Energy Transduction during Catalysis by *Escherichia coli* DNA Photolyase†

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ABSTRACT: Native DNA photolyase from *Escherichia coli* contains 1,5-dihydroFAD ($FADH_2$) plus 5,-10-methenyltetrahydropteroylpolyglutamate. Quantum yield and action spectral data for thymine dimer repair were obtained by using a novel multiple turnover approach under aerobic conditions. This method assumes that catalysis proceeds via a (rapid-equilibrium) ordered mechanism with light as the second substrate, as verified in steady state kinetic studies. The action spectrum observed with native enzyme matched its absorption spectrum and an action spectrum simulated based on an energy transfer mechanism where dimer repair is initiated either by direct excitation of $FADH_2$ or by pterin excitation followed by singlet–singlet energy transfer to $FADH_2$. The quantum yield observed for dimer repair with native enzyme ($\Phi_{Native} = 0.722 \pm 0.0414$) is similar to that observed with enzyme containing only $FADH_2$ ($\Phi_{EFADH_2} = 0.655 \pm 0.0256$), as expected owing to the high efficiency of energy transfer from the natural pterin to $FADH_2$ [$E_{ET} = 0.92$]. The quantum yield observed for dimer repair decreased (2.1-fold) when the natural pterin was partially (68.8%) replaced with 5,10-CH^+-H_4folate ($\Phi_{obs} = 0.342 \pm 0.0149$). This is consistent with the energy transfer mechanism ($\Phi_{calc} = 0.411 \pm 0.0118$) since a 2-fold lower energy transfer efficiency is observed when the natural pterin is replaced with 5,10-CH^+-H_4folate ($E_{ET} = 0.46$) (Lipman & Jorns, 1992). The action spectrum observed for 5,10-CH^+-H_4folate-supplemented enzyme matched a simulated action spectrum which exhibited a small (5 nm) hypsochromic shift as compared with the absorption spectrum ($\lambda_{max} = 385$ nm). [Larger shifts are observed in simulation studies when energy transfer is very inefficient (e.g., $\Delta = 20$ nm when $E_{ET} = 0.1$).] The results are in good agreement with data previously reported for DNA photorepair in *E. coli* (Harm, 1970; Jagger et al., 1969) but differ significantly as compared with in vitro data recently reported by Payne and Sancar (1990).

DNA photolyase binds UV-damaged DNA in a dark reaction and then repairs pyrimidine dimers when the complex is irradiated with visible light (Rupert, 1962). In Michaelis–Menten terms, the reaction appears to proceed via an ordered mechanism with light as the second substrate (eq 1, [L] = light intensity). The active form of *Escherichia coli* DNA

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftarrows}} ES \underset{k_3}{\overset{k_2[L]}{\rightleftarrows}} E^*S \xrightarrow{k_4} EP \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} E + P \qquad (1)$$

photolyase contains 1,5-dihydroFAD[1] ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4Pte(Glu)$_n$, n = 3–6] (Wang et al., 1988; Wang & Jorns, 1989; Jorns et al., 1990; Johnson et al., 1988). Either chromophore can act as a sensitizer in catalysis (Sancar et al., 1987). However, 5,10-CH^+-H_4Pte(Glu)$_n$ is not required when $FADH_2$ acts as a sensitizer but $FADH_2$ is required when 5,10-CH^+-H_4Pte(Glu)$_n$ acts as sensitizer (Jorns et al., 1990,1987b). Formation of an enzyme–substrate complex causes a selective quenching of $FADH_2$ fluorescence in a reaction that is fully reversible upon dimer repair, indicating that the excited singlet state of $FADH_2$ ($^1FADH_2^*$) acts as a catalytic intermediate (Jordan & Jorns, 1988). A decrease in pterin fluorescence quantum yield is observed in the presence of $FADH_2$, accompanied by an increase in $FADH_2$ fluorescence quantum yield, providing direct evidence for singlet–singlet energy transfer from excited pterin ($^1Pte^*$) to $FADH_2$ (Lipman & Jorns, 1992). These results and others indicate that $FADH_2$ is the chromophore which directly interacts with substrate whereas 5,10-CH^+-H_4Pte(Glu)$_n$ appears to function as an antenna, harvesting light energy which is then transferred to $FADH_2$ (Scheme I).

The action spectrum for a photosensitized reaction is obtained by plotting the photolytic cross section (PC = $\Phi\epsilon_{sensitizer}$) as a function of wavelength. For a reaction involving a single sensitizer, the quantum yield (Φ) is generally wavelength-independent and the action spectrum exhibits the same shape as the sensitizer absorption spectrum. In the case of native *E. coli* photolyase, the situation is complicated by the involvement of two sensitizers, but quantum yield (eq 2) and photolytic cross section (eq 3) values can be predicted based on the mechanism in Scheme I, using values measured for the quantum yield with enzyme containing only $FADH_2$ (Φ_{EFADH_2}), the efficiency of energy transfer from pterin to $FADH_2$ (E_{ET}), and the extinction coefficients of the enzyme's chromophores [ϵ_{FADH_2}, ϵ_{Pte}, $f_b = \epsilon_{Pte}/(\epsilon_{FADH_2} + \epsilon_{Pte})$]. Equation 3 predicts that action and absorption spectra observed for native enzyme should exhibit the same shape ($\lambda_{max} = 385$ nm)

$$\Phi_{Native} = \Phi_{EFADH_2}[1 + f_b(E_{ET} - 1)] \qquad (2)$$

$$PC_{Native} = \Phi_{EFADH_2}[\epsilon_{FADH_2} + E_{ET}\epsilon_{Pte}] \qquad (3)$$

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FADH*, blue neutral FAD radical; $FADH_2$, 1,5-dihydroFAD; 5,10-CH^+-H_4Pte(Glu)$_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate (5,-10-CH^+-H_4PteGlu$_1$), 5,10-methenyltetrahydrofolate; UV-oligo(dT)$_{18}$, dimer-containing oligothymidylate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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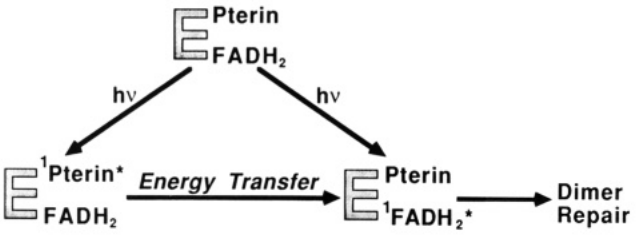
Scheme I: Proposed Energy Transfer Mechanism for DNA Photolyase



if $E_{ET} = 1$, whereas at the opposite extreme, if $E_{ET} = 0$ then the action spectrum shape should coincide with the absorption spectrum of the enzyme's $FADH_2$ component (λ_{max} = 360 nm). Recent studies with native enzyme show that energy transfer from the natural pterin to $FADH_2$ proceeds with an efficiency near unity (E_{ET} = 0.92). A 2-fold decrease in efficiency is observed when the natural pterin is replaced with 5,10-CH^+-H_4folate (E_{ET} = 0.46) (Lipman & Jorns, 1992).

In this paper we report quantum yield and action spectra studies conducted using a novel multiple turnover approach which permits rapid measurements under aerobic conditions. The method assumes that catalysis proceeds via an ordered or rapid-equilibrium ordered mechanism, as verified in steady-state kinetic studies. Quantum yield values and action spectra were obtained for a preparation of native enzyme that contained nearly stoichiometric amounts of its natural chromophores, with a preparation of pterin-depleted enzyme after enrichment with 5,10-CH^+-H_4folate and with a preparation of enzyme containing only $FADH_2$. The results with both pterin-containing preparations are in good agreement with predictions based on the mechanism in Scheme I and measured values for E_{ET} and Φ_{EFADH_2}. The results are also consistent with data obtained for DNA photorepair in early in vivo studies with *E. coli* (Harm, 1970; Jagger et al., 1969). In contrast, our results with 5,10-CH^+-H_4folate-supplemented enzyme differ significantly as compared with data recently reported (Payne & Sancar, 1990) for a similar enzyme preparation.

