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The Active Form of *Escherichia coli* DNA Photolyase Contains a Fully Reduced Flavin and Not a Flavin Radical, both in Vivo and in Vitro†

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ABSTRACT: *Escherichia coli* DNA photolyase is a flavoprotein that when purified is blue in color and contains a stable neutral radical FAD (E-FADH•). In the presence of a suitable electron donor (i.e., thiols, tyrosine, or NADH) the radical FAD adsorbs visible light and undergoes photoreduction to the fully reduced FAD ($E\text{-}FADH_2$). The in vitro quantum yield of dimer repair for E-FADH• is 0.07 while that of $E\text{-}FADH_2$ approaches the in vivo value of 1. Electron paramagnetic resonance studies on whole cells indicate that the in vivo form of photolyase is $E\text{-}FADH_2$ with enzyme containing radical FAD generated predominantly during the ammonium sulfate precipitation step of the purification. Activity measurements of E-FADH• using long-wavelength photoreactivating light indicate that enzyme containing FAD in the radical form is not active in dimer repair. Dimer repair observed with E-FADH• at shorter wavelengths is probably photoreduction of E-FADH• followed by dimer repair by $E\text{-}FADH_2$.

Irradiation of cells with UV light results in the formation of pyrimidine dimers between adjacent pyrimidines in DNA. DNA photolyases catalyze the light-dependent photoreversal of these mutagenic lesions, thus restoring the integrity of the DNA. Enzyme purified from *Escherichia coli* contains a stable neutral blue radical FAD and a second chromophore that has not yet been identified (Sancar & Sancar, 1984; Jorns et al., 1984).

The in vivo action spectrum of *E. coli* DNA photolyase has been determined by Harm (1970) and Jagger (Jagger et al., 1969). Harm reported a photoreactivation cross section ($\epsilon\phi$) of $(1.5\text{–}2.5) \times 10^4$ M^{-1} cm^{-1} at 384 nm. Assuming that it was unlikely that ϵ_{384} would be greater than 10^5 M^{-1} cm^{-1}, it was proposed that the quantum yield of photoreactivation (ϕ) must be between 0.1 and 1. Following purification of photolyase (Sancar et al., 1984) the molar extinction coefficient (ϵ) at 384 nm was determined to be 18.1×10^4 M^{-1} cm^{-1} (Jorns et al., 1987). By use of ϵ_{384} of purified photolyase, the in vivo quantum yield is estimated to be ~1. However, Sancar et al. (1987a) determined the in vitro photoreactivation cross section at 384 nm of purified radical FAD containing photolyase to be 1209 M^{-1} cm^{-1}, which gives an in vitro quantum yield of photoreactivation of 0.07. The disparity between the in vivo and in vitro quantum yields led us to suspect that the in vivo form of the enzyme was in some way different from the purified form. Several possibilities were ruled out (Sancar et al., 1987a). In particular, it was demonstrated that the discrepancy between the ϕ values obtained in vitro and those calculated in vivo was not due to the fact that the enzyme used for the in vitro experiments was purified from a strain that grossly overproduces photolyase. Other causes for the discrepancy were considered and investigated. The results of these investigations suggested that the purified blue radical form of photolyase (E-FADH•) was an artifact and that the in vivo form of photolyase contained a fully reduced FAD ($E\text{-}FADH_2$). In support of this it was found that dithionite-reduced photolyase has a photoreactivation cross section at 384 nm of 16 250 M^{-1} cm^{-1} (Sancar et al., 1987a). With ϵ_{384} = 16 100 M^{-1} cm^{-1} for the reduced enzyme (Jorns et al., 1987), ϕ was calculated to be 1.0 (Sancar et al., 1987a). This strongly suggested that the in vivo form of the enzyme contains a fully reduced FAD.

The observation that E-FADH• can undergo photoreduction (ϕ = 0.1) in the presence of certain appropriate electron donors (Heelis & Sancar, 1986; Heelis et al., 1987) led us to suspect that photolyase in the radical form may not be active and that the apparent photoreactivation activity observed with the blue radical flavin form of the enzyme is in fact photoreduction, followed by several rounds of dimer splitting.

In this paper we demonstrate that photolyase is not present as E-FADH• in vivo and that the radical flavin is generated during purification. Comparison of the photolytic cross section at 366 nm of E-FADH• and photoreduced enzyme, $E\text{-}FADH_2$, demonstrates that $E\text{-}FADH_2$ has a 4–8-fold higher quantum yield depending upon the electron donor present in the buffer

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for the E-FADH• experiments. Finally, we present evidence indicating that E-FADH• is not active in dimer repair and propose that the apparent dimer repair observed with E-FADH• is actually photoreduction to E-$FADH_2$, which then acts catalytically in dimer repair.

Experimental Procedures

Bacterial Strains and Plasmids. *E. coli* CSR603 (*recA1 uvrA6 phr1*) has been described previously (Sancar & Rupert, 1978). MS09 is CSR603 carrying $Flaci^Q$ and the *tac-phr* plasmid pMS969 (Sancar et al., 1984). *E. coli* GM33 and GM33/pAL7 were kindly provided by Dr. Ake Larsson of the Swedish University of Agricultural Sciences. pAL7 contains the genes for ribonucleotide reductase (*nrdA* and *nrdB*), and its construction is described elsewhere (Larsson & Sjöberg, 1986). MS09 was grown in Luria broth containing 20 μg/mL tetracycline. To induce the expression of photolyase, MS09 was grown to A_{600} = 0.8 at which time IPTG was added to 1 mM, and the incubation was continued overnight. GM33 was grown in Luria broth, and GM33/pAL7 was grown in Luria broth containing 200 μg/mL ampicillin.

