Evidence for a Singlet Intermediate in Catalysis by *Escherichia coli* DNA Photolyase and Evaluation of Substrate Binding Determinants†

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ABSTRACT: *Escherichia coli* DNA photolyase contains 1,5-dihydro-FAD ($FADH_2$) plus 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate). Both chromophores are fluorescent, and either can function as a sensitizer in catalysis. At 77 K separate fluorescence emission bands are observed for $FADH_2$ (λ_{max} = 505 nm, shoulder at 540 nm) and 5,10-CH^+-H_4folate (λ_{max} = 465, 440 nm) whereas at 5 °C only a shoulder at 505 nm is attributable to $FADH_2$. Formation of an enzyme–substrate complex with various dimer-containing oligothymidylates [UV-oligo$(dT)_n$] quenches the fluorescence due to $FADH_2$ at 5 °C or 77 K and also stabilizes $FADH_2$ against air oxidation. The fluorescence of 5,10-CH^+-H_4folate is unaffected by substrate. Reduction of the pterin chromophore eliminates the chromophore's fluorescence but does not affect catalytic activity or the ability of substrate to quench $FADH_2$ fluorescence. Quenching of $FADH_2$ fluorescence is fully reversible upon dimer repair. The results are consistent with the proposal that the singlet state of $FADH_2$ functions as an intermediate in catalysis. Fluorometric titrations indicate that the enzyme has a similar affinity for dimers in UV-oligo$(dT)_4$ ($K_D = 2.5 \times 10^{-7}$ M, ΔG = 8.4 kcal/mol at 5 °C) or UV-oligo$(dT)_6$, except for dimers located at the unphosphorylated 3′ end of the oligomers where binding is considerably weaker. The binding affinity observed with higher homologues [oligo$(dT)_n$, n = 9, 18] ($K_D \leq 10^{-8}$ M) is within the range previously reported for the binding of photolyase to dimers in DNA ($K_D \sim 10^{-8}$ M, ΔG = 10 kcal/mol). Studies with UV-oligo$(dT)_2$ provide an estimate of enzyme binding affinity for the unit $p\overline{TpT}$ ($K_D = 5.5 \times 10^{-5}$ M, ΔG = 5.4 kcal/mol) and indicate that about 50% of the binding energy observed with DNA as substrate can be attributed to the binding of the enzyme to this unit. A 1:1 binding stoichiometry with respect to dimer was observed except when the substrate tested contained more than 1 dimer per molecule. Binding affinity was then influenced by the ability of a substrate molecule to accommodate more than 1 molecule of photolyase. The latter was possible with UV-oligo$(dT)_{18}$ but not with UV-oligo$(dT)_9$.

Exposure of DNA to ultraviolet light results in the formation of cyclobutane dimers between adjacent pyrimidine residues. The dimers can be repaired by DNA photolyase in a rather unusual enzymatic reaction since catalysis requires visible light. The enzyme isolated from *Escherichia coli* contains two chromophores: a blue neutral FAD radical ($FADH^•$)[1] (Jorns et al., 1984) plus a second chromophore that has recently been identified as a pterin derivative (Wang et al., 1988; Johnson et al., 1988). The available evidence suggests that the pterin chromophore is 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) (Johnson et al., 1988). The catalytically and physiologically significant form of *E. coli* photolyase contains fully reduced flavin (1,5-dihydro-FAD, $FADH_2$) (λ_{max} = 360 nm) plus 5,10-CH^+-H_4folate) (λ_{max} = 390 nm) (Jorns et al., 1987a,b; Sancar et al., 1987a). This form is generated upon reduction of the flavin radical in the isolated enzyme with dithionite (Jorns et al., 1987a) or by photochemical reduction (Jorns et al., 1987b). Both chromophores in the catalytically significant form of photolyase are fluorescent (Jorns et al., 1987b). Our previous studies have shown that either $FADH_2$ or 5,10-CH^+-H_4folate can act as a photosensitizer in catalysis (Sancar et al., 1987a). Model studies indicate that the photosensitized cleavage of pyrimidine dimers is likely to involve electron transfer between an excited sensitizer and a pyrimidine dimer (Lamola, 1972). The nature of the excited state (singlet versus triplet) and the direction of the electron transfer are found to vary, depending on the nature of the sensitizer.

Evidence to determine whether singlet-state photochemistry might be important in the photolyase reaction was sought by examining the effect of dimer-containing oligothymidylates [UV-oligo$(dT)_n$] on the fluorescence of the enzyme's chromophores. [Turnover numbers obtained with UV-oligo$(dT)_n$ ($n \geq 4$) are identical with that observed with plasmid DNA as substrate (Jorns et al., 1985)]. In this paper we show that formation of an enzyme–substrate complex with UV-oligo-$(dT)_n$ results in a selective quenching of $FADH_2$ fluorescence. This observation, along with other data, supports the proposal that the singlet state of $FADH_2$ may be important as an intermediate in catalysis. Results obtained in fluorometric titrations with UV-oligo$(dT)_n$ provide information about binding energy determinants and the size of the contact site of the enzyme on the oligonucleotide.

Experimental Procedures

Materials. Oligothymidylates [oligo$(dT)_n$, n = 2, 4, 6, 9, 18] were obtained from P-L Biochemicals. Molecular weight protein standards from Pharmacia were a gift from Dr. John Swaney. Quinine sulfate dihydrate (standard reference ma-

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^•$, blue neutral FAD radical; $FADH_2$, 1,5-dihydro-FAD; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; oligo$(dT)_n$, oligothymidylate; UV-oligo$(dT)_n$, dimer-containing oligothymidylate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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terial 936) was obtained from The National Bureau of Standards. Fluorescein was obtained from Sigma.

Enzyme and Substrates. Purification of the blue form of *E. coli* photolyase was performed as previously described (Jorns et al., 1987a). Unless otherwise specified, all handling of the enzyme was done under yellow light. Dimer-containing oligothymidylates were prepared as previously described (Jorns et al., 1985). Dimer content and the percentage of dimers at the 3′ end of the oligomer were calculated as described by Jorns et al. (1985). Enzyme activity was measured with UV-oligo(dT)$_{18}$ as substrate, according to the method of Jorns et al. (1985). The concentration of the isolated enzyme was calculated on the basis of its absorbance at 580 nm [ϵ_{580} = 3.6 × 10^3 M^{-1} cm^{-1} (Jorns et al., 1984)].