EXPERIMENTAL PROCEDURES

Materials. Phenyl-Sepharose CL-4B was purchased from Pharmacia. Oligo(dT)$_{18}$ was purchased from the University of Pennsylvania DNA Synthesis Service. 5-Formyltetrahydrofolate and FAD were purchased from Sigma.

Preparation of Native Enzyme. E. coli photolyase was purified similarly to a previously described procedure (Jorns et al., 1987a) and stored at –70 °C in complete PRE buffer (50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol). The absorption spectrum of fully reduced enzyme (EPteFADH$_2$) was obtained after anaerobic photoreduction of the flavin radical in the isolated enzyme with yellow light (Westinghouse, F15T8/GO), as described by Jordan and Jorns (1988). For quantum yield measurements, a stock solution of EPteFADH$_2$ was prepared by mixing the isolated enzyme with excess dithionite (5 mM) in complete PRE buffer under aerobic conditions at 0 °C. (Dithionite stock solutions were prepared under anaerobic conditions.) Unless otherwise noted, the enzyme used for quantum yield studies contained 0.73 mol of 5,10-CH^+-H_4Pte(Glu)$_n$ plus 1.0 mol of $FADH_2$/mol of protein.

Apoenzyme Preparation and Reconstitution with $FADH_2$. Apophotolyase was prepared by a chromatographic procedure and then reconstituted with $FADH_2$ as described by Jorns et al. (1990). The reconstituted enzyme was stored in complete PRE buffer at –20 °C. $FADH_2$ was prepared by reduction of FAD with excess sodium dithionite (0.03 M). The flavin radical in the isolated reconstituted enzyme was converted to $FADH_2$ by photoreduction or by treatment with dithionite, as described for native enzyme. The reconstituted enzyme contained a stoichiometric amount of flavin and exhibited spectral properties similar to that previously described (Jorns et al., 1990).

Supplementation of Pterin-Depleted Photolyase with 5,10-CH^+-H_4folate. 5,10-CH^+-H_4folate was prepared as described by Rabinowitz (1963). A preparation of pterin-depleted enzyme (0.259 mol of pterin plus 0.880 mol of FADH$^\bullet$/mol of protein) was isolated using the same procedure, which typically yields enzyme with a much higher pterin content. The preparation was supplemented with 5,10-CH^+-H_4folate as described by Wang and Jorns (1989) to yield enzyme containing 0.83 mol of pterin (68.8% 5,10-CH^+-H_4folate, 31.2% native pterin) plus 0.875 mol of FADH$^\bullet$/mol of protein. The absorption spectrum of the fully reduced enzyme was recorded after photoreduction of the flavin radical to $FADH_2$ with yellow light, similar to that described above for native enzyme, except that the reaction was conducted under aerobic conditions in the presence of excess substrate [UV-oligo(dT)$_{18}$, 2.1-fold excess], which protects the fully reduced enzyme against air oxidation (Jordan & Jorns, 1988). Dimer photorepair does not occur during flavin photoreduction with the yellow light used in these studies which was filtered to eliminate possible stray light at shorter wavelengths. For quantum yield measurements, fully reduced enzyme was prepared by reduction with dithionite, as described for native enzyme.

Substrate Preparation. Unless otherwise noted, UV-irradiated oligo(dT)$_{18}$ [UV-oligo(dT)$_{18}$, 4.0–4.2 dimers/mol of oligomer] was used as the substrate for action spectra and quantum yield studies. Dimers were introduced by irradiating oligo(dT)$_{18}$ with germicidal light, as previously described (Jorns et al., 1985). Samples were then treated with black light, as described by Chanderkar and Jorns (1991), to eliminate the near-UV absorbance and fluorescence of a minor photoproduct formed during irradiation with germicidal light (Mitchell & Clarkson, 1984). An oligomer containing four thymines with a dimer formed between the middle residues (TT$\hat{}$TT) was prepared as previously described (Rycyna et al., 1988) and used for steady-state kinetic studies.

Absorption Spectroscopy. Spectra were recorded with a Perkin-Elmer Lambda 3 spectrophotometer. For determination of chromophore content, protein concentration was determined on the basis of absorbance at 280 nm ($\epsilon_{280} = 1.00 \times 10^5$ M^{-1} cm^{-1}), after correcting for contributions from bound chromophores, as previously described (Jorns et al., 1990; Wang & Jorns, 1989). The concentration of enzyme-bound FADH$^\bullet$ was determined based on its absorbance at 580 ($\epsilon_{580} = 4.8 \times 10^3$ M^{-1} cm^{-1}) (Wang & Jorns, 1989) and used to estimate the $FADH_2$ content after reduction of native enzyme. In reconstituted enzyme containing only $FADH_2$ (E$FADH_2$), the concentration of $FADH_2$ was determined based on its absorbance at 360 nm ($\epsilon_{360} = 6.00 \times 10^3$ M^{-1} cm^{-1}) (Jorns et al., 1990) and used to calculate extinction coefficients at other wavelengths. Pterin in native or 5,10-CH^+-H_4folate-supplemented enzyme was determined based on its absorption at 380 ($\epsilon_{380} = 25.9 \times 10^3$ M^{-1} cm^{-1}), after correction for contributions due to bound flavin, as previously described (Jorns et al., 1990). Extinction coefficients for these preparations were calculated based on chromophore composition using extinction coefficients determined for reconstituted enzyme containing only $FADH_2$ or pterin (Jorns et al., 1990; Lipman & Jorns, 1992).

Action Spectra Measurements. A collimated beam of light was generated using a Photon Technology International lamp housing (Model A1000), equipped with a parabolic reflector and a 150 W xenon lamp. An Oriel water filter (l = 9.5 cm, d = 3 in.), fitted with UV-grade silica windows, was placed immediately in front of the lamp housing and cooled with tap water. The beam size was reduced from 3 to 1.9 in. by passage through Oriel UV-grade fused silica plano convex lenses positioned at 59.5 (focal length = 20.0 cm, d = 4 in.) and 92.0 (focal length = 12.5 cm, d = 3 in.) cm from the front face of the lamp housing. Intensity modulation and wavelength selection were attained by passing the beam through a filter assembly, positioned 114.2 cm from the lamp housing, that contained various Oriel neutral density filters plus narrow band pass filters (Oriel, 10 nm bandwidth). The center of the beam was focused through a 15 × 25 mm slit located at the entrance to the cuvette holder assembly. The latter was constructed by encasing a Perkin-Elmer spectrophotometer cuvette holder with 10 cm of polyurethane insulation, cooled using a circulating ethylene glycol bath, and positioned such that the front face of the cuvette was 126.0 cm from the lamp housing. Light-proof barriers were positioned at various points within the optical bench, and the entire apparatus was encased to protect samples from stray light. Light intensity (erg mm^{-2} s^{-1}) was determined by ferrioxalate actinometry (Hatchard & Parker, 1956; Bowman & Demas, 1976). Similar results were obtained in measurements conducted at the beginning and end of each experiment.