Buffers. Photolyase storage buffer is 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), and 50% glycerol. Photolyase reaction buffer is the same as storage buffer except that it contains 100 μg/mL bovine serum albumin (BSA), no glycerol, and 100 mM NaCl instead of 50 mM NaCl. Buffer B contains 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, and either 100 mM or 2 M KCl. Buffer C contains 1 mM EDTA, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, and various concentrations of potassium phosphate, pH 6.8.

Photolyase Purification. A simplified purification procedure was adapted from the published procedure (Sancar et al., 1984). Briefly, cell-free extract was prepared as described. Proteins in the cell-free extract were then precipitated with 65% saturated ammonium sulfate. The precipitate was centrifuged and resuspended in a minimal volume of buffer B + 100 mM KCl and dialyzed against the same buffer overnight. The dialyzate was then loaded onto a Blue Sepharose (Sigma) column. The column was washed with buffer B + 100 mM KCl, and the photolyase was eluted with buffer B + 2 M KCl. The peak fractions were pooled and dialyzed into buffer C (20 mM potassium phosphate) and loaded onto a hydroxylapatite column (Bio-Gel HT). The column was washed with buffer C (100 mM potassium phosphate) and then eluted with a buffer C gradient (100–330 mM potassium phosphate). The peak fractions were pooled and dialyzed into photolyase storage buffer. For the electron paramagnetic resonance (EPR) measurements, both cell-free extract and fractions from the Blue Sepharose column were concentrated on Centricon 30 centrifuge columns to obtain a stronger EPR signal.

Substrate Preparation and Dimer Assay. pBR322 containing approximately six dimers per plasmid molecule was prepared as described previously (Sancar et al., 1985). Dimers were quantitated by the transformation assay, which has been described elsewhere (Sancar et al., 1987a,b) except the phenol extraction and ethanol precipitation steps were omitted. Competent cells were transformed with 25 μL of reaction mixture in the photoreactivation cross-section experiments and 40 μL in the E-FADH• activity experiments. All assays contained a 50-fold molar excess of photolyase over dimers and a pBR322 concentration of 25 μg/mL.

Photoreactivating Treatment. Photoreactivation cross-section measurements were done with continuous irradiation from a black (Sylvania F15T8BLB lamps) light having 90% of its energy output at 366 nm. The black light was covered with two Mylar sheets to decrease the transmission of contaminating 254-nm light. The fluence rate was measured with a UVX digital radiometer from Ultra-Violet Products, Inc., San Gabriel, CA. Fluence rates of 20 and 40 erg mm^{-2} s^{-1} were used for E-$FADH_2$ and E-FADH•, respectively. All photoreactivation treatment was performed on samples contained in 1.5-μL microfuge tubes. Photoreactivation using photoreduced photolyase (E-$FADH_2$) was done in a glovebag under an argon atmosphere. E-FADH• activity was measured with a 633-nm 5-mW HeNe laser (Spectra-Physics Stablite Model 120 S) and filtered camera flashes as photoreactivation light sources. The laser irradiation was done in quartz cuvettes. Because of dust in the atmosphere and in the solution, considerable scattering of the narrow laser beam occurred, resulting in the entire sample being irradiated at once (though probably not uniformly). A Haake circulating water bath was used to keep the enzyme at 4 °C during extensive laser irradiation. The filtered flash irradiation was conducted with three Vivitar 2500 camera flash units set to flash simultaneously. Oriel Long Pass 630-nm filters (51520) were attached to the flash units to prevent sample irradiation at wavelengths less than 600 nm. The unfiltered Vivitar flash system was used in both the laser and filtered flash experiments as a positive control for dimer repair. It has been previously demonstrated that a flash from a single Vivitar unit is of sufficient intensity to repair nearly all of the dimers that are enzyme bound at the moment of the flash (Sancar et al., 1987a). Photoreduction was done with either two Vivitar flash units with Oriel Long Pass 515-nm filters (51494) or three Vivitar flash units with Oriel Long Pass 630-nm filters (51520). The almost complete loss of absorption at 580 nm indicated that photoreduction was essentially complete.

Determination of Extinction Coefficients. We previously reported (Jorns et al., 1984) extinction coefficients at various near-UV wavelengths for the E-FADH• form of photolyase. Those estimates were based on apoprotein concentrations and appear to be underestimates. To obtain more accurate extinction coefficients, the extinction coefficient at 580 nm of radical FAD photolyase was determined on the basis of the content of flavin rather than on the content of apoprotein. First, a spectrum of radical FAD photolyase was taken where all FAD was enzyme bound and in the radical oxidation state. Then the enzyme was denatured with sodium dodecyl sulfate (SDS) releasing the FAD, which immediately becomes oxidized. The flavin concentration was determined by measurement of the absorbance at 450 nm and use of the published ϵ_{450} of oxidized FAD (1.12×10^4 M^{-1} cm^{-1}). With use of the A_{580} of the native enzyme and the flavin concentration, ϵ_{580} is calculated to be 5000 M^{-1} cm^{-1}, reflecting only the photolyase molecules containing radical FAD. Extinction coefficients at all other wavelengths were calculated relative to ϵ_{580}. Because of the observation that different batches of photolyase contain varying amounts of second chromophore, it is necessary to recalculate for every enzyme preparation the extinction coefficients at wavelengths where the second chromophore absorbs ($\lambda < 450$ nm).