Fluorescence and Phosphorescence Studies. Fluorescence and phosphorescence measurements were made with a Perkin-Elmer LS-5 luminescence spectrometer. Unless otherwise noted, fluorescence emission spectra at 5 °C were recorded by using a cuvette with a 1-cm path length. An EPR tube (i.d. = 3 mm) was used for fluorescence and phosphorescence measurements at 77 K. Low-temperature measurements were performed by using a liquid nitrogen filled optical Dewar flask that was specially designed to fit into the normal cell holder of the instrument. For fluorescence spectra recorded in an EPR tube at 5 °C the liquid nitrogen compartment of the optical Dewar flask was left empty and the temperature was maintained with the thermostated cell holder. All fluorescence spectra were recorded by using the same excitation and emission slits (10 nm). Results are reported with fluorescence intensity expressed in the same arbitrary units but are not corrected for differences in path length. Low-temperature fluorescence and phosphorescence emission spectra were obtained by using a buffer (buffer A) that froze to yield a clear glass at 77 K. Buffer A contained 50 mM Tris, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol. Most fluorescence emission spectra at 5 °C were recorded with buffer A, but similar results were obtained with buffer B (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.0 mM DDT, and 5% glycerol), which was used in some studies. Enzyme containing 5,10-CH$^+$-H$_4$folate plus FADH$_2$ was obtained by treating the isolated enzyme with excess dithionite at 5 °C under aerobic conditions, as previously described (Jorns et al., 1987b). Reaction of photolyase with sodium borohydride converts 5,10-CH$^+$-H$_4$folate to a stable, nonfluorescent tetrahydropterin derivative (Jorns et al., 1987b; Wang et al., 1988). Borohydride reduction of 5,10-CH$^+$-H$_4$folate is expected to generate 5-methyltetrahydrofolate (5-CH$_3$-H$_4$folate) (Foo et al., 1980). Enzyme containing 5-CH$_3$-H$_4$folate plus FADH$_2$ was prepared by reaction of the isolated enzyme with borohydride followed by dithionite treatment, as previously described (Jorns et al., 1987b). For low-temperature studies the desired enzyme form was prepared in a cuvette at 5 °C. The sample was then transferred to an EPR tube and frozen in liquid nitrogen. In some experiments the sample was divided into two parts before transfer to an EPR tube. One part was mixed with UV-oligo(dT)$_9$ while the other received only a comparable amount of buffer.

Fluorescence titration experiments were conducted with enzyme containing 5-CH$_3$-H$_4$folate plus FADH$_2$ in buffer C (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 50% glycerol). The smallest excitation slits available on the instrument (3 nm) were used in titration studies. Measurements were made by a brief exposure of the sample to the excitation beam. All points for a given titration were normally collected by using a single aliquot of enzyme. The minimal exposure of the sample to light during measurements did not cause significant dimer repair. Similar results were obtained by collecting fewer points from a given aliquot and then pooling results obtained from several aliquots of the same sample of enzyme. Unless otherwise noted, values for the dissociation constant and binding stoichiometry for enzyme–substrate complexes formed with UV-oligo(dT)$_n$ were calculated on the basis of the concentration of dimers. As discussed under Results, in the case of UV-oligo(dT)$_4$ and UV-oligo(dT)$_6$ the dimer concentration was corrected for weakly bound dimers at the 3′ end. The fraction of dimers at the 3′ end decreases as a function of chain length (Jorns et al., 1985). In the case of the higher homologues [oligo(dT)$_n$, n = 9, 18] the significance of 3′ dimers was further minimized by the fact that the oligomer preparations contained a greater number of dimers per molecule as compared with the number of molecules of photolyase bound per oligomer. The dimer concentration was not corrected for 3′ dimers in the case of the higher homologues.

Electrophoresis. Native gel gradient (4–30% acrylamide) electrophoresis studies were conducted at 4 °C, according to the procedure described by Anderson et al. (1977) except that the gel/running buffer was diluted 2-fold and azide was omitted. Enzyme–substrate complexes were formed by mixing the isolated, blue form of the enzyme with an equivalent amount of UV-oligo(dT)$_{18}$ or UV-oligo(dT)$_9$. The latter was estimated on the basis of the end point observed in titration studies. The gels were prerun for 1 h at 70 V and then run for 24 h at 150 V. The following proteins were used as standards: thyroglobulin (M_r 669 000), ferritin (M_r 440 000), catalase (M_r 232 000), lactate dehydrogenase (M_r 140 000), and albumin (M_r 66 000).

Quantum Yield Measurements. Quantum yields were determined by a procedure similar to that described by Sun et al. (1972) using fluorescein in 0.1 N NaOH as standard (ϕ = 0.84). Emission spectra (excitation λ = 366 nm) were recorded at 5 °C in buffer C and were corrected with quinine sulfate dihydrate as standard. Enzyme containing 5,10-CH$^+$-H$_4$folate plus FADH$_2$ was prepared by irradiating an anaerobic solution of the isolated, blue enzyme with yellow light (Westinghouse, F15T8/GO). Enzyme containing 5-CH$_3$-H$_4$folate plus FADH$_2$ was prepared by irradiating an anaerobic solution of the isolated enzyme with blue/black light (Sylvania F15T8/BLB) until both chromophores were reduced, as previously described (Jorns et al., 1987b). The fluorescence of the isolated enzyme (5,10-CH$^+$-H$_4$folate plus FADH$^\bullet$) was unaffected by air, and measurements with this enzyme form were made in aerobic buffer.

Aerobic Stability of Enzyme-Bound FADH$_2$. Enzyme containing FADH$_2$ plus 5,10-CH$^+$-H$_4$folate or FADH$_2$ plus 5-CH$_3$-H$_4$folate was mixed anaerobically with excess UV-oligo(dT)$_{18}$ (1.7 mol of oligomer/mol of enzyme) or with the same amount of dimer-free oligo(dT)$_{18}$. The samples were then made aerobic, and FADH$_2$ oxidation to FADH$^\bullet$ at 8 °C was monitored by following the increase in absorption at 580 nm with a Perkin-Elmer Lambda 3 spectrophotometer. Enzyme containing FADH$_2$ plus 5,10-CH$^+$-H$_4$folate or FADH$_2$ plus 5-CH$_3$-H$_4$folate was prepared by irradiating an anaerobic solution of the enzyme (1.10 × 10^{-5} M in buffer B containing 3 mM DTT) with yellow or blue/black light, respectively, at 4 °C. To test for the presence of a cofactor that might stabilize enzyme-bound FADH$_2$ in vivo, a crude extract of *E. coli* cells (10 mL) was prepared as previously described (Sancar, A., et al., 1984). A 1:1 dilution of the extract with methanol was incubated for 1.5 h at 0 °C and then centrifuged to remove

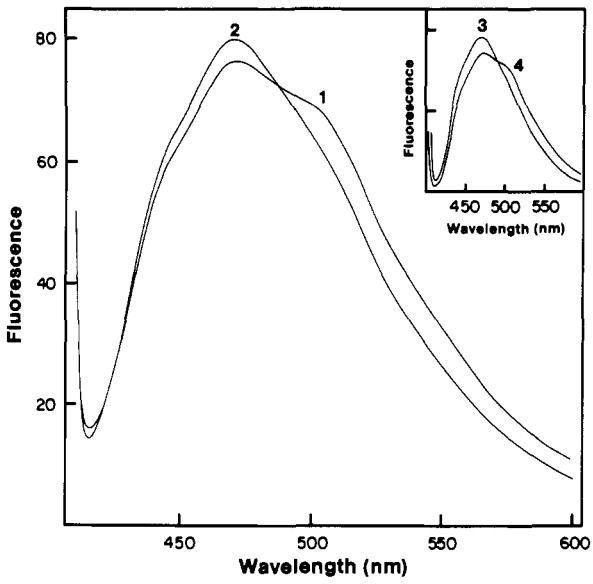


FIGURE 1: Effect of substrate on the fluorescence of photolyase containing 5,10-CH^+-H_4folate plus $FADH_2$ at 5 °C. Curve 1 shows the fluorescence emission spectrum (excitation λ = 390 nm) of the enzyme (1.07×10^{-5} M) in buffer A. Curve 2 was recorded after adding excess UV-oligo(dT)$_9$ (4.0 mol of dimer/mol of enzyme). Inset: Fluorescence emission spectra (excitation λ = 390 nm), recorded before and after dithionite reduction of FADH• in the isolated enzyme, are shown in curves 3 and 4, respectively. To facilitate comparison, curve 3 has been expanded 3.75-fold relative to curve 4.