All action spectra experiments were conducted under yellow light. Except as otherwise noted, reactions were conducted at 9.5 °C in 1.0-mL stoppered cuvettes under aerobic conditions in 50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl, 1 mM EDTA, 1.6 mM DTT, excess UV-oligo(dT)$_{18}$ (11.8–14.0 μM dimer) plus dithionite-reduced native enzyme (0.47 μM), pterin-depleted enzyme supplemented with 5,10-CH$^+$-H$_4$folate (0.97 μM), apoenzyme reconstituted with $FADH_2$ (0.12 μM), or native enzyme after pterin photodestruction (0.24 or 0.47 μM). The pterin in native enzyme was destroyed by anaerobic irradiation with black light under conditions which also result in flavin radical reduction (Jorns et al., 1987b). After irradiation, the preparation was mixed with 5 mM dithionite. With all preparations, the excess dithionite prevents $FADH_2$ oxidation during manipulation and storage of stock enzyme solutions and decomposes immediately after dilution (62-fold) into aerobic reaction mixtures. (For anaerobic experiments, a specially constructed 1.0-mL cuvette was made anaerobic by bubbling with argon and then enzyme was added from a side arm.) Reaction mixtures (310 μL) were exposed to photoreactivating light, and dimer repair was monitored spectrophotometrically (Jorns et al., 1985) by following the increase in absorbance at 260 nm ($\Delta\epsilon_{260}$ = 16.6 × 10^3 M^{-1} cm^{-1}) (Naylor & Gilham, 1966) as a function of the light dose. Quantum yield values at each wavelength were determined based on the observed velocity and measured values for enzyme absorbance and light intensity (see eq 6). Values for the photolytic cross section (PC = $\Phi\epsilon$) were calculated by using observed quantum yield values and extinction coefficients determined for substrate-free enzyme. The latter are acceptable, even though complete conversion to the ES complex is required for quantum yield studies, since substrate does not affect enzyme absorption (Jordan & Jorns, 1988; Payne & Sancar, 1990).

Action Spectra Simulations. Action spectra were simulated for enzyme containing stoichiometric amounts of $FADH_2$ and pterin at various energy transfer efficiencies (E_{ET}) using eq 3 and the known spectral properties of enzyme-bound pterin and $FADH_2$. In the derivation of eq 3, it is assumed that the efficiency of dimer repair by $^1FADH_2$* in native enzyme is unaffected by the presence of the pterin chromophore and can be estimated by the efficiency observed for enzyme containing only $FADH_2$ (Φ_{EFADH_2}). It is also assumed that dimer repair with native enzyme can be initiated by direct excitation of $FADH_2$ (path a, $\Phi_a = \Phi_{EFADH_2}$) or by pterin excitation followed by singlet–singlet energy transfer (path b, $\Phi_b = E_{ET}\Phi_{EFADH_2}$). The fraction of repair occurring by paths a [$f_a = \epsilon_{FADH_2}/(\epsilon_{FADH_2} + \epsilon_{Pte})$] and b [$f_b = \epsilon_{Pte}/(\epsilon_{FADH_2} + \epsilon_{Pte})$] will depend on the relative extinction coefficients of the chromophores and will vary depending on the wavelength of photoreactivating light. The observed quantum yield for native enzyme will be a weighted average, reflecting the fraction of repair occurring by paths a and b ($\Phi_{Native} = f_a\Phi_a + f_b\Phi_b$), and can be expressed as shown in eq 2. Equation 3 is obtained by multiplying eq 2 by the extinction coefficient of the enzyme ($\epsilon_{Native} = \epsilon_{FADH_2} + \epsilon_{Pte}$). Values for ϵ_{FADH_2} and ϵ_{Pte} were estimated based on the spectral properties observed for reconstituted enzyme containing only $FADH_2$ or 5,10-CH$^+$-H$_4$folate, respectively (Jorns et al., 1990; Lipman & Jorns, 1992).

The action spectrum for the preparation of native enzyme used in these studies (0.73 mol of 5,10-CH$^+$-H$_4$Pte(Glu)$_n$ plus 1.0 mol of $FADH_2$/mol of protein) was simulated using the value for E_{ET} determined in previous studies (E_{ET} = 0.92) (Lipman & Jorns, 1992). Values for ϵ_{Pte} were corrected for the somewhat less than stoichiometric amount of pterin in the preparation. In simulating the action spectrum for pterin-depleted enzyme supplemented with 5,10-CH$^+$H$_4$folate, values for ϵ_{FADH_2} and ϵ_{Pte} were corrected based on the observed chromophore content of the preparation [0.875 mol of $FADH_2$ plus 0.830 mol of pterin (68.8% 5,10-CH$^+$-H$_4$folate, 31.2% native pterin)/mol of protein]. Assuming a random chromophore distribution, the 5,10-CH$^+$-H$_4$folate-supplemented preparation will consist of the following mixture of enzyme forms: EPteFADH$_2$ [Pte = 5,10-CH$^+$-H$_4$folate (49.97%) or 5,10-CH$^+$-H$_4$Pte(Glu)$_n$ (22.66%)]; EPte (10.38%); EFADH$_2$ (14.88%); E (2.12%). The efficiency of energy transfer for the preparation (E_{ET} = 0.53) was estimated as a weighted average of contributions from the various pterin-containing forms: E_{ET} = 0.46 or 0.92 for EPteFADH$_2$ when Pte = 5,10-CH$^+$-H$_4$folate or 5,10-CH$^+$-H$_4$Pte(Glu)$_n$, respectively (Lipman & Jorns, 1992); E_{ET} = 0 for EPte.

Steady-State Kinetics with TT^TT as Substrate. Dimer repair was monitored at 9.5 °C under anaerobic conditions by following the increase in absorbance at 260 nm ($\Delta\epsilon_{260}$ = 16.6 × 10^3 M^{-1} cm^{-1}) in reaction mixtures containing 50 mM Tris-HCl, pH 7.2, 10 mM NaCl, 1 mM EDTA, 1.6 mM DTT, TT^TT (4.0–20.1 μM), plus dithionite-reduced native enzyme (0.366 μM). Light intensity at 390 nm was varied from 5.88 to 33.2 erg mm^{-2} s^{-1}. Kinetic parameters were determined by regression analysis of double-reciprocal plots, as described in Results or by fitting the data to eq 7 using the Marquardt–Levenberg algorithm. No weights were assigned to the data points.

Binding Studies with TT^TT. Native enzyme (3.1 μM) was reduced with dithionite (12.5 mM) and then titrated with TT^TT. Binding was monitored by measuring the quenching of $FADH_2$ fluorescence emission at 509 nm (excite 390 nm), similar to that described in previous studies with enzyme containing only $FADH_2$ (Jordan & Jorns, 1988; Jordan et al., 1989), except that the data was corrected for a small (5%) increase in pterin fluorescence, which accompanies the binding of substrate to native enzyme (Lipman & Jorns, 1992). The

latter was estimated by monitoring fluorescence emission at 470 nm, a wavelength where emission from $FADH_2$ is negligible (Lipman & Jorns, 1992). Titrations were performed in 50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 20% glycerol at 5 °C. These conditions were chosen for titration studies because enzyme stability and mixing are optimized, and previous studies have shown that similar values for dissociation constants of enzyme–substrate complexes are obtained under these conditions and under the somewhat different conditions used for steady-state kinetic studies (Jordan et al., 1989).

RESULTS

Action Spectra Determination Using a Multiple Turnover Approach under Aerobic Conditions. Two approaches have previously been used to determine action spectra for dimer repair with various photolyase preparations. In the single turnover approach, a large excess of enzyme is mixed with a small amount of substrate and the rate of dimer repair is monitored at limiting light intensities (Harm & Rupert, 1970). In a second approach, repair kinetics are monitored under turnover conditions at limiting light intensities and nonsaturating substrate concentrations (Eker et al., 1986). The accuracy of quantum yield values determined by the second method has been questioned (Kiener et al., 1989) because quantum yield calculations depend on values estimated for the rate of formation (k_1) and dissociation (k_{-1}) of the enzyme–substrate complex. Both methods require that the *entire* reaction be monitored and kinetic analysis is generally time-consuming, particularly since continuous assays may not be feasible. Also, anaerobic reaction conditions are required in studies with photolyase isolated from *E. coli* because enzyme-bound $FADH_2$ is air sensitive in the absence of excess substrate (Jordan & Jorns, 1988).

