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Photochemical Properties of *Escherichia coli* DNA Photolyase: A Flash Photolysis Study†

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ABSTRACT: *Escherichia coli* DNA photolyase contains a stable flavin neutral blue radical that is involved in photosensitized repair of pyrimidine dimers in DNA. We have investigated the effect of illumination on the radical using light of $\lambda > 520$ nm from either a camera flash or laser. We find that both types of irradiations result in the photoreduction of the flavin radical with a quantum yield of 0.10 ± 0.02. While photoreduction with the camera flash is minimal in the absence of an electron donor (dithiothreitol), laser flash photolysis at 532 nm reduces the flavin to the same extent in the presence or absence or an electron donor. Thus, it is concluded that the primary step in photoreduction involves an electron donor that is a constituent of the enzyme itself. Laser flash photolysis produces a transient absorption band at 420 nm that probably represents the absorption of the lowest excited doublet state (${}^{2}_{1}\Pi\Pi^*$) of the radical and decays with first-order kinetics with $k_1 = 0.8 \times 10^6$ s^{-1}. The photoreduction data combined with the results of recent studies on the activity of dithionite-reduced enzyme suggest that electron donation by excited states of E–FADH$_2$ is the mechanism of flavin photosensitized dimer repair by *E. coli* DNA photolyase.

Photolyases are enzymes that catalyze the light-dependent opening of the cyclobutane ring in pyrimidine dimers in DNA, thereby restoring the pyrimidine monomers. The spectroscopic properties of *Escherichia coli* photolyase indicate that the enzyme contains FAD[1] in the blue neutral radical form (Jorns et al., 1984). In addition, spectroscopic data suggest that the enzyme contains a second chromophore with an absorption maxima at 390 nm and a fluorescence emission maximum at 480 nm. The chemical nature of the "second chromophore" has, however, proved elusive, due to its instability upon separation from the apoenzyme.

Action spectra of both the natural (blue semiquinone) and fully reduced enzyme indicate that light absorption by the flavin chromophore is involved in photolyase activity (Sancar et al., 1986). In addition, blue enzyme preparations with much reduced levels of the second chromophore show essentially the same specific activity, while the photolyase enzyme containing oxidized FAD possesses no photolyase activity (Jorns et al., 1986).

Clearly, therefore, a knowledge of the photochemical properties of the blue semiquinone of photolyase is of importance in understanding the mechanism of photolyase action. This is particularly true, as in contrast to oxidized flavins, for which a wealth of data is available (Heelis, 1982), little is known about the photochemistry of the flavin semiquinone.

In this study, the flash photolysis technique is employed in order to understand the primary photochemical processes following light absorption by the flavin chromophore of *E. coli* DNA photolyase.

EXPERIMENTAL PROCEDURES

DNA photolyase was prepared as previously described (Sancar & Sancar, 1984), and the "blue enzyme" used in this study contained less than 10% of FAD in the oxidized form, as determined spectrophotometrically. Enzyme samples were stored at −20 °C in storage buffer containing 0.05 M Tris, pH 7.4, 0.05 M NaCl, 10^{-3} M EDTA, 0.01 M DTT, and 50% glycerol. Standard buffer was identical with the storage buffer except that no glycerol was present and the NaCl concentration was 0.1 M. Enzyme concentrations used in the photolysis experiments were in the range $(2–5) \times 10^{-5}$ M with respect to the flavin coenzyme. Chemicals were purchased from either Sigma or British Drug House and were the purest grade available.

The laser flash photolysis system was based on a JK Lasers System 2000 neodymium:YAG laser emitting pulses at 532 nm with energies in the range 10–200 mJ and a pulse duration of 20 ns. A pulsed Xe lamp, which produced light of constant intensity for 400 μs after the laser pulse, was used as the analyzing light source. Measurements of transient changes in the absorbance at wavelengths in the range of 300–700 nm were recorded on a Phillips PM3311 digital oscilloscope and then transferred to an LSI/2 computer and stored on disc for further analysis. Pulse-to-pulse variations in the laser intensity were corrected for by monitoring the integrated laser intensity on each pulse. Samples were contained in quartz flow cells of 1-cm optical path length. Appropriate filters were placed in the analyzing light path to reduce photolysis.

Transient quantum yields were determined by the comparative technique (Bensasson et al., 1978). The ruthenium tris(bipyridyl) triplet-state absorption was employed as a standard with $\epsilon_{360} = 10^4$ M^{-1} cm^{-1} and $\phi = 1.0$ (Rougee et al., 1982).

