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Determination of Rates and Yields of Interchromophore (Folate → Flavin) Energy Transfer and Intermolecular (Flavin → DNA) Electron Transfer in *Escherichia coli* Photolyase by Time-Resolved Fluorescence and Absorption Spectroscopy†

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ABSTRACT: *Escherichia coli* DNA photolyase, which photorepairs cyclobutane pyrimidine dimers, contains two chromophore cofactors, 1,5-dihydroflavin adenine dinucleotide ($FADH_2$) and 5,10-methenyltetrahydrofolate (MTHF). Previous work has shown that MTHF is the primary photoreceptor which transfers energy to the $FADH_2$ cofactor; the $FADH_2$ singlet excited state then repairs the photodimer by electron transfer. In this study, we have determined the rate constants for these photophysical processes by time-resolved fluorescence and absorption spectroscopy. From time-resolved fluorescence, we find that energy transfer from MTHF to $FADH_2$ and FADH° occurs at rates of 4.6×10^9 and 3.0×10^{10} s^{-1}, respectively, and electron transfer from $FADH_2$ to a pyrimidine dimer occurs at a rate of 5.5×10^9 s^{-1}. Using Förster theory for long-range energy transfer and assuming $K^2 = {}^2/_3$, the interchromophore distances were estimated to be 22 Å in the case of the MTHF-$FADH_2$ pair and 21 Å for the MTHF-FADH° pair. Picosecond absorption spectroscopy identified an MTHF single state which decays to yield the first excited singlet state of $FADH_2$. The lifetimes of MTHF and $FADH_2$ singlets and the rates of interchromophore energy transfer, as well as the rate of electron transfer from $FADH_2$ to DNA measured by time-resolved fluorescence, were in excellent agreement with the values obtained by picosecond laser flash photolysis. Similarly, fluorescence or absorption lifetime studies of the folate-depleted enzyme with and without photodimer suggest that $FADH_2$, in its singlet excited state, transfers an electron to the dimer with 89% efficiency. The distance between $FADH_2$ and the photodimer was calculated to be ca. 14 Å.

The *cis-syn*-cyclobutadipyrimidine (pyrimidine dimer) is the major photoproduct produced in DNA by ultraviolet light (Wang, 1976). This form of UV damage is subject to repair by DNA photolyases, enzymes that bind to damaged DNA and split the cyclobutane ring of the dimer by utilizing the energy of near-UV or visible light. The enzyme isolated from *Escherichia coli* contains two chromophores, flavin adenine dinucleotide (Sanar & Sancar, 1984) and 5,10-methenyltetrahydrofolylpolyglutamate (MTHF;[1] Johnson et al., 1988). The flavin cofactor is apparently in the dihydro ($FADH_2$) form in vivo but becomes oxidized to the neutral radical form (FADH°) during purification (Jorns et al., 1984; Payne et al., 1987).

Previous work on the roles of two chromophores has shown that enzyme containing the flavin blue neutral radical is catalytically inert (Payne et al., 1987; Sancar et al., 1987).

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[1] Abbreviations: Pyr<>Pyr, T<>T, and U<>U, cyclobutadipyrimidines of the indicated compositions; *c-s*-T<>T, *cis-syn*-thymine dimer; MTHF, methenyltetrahydrofolate; E-$FADH_2$, E-$FADH_2$-MTHF, etc., photolyase containing the indicated chromophore(s); DTT, dithiothreitol; ϕ_F, ϕ_{eT}, ϕ_{ET}, and ϕ_{spl}, quantum yields for fluorescence, energy transfer, electron transfer, and splitting of cyclobutane ring, respectively.