In the alternate approach used in these studies, *initial* rates of dimer repair are monitored during turnover at saturating dimer and limiting light using a simple spectrophotometric assay. For the sequential mechanism shown in eq 1, the observed velocity at saturating dimer and low light intensity ([L], erg mm^{-2} s^{-1}) is equal to the quantum yield for dimer repair [$\Phi = k_4/(k_4 + k_3)$] multiplied by the rate of conversion of the ES complex to E*S (eq 4, $[ES] = [E]_{total}$). Equation 6 is derived from eq 4 using the known relationship shown in eq 5. Preliminary studies were conducted with native photolyase to determine conditions where eq 6 is valid. Dimer repair rates must be independent of substrate concentration and directly proportional to light intensity and enzyme concentration. UV-irradiated oligo(dT)$_{18}$ [UV-oligo(dT)$_{18}$, 4.0–4.2 dimers/oligomer] was selected as the substrate because it is tightly bound to photolyase ($K_d \leq 10^{-8}$) (Jordan et al., 1989) and similar rates of dimer repair were previously reported in assays using black light at dimer concentrations from 0.8 to 7.8 μM (Jorns et al., 1985). The latter was confirmed in studies using light at 380 nm ([L] = 9.95 erg $mm^{-2}s^{-1}$) where reaction rates were unaffected when the dimer concentration was varied from 4.8 to 24.2 μM. In other experiments under the same irradiation conditions, the observed repair rate at 8.7 μM dimer was directly proportional to the enzyme concentration when the latter was varied from 0.21 to 1.72 μM (correlation coefficient = 0.97, data not shown). In experiments conducted with light at 390 nm, reaction rates observed at 10.3 μM dimer were directly proportional to light intensity in the range from 29.4 to 233 erg mm^{-2} s^{-1} (correlation coefficient = 0.99, data not shown). The Michaelis constant for light intensity [$K_L = k_5(k_4 + k_3)/k_2(k_4 + k_5)$] should vary depending on wavelength since it is inversely proportional to k_2 and, therefore, inversely proportional to $\epsilon\lambda$ (see eq 5). However, limiting light intensities at 390 nm, a wavelength near the absorption maximum of native enzyme, should also be limiting at other wavelengths because values for $\epsilon\lambda$ reach a maximum near 390 nm, resulting in minimal values for K_L.

$$v_{obs} = k_2[L][E]_{total}(k_4/k_4 + k_3) = k_2[L][E]_{total}\Phi \quad (4)$$

$$k_2 = \lambda(nm)\epsilon/5.2 \times 10^9 \quad (5)$$

$$v_{obs} = [E]_{total}[L]\lambda\epsilon\Phi/5.2 \times 10^9 \quad (6)$$

All of the enzyme is converted to the enzyme–substrate complex under conditions where eq 6 is valid. Since enzyme-bound $FADH_2$ in the complex is stable against air oxidation (Jordan & Jorns, 1988), it was expected that oxygen would not interfere with measurements under these conditions. This hypothesis was confirmed in studies conducted with native enzyme (0.47 μM) or with reconstituted enzyme containing only $FADH_2$ (0.28 μM) where similar rates of dimer repair were observed under aerobic or anaerobic conditions (108% or 99.4% of aerobic rate, respectively) in experiments conducted with 11.8 μM dimer and light at 380 (13.0 erg mm^{-2} s^{-1}) or 360 (180 erg mm^{-2} s^{-1}) nm, respectively.

To evaluate the aerobic multiple turnover approach, action spectra were determined with enzyme preparations containing only $FADH_2$ ($EFADH_2$) since complications due to energy transfer from pterin are eliminated and the action spectrum should exhibit the same shape as the absorption spectrum. For these studies, $EFADH_2$ was prepared by reconstituting apoenzyme with $FADH_2$ or by photodestruction of the pterin chromophore in native enzyme as described in Experimental Procedures. Similar results were obtained using $EFADH_2$ prepared via either method, and the data from the two preparations were averaged. Both absorption and action spectra obtained for $EFADH_2$ exhibit a maximum at 360 nm, and there is fairly good agreement between the shapes of the two spectra (Figure 1). Quantum yield values determined at nine different wavelengths in the range from 320 to 426 nm are reasonably constant (Table I). The average quantum yield observed in these studies ($\Phi_{EFADH_2} = 0.655 \pm 0.0256$) is in good agreement with a value previously determined (Payne & Sancar, 1990) using the single turnover method under anaerobic conditions ($\Phi_{EFADH_2} = 0.689 \pm 0.091$).

Action Spectrum of Native Enzyme. Studies with native enzyme were conducted with a preparation containing 0.73 mol of pterin/mol of protein plus a stoichiometric amount of flavin. The absorption spectrum of the fully reduced enzyme ($EPteFADH_2$), determined after photoreduction of the flavin radical in the isolated enzyme, exhibited a peak at 380 nm (Figure 2). (A maximum at 385 nm is expected for enzyme containing equimolar amounts of both chromophores.) The absorption spectrum observed for native enzyme after photoreduction is virtually superimposable with a spectrum calculated on the basis of the chromophore content of the preparation and spectral properties observed for reconstituted enzyme containing only $FADH_2$ or pterin (Figure 2, inset). The results rule out possible ground-state interactions between the chromophores in native enzyme that might affect chromophore absorption (e.g., charge transfer complex).

Quantum yield values determined for native enzyme at nine different wavelengths are reasonably constant (Table II), except for deviations near the extremes of the tested range

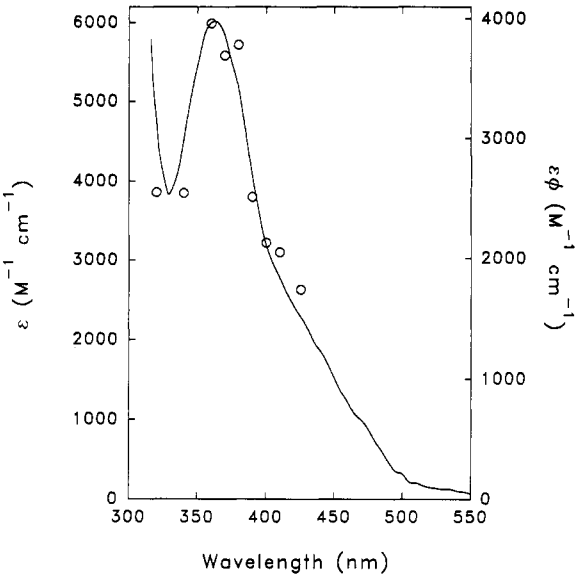


FIGURE 1: Comparison of absorption and action spectra obtained for enzyme containing only $FADH_2$ ($EFADH_2$). The solid curve is the absorption spectrum of $EFADH_2$ in 50 mM Tris-HCl, pH 7.4, containing 18 mM NaCl, 1 mM EDTA, 10 mM DTT, and 20% glycerol at 5 °C. Photolytic cross section values (open circles) were calculated by using extinction coefficients determined with reconstituted enzyme and quantum yield values averaged from studies using $EFADH_2$ prepared via apoenzyme reconstitution or pterin photodestruction, as described in the text.