EPR. Electron paramagnetic resonance spectra were recorded with a Varian E-109 spectrometer at X-band frequencies. The magnetic field was calibrated with an NMR gaussmeter (Magnion Model G-502) and a Hewlett Packard 5340A precision frequency counter. The Klystron frequency was measured directly with the frequency counter at all times. A microcomputer interfaced to the spectrometer provided

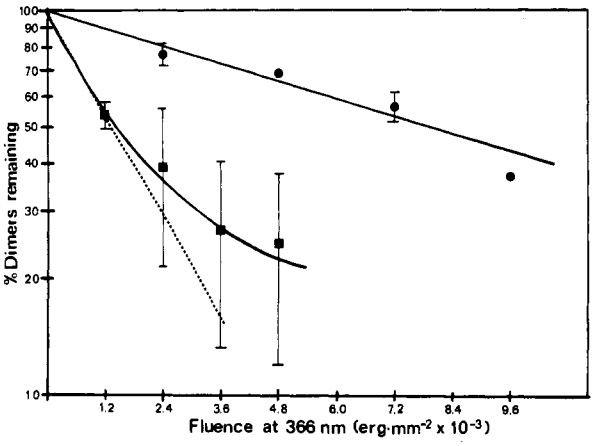

FIGURE 1: Fraction of total dimers remaining as a function of fluence at 366 nm. A 50-fold excess of (●) E-FADH• or (■) photoreduced E-FADH• (E-$FADH_2$) was incubated with dimer containing pBR322 and exposed to black light (λ_{max} = 366 nm) at a fluence rate of 40 erg mm^{-2} s^{-1} for E-FADH• and 20 erg mm^{-2} s^{-1} for E-$FADH_2$. The number of dimers was quantitated as described under Experimental Procedures.

spectral storage and manipulation. All EPR spectra were recorded with a modulation amplitude of 1 G, a modulation frequency of 100 kHz, a microwave power of 10 dB, and a Klystron frequency of approximately 9.135 GHz. All samples were run at 77 K in quartz tubes.

RESULTS

Quantum Yield for Dimer Repair by Photoreduced Photolyase (E-$FADH_2$). The photoreactivation cross section of photoreduced enzyme (E-$FADH_2$) at 366 nm was determined and compared with that found in a parallel experiment for radical FAD photolyase (E-FADH•). E-FADH• in photolyase storage buffer was placed in an anaerobic cuvette and made anaerobic by blowing argon over the surface for 20 min. To photoreduce the enzyme, the cuvette was then given 300 flashes with two or three Vivitar flash units set to flash simultaneously. The flash units were covered by Oriel $\lambda > 515$ nm or $\lambda > 630$ nm Long Pass filters to ensure that the photolyase was exposed only to light corresponding to the long-wavelength flavin radical absorption and to prevent the photodecomposition of the second chromophore by lower wavelengths (Heelis et al., 1987). The absorption spectrum taken after the filtered flashes showed complete disappearance of the long-wavelength absorption peak, indicating that photoreduction was essentially complete (Heelis & Sancar, 1986; Heelis et al., 1987).

Excess photoreduced enzyme was then added to irradiated pBR322 in standard reaction buffer (without BSA) under conditions where all dimers are bound (as determined by single flash photolysis; Sancar et al., 1987a). The reaction mixture was then exposed to black light (366 nm) for varying lengths of time. Dimer repair was quantitated as a function of photoreactivating fluence and plotted on a Rupert plot (Figure 1). A Rupert plot is a plot of log (fraction dimers remaining) vs photoreactivation fluence received by the sample. The slope of the resulting curve is equal to the negative photolytic constant ($-k_p$), which is a measure of the photoreactivation efficiency and proportional to the photoreactivation cross section ($\epsilon\phi$) at a given wavelength. The data from a parallel experiment performed aerobically with E-FADH• were also plotted for comparison. The photoreactivation cross section ($\epsilon\phi$) was calculated for both forms of the enzyme as previously outlined (G. Sancar et al., 1987a), with $\epsilon\phi$ = 7370 M^{-1} cm^{-1} obtained for the photoreduced enzyme and $\epsilon\phi$ = 1257 M^{-1} cm^{-1} obtained for radical FAD photolyase by irradiation at 366 nm.