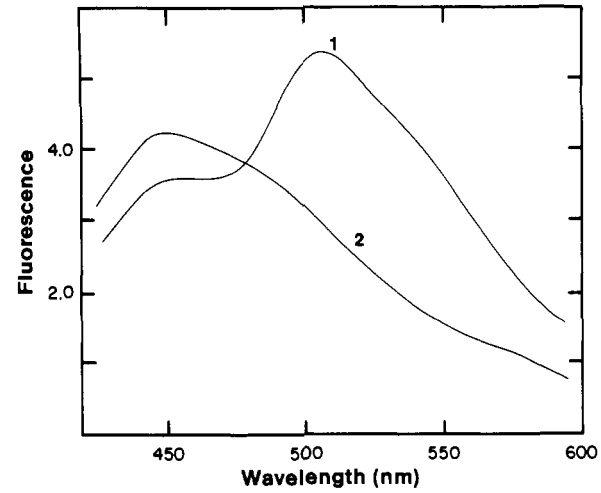


FIGURE 2: Effect of substrate on the fluorescence of photolyase containing 5-CH_3-H_4folate plus $FADH_2$ at 5 °C. Curve 1 shows the fluorescence emission spectrum (excitation λ = 390 nm) of the enzyme (1.08×10^{-5} M) in buffer B. Curve 2 was recorded after adding excess UV-irradiated oligo(dT)$_9$ (1.8 mol of dimer/mol of enzyme).

denatured protein. The methanol was evaporated under a stream of nitrogen, and the sample was lyophilized. The residue was dissolved in water (0.5 mL). The concentration of the postulated cofactor was estimated by assuming that the initial crude extract contained a 1:1 complex of the cofactor with photolyase. The photolyase concentration in the crude extract was estimated on the basis of the yield of purified enzyme. An anaerobic solution of enzyme containing $FADH_2$ plus 5-CH_3-H_4folate was mixed with an aliquot of cell extract estimated to contain an equivalent amount of cofactor. The sample was then made aerobic. The reoxidation of $FADH_2$ was monitored at 580 nm. The enzyme for this experiment was prepared by anaerobic photoreduction with blue/black light in buffer B containing 100 mM EDTA.

RESULTS

Effect of Substrate on Photolyase Fluorescence. If the singlet state of one or both of the enzyme's chromophores is a reactive species in dimer repair, formation of an enzyme–substrate complex might result in a quenching of the fluorescence of the chromophore. The fluorescence of the isolated enzyme (emission λ_{max} = 470 nm) is due entirely to 5,10-CH^+-H_4folate since the flavin radical is nonfluorescent (Jorns et al., 1984). Although the isolated enzyme has a high affinity for substrate (Sancar et al., 1987c), no effect on enzyme fluorescence was observed upon addition of excess UV-oligo(dT)$_9$. Reaction with dithionite converts the flavin radical in the isolated enzyme to $FADH_2$ which is fluorescent (emission λ_{max} = 505 nm) and also enhances the fluorescence due to 5,10-CH^+-H_4folate (Jorns et al., 1987b). 5,10-CH^+-H_4folate is the major fluorophore in dithionite-reduced enzyme. This form of the enzyme exhibits an emission peak at 470 nm due to 5,10-CH^+-H_4folate plus a pronounced shoulder at 505 nm due to $FADH_2$ (Figure 1, curve 1). The shoulder at 505 nm was virtually eliminated upon formation of a complex with UV-oligo(dT)$_9$ (Figure 1, curve 2). Except for a difference in intensity, the emission spectrum observed for the enzyme–substrate complex was very similar to that observed for the isolated form of photolyase where fluorescence is due only to 5,10-CH^+-H_4folate (Figure 1, curve 3). The results suggested that formation of the enzyme–substrate complex resulted in a selective quenching of the fluorescence due to $FADH_2$. Further evidence was sought in experiments with enzyme containing $FADH_2$ as the major fluorophore. This form of the enzyme was prepared by reaction of the isolated enzyme with borohydride followed by dithionite reduction of the flavin radical. Reaction with borohydride converts 5,10-CH^+-H_4folate to a nonfluorescent tetrahydropterin derivative that does not absorb at $\lambda > 300$ nm (Jorns et al., 1987b; Wang et al., 1988). Borohydride reduction of 5,10-CH^+-H_4folate is expected to generate 5-CH_3-H_4folate (Foo et al., 1980). The resulting enzyme preparation is fully active (Jorns et al., 1987b). It showed an emission maximum at 505 nm due to $FADH_2$ plus a shoulder around 450 nm (Figure 2, curve 1). (The shoulder was not always observed and was probably due to residual 5,10-CH^+-H_4folate that is highly fluorescent as compared with $FADH_2$.) Quenching of the emission due to $FADH_2$ occurred upon formation of the enzyme–substrate complex with UV-oligo(dT)$_9$, as evidenced by the loss of the emission band at 505 nm (Figure 2, curve 2). The fluorescence quenching observed in these experiments was not due to oxidation of $FADH_2$ to a nonfluorescent flavin radical since the characteristic absorption of the radical in the 500–700-nm region was not detectable in spectra recorded for $EFADH_2$–substrate complexes.

The catalytic relevance of the observed quenching reactions was suggested by the following observations: (1) A quenching of the fluorescence of $FADH_2$, similar to that described with UV-oligo(dT)$_9$, was observed with both longer and shorter chain oligomers (vide infra); (2) no quenching was observed with dimer-free oligothymidylates; (3) the quenching observed with dimer-containing oligothymidylates was reversible upon exposure of the enzyme–substrate complex to the photoreactivating light used in a normal catalytic assay.

Additional studies were conducted to determine whether the effects observed with substrate at 5 °C could be duplicated in the frozen state at liquid nitrogen temperatures since these data might prove useful in connection with low-temperature phosphorescence studies. The fluorescence emission spectrum, recorded at 77 K for enzyme containing $FADH_2$ plus the nonfluorescent 5-CH_3-H_4folate, showed two significant dif-

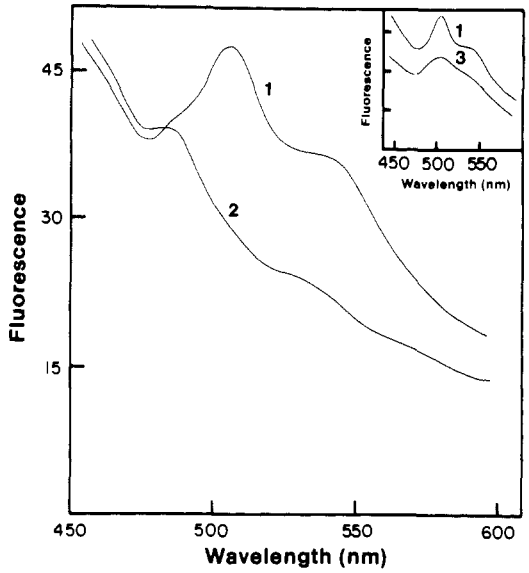


FIGURE 3: Effect of low temperature and effect of substrate at low temperature on the fluorescence of photolyase containing 5-CH_3-H_4folate plus $FADH_2$. Curve 1 shows the fluorescence emission spectrum (excitation λ = 390 nm) of the enzyme (1.04×10^{-5} M) in buffer A at 77 K. Curve 2 was recorded for an aliquot of the same enzyme preparation in the presence of UV-oligo(dT)$_9$ (2.5 mol of dimer/mol of enzyme). Curve 1 is reproduced in the inset for comparison with a spectrum (curve 3) recorded at 5 °C before the sample was frozen. Curve 3 is shown expanded 4.4-fold relative to curve 1.

ferences as compared with the spectrum obtained at 5 °C: (1) The intensity of the emission band due to $FADH_2$ at 505 nm was 6.5-fold greater at 77 K; (2) a pronounced shoulder at 540 nm was detectable only at low temperature (Figure 3, curve 1). The spectrum of the enzyme–substrate complex, recorded at 77 K (Figure 3, curve 2), showed quenching of $FADH_2$ fluorescence, similar to results obtained at 5 °C.