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However, irradiation of the enzyme with photoreactivating light results in an intermolecular hydrogen-atom transfer from a Trp residue of the apoenzyme to FADH°; the resulting Trp radical in turn is reduced by an external electron donor such as dithiothreitol or NADH (Heelis & Sancar, 1986; Heelis et al., 1987, 1990). The photoreduction of FADH° to $FADH_2$ occurs with a quantum yield of about 0.1 (Heelis et al., 1987), and the photoreduced enzyme repairs Pyr<>Pyr with an average quantum yield of 0.69 per photon absorbed by $FADH_2$ (Payne & Sancar, 1990). Since the photoreactivating light absorbed by the chromophores is not energetic enough for dimer splitting to be accomplished by direct energy transfer (energy transfer from photoexcited chromophores to the Pyr<>Pyr is highly endothermic), it has been suggested that the mechanism of photorepair by DNA photolyases involves photoinduced electron transfer between the chromophore and Pyr<>Pyr (Lamola, 1972; Rokita & Walsh, 1984; Van Camp et al., 1987; Sancar & Sancar, 1988; Witmer et al., 1989; Pac et al., 1990). Evidence for photoinduced electron transfer in photoreactivation was recently obtained by the observation of a radical intermediate in the picosecond laser photolysis on the *E. coli* photolyase–photodimer (U<>U) complex (Okamura et al., 1991).

It has been shown that $FADH_2$ in photolyase can repair Pyr<>Pyr in the absence of MTHF (Heelis et al., 1987); however, enzyme bound MTHF cannot repair Pyr<>Pyr in the absence of $FADH_2$ (Jorns et al., 1990; Payne & Sancar, 1990). Therefore, it has been proposed that the sole function of MTHF is to absorb a photon and transfer its excitation to $FADH_2$ with high quantum yield (Heelis et al., 1987; Payne & Sancar, 1990; Jorns et al., 1990).

In this study, we have employed steady-state and time-resolved fluorescence spectroscopy, together with picosecond laser flash photolysis, to derive kinetic data for interchromophore energy transfer and enzyme–substrate electron transfer. These data, combined with data on spectroscopic properties of free MTHF in various solvent systems and the polarization state of chromophore fluorescence, have enabled us to estimate the interchromophore distance.

Materials and Methods

Substrate and Cofactor. The thymidylate photodimer, dT<p>dT, was prepared as described by Liu and Yang (1978). Photoclosing of oligo$(dT)_{15}$ was done in the same way as for the TpT photodimer, except that irradiation was stopped either at 15% or 75% dimerization levels to obtain substrates with different T<>T densities. This acetone-sensitized photoreaction effectively prevented formation of photoadducts other than photodimers. The T<>T concentration in the oligo$(dT)_{15}$ substrate was calculated on the basis of $\epsilon_{260} = 8300$ for a thymine in the oligomer and no absorption by the photodimer at this wavelength.

MTHF was prepared in two different ways. In one method (Hamm-Alvarez et al., 1989) 5 mg of folinic acid (5-formyltetrahydrofolate) was dissolved in 1.2 mL of 0.01 N HCl and incubated at 4 °C in the dark. The incubation was continued until the ratio of absorption at 355 nm to that at 280 nm was greater than 1.6. At that point the solution was frozen and kept at −20 °C until use in reconstitution experiments with photolyase. In the other method, 3 mg of folinic acid was added to a 10-mL solution of $HClO_4$–alcohol (1:9 by vol) and incubated for 3 h in the dark at room temperature; the MTHF prepared by this method was used for solvent-dependent fluorescence experiments.

Photolyase (E-FADH°-MTHF, E-$FADH_2$-MTHF, E-MTHF, E-$FADH_2$, and Apoenzyme). *E. coli* DNA photolyase was purified as described previously (Sancar et al., 1984). Enzyme purified by this method contains flavin neutral radical and less than stoichiometric amount of MTHF. Photolyase containing stoichiometric amounts of both chromophores was prepared by supplementing the enzyme with MTHF as described by Hamm-Alvarez et al. (1989). E-FADH°-MTHF was converted to E-$FADH_2$-MTHF by photoreduction with filtered camera flash ($\lambda > 630$ nm) in the presence of dithiothreitol. E-$FADH_2$ was prepared by photoreduction of FADH° concomitant with selective photodecomposition of MTHF with filtered ($\lambda > 345$ nm) camera flash (Heelis et al., 1987). The E-MTHF form was prepared by reconstitution of photolyase with apoenzyme plus MTHF as described above for supplementation of the natural enzyme with MTHF.