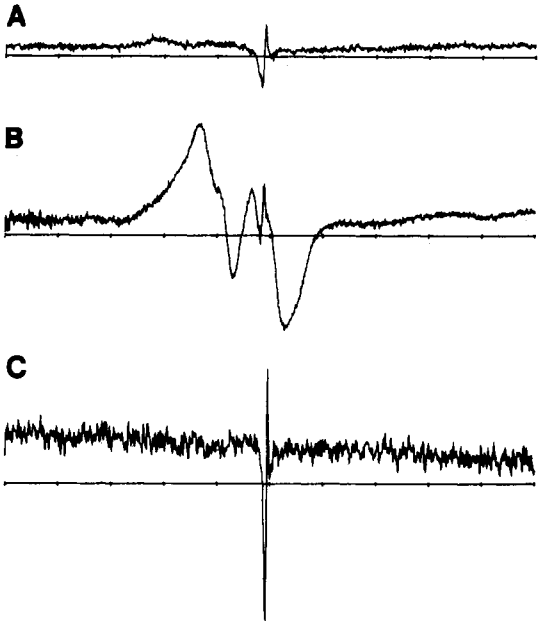

FIGURE 2: Electron paramagnetic resonance spectra of *E. coli* cell suspensions: (A) MS09 (induced with 1 mM IPTG) photolyase overproducer; (B) GM33/pAL7 ribonucleotide reductase overproducer; (C) buffer. All spectra were centered at 3260 G. Spectra A and B were recorded at a gain of 8×10^3 and with 200-G sweep width and a 0.128-s time constant. Spectrum C was recorded at a gain of 5×10^4 and with a 400-G sweep width and 0.25-s time constant.

With ϵ_{366} = 21 500 and 16 661 M^{-1} cm^{-1} for E-FADH• and E-$FADH_2$, respectively, the quantum yields of photoreactivation are calculated to be 0.059 for E-FADH• and 0.44 for E-$FADH_2$. (The extinction coefficients given for both forms include the spectral contributions of the flavin and the second chromophore.) Because of the difficulties in maintaining anaerobic conditions and performing glovebag irradiations for the photoreduced enzyme measurements (as evidenced by the error bars), only the first point, which is very reproducible, was used in the calculation of ϕ.

Electron Paramagnetic Resonance. EPR measurements on whole cells have been used to detect stable free radical proteins in vivo. It has previously been shown that the *nrdB* gene product (ribonucleotide reductase subunit B2) contains a stable tyrosine radical. When this protein is overexpressed in *E. coli* cells, it is possible to detect an EPR signal originating from the tyrosine radical (Larsson & Sjöberg, 1986). Therefore, one would expect a photolyase-overproducing strain to show an EPR signal if the flavin was present in the same neutral radical form in vivo as it is when photolyase is purified. *E. coli* MS09 and GM33/pAL7, which overproduce photolyase and ribonucleotide reductase, respectively, were grown as described under Experimental Procedures. Cells were centrifuged, and the pellet was forced into the EPR tube with a 10-cm^3 syringe. The cells containing the stable tyrosine radical give a strong radical signal (Figure 2B) essentially identical with the spectrum obtained by Larsson and Sjöberg (1986). However, IPTG-induced MS09 cells show no such signal (Figure 2A), indicating that photolyase contains no flavin radical in vivo. Furthermore, neither freeze-thawing nor sonication of the cells resulted in a change in the EPR spectra obtained (data not shown). All spectra contained a small sharp signal from a buffer component superimposed on the spectrum. This is shown at a 6.25-fold higher gain (Figure 2C) and is not characteristic of flavin radicals as the line width (3 G) is much smaller than would be expected for neutral flavoprotein radical (19 G) (Palmer et al., 1971). The absence of a radical signal from photolyase-overproducing cells is not due to a lesser

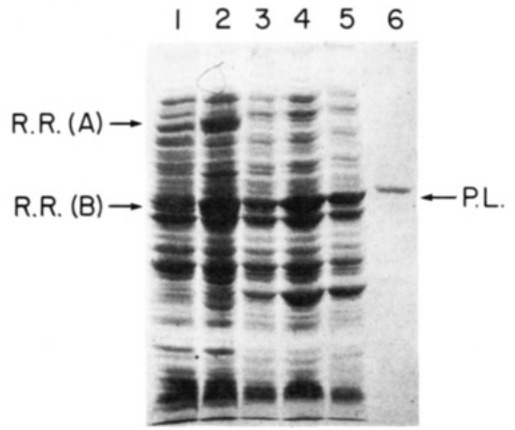

FIGURE 3: Analysis of EPR samples on 10% SDS–polyacrylamide gel: (lane 1) GM33; (lane 2) GM33/pAL7; (lane 3) MS09 (uninduced); (lane 4) MS09 (induced with 1 mM IPTG); (lanes 5 and 6) relative amounts of protein present in EPR tube for measurements on MS09 pellet (shown in Figure 2A) and purified radical FAD photolyase, respectively. P.L. = 54-kDa photolyase; R.R. (A) = 87-kDa *nrdA* gene product (B1 subunit of ribonucleotide reductase; R.R. (B) = 43.5-kDa *nrdB* gene product (B2 subunit of ribonucleotide reductase).

amount of this enzyme in the cell compared to ribonucleotide reductase. SDS–polyacrylamide gel electrophoresis of both overproducers indicates the presence of comparable amounts of the two proteins (Figure 3, lanes 2 and 4). Furthermore, the quantity of photolyase was sufficient to detect a signal if the flavin had been in the radical state. This is demonstrated in Figure 3, lanes 5 and 6, where the relative amounts of photolyase present in the EPR tubes for the MS09 pellet spectrum and a purified photolyase spectrum, respectively, are shown. The concentration of purified photolyase in lane 6 was enough to give a radical EPR signal (data not shown), whereas the much higher concentration of photolyase present in the MS09 pellet did not give a signal. The host strain for the photolyase-overproducing plasmid (CSR603/Flaci^{Q}) gave an EPR spectrum identical with that of induced MS09 (data not shown). Both spectra were similar to that obtained for the host strain for the ribonucleotide reductase overproducing plasmid (GM33) (data not shown).