Table I: Efficiency of Dimer Repair with Enzyme Containing only $FADH_2$

wavelength, nm	intensity, erg mm^{-2} s^{-1}	ϵ, M^{-1} cm^{-1}	Φ_{EFADH_2}
426	100.2	2300	0.756
410	208.5	2780	0.737
400	250.0	3200	0.665
390	209.9	4070	0.618
380	175.6	5170	0.731
370	159.5	5840	0.632
360	130.5	6000	0.660
340	111.0	4500	0.566
320	116.0	4780	0.534
			av 0.655 ± 0.0256

(320–426 nm), similar to that observed for $EFADH_2$. On the basis of the mechanism in Scheme I, native enzyme should exhibit wavelength-dependent quantum yield values. However, calculations using eq 2 and known values for E_{ET} and f_b show that a less than 3% variation in quantum yield values is actually expected for native enzyme in the tested wavelength range. The average quantum yield observed for native enzyme (Φ_{Native} = 0.722 ± 0.0414) is not significantly different from that observed for enzyme containing only $FADH_2$ (Φ_{EFADH_2} = 0.655 ± 0.0256). This result is not surprising, given the error level of our measurements, since a quantum yield value calculated for $EFADH_2$ ($\Phi_{EFADH_2(calc)}$ = 0.767) using eq 2 and the observed value for Φ_{Native} is only 6% larger than the value obtained for native enzyme.

The action spectrum obtained for native enzyme (Figure 2, open circles) exhibits a peak at 380 nm. Its shape matches the enzyme's absorption spectrum (Figure 2, solid curve) and an action spectrum simulated based on the mechanism in Scheme I and the known efficiency of energy transfer from the native pterin to $FADH_2$ (E_{ET} = 0.92) (Figure 2, closed triangles). The photolytic cross section values observed with native enzyme superimpose with a simulated spectrum calculated by using a value for Φ_{EFADH_2} (0.77) that is 18% larger than the observed value.

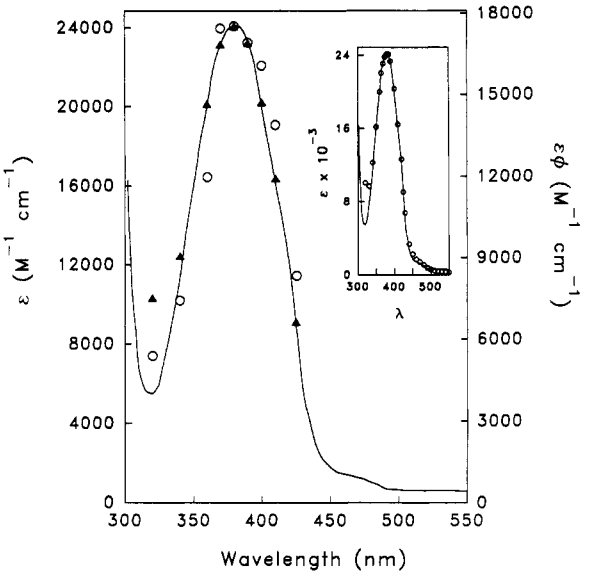


FIGURE 2: Comparison of absorption and action spectra obtained for native enzyme ($EPteFADH_2$). The solid curve is the absorption spectrum of native enzyme in 50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 50% glycerol at 5 °C obtained after yellow light photoreduction of the flavin radical in the isolated enzyme. Photolytic cross section values for native enzyme (open circles) were determined as described in the text using the data in Table II. A simulated action spectrum (solid triangles) was calculated, using eq 3 with E_{ET} = 0.92 (Lipman & Jorns, 1992) and Φ_{FADH_2} = 0.77. The latter is 18% larger than the observed value (Table I) but was chosen to facilitate comparison of the shapes of observed versus simulated action spectra. The inset compares the absorption spectrum observed for native enzyme (solid trace) with a spectrum calculated (open circles) using extinction coefficients determined with reconstituted enzyme preparations containing only $FADH_2$ or pterin, as described in the text.

Table II: Efficiency of Dimer Repair with Native Photolyase

wavelength, nm	intensity, erg mm^{-2} s^{-1}	ϵ, M^{-1} cm^{-1}	Φ_{Native}
426	30.5	9070	0.918
410	17.8	16400	0.844
400	16.8	20300	0.791
390	14.8	23300	0.726
380	12.4	24100	0.728
370	20.5	23100	0.756
360	26.2	20000	0.599
340	40.1	12300	0.604
320	80.7	10100	0.534
			av 0.722 ± 0.0414

Action Spectrum of Pterin-Depleted Photolyase Supplemented with 5,10-CH$^+$-H$_4$folate. The results obtained with native enzyme differ significantly from data recently reported (Payne & Sancar, 1990) for a preparation of pterin-depleted photolyase that had been supplemented with 5,10-CH$^+$-H$_4$folate to yield enzyme containing stoichiometric amounts of $FADH_2$ and pterin (50–80% 5,10-CH$^+$-H$_4$folate). Although efficient energy transfer was estimated for the 5,10-CH$^+$-H$_4$folate-supplemented preparation based on photolytic cross section data (E_{ET} = 0.805), the action spectrum (λ_{max} = 366 nm, see Figure 3, open squares) did not match the enzyme's absorption spectrum but instead more closely resembled the spectrum of the $FADH_2$ component (λ_{max} = 360 nm), a feature expected for the mechanism in Scheme I only in the case of inefficient energy transfer. For example, action spectra calculated in simulation studies exhibit maxima that shift from 360 to 380 nm as E_{ET} is increased from 0 to 0.4 (Figure 3, inset). A maximum at 365 is observed when E_{ET} = 0.1 (see Figure 3, dashed spectrum). When $E_{ET} \geq 0.8$,

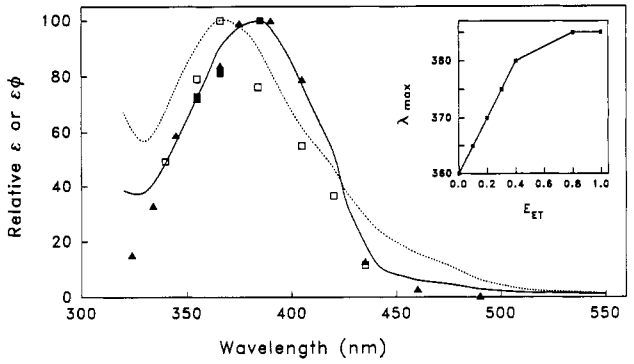


FIGURE 3: Action spectra simulations and comparison of native enzyme absorption with the action spectrum for in vivo DNA photorepair. The solid trace is the absorption spectrum calculated for enzyme containing stoichiometric amounts of pterin and $FADH_2$ (λ_{max} = 385 nm, ϵ_{385} = 31260 M^{-1} cm^{-1}). The absorption spectrum superimposes with an action spectrum (not shown) simulated for the case where E_{ET} = 1.0 when the spectra are normalized to 100% at 385 nm. The dashed trace is an action spectrum simulated for the case where E_{ET} = 0.1, normalized to 100% at its maximum (λ_{max} = 365 nm). Relative photolytic cross section data (normalized at 390 nm) reported by Jagger et al. (1969) for photoreactivation in *E. coli* strain B_{s-1} are plotted as solid squares. Absolute photolytic cross section data obtained by Harm (1970) with *E. coli* strain B_{s-1} at 385, 365, and 355 nm ($\epsilon\Phi$ = 24000, 19500, and 17500 M^{-1} cm^{-1} at 385, 366, and 355 nm, respectively) were normalized to 100% at 385 nm and plotted as solid triangles. In vitro photolytic cross section data reported by Payne and Sancar (1990) for 5,10-CH^+-H_4folate-supplemented enzyme were normalized to 100% at 366 nm (λ_{max} = 366 nm, $\epsilon\Phi$ = 19460 M^{-1} cm^{-1}) and plotted as open squares. The inset shows a plot of maxima in action spectra simulated for enzyme containing stoichiometric amounts of pterin and $FADH_2$ using various values for E_{ET}.