Apoenzyme was prepared by the method of Husain and Massey (1978) with some modifications (Payne et al., 1990). Native photolyase at 200 μM was dialyzed against 50 mM Tris-HCl, pH 4.0, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 2 M KBr at 4 °C. The buffer was changed daily, and the absorption of the enzyme was monitored. After a 5-day dialysis, the enzyme contained about 5% E-FAD. As it has been shown that this form of the enzyme binds DNA while apoenzyme and E-MTHF do not (Payne et al., 1990), the apoenzyme was purified away from the remaining holoenzyme by first dialyzing the mixture into low-salt buffer (100 mM KCl without KBr) followed by chromatography on a DNA cellulose column. The 10-mL DNA cellulose column (Sigma) was equilibrated with 50 mM Tris-HCl, pH 4.0, 100 mM KCl, 1 mM EDTA, and 50% glycerol. After loading the apoenzyme preparation, the column was developed with the same buffer. The apoenzyme came off in the wash while E-FAD was retained. Fractions containing apoenzyme were pooled, concentrated, and dialyzed overnight against photolyase storage buffer.

Steady-State Fluorescence. The steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MPF-3 fluorescence spectrophotometer. All spectra were taken with a 1-cm path length quartz cuvette at 15 + 1 °C, unless otherwise noted. Emission spectra were corrected with quinine sulfate dihydrate as a standard. The excitation and emission bandwidths were 4 nm. Fluorescence quantum yields were determined using quinine sulfate in 0.1 N H_2SO_4 as a standard with a quantum yield of 0.70 at 23 °C (Scott et al., 1970).

For fluorescence polarization studies, the fluorimeter was equipped with a Hitachi polarizer. The sample preparation and irradiation conditions for polarization studies were as follows: MTHF in 0.1 N HCl and 95% glycerol, $\lambda_{ex} = 360$ nm, and $\lambda_{em} = 450$ nm; E-FAD_{ox}:defolated enzyme in which ~90% of flavin was oxidized after about 1 year of storage at −20 °C under aerobic conditions; $\lambda_{ex} = 450$ nm and $\lambda_{em} = 620$ nm to isolate FAD_{ox} emission from the emission of any residual enzyme-bound or free folate fluorescence; E-$FADH_2$, enzyme defolated by 360 nm and flavin reduced by dithionite, $\lambda_{ex} = 380$ nm, and $\lambda_{em} = 520$ nm. Polarization of folate fluorescence was investigated in the following samples: E-MTHF-$FADH_2$ which was prepared by dithionite reduction of the "blue enzyme", $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm; even though $FADH_2$ contributes to luminescence at these wavelengths, the fluorescence intensity is negligible compared to MTHF fluorescence and therefore it was assumed that all emission at 460 nm was due to MTHF; E-FADH°-MTHF, ordinary "blue enzyme", $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm; E-FAD_{ox}-MTHF, "yellow photolyase", obtained by long-term storage, $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm. Polarization was measured as described by Kurtin and Song (1968), and ro-

Table I: Effect of Environment on Folate Spectroscopy[a]

chromophore (solvent)	ϵ	cp	λ_{max} (abs)	λ_{max} (ems)	ϕ_F	τ
MTHF (H_2O)	78.5	1.1	354	463 ± 1	0.012	<30
MTHF (methanol)	32.6	0.6	358	463 ± 1	0.027	40
MTHF (ethanol)	24.3	1.3	358	463 ± 1	0.026	57
MTHF (2-propanol)	18.3	2.4	358	463 ± 1	0.037	75
MTHF (1-pentanol)	13.9	3.7	360	463 ± 1	0.045	105
E-MTHF (standard buffer, pH 7.4)	60.0	6.0	384	469 ± 1	0.320	354
E-FADH°-MTHF (standard buffer)[b]	60.0	6.0	384	469 ± 1	0.019	<30
E-$FADH_2$-MTHF (standard buffer)[b]	60.0	6.0	384	469 ± 1	0.072	134