Having determined that photolyase does not contain flavin radical in vivo, we decided to ascertain what specific step in the purification procedure was responsible for the generation of the flavin neutral radical. We had already determined that lysing the cells did not generate radical FAD, and consequently, EPR measurements were performed on subsequent steps of the purification. The results are shown in Figure 4. A slight indication of the characteristic 19-G neutral flavin radical signal is seen in the concentrated cell-free extract (Figure 4A) after it has been stored at 4 °C for 3 days. (Again, the sharp buffer component shown in Figure 4D is superimposed on all spectra.) However, the spectrum shown in Figure 4B taken after the ammonium sulfate precipitation (and dialysis into buffer B + 100 mM KCl) clearly shows a significant amount of radical of the characteristic 19-G line width and expected field set of neutral flavoprotein radicals (Palmer et al., 1971). The signal is virtually identical with that obtained previously with purified photolyase (Jorns et al., 1984). The EPR spectrum (6.25-fold lower gain) of concentrated 80–90% pure photolyase (Figure 4C) obtained after the Blue Sepharose column step also clearly shows extensive radical formation. The relative amounts of photolyase present in the EPR tubes for the spectra in Figure 4 are compared by analysis on an SDS–polyacrylamide gel (Figure 5). An inspection of lanes 1 and 2 demonstrates that the amount of

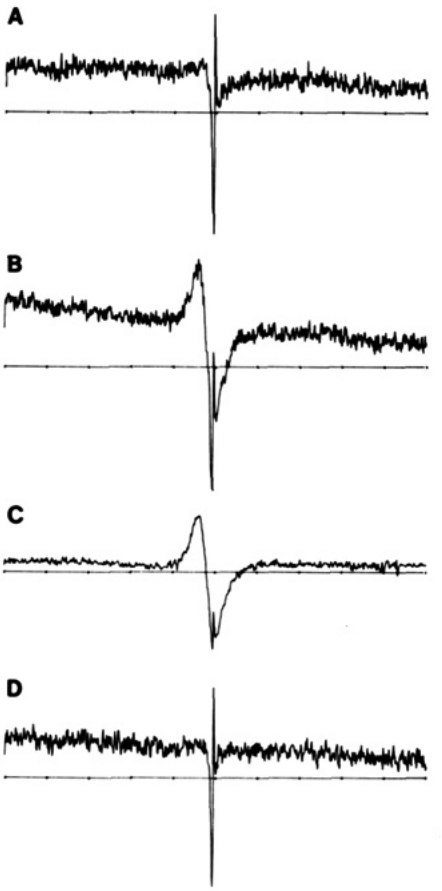

FIGURE 4: Electron paramagnetic resonance spectra of partially pure photolyase at various steps in the purification procedure: (A) concentrated cell-free extract; (B) ammonium sulfate precipitate; (C) concentrated Blue Sepharose column fractions; (D) buffer. All spectra were centered at 3260 G with 400-G sweep widths. Spectra A, B, and D were recorded at a gain of 5×10^4 and a time constant of 0.25 s. Spectrum C was recorded with a gain of 8×10^3 and a 0.128-s time constant.

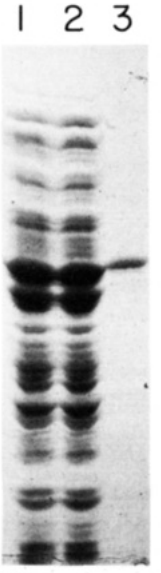

FIGURE 5: Analysis by 10% SDS–polyacrylamide gel electrophoresis of photolyase at various purification steps. (Lanes 1–3) Relative amounts of protein in the EPR tube for the spectra shown in Figure 4A–C. (1) Concentrated-cell free extract; (2) ammonium sulfate precipitate; (3) concentrated Blue Sepharose column fractions (10-fold dilution).

photolyase in the concentrated cell-free extract was essentially equal to that present in the ammonium sulfate precipitate. This indicates that the radical signal in Figure 4B was not due

Table I: Summary of Photolytic Cross-Section Measurements

sample	photoreactivating light source (wavelength)	$\epsilon\phi$ (M^{-1} cm^{-1})	ϵ (M^{-1} cm^{-1})[a]	ϕ
E-FADH• (2-mercaptoethanol)	black light (366 nm)	1257	21 500	0.059
E-FADH• (dithiothreitol)	black light (366 nm)	2390	21 500	0.11
E-$FADH_2$ (photoreduced)	black light (366 nm)	7370	16 661	0.44
E-FADH•	laser (633 nm)[b]	2.60	4080	6.36×10^{-4}
E-FADH•	filtered flashes (630-nm Long Pass filters)	0.581	4080	1.42×10^{-4}

[a] Revised estimates—see Experimental Procedures. [b] Average of two experiments.

to a greater amount of photolyase present but rather to the presence of increased amounts of photolyase in the radical FAD form.