Conventional photolysis experiments were carried out with a Starblitz 2400 TS photographic flash unit on manual mode and will be termed camera flash experiments in order to distinguish them from the laser flash photolysis experiments.

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[1] Abbreviations: FAD, flavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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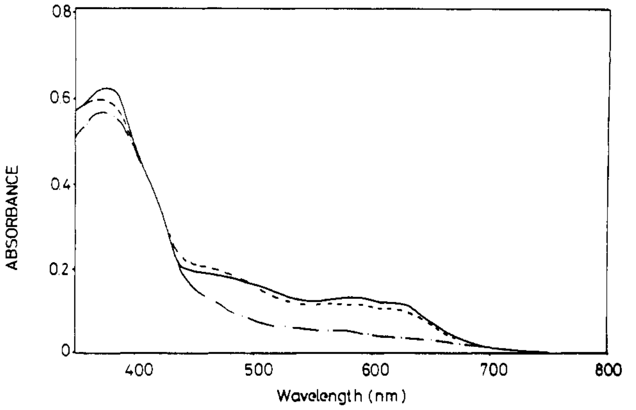


FIGURE 1: Camera flash photolysis of blue enzyme (3.4 × 10^{-5} M) in standard buffer under aerobic conditions: (—) before photolysis; (-•-) after 21 flashes; (--) after 21 flashes followed by 30-min storage in the dark at 18 °C.

In all cases, the light output was filtered to exclude wavelengths shorter than 520 nm to ensure absorption by the flavosemiquinone only.

Concentrated enzyme samples in storage buffer were diluted 20-fold by either standard assay buffer or 0.01 M NaCl as described under Results and Discussion. In some cases, EDTA, dithiothreitol, or Tris buffer was dissolved in the 0.1 M NaCl diluent. Experiments were carried out at 4 ± 2 °C unless otherwise stated. Deoxygenation was accomplished by directing a stream of oxygen-free nitrogen onto the solution for 45 min with sufficient force as to depress the solution surface.

RESULTS AND DISCUSSION

Camera Flash Photolysis. The absorption spectrum of the blue enzyme is shown in Figure 1 and is essentially identical with that reported previously (Jorns et al., 1984). The absorption at longer wavelengths (λ >420 nm) is solely due to the neutral flavosemiquinone, while the absorption peak at 380 nm represents the combined absorption of the radical and the so-called second chromophore (SC). Camera flash experiments in standard assay buffer under aerobic conditions produced a progressive loss of the long-wavelength absorption due to the radical with increasing numbers of flashes. However, after 30-min storage in the dark at 4 °C, a near complete return to the original spectrum is observed (Figure 1). Identical camera flash experiments were carried out under anaerobic conditions with essentially identical results with respect to the spectral changes produced. However, in this case, no change in the absorption spectrum was observed upon storage in the dark. The effect of oxygen on the kinetics of recovery of the radical absorption was investigatged in more detail. Figure 2 shows the recovery of the absorption of the radical at 580 nm as a function of time at 18 °C, before and after 10 flashes under aerobic conditions (curve 1). The corresponding first-order kinetic plot is shown in Figure 2 (inset) and yields a first-order rate constant of 4 × 10^{-3} s^{-1} ($t_{1/2}$ = 180 s). This can be compared to the reoxidation of non-enzyme-bound reduced flavins, which occurs in less than 1 s (Holmström, 1964). The reoxidation of the reduced enzyme was also followed at 4 °C. In this case the first-order rate constant was 1.34 × 10^{-3} s^{-1} ($t_1/_2$ = 514 s). In addition to temperature effects, we have also observed a batch effect on the reoxidation rate. Thus, with freshly prepared minimally handled batches we obtain $t_{1/2}$ for reoxidation of ~100 and 15 min at 4 and 18 °C, respectively (data not shown). However, there was no difference in the photoreduction properties of freshly prepared and stored enzymes.