[a] All spectra were taken at 15 °C. ϵ = dielectric constant. cp = viscosity of solvent in centipoise. ϕ_F = fluorescence quantum yield at λ_{ex} = 355 nm. λ_{max} (abs) = absorption maximum in wavelength (nm). λ_{max} (ems) = fluorescence emission maximum in wavelength (nm). τ = fluorescence lifetime in picoseconds (ps). The alcohol solution contains 10% of 35% $HClO_4$. The fluorescence quantum yield of E-MTHF remained about the same at pH 5.0–7.4. [b] Corrections were made to subtract the fraction of the light absorbed by the flavin.

tational correlation times were calculated using the Weber equation (Weber, 1966):

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + \tau_f/\tau_c) \quad (1)$$

where P = observed polarization, P_0 = intrinsic fluorescence polarization in rigid media, τ_f = fluorescence lifetime, and τ_c = rotational correlation time.

Fluorescence Lifetime and Picosecond Flash Photolysis. Fluorescence lifetimes were measured at the Center for Fast Kinetics Research (CFKR) at the University of Texas, Austin, TX, by time-correlated single photon counting, following Nd:YAG laser flash photolysis at 355 nm. The fluorescence emission was filtered through filters passing $\lambda > 470$ nm. Data analysis was carried out with a PDP 11/70 computer. The instrument response function was 80 ps FWHM. For a typical lifetime measurement, an aliquot of 1.0 mL of photolyase solution in storage buffer was introduced into a 1-cm path length quartz cell and stoppered with a rubber septum cap. The solution was then deoxygenated by blowing nitrogen onto the sample for 30 min at 5 °C. For fluorescence lifetime measurement of E-$FADH_2$, the deoxygenated solution was irradiated with filtered ($\lambda > 330$ nm) output of 500 W Hg–Xe lamp in order to remove any residual folate and, at the same time, to reduce the radical flavin.

For the fluorescence lifetime measurements of E-$FADH_2$ in the presence of substrate, either *c-s*-T<>T or oligo-$(dT)_{15}$-T<>T was dissolved in 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1.0 mM DTT, and 50% glycerol and deoxygenated by bubbling nitrogen through the solution for 30 min prior to mixing with the enzyme solution. The enzyme concentrations used with these two substrates were 2×10^{-4} M and 1×10^{-4} M, respectively. The concentration of the enzyme was calculated on the basis of its flavin radical absorbance at 580 nm [$\epsilon = 4.5 \times 10^3$ (Payne et al., 1987)].

Transient absorption spectra were obtained by picosecond flash photolysis as described previously (Okamura et al., 1989, 1991).

Results

Electronic Energy Transfer

Three out of four photons utilized by photolyase in photorepair are absorbed by MTHF, which apparently transfers electronic energy to $FADH_2$ with high quantum yield (Payne & Sancar, 1990). To better define the role of MTHF, its spectroscopic properties were investigated by time-resolved and steady-state techniques.

(A) Time-Resolved and Steady-State Fluorescence of Free MTHF. The fluoresence properties of enzyme-bound MTHF differ in important aspects from those of free MTHF. With excitation at λ_{ex} = 355 nm, the corrected fluorescence emission spectrum of enzyme-bound MTHF exhibited maximum emission at λ_{max} = 468–470 nm, while that of free MTHF exhibited a maximum at λ_{max} = 462–465 nm. More significantly, the fluorescence quantum yield of enzyme-bound MTHF, ϕ_F (355 nm) = 0.32, was found to be much higher than that of free MTHF, ϕ_F (355 nm) = 0.036 under identical conditions (pH 5.0). In order to have a better understanding of the factors important for the high quantum yield observed for the enzyme-bound MTHF, time-resolved and steady-state fluorescence experiments were performed in a series of solvents of differing polarities and viscosities. Two components were detected for MTHF in all cases. The shorter lived component ($\tau \leq 30$–105 ps; $A \sim 0.94$) was taken as the true folate emission rather than the very weak second component (τ = 1–2 ns; $A \sim 0.06$). The latter almost certainly represents folate oxidation products (e.g., dihydrofolates), because it increased substantially upon prolonged storage of solutions. As shown in Table I, the fluorescence lifetime as well as quantum yield of free folate are highly solvent dependent. For the solvents employed, the lifetime of MTHF varied by a factor of ca. 4, from a low of <30 ps (H_2O) to a high of 105 ps (1-pentanol). Similarly, the fluorescence quantum yield increased from 0.012 in water to 0.045 in 1-pentanol, which is less polar and more viscous. These results show that both solvent polarity and viscosity can influence the fluorescence quantum yield of MTHF.

