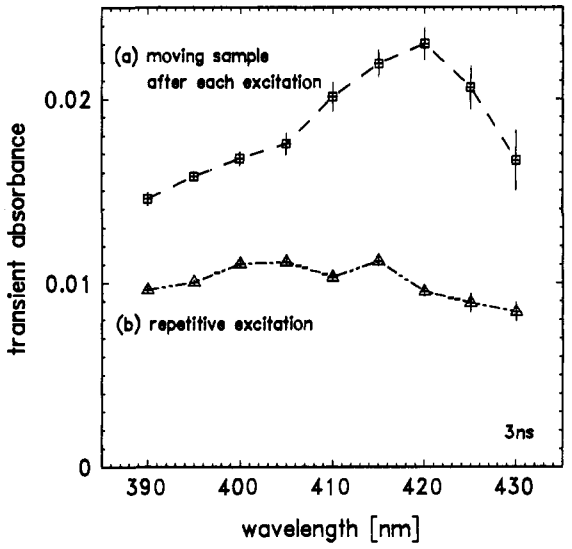


**Figure 1.** Light-induced transient absorption spectra of *E. coli* photolyase (0.2 mM) in the presence of 5-fold molar excess of U◇T. Optical path length was 2 mm. The spectra were taken 3 ns after excitation: (a) with moving the sample after each excitation and (b) 6-Hz repetitive excitation of the same spot leading to depletion of the substrate. Each point represents the average over 20 measurements with the error bars indicating the standard deviation. "Transient absorbance" is the difference spectra of absorption after the flash relative to that before the flash.

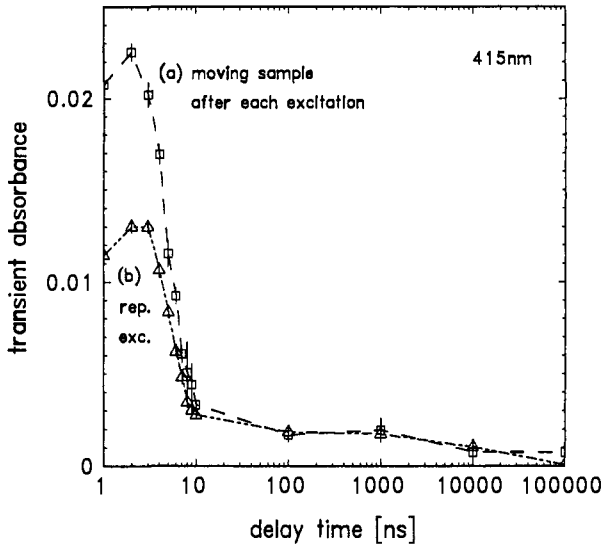


**Figure 2.** Decay of the light-induced transient absorption at 415 nm in *E. coli* photolyase in the presence of a 5-fold excess of U◇T. Conditions as in Figure 1, except that 80 single measurements were averaged here. Note the logarithmic scale of the time axis.

In fact, when absorption at 400 nm was monitored, a decrease in the transient absorption signal was observed in the presence of T◇T, compared to a sample containing no substrate or a sample with substrate subjected to repetitive excitation of the same volume. Thus, the decrease in the transient signal at 400 nm in the presence of T◇T is attributable to a decrease in the lifetime of the excited singlet state of the reduced flavin, in agreement with the time-resolved fluorescence studies.[4b] The fact that the 400-nm species previously observed with U◇U is also observed with U◇T but not with T◇T is consistent with the pyrimidine rather than the flavin moiety being responsible for the transient absorption. The lack of the 400–420-nm signal with T◇T could be due either to a shorter lifetime of the transient species when splitting T◇T or to a shift of the absorbance of the transient to the outside of the spectral region probed. In fact, pulse radiolysis studies of the radical anions of C5(H)–C6(H) dihydropyrimidines, which are isoelectronic with the structure of the proposed intermediate,[2b] show a 30-nm red shift in the absorption of the C(6)H adduct of uracil (410 nm) compared to that of thymine (380 nm).[12] A third possibility that repair does not occur with T◇T can be ruled out from repair quantum yield[3d,10b] and time-resolved emission studies.[4b] Thus, we can safely conclude that the transient absorption signal detected with U◇U and U◇T represents a reaction intermediate. Furthermore, having determined the lifetime of this transient, we can also conclude that the 420-nm species observed here cannot be identical to the paramagnetic species detected by flash EPR measurements[6] because the experimental setup used in those measurements could not detect paramagnetic species with a lifetime less than ~50 μs.

If the 420-nm transient signal indeed originates from a radical, its decay could be affected by an external magnetic field.[13] However, application of a magnetic field (700 G) to our sample did not affect the 420-nm signal within the resolution of our experimental system. The absence of a magnetic field effect, however, can be due either to the short lifetime as related to the relevant hyperfine couplings or to a large exchange interaction, favored by close proximity of the recombining pair.

To summarize, we have found that nanosecond flash photolysis of photolyase–U◇T complex but not photolyase–T◇T generates a 420-nm transient species with a lifetime of 0.5–2 ns. This observation, combined with the reported absorption of the C6-(H) adduct of uracil (410 nm),[12] suggests that the transient absorbance we detect in our enzyme–substrate system originates from C(5)–C(6) bond-saturated (di)pyrimidines and may be an early intermediate on the path of converting a cyclobutadipyrimidine into two pyrimidines by a radical mechanism.

(12) Deeble, D. J.; Das, S.; Von Sonntag, C. *J. Phys. Chem.* **1985**, *89*, 5784.

(13) In spatially separated radical pairs, transition between quasidegenerate singlet and triplet states can be induced by the magnetic hyperfine interaction of the unpaired electrons with their respective nuclei. An external magnetic field affects this singlet–triplet mixing via the Zeeman effect and thereby can influence the recombination dynamics of the radical pair and the yield of singlet and triplet recombination products, respectively. For example, this radical pair mechanism governs the recombination of the primary radical pair ($P^+H^-$) in photosynthetic reaction centers, see: (a) Haberkorn, R.; Michel-Beyerle, M. E. *Biophys. J.* **1979**, *26*, 489. (b) Ogrodnik, A.; Krüger, H. W.; Orthuber, H.; Haberkorn, R.; Michel-Beyerle, M. E. *Biophys. J.* **1982**, *39*, 91.