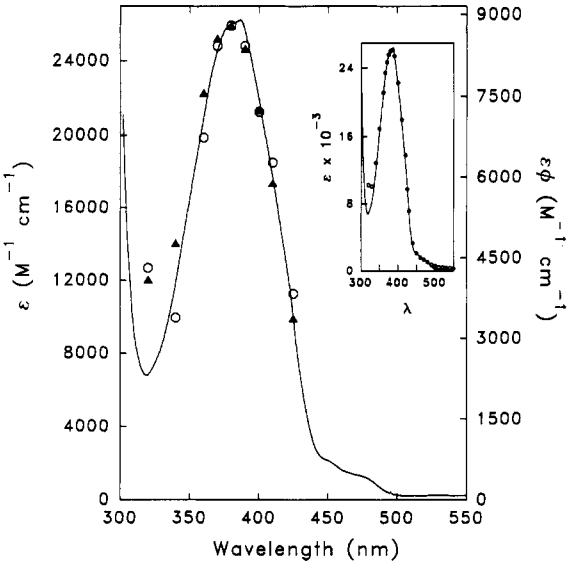


FIGURE 4: Comparison of action and absorption spectra obtained for 5,10-CH^+-H_4folate-supplemented enzyme [EPte(supp)$FADH_2$]. The solid curve is the absorption spectrum of 5,10-CH^+-H_4folate-supplemented enzyme in 50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 50% glycerol at 5 °C obtained after yellow light photoreduction of the flavin radical in the isolated enzyme. The observed action spectrum (open circles) was determined as described in the text using the data in Table III. A simulated action spectrum (solid triangles) was calculated using eq 3 with E_{ET} = 0.53, estimated as described in the text, and Φ_{FADH_2} = 0.554. The latter value is 15% smaller than the observed value (Table I) but was chosen to facilitate comparison of the shapes of observed versus simulated action spectra. The inset compares the absorption spectrum observed for 5,10-CH^+-H_4folate-supplemented enzyme (solid trace) with a spectrum calculated (open circles) using extinction coefficients determined with reconstituted enzyme preparations containing only $FADH_2$ or pterin, as described in the text.

simulated action spectra exhibit a maximum at 385 nm and a shape virtually indistinguishable from the absorption spectrum.

The efficiency of energy transfer from pterin to $FADH_2$ is decreased 2-fold when the natural pterin (E_{ET} = 0.92) is replaced with 5,10-CH^+-H_4folate (E_{ET} = 0.46) (Lipman & Jorns, 1992). However, simulation studies suggested that this difference should cause only a minor perturbation in the shape of the action spectrum and prompted studies to reevaluate the action spectrum with 5,10-CH^+-H_4folate-supplemented enzyme. A preparation of pterin-depleted enzyme (0.259 mol of pterin plus 0.880 mol of flavin/mol of protein) was supplemented with 5,10-CH^+-H_4folate to yield enzyme containing 0.830 mol of pterin (68.8% 5,10-CH^+-H_4folate, 31.2% native pterin) plus 0.875 mol of flavin/mol of protein. The absorption spectrum, observed for the 5,10-CH^+-H_4folate-supplemented preparation [EPte(supp)$FADH_2$] after photoreduction of the flavin radical in the isolated enzyme, exhibited a maximum at 385 nm, as expected based on the chromophore content of the preparation (see Figure 4, inset).

Similar quantum yield values were obtained for the preparation of 5,10-CH^+-H_4folate-supplemented enzyme at various wavelengths in the range from 320 to 426 nm (Table III). The average quantum yield value ($\Phi_{EPte(supp)FADH_2}$ = 0.342 ± 0.0149) is 2.1-fold smaller than observed for native enzyme (Φ_{native} = 0.722 ± 0.0414). Quantum yield values observed for 5,10-CH^+-H_4folate-supplemented enzyme are in fairly good agreement with values calculated using eq 2 (Φ_{calc} = 0.411 ± 0.0118, see Table III). In these calculations, observed values for Φ_{EFADH_2} (Table I) were used, and the efficiency of energy transfer (E_{ET} = 0.53) was estimated as a weighted average of contributions from the various pterin-containing species present in the preparation of 5,10-CH^+-H_4folate-supplemented enzyme, as described in Experimental Procedures. The action spectrum observed for 5,10-CH^+-H_4folate-supplemented enzyme (Figure 4, open circles) and a simulated action spectrum (Figure 4, solid triangles) both exhibit a peak at 380 nm, whereas the absorption maximum appears at a slightly longer wavelength (λ_{max} = 385 nm). Although the small hypsochromic shift in the action spectrum is apparent in high-resolution simulation studies (not shown), the 5-nm difference is beyond the resolution of the observed action spectra data.

Table III: Efficiency of Dimer Repair with 5,10-CH^+-H_4folate-Supplemented Enzyme

wavelength, nm	intensity, erg mm^{-2} s^{-1}	ε, M^{-1} cm^{-1}	Φ_{obs}	Φ_{calc}[a]
426	25.3	9700	0.394	0.473
410	19.7	18000	0.349	0.436
400	15.4	22300	0.324	0.391
390	9.75	25400	0.331	0.367
380	8.16	26000	0.338	0.446
370	12.1	24700	0.341	0.395
360	14.0	21200	0.319	0.426
340	26.9	12800	0.264	0.381
320	24.0	10200	0.421	0.385
			av 0.342 ± 0.0149	0.411 ± 0.0118

[a] Values were calculated as described in the text.

Steady-State Kinetics with TT^TT. The approach used in these studies to determine quantum yields and action spectra assumes that catalysis proceeds via an ordered mechanism, as suggested by previous studies (Rupert, 1962). Although various alternate mechanisms would appear incompatible with the fact that the dimer is known to react with a very short-

lived excited state species ($^1FADH_2$*, τ = 1.4 ns) (Kim et al., 1991; Jordan & Jorns, 1988), the steady-state kinetics of the enzyme have never been directly evaluated in studies where both substrates, dimer and light, are varied. An oligomer containing four thymines with a dimer formed between the middle residues (TT^TT) was selected for these studies since turnover can be monitored using a simple spectrophotometric assay at concentrations of TT^TT near its apparent Michaelis constant. A more sensitive assay would be required for comparable studies with UV-oligo(dT)$_{18}$, which is more tightly bound to photolyase, although similar reaction rates are observed at saturating concentrations of either substrate (Jordan et al., 1989). Light intensity was varied over a range (5.88–33.2 erg mm^{-2} s^{-1} at 390 nm) which permitted measurements at native enzyme concentrations comparable to those used for action spectra studies. Reactions were conducted under anaerobic conditions since enzyme-bound $FADH_2$ is air sensitive in the absence of excess dimer substrate.

Linear double-reciprocal plots were obtained with TT^TT as the variable substrate and light as the variable fixed substrate, as judged by regression analysis. Secondary plots of the slopes and intercepts versus the reciprocal of light intensity yielded straight lines which passed through the origin (Figure 5A, inset). Double-reciprocal plots with light as the variable substrate and TT^TT as the variable fixed substrate were linear and yielded a family of lines intersecting at the origin. A secondary plot of slopes versus the reciprocal of TT^TT concentration was linear with a positive intercept on the $1/v$ axis and a negative intercept on the horizontal axis (Figure 5B, inset). The observed patterns are consistent with a rapid-equilibrium ordered mechanism where the second substrate, light, is limiting ([L] $\ll K_L$) (eq 7, [S] = [TT^TT]).

$$[E]_{total}/v = 1/k_{cat}(K_L/[L] + K_{iS}K_L/[L][S]) \quad (7)$$

Values for K_{iS} and K_L/k_{cat} were estimated from linear regression analysis of the secondary plots (correlation coefficients >0.96) or by fitting the data directly to eq 7. Similar results were obtained by either method (Table IV). The lines drawn in the primary double-reciprocal plots in Figure 5 show the fit of the data to eq 7.