In addition to enhanced fluorescence intensity, a rather striking change in the shape of the fluorescence emission spectrum was observed at low temperature for enzyme containing $FADH_2$ plus 5,10-CH^+-H_4folate. At 77 K the emission bands of the two chromophores were clearly resolved (Figure 4, curve 2), in contrast to the spectrum recorded at 5 °C which showed only a single band at 470 nm due to 5,10-CH^+-H_4-folate plus a shoulder at 505 nm due to $FADH_2$ (Figure 4, curve 1). At 77 K the fluorescence due to $FADH_2$ appeared as a well-defined peak at 505 nm with a pronounced shoulder at 540 nm. The shape of the $FADH_2$ emission band was unaffected by the redox state of the pterin chromophore, but the intensity of the emission at 505 nm was about 5-fold greater when the pterin was present as 5,10-CH^+-H_4folate (Figure 4, curve 2) as compared with enzyme containing 5-CH_3-H_4folate (Figure 3, curve 1). The emission spectrum obtained for enzyme containing 5,10-CH^+-H_4folate plus $FADH_2$ at 77 K showed an additional pair of bands at 465 and 440 nm (Figure 4, curve 2). Several observations indicate that these bands were due to 5,10-CH^+-H_4folate. First, the bands were absent when the pterin chromophore was converted to the nonfluorescent 5-CH_3-H_4folate (Figure 3, curve 1). A similar pair of bands was observed at low temperature with enzyme containing 5,10-CH^+-H_4folate plus the nonfluorescent flavin radical (Figure 5, curve 3). The emission spectrum, recorded at 77 K for the enzyme–substrate complex formed with enzyme containing 5,10-CH^+-H_4folate plus $FADH_2$, showed a quenching of the fluorescence due to $FADH_2$, whereas no effect was observed for the bands due to 5,10-CH^+-H_4folate (Figure 5, curve 2). The residual fluorescence observed for the enzyme–substrate complex showed emission

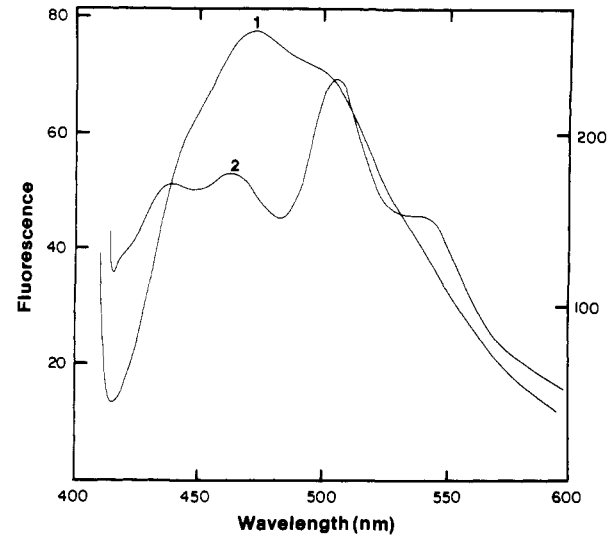


FIGURE 4: Effect of temperature on the fluorescence of photolyase containing 5,10-CH^+-H_4folate plus $FADH_2$. Curve 1 shows the emission spectrum (excitation λ = 390 nm) of the enzyme (1.11×10^{-5} M) in buffer A at 5 °C. Curve 2 shows the spectrum of the same sample recorded at 77 K. Both spectra were recorded in an EPR tube.

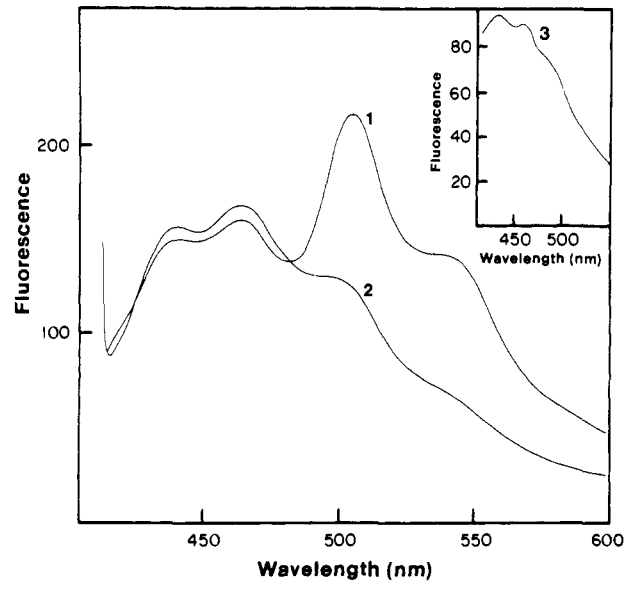


FIGURE 5: Effect of substrate on the fluorescence of photolyase containing 5,10-CH^+-H_4folate plus $FADH_2$ at 77 K. Curve 1 shows the fluorescence emission spectrum (excitation λ = 390 nm) of the enzyme (1.11×10^{-5} M) in buffer A at 77 K. Curve 2 was recorded for an aliquot of the same enzyme preparation in the presence of UV-oligo(dT)$_9$ (4.0 mol of dimer/mol of enzyme). Inset: Curve 3 shows the fluorescence emission spectrum of the isolated enzyme (1.11×10^{-5} M), which contains 5,10-CH^+-H_4folate plus $FADH^\bullet$ in buffer A at 77 K.

maxima that were very similar to that observed at low temperatures for a preparation of substrate-free enzyme that contained 5,10-CH^+-H_4folate as the only fluorophore (Figure 5, curve 3). The results showed that the quenching of $FADH_2$ emission in enzyme–substrate complexes was readily detectable at 77 K.

Binding Affinity and Stoichiometry for Various Enzyme–Substrate Complexes. The quenching of the fluorescence due to enzyme-bound $FADH_2$, observed upon formation of enzyme–substrate complexes with various dimer-containing oligothymidylates, suggested that fluorescence titrations might be used to measure binding affinity and stoichiometry as a function of chain length. To enhance the sensitivity of the method, titrations were conducted with enzyme containing

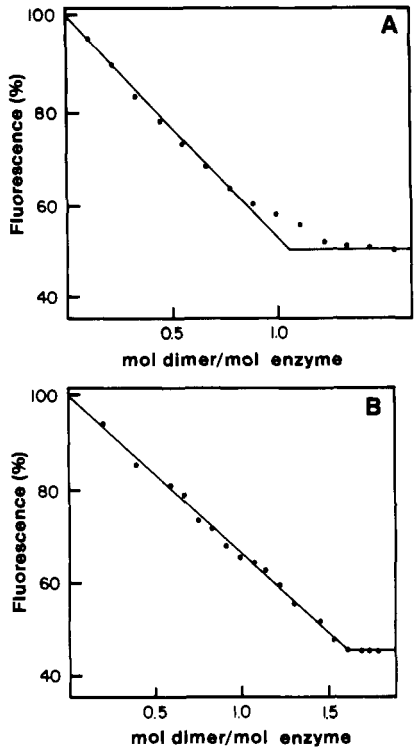


FIGURE 6: Fluorometric titrations with UV-oligo(dT)$_4$ and UV-oligo(dT)$_{18}$. Enzyme containing 5-CH_3-H_4folate plus $FADH_2$ was titrated with UV-oligo(dT)$_4$ (panel A) or UV-oligo(dT)$_{18}$ (panel B) in buffer C at 5 °C. The enzyme concentration was 1.29×10^{-5} M (panel A) or 1.53×10^{-5} M (panel B). Fluorescence emission was measured at 540 nm with excitation at 390 nm. In the case of UV-oligo(dT)$_4$ the amount of dimer added was corrected for dimers at the 3′ end, as described in the text. The correction is negligible in the case of UV-oligo(dT)$_{18}$ (Jorns et al., 1985) and was not done.