The absorption spectrum of MTHF, similarly, indicated that a bathochromic shift can be caused by either low solvent polarity or high viscosity. In water, MTHF has a λ_{max} at 354 nm, while in 99.5% glycerol or 99.5% dioxane λ_{max} is at 368 nm. The absorption λ_{max} of enzyme-bound MTHF is at 384 nm, suggesting some specific interactions (conceivably a negative charge on the polypeptide with the positive charge on the MTHF methenyl bridge) with the apoenzyme in addition to the effects of rigidity and low polarity that may be imposed on the cofactor by the enzyme-binding site.

(B) Fluorescence Lifetime of Enzyme-Bound MTHF. Figure 1 shows oscilloscopic traces of fluorescence decay of MTHF in E-MTHF, E-$FADH_2$-MTHF, and E-FADH°-MTHF. As with free MTHF, two-component fits were necessary, with the short-lived component taken as the true folate emission. Additional evidence for this assignment is that UV irradiation decreased the intensity of this component in line with the MTHF absorption at 384 nm. The χ^2 values were in the range 1.5–3. Although relatively high, they are reasonable given the complexity of the system. From this figure, fluorescence lifetimes of 354, 134, and <30 ps, respectively, are obtained (Table I). The significant shortening of fluorescence lifetime in the presence of flavin is indicative of efficient energy transfer from MTHF to flavin.

The rate constants for energy transfer can be obtained directly from the excited state fluorescence lifetimes. The fluorescence lifetimes in the absence and presence of the flavin cofactor, τ_1 and τ_2, respectively, are related to the rate constant