Photoreactivation Activity of E-FADH•. Although neutral blue radical FAD photolyase (E-FADH•) has been routinely used in dimer repair assays, it is not known whether it is actually able, in radical form, to catalyze photoreversal of the dimer. The ability of the flavin radical to be photoreduced in the presence of an electron donor (Heelis & Sancar, 1986), the apparent increase in specific activity and quantum yield of fully reduced FAD enzyme (E-$FADH_2$) (Jorns et al., 1987; Sancar et al., 1987a), and the indication that photolyase is fully reduced in vivo suggest that only the fully reduced form of the enzyme is active. We reasoned that, in a dimer repair assay using photoreactivating light at a wavelength that is absorbed only by E-FADH• but not E-$FADH_2$ or the second chromophore, only repair resulting from the action of E-FADH• would be observed. This hypothesis was tested with two sources emitting long-wavelength visible light, a HeNe laser emitting monochromatic light of wavelength 633 nm, and filtered camera flashes. The fluence rate of the laser was determined by measuring the extent of photoreduction in a 3-h irradiation of photolyase in an anaerobic cuvette. The results are shown in Figure 6. The loss of absorbance at 580 nm indicates 37% photoreduction occurs in a 3-h irradiation. As photoreduction is irreversible under anaerobic conditions (Heelis & Sancar, 1986), with a quantum yield of photoreduction of 0.1 (Heelis & Sancar, 1986; Heelis et al., 1987) an average laser fluence rate of 2 erg mm^{-2} s^{-1} is calculated. Next, the photolytic cross section at 633 nm was determined with essentially the same method used for determination of the photolytic cross section at 366 nm. We had previously determined the action spectrum for E-FADH• (Sancar et al., 1987a), which revealed a small but significant amount of dimer repair at long wavelengths (ϕ_{625nm} = 0.003). However, we suspected that during the extensive irradiation with 625-nm light stray light of lower wavelengths may have caused the small amount of repair. As the laser is monochromatic, no contaminating light is expected. Therefore, we irradiated the sample with the 633-nm laser for 124 h (second experiment—139 h) to give a fluence sufficient to repair 63% of the dimers with a quantum yield of 3×10^{-3}. Because of the long duration of irradiation, 20% glycerol was added to the reaction mixture, and the cuvette was cooled to 4 °C. To ensure that the enzyme was stable under these conditions, part of the sample was given one white flash with the camera flash unit before and after the laser irradiation, and the two resulting repair efficiencies were compared.

A similar experiment was done with 4000 filtered (Oriel Long Pass 630-nm filters) flashes from the three simultaneous flash units. A fluence delivered per flash of 207 erg/mm^2 was determined by using the extent of photoreduction of E-FADH• as an actinometer as done for the laser fluence rate determination.

The results of all experiments show a small but significant amount of repair with long-wavelength visible light. With ϵ_{633} = 4080 M^{-1} cm^{-1} for E-FADH•, the quantum yield of dimer repair (by E-FADH•) is calculated to be 3×10^{-4} to 1×10^{-3} for the laser irradiations and 1.4×10^{-4} for the filtered flash irradiation. Furthermore, the extensive long-wavelength irradiation did not inactivate the photolyase, as demonstrated by comparison of the repair with one white flash before and after the irradiation. In all experiments the single white flash repair was significantly greater for the samples that had been preilluminated with long-wavelength light, with 90% of the dimers repaired compared with 70%. This is almost certainly a result of photoreduction of the enzyme to the apparently more active (E-$FADH_2$) form.

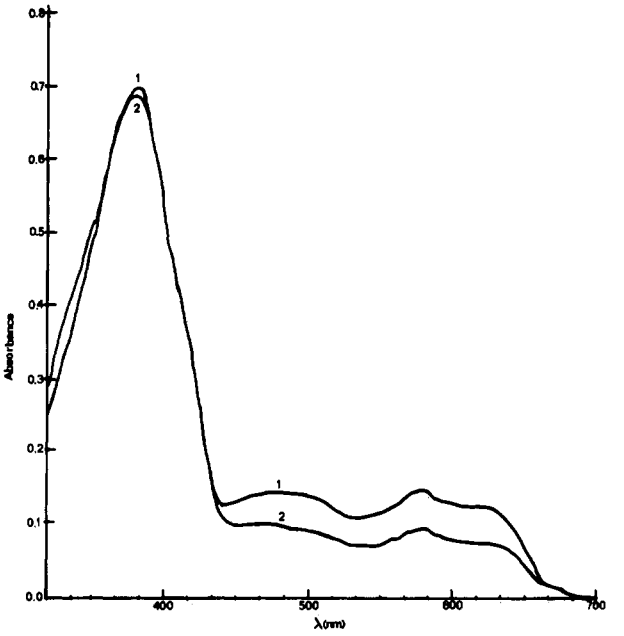

FIGURE 6: Absorption spectra of E-FADH• (1.71 mg/mL) before irradiation (curve 1) and after 3 h of irradiation in an anaerobic cuvette by a 633-nm HeNe laser (curve 2). The cuvette was made anaerobic by blowing argon over the surface of the photolyase for 20 min. During the irradiation the cuvette was cooled to 4 °C by a circulating water bath.

The results of all of the photoreactivation cross-section experiments are given in Table I. The quantum yield of dimer repair at 366 nm of the fully reduced FAD form of photolyase is more than 3000-fold greater than the apparent quantum yield obtained with filtered flashes and nearly 700-fold greater than that obtained with 633-nm laser irradiation.