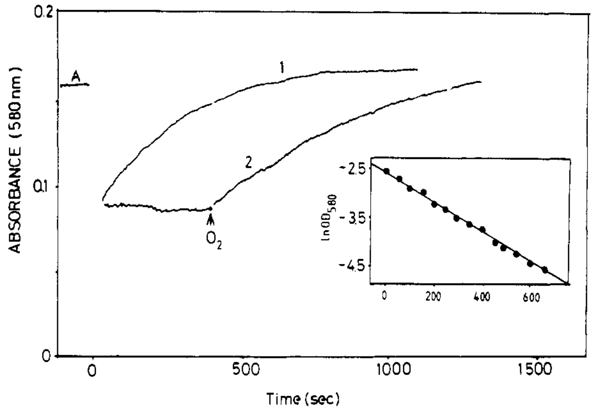


FIGURE 2: Kinetic plots of the recovery of the radical absorbance at 580 nm after camera flash photolysis at 18 °C: section A, before flashing; curve 1, aerobic sample after 10 flashes; curve 2, anaerobic sample, after 10 flashes, cuvette opened and oxygen blown over the surface after 400 s; inset, first-order kinetic plot of recovery under aerobic conditions.

Corresponding camera flash experiments under anaerobic conditions are also shown in Figure 2 (curve 2). As can be seen, no recovery of the radical absorption is observed until the cell was opened and oxygen blown over the surface of the solution.

The above observations are consistent with a one-electron photoreduction of the flavosemiquinone (E–FADH•) to the fully reduced form (E–$FADH_2$). Fully reduced flavins are known to have an absorption maxima at 340–350 nm, the exact position depending somewhat on their precise structure and environment, with a long tail extending to 500 nm. This is consistent with the absorption spectrum observed after the camera flash experiments although the absorption maximum at 380 nm is largely due to the second chromophore. A very similar spectrum is produced by stoichiometric dithionite reduction of the blue enzyme. Similarly, reoxidation to the blue radical is also observed (Jorns et al., 1986). Reoxidation of non-enzyme-bound reduced flavins by molecular oxygen occurs very rapidly (Holmström, 1964). However, the much slower rate of reoxidation of E–$FADH_2$ observed here is entirely consistent with the slower reactions of enzyme-bound flavin species, e.g., the reaction of the semiquinone or fully reduced flavodoxin with oxygen (Edmonson & Tollin, 1983; Massey et al., 1969).

The nature of the electron donor involved in the photoreduction of the radical was investigated by flash photolysis of the blue enzyme under aerobic conditions after dilution with 0.1 M NaCl rather than assay buffer or 0.1 M NaCl containing either EDTA, DTT, of Tris buffer. Figure 3 shows the absorbance of the enzyme at 580 nm as a function of the number of flashes. As can be seen, minimal photoreduction occurs with NaCl alone, a small degree of photoreduction with Tris buffer or EDTA, but a pronounced reduction with DTT. An essentially identical acceleration of photoreduction by DTT compared to NaCl alone is also observed under anaerobic conditions.

These observations are consistent with a mechanism involving a one-electron or hydrogen atom transfer from the thiol group(s) of DTT to an excited state of the flavin radical (E–FADH•*, eq 1). Although the absorption spectra of the

$$\begin{aligned}&\text{E–FADH}^{\bullet *} + \text{RSH} \rightarrow \\ &\qquad \text{E–FADH}_2 + \text{RS}^{\bullet} \text{ or E–FADH}^{-} + \text{RSH}^{\bullet +} \quad (1)\end{aligned}$$

neutral or anionic forms of free reduced flavins are spectrally distinct (Ghisla et al., 1974), the differences are too small to

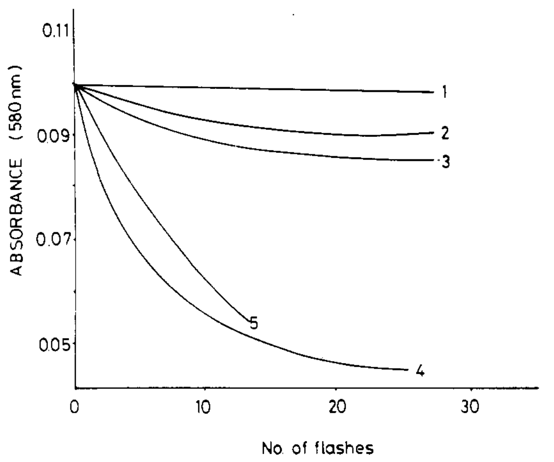


FIGURE 3: Camera flash photolysis of the blue enzyme under aerobic conditions. The absorbance of the radical at 580 nm as a function of the number of flashes is shown (the time interval between each flash is constant and equals 10 s). Curves 1–4 are the stock enzyme diluted 20-fold with 0.1 M NaCl containing (curve 1) no additions, (curve 2) Tris buffer, 0.066 M, (curve 3) EDTA, 2.5×10^{-3} M, (curve 4) DTT, 0.027 M, or (curve 5) stock enzyme diluted with standard buffer.