$FADH_2$ plus the nonfluorescent 5-CH_3-H_4folate. Figure 6A shows a fluorescence titration curve obtained with UV-oligo(dT)$_4$. The value calculated for the stoichiometry (1.6 mol of dimer/mol of enzyme) appeared somewhat high as compared with other studies which showed that the enzyme had a single binding site for substrate (Sancar et al., 1987c). However, 33% of the dimers in UV-oligo(dT)$_4$ are located at the unphosphorylated 3′ end of the oligomer (Jorns et al., 1985). Our previous catalytic studies with UV-oligo(dT)$_n$ ($n \geq 4$) showed that turnover was unaffected by the position of the dimer along the oligomer chain except for dimers located at the 3′ end where repair was not detectable (Jorns et al., 1985). When the binding of the enzyme to dimers at the 3′ end of UV-oligo(dT)$_4$ was assumed to be negligibly weak as compared to binding at other positional isomers, the calculated stoichiometry (1.1 mol of dimer/mol of enzyme) was in good agreement with the expected value. Different points near the end of the titration with UV-oligo(dT)$_4$ were used to calculate values for the dissociation constant of the enzyme–substrate complex. The calculated values were in good agreement with each other [K_D (range) = 2.3×10^{-7}–2.9×10^{-7} M; K_D (average) = 2.5×10^{-7} M] when the dimer concentration was corrected for dimers at the 3′ end. The results support the hypothesis that the binding of the enzyme to dimers at the 3′ end of UV-oligo(dT)$_4$ is negligible as compared with other positions in the oligomer. Further evidence was sought in binding studies with UV-oligo(dT)$_2$ since the only dimer possible in this molecule (p$\overline{\text{TpT}}$) is located at the 3′ end. Consistent with our hypothesis, the affinity of the enzyme for UV-oligo(dT)$_2$ ($K_D = 5.5 \times 10^{-5}$ M) was found to be more than 2 orders of magnitude weaker as compared to the affinity of the enzyme for dimers at positions in UV-oligo(dT)$_4$ other than the 3′ end. Titration of the enzyme with UV-oligo(dT)$_6$ (data not shown) yielded a stoichiometry close to unity (1.1 mol of dimer/mol of enzyme) when the substrate concentration was corrected for dimers at the 3′ end [20% (Jorns et al., 1985)]. The value obtained for the dissociation constant with UV-oligo(dT)$_6$ ($K_D = 2.6 \times 10^{-7}$ M) was similar to that observed for UV-oligo(dT)$_4$. That the enzyme exhibits a similar affinity for dimers in these two oligomers is consistent with results obtained in catalytic studies (Jorns et al., 1985) where a similar value was observed with either oligomer for the minimal substrate concentration required to saturate the enzyme (~10 μM with respect to dimer). The catalytic studies suggested that tighter binding could be expected with UV-oligo(dT)$_9$ or UV-oligo(dT)$_{18}$ since the minimal substrate concentration required for saturation was about 5-fold smaller (Jorns et al., 1985). This prediction was verified by results obtained in fluorometric titrations with UV-oligo(dT)$_{18}$ (Figure 6B) and UV-oligo(dT)$_9$ (data not shown). With these oligomers, complex formation with photolyase was found to be directly proportional to the amount of added dimer. No curvature was detectable near the end of the titration, which meant that the data could not be used to calculate values for the dissociation constant. At the enzyme concentration used in these studies (12–15 μM) it can be calculated that, when $K_D = 2.5 \times 10^{-7}$ M, a deviation from linearity would be readily detectable near the end of the titration (e.g., see Figure 6A), whereas when $K_D = 10^{-8}$ M, only a small deviation would be expected (2% at 95% complex formation). Since the sensitivity of the fluorometric method did not permit measurements at lower enzyme concentrations, we can only estimate the value for the dissociation constant with UV-oligo(dT)$_9$ or UV-oligo(dT)$_{18}$ ($K_D \leq 10^{-8}$ M).

Titration studies with UV-oligo(dT)$_n$ ($n \geq 4$) were conducted with oligonucleotide preparations that contained a similar percentage (28–32%) of thymine residues as dimers. As a consequence, the number of dimers per oligomer was greater than 1 in the case of the higher homologues [1.7 mol of dimer/mol of oligo(dT)$_9$; 3.5 mol of dimer/mol of oligo(dT)$_{18}$] but not in the case of the lower homologues [0.56 mol of dimer/mol of oligo(dT)$_4$; 0.93 mol of dimer/mol of oligo(dT)$_6$]. This factor is important in interpreting the binding stoichiometry observed with UV-oligo(dT)$_{18}$ and UV-oligo(dT)$_9$. In the case of UV-oligo(dT)$_9$, an apparent stoichiometry of 1.5 mol of dimer/mol of enzyme was observed. However, the observed stoichiometry with respect to dimers corresponded to the binding of only 1 molecule of photolyase per oligomer [1.1 mol of enzyme/mol of UV-oligo(dT)$_9$]. The results suggested that, even though there was more than one dimer present, a molecule of oligo(dT)$_9$ was too small to accommodate more than 1 molecule of enzyme. This did not appear to be the case with UV-oligo(dT)$_{18}$ since the observed stoichiometry with respect to dimers (1.6 mol of dimer/mol of enzyme) was found to correspond to the binding of close to 2 molecules of photolyase per oligomer [2.2 mol of enzyme/mol of UV-oligo(dT)$_{18}$]. The results suggested that complex formation with UV-oligo(dT)$_{18}$ should be accompanied by a doubling of the molecular weight of the enzyme. Evidence to evaluate this hypothesis was sought by determining the molecular weight of the enzyme–substrate complexes using the native gel gradient electrophoresis technique (Anderson et al., 1977). Since substrate binding is unaffected by the redox state of the flavin chromophore (Sancar et al., 1987c), complexes were prepared by using the isolated, flavin radical containing form of the enzyme. The gels were developed under

Table I: Fluorescence Quantum Yield for Various Forms of Photolyase[a]

chromophore composition		
pterin	flavin	quantum yield
5,10-CH^+-H_4folate	$FADH^\bullet$	0.011
5-CH_3-H_4folate	$FADH_2$	0.015
5,10-CH^+-H_4folate	$FADH_2$	0.056

[a] Quantum yields were measured at 5 °C as described under Experimental Procedures.

conditions such that the observed R_f values were insensitive to protein charge. This was evidenced by the linear relationship observed between log molecular weight and R_f value for a group of protein standards (data not shown). In agreement with the prediction from titration studies, the molecular weight determined for the complex formed with photolyase and UV-oligo(dT)$_{18}$ (M_r 118 000) was nearly 2-fold larger as compared to the value obtained for the molecular weight of the complex formed with UV-oligo(dT)$_9$ (M_r 63 000). The procedure could not be used with uncomplexed photolyase since the free enzyme was labile under the experimental conditions. However, the molecular weight determined for the enzyme complex with UV-oligo(dT)$_9$ was in reasonable agreement with the molecular weight calculated (M_r 55 500) on the basis of the contribution from the polypeptide chain [M_r 53 994, as determined from the DNA sequence (Sancar, G. B., et al., 1984)] plus the contribution from the enzyme's two chromophores.