In studies with UV-oligo(dT)$_{18}$, quantum yield values were determined based on the velocity observed at saturating dimer and limiting light intensity where $v_{obs} = [E]_{total}[L]k_{cat}/K_L$. The quantum yield for dimer repair with TT^TT as substrate was estimated by using values obtained for K_L/k_{cat} from regression analysis (Φ = 0.571 ± 0.043) or by fitting the steady state data to eq 7 (Φ = 0.551 ± 0.041). The results obtained with TT^TT are fairly comparable to those observed with UV-oligo(dT)$_{18}$ (Φ = 0.722 ± 0.0414). Our results with native enzyme agree with previous studies with $EFADH_2$ where similar quantum yield values were obtained for dimer repair with oligothymidylate substrates of varying chain length (Kim & Sancar, 1991).

The data in Figure 5A suggest that it should be possible to estimate K_{iS} by varying [TT^TT] at a single light intensity since double-reciprocal plots of velocity versus [TT^TT] at various light intensities appear to form a family of lines intersecting the horizontal axis at $1/[TT^TT] = -1/K_{iS}$. Consistent with this hypothesis, a value estimated for K_{iS} from previous studies with TT^TT at a fixed intensity of black light (K_{iS} = 8.0 μM at [I] $\approx$ 450 erg mm^{-2} s^{-1}) (Jordan et al., 1989) is similar to results obtained in the present studies (K_{iS} = 8.89 or 8.51 μM, depending on the analysis method) where data were collected over a range of much lower light intensities (5.88–33.2 erg mm^{-2} s^{-1}). However, these estimates for K_{iS} are more than 1 order of magnitude larger than a value previously reported for the dissociation constant of the complex formed with $EFADH_2$ and TT^TT (K_d = 0.37 μM) (Jordan et al., 1989). Since K_{iS} should correspond to the dissociation constant of the enzyme·TT^TT complex, studies were conducted to determine whether the complex was less stable when formed with native enzyme instead of $EFADH_2$. Similar values for the dissociation constant were obtained in two separate titration experiments with TT^TT and native enzyme [K_d = 0.96 ± 0.037 μM (trial 1) or 0.98 ± 0.013 μM (trial 2)]. In each case, the data gave a good fit when compared with simulated titration curves, as illustrated in Figure 6 with the data from trial 1. The value obtained for the dissociation constant with native enzyme, although somewhat larger than

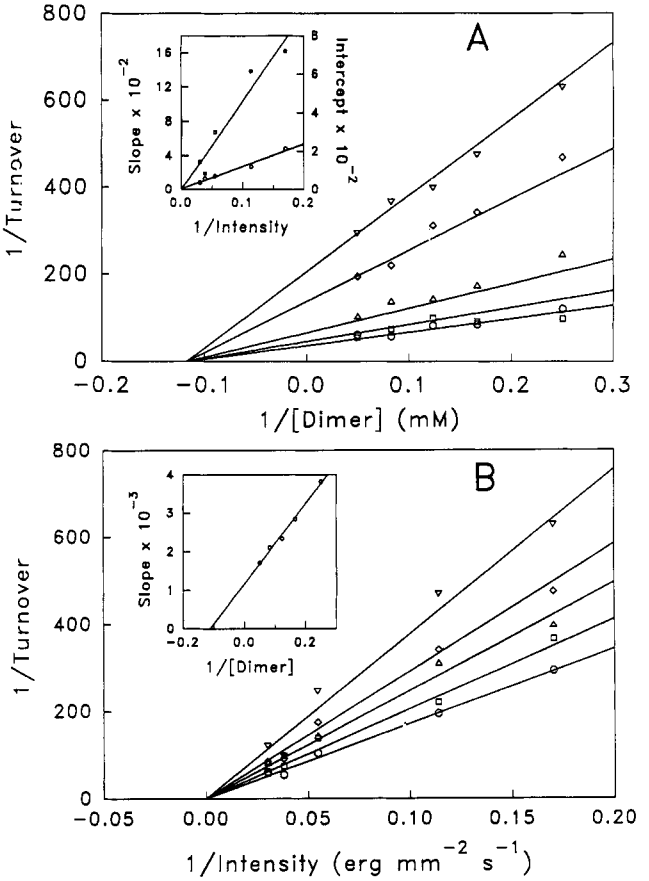


FIGURE 5: Steady-state kinetics with TT^TT as substrate. Turnover numbers [mol of dimer repaired/(s·mol of enzyme)] were measured under anaerobic conditions using light at 390 nm, as detailed in Experimental Procedures. Panel A shows a double-reciprocal plot of turnover number versus TT^TT concentration at various light intensities [33.2 (circles), 26.3 (squares), 18.3 (triangles up), 8.78 (diamonds), and 5.88 (triangles down) erg mm^{-2} s^{-1}, respectively]. Panel B shows a double-reciprocal plot of turnover number versus light intensity at various TT^TT concentrations [20.1 (circles), 12.1 (squares), 8.1 (triangles up), 6.0 (diamonds), and 4.0 (triangles down) μM, respectively]. The lines drawn in the primary plots are based on a fit of the data to eq 7. The slopes and intercepts plotted in the insets were determined from linear regression analysis of the data in the primary plots.

Table IV: Steady-State and Quantum Yield Values for Dimer Repair with TT^TT as Substrate[a]

parameter	regression analysis	fit to eq 7
K_L/k_{cat}, erg mm^{-2}	1170 ± 87.8	1210 ± 89.4
K_{iS}, mM	8.89 ± 1.15	8.51 ± 1.08
Φ	0.571 ± 0.043	0.551 ± 0.041

[a] Values were determined by regression analysis or by fitting the data to eq 7, as described in the text.

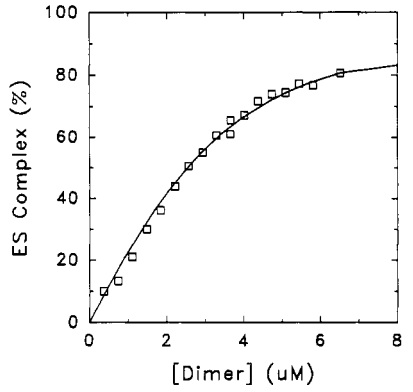


FIGURE 6: Titration of native enzyme (EPteFADH$_2$) with TT^TT. Binding of TT^TT to native enzyme (3.1 μM) was monitored by following the quenching of FADH$_2$ fluorescence emission at 509 nm, as detailed in Experimental Procedures. Excess UV-oligo(dT)$_{18}$ (5.5 μM with respect to dimer) was added near the end of the titration ([E·TT^TT] > 80%[E]$_{total}$) to determine the end point. The data are shown by open squares. The solid trace is a titration curve calculated for case where K_d = 0.96 μM.

observed with EFADH$_2$, is still significantly smaller than the values estimated for K_{iS}. The reason for this discrepancy is unclear.