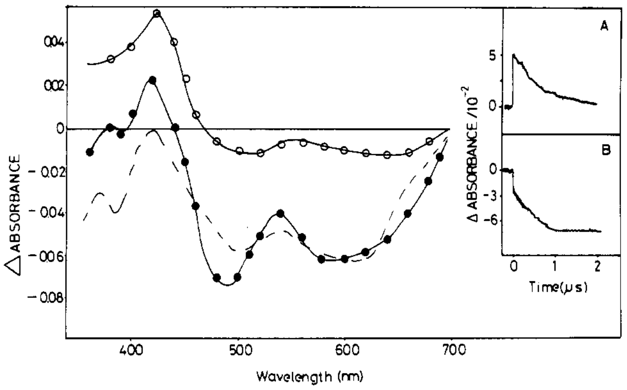


FIGURE 4: Laser flash photolysis of the blue enzyme under anaerobic conditions in standard buffer with excitation at 532 nm: transient difference spectrum after 0.05 (○) and 1.0 μs (●). For comparison, the difference spectrum observed after one camera flash normalized at 600 nm is also shown (– –). Insets are typical oscilloscope traces observed at 430 (A) and 580 nm (B).

provide an assignment of the enzyme-bound species in this case, as the spectrum is complicated by the presence of the second chromophore.

Laser Flash Photolysis. Laser flash photolysis of the blue enzyme in standard buffer was carried out with 532-nm excitation under anaerobic conditions. Immediately (0.05 μs) after the laser pulse a transient absorption (λ_{max} 420 nm) is observed (Figure 4). This transient species decays by first-order kinetics with $k_1 = 0.8 \times 10^6$ s^{-1}. Concomitant with the decay at 420 nm (Figure 4, inset A), a depletion appears from 450 to 700 nm (Figure 4) with a small positive absorption at 420 nm (Figure 4, inset B). No further change in absorption occurs up to 200 μs after the laser pulse.

The short lifetime of the initial transient species (λ_{max} 420 nm) suggests that it may represent the absorption of an excited state of the radical. Light absorption by flavin radicals occurs primarily via Π–Π* transitions (Edmonson & Tollin, 1983). Hence, excitation into the long wavelength absorption band of the radical is expected to produce the lowest excited doublet state ($^2_1\Pi\Pi^*$). Intersystem crossing to form the lowest excited quartet ($^4_1\Pi\Pi^*$) is unlikely to occur, as theoretical considerations of aromatic radicals such as benzyl (Bromberg & Meisel, 1985) suggest that such a state would be higher in energy than the lowest excited doublet state. However, even if excitation were to produce the second excited doublet ($^2_2\Pi\Pi^*$), intersystem crossing from $^2_2\Pi\Pi^* \rightarrow {}^4_1\Pi\Pi^*$ would be unlikely, due to the spin-forbidden nature of such a process, which would not compete with the highly allowed $^2_2\Pi\Pi^* \rightarrow {}^2_1\Pi\Pi^*$ transition. Of interest is that the lifetime of the lowest excited doublet state of benzyl and related radicals is of the order of 1 μs, due to the partially forbidden nature of the transitions from the lowest excited doublet to the ground-state doublet (Bromberg & Meisel, 1985). This compares well with the lifetime of the 420-nm transient species.

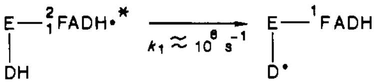
The transient absorption spectrum after 1 μs can be compared to the difference spectrum measured by the conventional spectrophotometer after one camera flash (Figure 4). As can be seen, both difference spectra show a marked similarity. Hence, it is suggested that both spectra represent essentially the same process, i.e., one-electron reduction of E–FADH•. Hence, eq 1 can be rewritten as

$$\text{E–}^2\text{FADH}^{\bullet *} + {}^1\text{RSH} \rightarrow \text{E–}^1\text{FADH}_2 + {}^2\text{RS}^{\bullet} \quad (2)$$

Upon the assumption that the laser-induced transient spectrum after 1.0 μs represents the E–FADH• → E–FADH$_2$ difference spectrum, then the value of the difference extinction coefficient ($\Delta\epsilon$) at any wavelength (λ) is equal to ϵ^{λ}(E–FADH$_2$) – ϵ^{λ}(E–FADH•). At 580 nm, ϵ(E–FADH•) = 3600 M^{-1} cm^{-1} (Jorns et al., 1984), and E–FADH$_2$ is not expected to absorb at λ >500 nm. Hence, by use of a value of $\Delta\epsilon$ = –3600 M^{-1} cm^{-1} a quantum yield of 0.10 ± 0.02 for the reduction of E–FADH•* was determined with ruthenium tris(bipyridyl) as the standard.