Search for Triplet-State Photochemistry. The inability of substrate to quench the fluorescence due to 5,10-CH^+-H_4folate raised the possibility that the triplet state of 5,10-CH^+-H_4folate might function as a reactive species in catalysis. A preliminary attempt to evaluate this hypothesis, via phosphorescence studies at 77 K, was prompted by the observation that the quenching of $FADH_2$ fluorescence in enzyme–substrate complexes was readily detectable at low temperatures. Various enzyme forms were prepared, similar to that described for low-temperature fluorescence studies. However, none of the samples showed any detectable phosphorescence either in the presence or in the absence of substrate. [The characteristic phosphorescence emission of riboflavin at 605 nm (ϕ = 0.0012 at 77 K; Bowd et al., 1968) was readily detected in control studies conducted under similar conditions.] The absence of phosphorescence could mean that triplet formation was negligible or that triplets were formed but decayed preferentially via nonradiative processes.

Triplet formation must be negligible in the case of a chromophore that exhibits a fluorescence quantum yield near 1. This suggested that evidence regarding the triplet yield of the photolyase chromophores might be obtained by measuring the fluorescence quantum yield of the enzyme with the chromophores in various redox states. Similar values were obtained for the quantum yield with enzyme preparations containing either 5,10-CH^+-H_4folate or $FADH_2$ as the only fluorophore (Table I). The value observed for the quantum yield was about 5-fold larger with enzyme containing 5,10-CH^+-H_4folate plus $FADH_2$ where both of the chromophores contribute to the observed fluorescence. However, all values for the quantum yield were considerably less than 1. As a control, the quantum yield for riboflavin was measured (ϕ = 0.24) and found to be in good agreement with the value (ϕ = 0.25) reported by Moore et al. (1977). Since the quantum yield for singlet formation is always 1, the low fluorescence quantum yield values observed for the photolyase chromophores would indicate that, in the absence of substrate, significant radiationless deactivation of the singlet occurs and/or that interconversion of the singlet to the triplet is significant.

Effect of Substrate on the Stability of the Reduced Flavin in Photolyase. The observed quenching of $FADH_2$ fluorescence in enzyme–substrate complexes prompted studies to determine whether substrate might affect other properties of the reduced flavin. No effect was observed on the absorption spectrum of $FADH_2$. To examine the effect of substrate on the reactivity of $FADH_2$ with oxygen, enzyme preparations containing $FADH_2$ plus 5,10-CH^+-H_4folate or $FADH_2$ plus 5-CH_3-H_4folate were mixed with excess UV-oligo(dT)$_{18}$ under anaerobic conditions, as detailed under Experimental Procedures. The samples were then made aerobic, and the rate of reoxidation of $FADH_2$ to $FADH^\bullet$ was monitored at 8 °C. Control samples were mixed with a comparable amount of dimer-free oligo(dT)$_{18}$. In the presence of dimer-free oligo(dT)$_{18}$, reoxidation of $FADH_2$ was complete within 30 min in enzyme containing either 5,10-CH^+-H_4folate or 5-CH_3-H_4folate. In contrast, a pronounced increase in stability was observed for $FADH_2$ in the presence of UV-oligo(dT)$_{18}$. No oxidation of $FADH_2$ was detectable after a 1-h incubation in the dark with enzyme–substrate complexes containing either 5,10-CH^+-H_4folate or 5-CH_3-H_4folate. This enhanced stability was lost after exposure (2 min) of the enzyme–substrate complexes to photoreactivating (blue/black) light. After dimer repair, reoxidation of $FADH_2$ occurred at a rate comparable to that observed for the control samples. Our previous studies have shown that in vivo the flavin chromophore in *E. coli* photolyase exists as $FADH_2$. The flavin radical, found in purified enzyme, is generated by oxidation of $FADH_2$ during enzyme isolation (Sancar et al., 1987a). The observed stabilization of $FADH_2$ by substrate suggested that $FADH_2$ might be stabilized in vivo by a cofactor that was lost during the isolation of the enzyme. Since enzyme containing $FADH_2$ is present in the initial extract of *E. coli* cells (Payne et al., 1987), a crude enzyme extract was denatured and used to prepare a concentrated, protein-free extract that might contain the postulated cofactor. However, no evidence for stabilization of $FADH_2$ against air oxidation was obtained when an aliquot of concentrated extract was tested with enzyme containing $FADH_2$ plus 5-CH_3-H_4folate. It is conceivable that the apparent stability of enzyme-bound $FADH_2$ in vivo is due to a cellular system that reduces any oxidized photolyase molecules.

Discussion

In this paper we have shown that the formation of an enzyme–substrate complex with *E. coli* photolyase and dimer-containing oligothymidylates results in a quenching of $FADH_2$ fluorescence whereas the fluorescence due to 5,10-CH^+-H_4folate was unaffected. The selective quenching of $FADH_2$ fluorescence was detectable at either 5 °C or in the frozen state at 77 K. With the physiologically significant form of the enzyme, the effect of substrate was more dramatic at 77 K. This is because separate emission bands were observed for $FADH_2$ and 5,10-CH^+-H_4folate at low temperature but not at 5 °C where only a shoulder was attributable to $FADH_2$. Both chromophores showed an increase in fluorescence intensity in going from a liquid at 5 °C to a solid solution at 77 K as well as the appearance of pronounced shoulders. The latter may reflect a partial resolution of vibrational transitions. The increase in fluorescence intensity is probably due to a decrease in radiationless deactivation of the singlet that would be expected at low temperature. In addition to the effect on $FADH_2$ fluorescence, the binding of substrate also resulted in a dramatic stabilization of $FADH_2$ against air oxidation. This property may be important physiologically since, in the absence of photoreactivating light, the presence of dimers in DNA could at least serve to maintain the enzyme in its cat-

alytically active form. Studies with flavoenzymes that catalyze oxidation–reduction reactions show that the redox properties of the flavin prosthetic group may also be regulated by substrate binding (Van den Berghe-Snorek & Stankovich, 1984).