Also of interest is the apparent increase in quantum yield at 366 nm of the radical FAD photolyase seen when dithiothreitol rather than 2-mercaptoethanol was present in the reaction. Dithiothreitol has been shown to be twice as efficient in electron donation during photoreduction (Heelis et al., 1987). Therefore, the apparent quantum yield of dimer repair seen for E-FADH• with the black light is dependent on the electron donor present.

Discussion

For several years after the purification of large quantities of the scarce *E. coli* DNA photolyase (Sancar et al., 1984), it was believed that the purified form of the enzyme (neutral blue radical FAD) was the active form. The only indication

to the contrary was the lack of blue color in colonies of the overproducing strain where 15% of the total cellular protein was photolyase. Subsequent study of the in vitro action spectrum of E-FADH$^\bullet$ (Sancar et al., 1987a) revealed a puzzling enigma when the in vitro and in vivo quantum yields were compared. The apparent 20-fold lower in vitro quantum yield combined with several other observations suggested that the E-FADH$_2$ form of photolyase may actually be the in vivo form of the enzyme. Flash photolysis studies of E-FADH$^\bullet$ demonstrated that in the presence of DTT (and other electron donors) the enzyme becomes photoreduced upon irradiation at wavelengths where E-FADH$^\bullet$ absorbs (Heelis & Sancar, 1986; Heelis et al., 1987). As the standard reaction buffer contains thiol electron donors (dithiothreitol or 2-mercaptoethanol), we considered that the apparent repair observed with E-FADH$^\bullet$ may, in fact, be preceded by photoreduction of the radical.

This study demonstrates that *E. coli* DNA photolyase does not contain a radical FAD in vivo. Whole cells of an *E. coli* strain containing a plasmid that overproduces the tyrosine radical containing subunit of ribonucleotide reductase give a strong EPR signal. However, similar measurements with the photolyase-overproducing strain fail to reveal the presence of a radical even though the relative concentrations of photolyase and ribonucleotide reductase in the cells are roughly the same. We have also demonstrated the specific step of purification that is responsible for the destabilization of the in vivo form of photolyase, resulting in significant radical formation. The EPR data are consistent with the intrinsic FAD chromophore existing in either the fully oxidized or fully reduced oxidation state in vivo. However, photolyase containing fully oxidized FAD is inactive in dimer photoreversal (Jorns et al., 1987). Therefore, we conclude that photolyase is fully reduced in vivo. It is interesting to note that the yeast photolyase from *Saccharomyces cerevisiae* also contains an FAD. However, unlike the *E. coli* enzyme, the yeast enzyme is isolated with the FAD in the fully reduced state (Sancar & Smith, 1987).

Photolyase containing neutral blue radical FAD is unable to catalyze dimer repair. If one assumes that photosensitized reactions occur from the lowest excited state, then excitation at any wavelength in the UV–visible spectrum that the chromophore absorbs should catalyze the reaction with the same quantum yield (ϕ) (Turro, 1978). The action spectrum of E-FADH$^\bullet$ does not give a constant quantum yield over all wavelengths (Sancar et al., 1987a). At 625 nm a small but detectable amount of dimer repair was observed with a quantum yield of 3×10^{-3}. It was proposed that the lengthy irradiation resulted in absorption of lower wavelength contaminating light where the fully reduced FAD photolyase certainly absorbs. However, our results using a monochromatic light source (633-nm laser) wherein no contaminating photons would occur indicate that a small but significant amount of dimer repair does, in fact, take place. Upon comparison of the quantum yields (ϕ) calculated from extinction coefficients for E-FADH$^\bullet$, a decreasing trend is observed with increasing photoreactivation wavelength if we consider the filter flash as the longest wavelength (as most of the transmitted light is at wavelengths greater than 630 nm). ϕ values at 625 nm [obtained by Sancar et al. (1987a)], 633 nm (laser), and $\lambda > 633$ nm (filtered flash) are 3×10^{-3}, 6.36×10^{-4}, and 1.42×10^{-4}, respectively. The fact that the apparent quantum yield for E-FADH$^\bullet$ decreases drastically with increasing photoreactivating wavelengths and $\epsilon\phi$ does not match the E-FADH$^\bullet$ absorption spectrum is a strong indication that it is not the species that catalyzes dimer repair. A much more attractive alternative is that E-FADH$_2$ possesses an extremely long absorption tail extending out to very long visible wavelengths that slowly approaches zero and is responsible for catalyzing dimer repair throughout the near-UV–visible spectrum with a constant quantum yield. However, we cannot rule out the possibility that E-FADH$^\bullet$ is able to repair dimers with an extremely low quantum yield. Measurement of the reduction potentials of the E-FADH$_2$/E-FADH$^\bullet$ and E-FADH$^\bullet$/E-FAD$_{ox}$ pairs will determine whether E-FADH$^\bullet$ is capable of donating an electron to the dimer in this system.