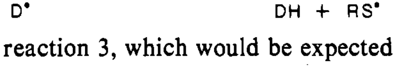
Laser flash photolysis of the blue enzyme diluted with 0.1 M NaCl showed essentially identical results with the enzyme in standard buffer, both with respect to the initial and 1-μs transient spectrum and kinetics. In addition, an essentially identical quantum yield of reduction was determined. These results are, however, inconsistent with the camera flash experiments, which showed that only minimal photoreduction occurs after dilution with 0.1 M NaCl.

It is suggested, therefore, that the primary step in photoreduction (eq 3) does not in fact involve DTT or other buffer

 (3)

$$\begin{array}{l}\text{E—}^2_1\text{FADH}\cdot^*\\ |\\ \text{DH}\end{array} \xrightarrow[k_1 \approx 10^6\ \text{s}^{-1}]{} \begin{array}{l}\text{E—}^1\text{FADH}_2\\ |\\ \text{D}^{\bullet}\end{array} \quad (3)$$

components but instead the electron donor is a constituent of the enzyme itself, possibly an amino acid residue (DH) or even the second chromophore. The role of DTT therefore may well be to reduce the D• radical species (eq 4), thus preventing the

 (4)

$$\begin{array}{l}\text{E—FADH}_2\\ |\\ \text{D}^{\bullet}\end{array} + \text{RSH} \longrightarrow \begin{array}{l}\text{E—FADH}_2\\ |\\ \text{DH} + \text{RS}^{\bullet}\end{array} \quad (4)$$

reversal of reaction 3, which would be expected on thermodynamic grounds. Reactions 3 and 4 are also consistent with the observation that the rate of decay of the initial transient species is unaffected by DTT, whereas reaction 2 predicts that its lifetime would be reduced by DTT. The rate of reaction 4 is unknown; however, it is not observed in the laser flash experiments up to 200 μs after the flash but would have to be complete by approximately 2 s (the minimum time needed for conventional spectral measurements following the camera flash).

That the two types of flash photolysis difference spectra shown in Figure 4 are not identical could be due to a number of reasons. Notwithstanding the different experimental techniques and the lower resolution of the transient spectrum, the differences are believed to be significant. One possible explanation is that the transient spectrum represents a different protolytic form from that observed after several seconds with the conventional spectrophotometer. An initial one-electron (as opposed to an hydrogen atom) reduction of E–FADH•* would yield initially the anionic reduced flavin E–FADH$^-$, which may protonate on a longer time scale (than that available on the laser flash photolysis instrument) to yield E–FADH$_2$. Of course, an initial hydrogen atom uptake followed by deprotonation would be equally possible. However, as the pK_a of the enzyme-bound reduced flavin is not known, no distinction can be made at present.

A further possibility is that again spectral changes occur between the two different times of measurement, but due in this case to reorientation of the flavin chromophore relative to the enzyme structure. Such a process has been postulated to explain the small but significant changes in spectra of the blue flavosemiquinone after one-electron reduction of the egg white riboflavin binding protein (Faraggi & Klapper, 1979).

These results have important implications regarding the mechanism of photorepair of pyrimidine dimers by *E. coli* DNA photolyase. Model studies with photosensitizers have shown that thymine dimers can be monomerized with either electron donors or acceptors. However, the clear tendency of E–FADH• to become photoreduced observed in this study suggests that electron donation by E–FADH•* to pyrimidine is unlikely. Several alternative mechanisms involving reduction of E–FADH• can however be envisaged, including (a) electron abstraction from pyrimidine dimers by E–FADH•* and (b) photoreduction of E–FADH•*, followed by either dark- or light-induced electron donation by E–FADH$_2$ to pyrimidine dimers. Process a would however have to be competitive with the proposed intraenzyme electron-transfer process (eq 4). Process(es) b would be consistent with the increase in repair activity found in the presence of DTT, now explicable in terms of the photoreduction of E–FADH• observed in this work. In addition, as the dithionite-reduced enzyme does not repair dimers without light (Jorns et al., 1986), electron donation by excited states of E–FADH$_2$ is suggested. Work is now in progress to clarify the photochemical properties of the reduced enzyme.

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Registry No. DNA photolyase, 37290-70-3.

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