Thermodynamic Determinants and Spacial Aspects of Substrate Binding. The quenching of $FADH_2$ fluorescence by substrate was used to probe the effect of chain length on the binding affinity of the enzyme for dimers in various oligothymidylates. The value observed for the dissociation constant with UV-oligo(dT)$_2$ (p$\overline{\text{TpT}}$) ($K_D = 5.5 \times 10^{-5}$ M) corresponds to a free energy change of 5.4 kcal/mol at 5 °C. Comparison with results obtained for the binding of the enzyme to dimers in plasmid DNA ($K_D \sim 10^{-8}$ M, $\Delta G \sim 10$ kcal/mol) (Sancar et al., 1987c) indicates that about 50% of the binding energy observed with DNA as substrate must be due to the binding of the enzyme to the unit p$\overline{\text{TpT}}$. The additional binding energy observed with DNA is attributable to the interaction of the enzyme with a limited region immediately surrounding the dimer, as evidenced, in part, by comparison of the binding affinity observed with DNA with results obtained with UV-oligo(dT)$_9$ or UV-oligo(dT)$_{18}$ ($K_D \leq 10^{-8}$ M). Analysis of the results obtained with UV-oligo(dT)$_4$ provides further insight regarding binding determinants. Three possible isomers are generated by the introduction of a single dimer into oligo(dT)$_4$ (A, p$\overline{\text{TpT}}$pTpT; B, pTp$\overline{\text{TpT}}$pT; C, pTpTp$\overline{\text{TpT}}$). Our titration studies indicate that the enzyme exhibits a similar affinity for dimers in isomers A and B ($K_D = 2.5 \times 10^{-7}$ M, $\Delta G = 8.4$ kcal/mol) whereas, by comparison, the affinity of the enzyme for dimers in isomer C is negligibly small. A 200-fold lower affinity is estimated for binding to the dimer in isomer C, on the basis of results obtained with UV-oligo(dT)$_2$. A common structural unit (p$\overline{\text{TpT}}$pT) is shared by isomers A and B but is only partially represented (p$\overline{\text{TpT}}$) in isomer C. The results suggest that the interaction of the enzyme with the unit p$\overline{\text{TpT}}$pT could account for about 80% of the binding energy observed with DNA as substrate. In this case, the difference in binding energy observed for UV-oligo(dT)$_4$ versus UV-oligo(dT)$_2$ (3.0 kcal/mol) could be attributed to interaction of the enzyme with the phosphate and base 3′ to the dimer. According to Record et al. (1976), the dissociation of a lysine–phosphate electrostatic interaction would be associated with a free energy change of 1.1 kcal/mol at the salt concentration used in our fluorometric titration studies. Electrostatic interaction of photolyase with the 3′-phosphate would, therefore, leave about 2 kcal/mol for interaction of the enzyme with the base 3′ to the dimer. It was found in previous studies that the binding of photolyase to a dimer in DNA involves electrostatic interaction with 1.6 ± 0.3 phosphate residues. Electrostatic interaction with the phosphates 5′ and 3′ to the dimer (*p*$\overline{\text{TpT}}$*p*) but not with the phosphate within the dimer is consistent with results obtained in catalytic (Jorns et al., 1985) and footprint analysis (Husain et al., 1987) studies.

While the unit p$\overline{\text{TpT}}$pN appears to be a major binding energy determinant, the actual contact site between the enzyme and its substrate is probably somewhat larger. Results obtained in various footprint analysis studies (Husain et al., 1987) provide an estimate of the contact unit (Np$\overline{\text{TpT}}$pNpNpN). It is relevant to consider whether this contact unit can explain the difference in binding stoichiometry observed with UV-oligo(dT)$_9$ versus UV-oligo(dT)$_{18}$. Both titration and molecular weight studies showed that only 1 molecule of photolyase would bind per molecule of UV-oligo(dT)$_9$ even though the oligomer preparation contained more than 1 dimer per oligomer [1.7 dimer/oligo(dT)$_9$]. On the other hand, it was found that 2 molecules of photolyase would bind per UV-oligo(dT)$_{18}$ in studies conducted with a substrate preparation that contained 3.5 dimers per oligomer. There are 21 possible isomers of UV-oligo(dT)$_9$ that contain 2 dimers/oligomer. On the basis of the contact unit, Np$\overline{\text{TpT}}$pNpNpN, it is estimated that only one out of the 21 possible isomers (<5%) could accommodate 2 molecules of photolyase. There are 118 possible isomers of UV-oligo(dT)$_{18}$ that contain 2 dimers/oligomer. Using the same contact unit, it is estimated that 56 of the possible isomers (47%) could bind 2 molecules of photolyase. The fraction of the isomers that can accommodate two molecules of photolyase increases when the number of dimers per oligo(dT)$_{18}$ is increased. For example, there are 519 possible isomers of UV-oligo(dT)$_{18}$ that contain 3 dimers/oligomer. It is estimated that 438 of these isomers (84%) could bind 2 molecules of photolyase. A still larger fraction would be expected for the preparation of UV-oligo(dT)$_{18}$ used for titration studies since the dimer content [3.5 dimers/oligo(dT)$_{18}$] was higher.

Excited States and Electron Transfer in Photorepair. The observed quenching of $FADH_2$ fluorescence in enzyme–substrate complexes was fully reversible upon dimer repair and independent of the redox state of the pterin chromophore. The latter finding is consistent with our previous studies (Jorns et al., 1987a,b), which show that the pterin chromophore is not required when $FADH_2$ acts as the sensitizer in catalysis. It is conceivable that the quenching of $FADH_2$ fluorescence observed at 5 °C or 77 K might reflect enhanced radiationless deactivation of the singlet to the ground state. However, at 23 °C we have previously shown that every quantum of light that is absorbed by $FADH_2$ is used to split dimers (Sancar et al., 1987a) and substrate also quenches $FADH_2$ fluorescence at this temperature (Jordan and Jorns, unpublished observations). The results suggest that the singlet excited state of $FADH_2$ ($^1FADH_2$*) is an intermediate in catalysis. We propose that $^1FADH_2$* donates an electron to the pyrimidine dimer ($\overline{\text{TT}}$), generating FADH$^\bullet$ plus a dimer radical anion (eq 2). The latter is known to be unstable (Lamola, 1972). $FADH_2$ may be regenerated after cleavage of the dimer radical as shown in eq 3 and 4, or via an alternative mechanism (see Scheme II). Electron donation from a singlet-state sensitizer

$$FADH_2 \xrightarrow{h\nu} {}^1FADH_2^* \quad (1)$$

$$^1FADH_2^* + \overline{\text{TT}} \rightarrow FADH^\bullet + \overline{\text{TT}}^{\bullet-} + H^+ \quad (2)$$

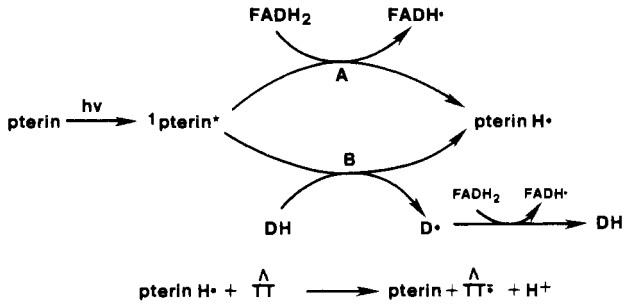
$$\overline{\text{TT}}^{\bullet-} \rightarrow T + T^{\bullet-} \quad (3)$$

$$T^{\bullet-} + FADH^\bullet + H^+ \rightarrow T + FADH_2 \quad (4)$$

has been proposed for the cleavage of thymine dimers observed with various indole derivatives, including a covalent model compound where the pyrimidine dimer was covalently linked to the sensitizer (Van Camp et al., 1987). The fluorescence typically seen for indole derivatives was quenched in the covalent model compound, analogous to that observed for $FADH_2$ fluorescence in photolyase–substrate complexes.