DISCUSSION

The action spectrum observed for a preparation of native *E. coli* photolyase matched the enzyme's absorption spectrum and an action spectrum simulated based on the mechanism in Scheme I. The quantum yield observed for dimer repair (Φ_{Native} = 0.722 ± 0.0414) is not significantly different from that observed with enzyme containing only FADH$_2$ (Φ_{EFADH_2} = 0.655 ± 0.0256). In fact, only a small (6%) difference in dimer repair efficiency is predicted for these enzyme forms owing to the high efficiency observed for energy transfer from the natural pterin to FADH$_2$ [E_{ET} = 0.92 (Lipman & Jorns, 1992)]. Studies to evaluate the effect of substituting the natural pterin with 5,10-CH$^+$-H$_4$folate were prompted, in part, by the 2-fold lower energy transfer efficiency observed with 5,10-CH$^+$-H$_4$folate (E_{ET} = 0.46) (Lipman & Jorns, 1992). For these studies, a preparation of pterin-depleted enzyme was supplemented with 5,10-CH$^+$-H$_4$folate to yield enzyme containing equimolar amounts of pterin (68.8% 5,10-CH$^+$-H$_4$folate) and FADH$_2$. The efficiency of energy transfer (E_{ET} = 0.53) was estimated as a weighted average of contributions from the various pterin-containing forms in the preparation. Although 2.1-fold smaller than observed with native enzyme, the quantum yield observed for 5,10-CH$^+$-H$_4$folate-supplemented enzyme ($\Phi_{EPte(supp)FADH_2}$ = 0.342 ± 0.0149) was in fairly good agreement with a value calculated (Φ_{calc} = 0.411 ± 0.0118) based on the mechanism in Scheme I. The action spectrum observed for 5,10-CH$^+$-H$_4$folate-supplemented enzyme matched a simulated action spectrum which exhibited a small (5 nm) hypsochromic shift as compared with the absorption spectrum (λ_{max} = 385 nm). That a substantial decrease in the efficiency of energy transfer had only a minor effect on the position of the action spectrum is consistent with simulation studies which show that pronounced changes are expected only in the case of very inefficient energy transfer (e.g., λ_{max} = 365 nm when E_{ET} = 0.1). This property derives from the fact that both action and absorption spectra are dominated by the pterin, owing to the chromophore's higher extinction (λ_{max} = 390 nm, ϵ_{390} = 26370 M^{-1} cm^{-1}) as compared with FADH$_2$ (λ_{max} = 360 nm, ϵ_{360} = 6000 M^{-1} cm^{-1}).

The shape of the action spectrum previously observed (Jagger et al., 1969; Harm, 1970) for DNA repair in in vivo studies with *E. coli* (Figure 3, filled symbols) matches the absorption spectrum calculated for enzyme containing equimolar amounts of FADH$_2$ and pterin (Figure 3, solid curve, λ_{max} = 385 nm). Quantum yield values for in vivo photorepair were calculated using photolytic cross section data reported by Harm (1970) and extinction coefficients determined for enzyme containing stoichiometric amounts of pterin and FADH$_2$. Similar quantum yield values were obtained at different wavelengths (Φ = 0.768, 0.698, and 0.773 at 385, 366, and 355 nm, respectively) and the average value ($\Phi_{in\ vivo}$ = 0.746) is in remarkably good agreement with the value observed with native enzyme (Φ_{Native} = 0.722). The results indicate that efficient interchromophore energy transfer occurs during in vivo photorepair, similar to that observed with the isolated enzyme.

An action spectrum recently reported for a preparation of 5,10-CH$^+$-H$_4$folate-supplemented enzyme (λ_{max} = 366 nm) more closely resembles the absorption spectrum of the enzyme's FADH$_2$ component and differs significantly as compared with the spectrum simulated on the basis of the mechanism in Scheme I. A mechanism involving exciplex formation was suggested to account for this discrepancy (Payne & Sancar, 1990). However, fluorescence emission maxima of enzyme-bound FADH$_2$ (λ_{max} = 509 nm) and pterin (λ_{max} = 470 nm) are the same in preparations containing one or both chromophores, and the latter preparations fail to exhibit a new red-shifted emission band expected in the case of exciplex formation (Lipman & Jorns, 1992; Jorns et al., 1990). Using a different method, the action spectrum obtained in the present studies with a similar enzyme preparation was in good agreement with the simulated action spectrum. It is unlikely that the difference in results is attributable to the difference in the methodology (i.e., aerobic multiple turnover versus anaerobic single turnover) since, in the case of enzyme containing only FADH$_2$, similar action spectra and quantum yield values (Φ_{EFADH_2} = 0.655 ± 0.0256 or 0.689 ± 0.091, respectively) were obtained in the two studies.

The aerobic multiple turnover approach used in these studies assumes that catalysis proceeds via an ordered mechanism with light as the second substrate. Results obtained in steady-state kinetic studies with TT^TT and light at 390 nm (5.88–33.2 erg mm^{-2} s^{-1}) are consistent with a rapid-equilibrium ordered mechanism where the second substrate, light, was limiting ([L] ≪ K_L) (eq 7). In studies with UV-oligo(dT)$_{18}$ and light at 390 nm, the observed rate of dimer repair was directly proportional to light intensity when the latter was varied from 29.4 erg mm^{-2} s^{-1} to a value (233 erg mm^{-2} s^{-1}) close to the upper limit of the light source. The results suggest that the Michaelis constant for light intensity is quite large, in apparent disagreement with a previous claim (Sancar et al., 1987) that light at 384 nm is saturating at 70 erg mm^{-2} s^{-1}. A rough estimate for K_L (1200 erg mm^{-2} s^{-1}) can be calculated using the value obtained in this study for K_L/k_{cat} with TT^TT as substrate and a value recently reported for k_{cat} (50 min^{-1}) with an unspecified substrate (Li & Sancar, 1991). In previous studies with various oligothymidylate substrates, including UV-oligo(dT)$_{18}$, it was assumed that black light ($\lambda_{max} \approx$ 355 nm) was saturating at an intensity of about 450 erg mm^{-2} s^{-1} (Jordan et al., 1989). This assumption is probably incorrect, as judged by results obtained in the present studies, particularly since values for K_L are expected to reach a minimum near 390 nm.

The steady-state data with TT^TT clearly rule out various alternate mechanisms, such as ping-pong or random mechanisms. However, as pointed out by Huang (see appendix in Kwiatkowski et al., 1990), an ordered two-substrate reaction can exhibit apparent rapid-equilibrium ordered kinetics under certain conditions (e.g., $K_{iB} \ll K_B$, where B is the second substrate). Also, the rate equation for an ordered mechanism ($[E]_{total}/v = 1/k_{cat}(1 + K_A/[A] + K_B/[B] + K_{iA}K_B/[A][B])$ will reduce to a form equivalent to eq 7 if $[B] \ll K_B$ and $K_{iA} = K_A$.

Various studies can be conducted to distinguish between a true versus pseudo rapid-equilibrium ordered mechanism. However, for the present investigation, this distinction is relevant only if it affects quantum yield and action spectra calculations. To evaluate this possibility, it was assumed that dimer binding in the mechanism shown in eq 1 achieves rapid equilibrium during turnover whereas all other species reach steady-state concentrations. The rate equation, derived by using the method described by Cha (1968), exhibited the format expected for a rapid-equilibrium ordered mechanism ($[E]_{total}/v = 1/k_{cat}(1 + K_L/[L] + K_LK_{iS}/[L][S])$. Interestingly, expressions for $k_{cat}[k_4k_5/(k_4 + k_5)]$ and $K_L[k_5(k_4 + k_3)/k_2(k_4 + k_5)]$ were identical to those obtained for the ordered mechanism. As a consequence, the same expression was obtained for either mechanism when the velocity at saturating dimer and limiting light ($v = [E]_{total}[L]k_{cat}/K_L$) was expressed in terms of rate constants (see eq 4). This means that the aerobic multiple turnover method for quantum yield and action spectra determination is applicable for both ordered and rapid-equilibrium ordered mechanisms.

In summary, we show that quantum yield and action spectral data for dimer repair with various forms of *E. coli* DNA photolyase can be obtained using a novel multiple turnover approach under aerobic conditions. The data are fully consistent with predictions based on a singlet–singlet energy transfer mechanism where the enzyme's pterin chromophore acts as an antenna to harvest light energy, which is then transferred to the $FADH_2$ reaction center.

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