Finally, we have demonstrated that photoreduced photolyase has a quantum yield that approaches the in vivo value (and the value obtained with dithionite-reduced photolyase) of 1.0 (Harm, 1970; Sancar et al., 1987a). This is in support of our proposal that the apparent repair of dimers observed with the E-FADH$^\bullet$ in vitro is a result of photoreduction to the fully reduced form of photolyase, followed by several rounds of dimer repair. We suspect that the quantum yield of photoreduced photolyase is actually identical with that of dithionite-reduced photolyase and that this fact is obscured by the variation in experimental conditions used to determine these values. For the data presented in this paper, the anaerobiosis was not complete, resulting in significant reoxidation of E-FADH$_2$ to E-FADH$^\bullet$ during the photoreactivation treatment, as is evident by a decrease in the slope of the photoreactivation curve with longer irradiation times. It should be emphasized, therefore, that the quantum yield of 0.4 for photoreduced photolyase reported in this paper is probably an underestimate and that a higher quantum yield will be obtained under strictly anaerobic conditions. These reservations notwithstanding, the data presented in this paper clearly show that *E. coli* photolyase, in addition to the second chromophore, contains fully reduced FAD in vivo. Furthermore, our data show that the E-FADH$^\bullet$ form of the enzyme is a purification artifact and the flavin radical in this form of the enzyme cannot act as a photocatalyst in splitting pyrimidine dimers.

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Phosphorothioate Analogues of 2′,5′-Oligoadenylate. Enzymatically Synthesized 2′,5′-Phosphorothioate Dimer and Trimer: Unequivocal Structural Assignment and Activation of 2′,5′-Oligoadenylate-Dependent Endoribonuclease†

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ABSTRACT: In continued studies to elucidate the requirements for binding to and activation of the 2′,5′-oligoadenylate-dependent endoribonuclease (RNase L), chirality has been introduced into the 2′,5′-oligoadenylate (2-5A, p_3A_n) molecule to give the R_P configuration in the 2′,5′-internucleotide backbone and the S_P configuration in the α-phosphorus of the pyrophosphoryl moiety of the 5′-terminus. This was accomplished by the enzymatic conversion of (S_P)-ATPαS to the 2′,5′-phosphorothioate dimer and trimer by the 2-5A synthetase from lysed rabbit reticulocytes. The most striking finding reported here is the ability of the 2′,5′-phosphorothioate *dimer* 5′-triphosphate (i.e., $p_3A_2\alpha S$) to *bind to and activate* RNase L. $p_3A_2\alpha S$ displaces the $p_3A_4[^{32}P]pCp$ probe from RNase L with an IC_{50} of 5×10^{-7} M, compared to an IC_{50} of 5×10^{-9} M for authentic p_3A_3. Further, $p_3A_2\alpha S$ activates RNase L to hydrolyze poly(U)-3′-$[^{32}P]pCp$ (20% at 2×10^{-7} M), whereas authentic p_3A_2 is unable to activate the enzyme. Similarly, the enzymatically synthesized $p_3A_2\alpha S$ at 10^{-6} M activated RNase L to degrade 18S and 28S rRNA, whereas authentic p_3A_2 was devoid of activity. $p_3A_3\alpha S$ was as active as authentic p_3A_3 in the core–cellulose and rRNA cleavage assays. The absolute structural and configurational assignment of the enzymatically synthesized $p_3A_2\alpha S$ and $p_3A_3\alpha S$ was accomplished by high-performance liquid chromatography, charge separation, enzymatic hydrolyses, and comparison to fully characterized chemically synthesized (R_P)- and (S_P)-2′,5′-phosphorothioate dimer and trimer cores. The absolute structural assignment for $p_3A_2\alpha S$ is 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)adenosine, and for $p_3A_3\alpha S$ it is 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)-(R_P)-*P*-thioadenylyl(2′–5′)adenosine. These assignments confirm the previous suggestion of an R_P configuration at the 2′,5′-internucleotide linkages of enzymatically synthesized $p_3A_3\alpha S$ [Lee, C., & Suhadolnik, R. J. (1985) *Biochemistry 24*, 551–555].

The 2′,5′-oligoadenylate [2′,5′-ppp(Ap)$_n$A; 2-5A][1] system is widely accepted to be involved in the antiviral mechanism of interferon [consult Johnston and Torrence (1984) and Lengyel (1982) for recent reviews] and may also be involved in the regulation of cell growth and differentiation (Etienne-Smekens et al., 1983; Ferbus et al., 1984; Jacobsen et al., 1983; Bayard et al., 1986; Wells & Mallucci, 1985; Zullo et al., 1985). 2-5A synthesized from ATP by 2′,5′-oligoadenylate synthetase [ATP:(2′–5′)oligo(A) adenylyltransferase (EC 2.7.7.19)] exerts its biological effects by binding to and activating its only known target enzyme, the unique 2-5A-dependent endoribonuclease (RNase L, EC 3.1.27), which cleaves viral and cellular mRNA or rRNA, thereby inhibiting protein synthesis (Hovanessian & Kerr, 1979; Kerr & Brown, 1978). 2-5A, however, is metabolically unstable and is degraded by a cellular 2′-phosphodiesterase (2′-PDE) and phosphatases (Knight et al., 1981; Minks et al., 1979; Williams et al., 1978).

The literature is replete with structurally modified 2-5A molecules with modifications in the adenyl or ribosyl moiety designed to explore the biological role of the 2-5A synthetase/RNase L system. Our strategy for the examination of the requirements for binding to and activation of RNase L has involved backbone modification of the 2-5A molecule,

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