That substrate did not affect 5,10-CH^+-H_4folate fluorescence is compatible with a triplet intermediate (^{3}pterin*). However, we have previously shown that photolyase catalysis is unaffected by oxygen, a known triplet quencher (Jorns et al., 1987a). Also, preliminary flash photolysis studies indicate

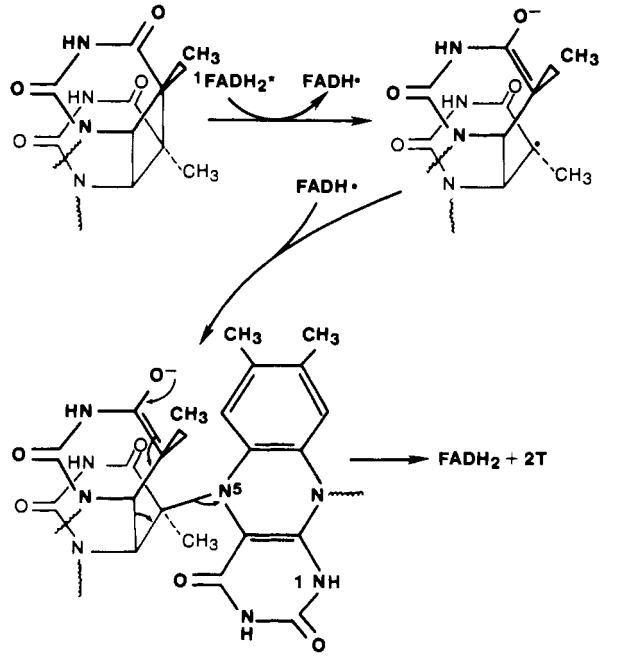
Scheme I



that, if a triplet intermediate is important in catalysis, it must have a lifetime less than 2 μs (Tollin, Jordan, Hazzard, and Jorns, unpublished observations). That substrate did not affect 5,10-CH^+-H_4folate fluorescence does not exclude a singlet intermediate (^{1}pterin*), provided that the singlet reacts with a component of the enzyme instead of directly interacting with substrate. This requirement could be satisfied by energy transfer from ^{1}pterin* to $FADH_2$. While energy transfer could also explain why $FADH_2$ is required for dimer repair when 5,10-CH^+-H_4folate acts as sensitizer (Jorns et al., 1987a), the process is energetically unlikely (Jorns et al., 1987b) and can be ruled out. In an alternative mechanism, shown in Scheme I, a pterin radical (pterinH$^\bullet$) might be formed by electron abstraction by ^{1}pterin* from $FADH_2$ (path A) or from an amino acid residue (DH) (path B). This mechanism requires that pterinH$^\bullet$ act as a potent reductant that is able to transfer an electron to the pyrimidine dimer. In the case of path B, an unproductive collapse of the initial pterinH$^\bullet$/D$^\bullet$ radical pair is prevented by rapid reduction of the amino acid radical by $FADH_2$. In either path, the catalytic cycle is completed by cleavage of the unstable dimer radical and reduction of FADH$^\bullet$ as shown in eq 3 and 4 or by the alternative mechanism shown in Scheme II. With respect to path B in Scheme I, it may be relevant that the existence of an amino acid donor at the active site of photolyase has been proposed on the basis of results obtained for the photoreduction of FADH$^\bullet$ to $FADH_2$ (Heelis et al., 1987).

Comparison of Two Classes of Photorepair Enzymes: Mechanistic Implications. The available evidence suggests that photorepair enzymes can be divided into two classes on the basis of the nature of their chromophores. *E. coli* photolyase is an example of a class I enzyme. Another example is provided by yeast photolyase which also contains $FADH_2$ (Jorns et al., 1987a; Sancar et al., 1987b) plus 5,10-CH^+-H_4folate (Johnson et al., 1988). The second class of photorepair enzymes contains $FADH_2$ plus an 8-hydroxy-5-deazaflavin derivative. Photolyase from the green alga *Scenedesmus acutus* is an example of a class II enzyme (Eker et al., 1988). A second example is provided by photolyase from *Streptomyces griseus* (Eker et al., 1986, 1987). Since both classes of photorepair enzymes contain $FADH_2$, it is tempting to speculate that the mechanism of dimer repair is similar and that the pterin and 5-deazaflavin chromophores have analogous functions in catalysis. The latter hypothesis is consistent with the fact that both the pterin and 5-deazaflavin chromophores are known to function as sensitizers in catalysis (Sancar et al., 1987a; Eker et al., 1988). In this paper we have suggested that a highly reactive pterin radical may be generated photochemically that is able to transfer an electron to the pyrimidine dimer in a dark reaction. That the 5-deazaflavin chromophore in class II enzymes might behave analogously is suggested by the fact that 5-deazaflavin radicals are photochemically accessible and act as potent, one-electron reductants (Massey & Hemmerich, 1978).

Scheme II



Enzymic dimer repair is a highly efficient process, as evidenced by the quantum yield (ϕ = 1.0) observed with photolyase from *E. coli* (Sancar et al., 1987a) or from *S. griseus* (Eker et al., 1986). On the other hand, model studies indicate that the splitting of thymine dimer radical anions is rapid [$\tau \sim 50$ μs (Santus et al., 1972)] but that the process is inefficient. A splitting efficiency of around 0.05 was estimated for the cleavage of dimer radical anions formed by reaction of thymine dimers with solvated electrons in pulse radiolysis experiments (Lamola, 1972). A quantum yield of approximately 0.04 was found for the photochemical splitting of pyrimidine dimers covalently bound to indole. This low quantum yield was attributable to inefficient splitting since inefficient electron transfer from excited indole to the covalently linked dimer could be ruled out (Van Camp et al., 1987). Inefficient splitting of dimer radical anions is not due to competition from decomposition reactions since no products other than monomers are detectable (Lamola, 1972). Inefficient splitting is, therefore, probably due to electron loss before monomerization can occur. The presence of $FADH_2$ as a common element in photorepair enzymes suggests that the chromophore may be significant with respect to the high quantum yield observed for enzymic photorepair. The second chromophore in these enzymes does not appear to be important, as suggested by the fact that the quantum yield observed for *E. coli* photolyase is unaffected by elimination of the pterin chromophore (Heelis et al., 1987). As an alternative to the mechanism shown in eq 3 and 4, we suggest that efficient dimer radical cleavage might be achieved by formation of a covalent flavin–dimer adduct (Scheme II). It is proposed that an initially formed dimer radical anion ($\overline{TT}^{\bullet-}$, structure not shown) is converted to a partially cleaved species (T–T$^{\bullet-}$, structure shown in Scheme II) in a reversible reaction. (Although not indicated, a change in the relative orientation of pyrimidine rings would be expected upon conversion of $\overline{TT}$ to T–T$^{\bullet-}$.) Collapse of the T–T$^{\bullet-}$/FADH$^\bullet$ radical pair yields a covalent adduct which can then rearrange to yield $FADH_2$ plus pyrimidine monomers. Formation of a covalent adduct could enhance the efficiency of dimer cleavage by eliminating back-transfer of an electron to the donor before cleavage could be completed. The splitting

of $\overline{TT}^{\bullet-}$, via a stepwise mechanism involving $T-T^{\bullet-}$ as an intermediate, has recently been suggested by Hartman et al. (1987) on the basis of molecular orbital calculations and application of Woodward–Hoffmann orbital symmetry conservation rules. $T-T^{\bullet-}$ is stabilized by electron delocalization involving the carbonyl group at position 4 of the pyrimidine ring. A comparable intermediate cannot be formed when thymine is replaced by cytosine. This is not disturbing since *E. coli* photolyase does not repair cytosine dimers (Myles et al., 1987). Reduced flavin–substrate adducts have been postulated as intermediates in oxidation–reduction reactions catalyzed by flavoenzymes. Adduct formation is well documented in the case of lactate oxidase (Ghisla & Massey, 1980). The catalytic intermediate in the lactate oxidase reaction has been identified as a 5-substituted 1,5-dihydro-FMN derivative [FMNHR, R = $-C(CH_3)(OH)CO_2H$] which rearranges to form pyruvate plus 1,5-dihydro-FMN in a reaction analogous to that proposed in Scheme II for the photolyase reaction.

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Registry No. $FADH_2$, 1910-41-4; DNA photolyase, 37290-70-3.

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