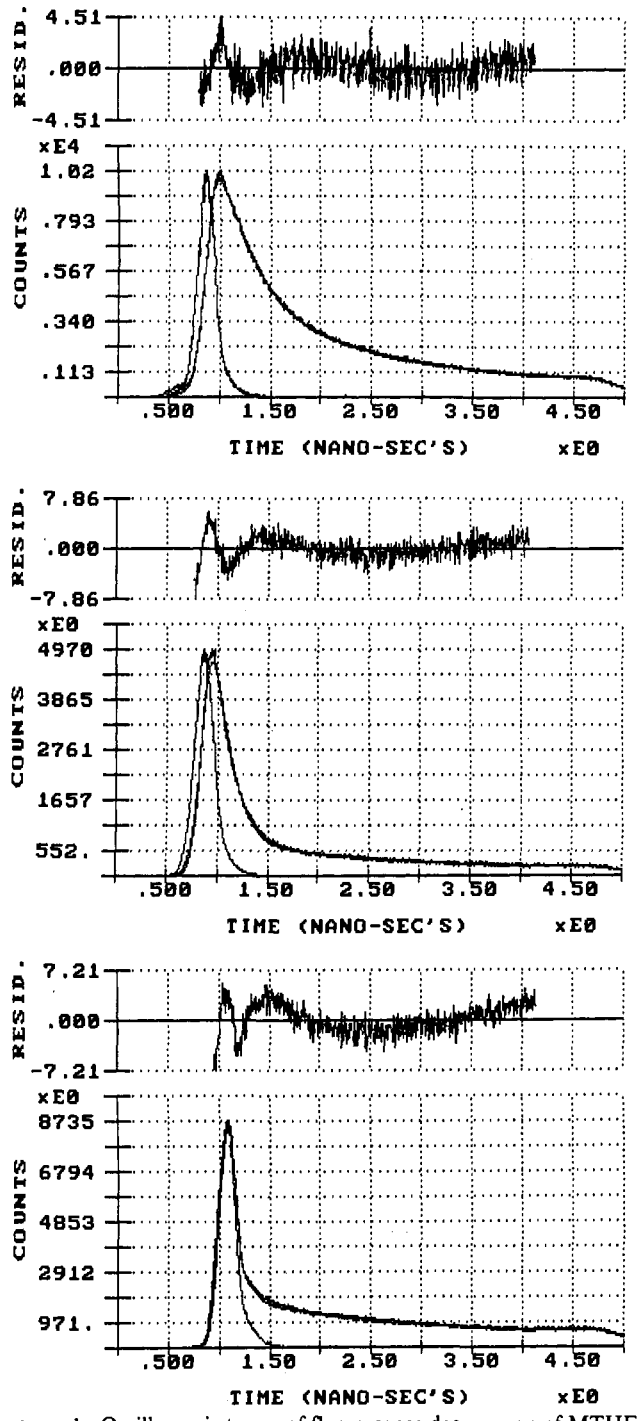

FIGURE 1: Oscilloscopic traces of fluorescence decay curve of MTHF in the presence and absence of flavin. Biexponential fits were used with the longer lived component (τ_2 = 2.4 ns) taken as folate oxidation products (eg. dihydrofolates) since it increased substantially upon prolonged storage of solutions. Two component analyses gave the best fitting with χ^2 values in the range of 1.5 to ~3.0. (A, top) E-MTHF (τ_1 = 354 ps); (B, middle) E-FADH$_2$-MTHF (τ_1 = 134 ps); (C, bottom) E-FADH°-MTHF (τ_1 < 30 ps). The enzyme concentration was ca. 10^{-4} M in storage buffer. The excitation wavelength was 355 nm, and emission was monitored at 470 nm.

for energy transfer k_{eT}, the rate constant for fluorescence emission k_F, and the sum of the rate constants for all nonradiative processes other than energy transfer k_I (which we assume not to be affected by the flavin) by

$$\tau_1 = 1/(k_F + k_I) \quad (2)$$

$$\tau_2 = 1/(k_F + k_I + k_{eT}) \quad (3)$$

and therefore

$$k_{eT} = 1/\tau_2 - 1/\tau_1 \quad (4)$$

The energy transfer rate constants determined in this way were $k_{eT} = 4.6 \times 10^9$ and 3×10^{10} s^{-1}, for energy transfer to FADH$_2$ and FADH°, respectively. The value for k_F can be calculated from

$$k_F = \phi_1/\tau_1 \quad (5)$$

which upon substitution of ϕ_1 and τ_1 values from Table I gives $k_F = 9 \times 10^8$ s^{-1}. By combining eqs 2 and 5, $k_I = 1.9 \times 10^9$ s^{-1} is obtained from

$$k_I = (1 - \phi_1)/\tau_1 \quad (6)$$

The efficiency of energy transfer based on fluorescence lifetimes of E-MTHF, E-FADH°-MTHF, and E-FADH$_2$-MTHF is given by

$$\phi_{eT} = 1 - \tau_2/\tau_1 \quad (7)$$

where τ_2 is the fluorescence lifetime of MTHF in the prescence of either FADH° or FADH$_2$. Substituting the values from Table I, we obtain efficiencies of energy transfer of 0.62 to FADH$_2$ and >0.92 to FADH°. For comparison, the efficiencies of energy transfer from MTHF to FADH° of ca. 1.0 (Heelis et al., 1987) and 0.93 (Jorns et al., 1990) have been reported from photoreduction and steady-state fluorescence quenching measurements, respectively. Similarly, absolute action spectrum measurements yielded a quantum yield of 0.8 (Payne & Sancar, 1990), and steady-state fluorescence quenching gave a value of 0.58 (Jorns et al., 1990) for energy transfer from MTHF to FADH$_2$.

(C) Interchromophore Distance. The singlet energy transfer has been used to estimate the proximity relationships in a wide variety of macromolecules (Stryer, 1978; Kasprzak et al., 1998; Griep & McHenry, 1990). The dependence of the efficiency of transfer on the distance between the energy donor and acceptor is predicted by the Förster's theory (Förster, 1965). According to this theory, the efficiency of transfer (ϕ_{eT}) is related to the distance (r) between the donor and acceptor by

$$\phi_{eT} = R^6/(R^6 + r^6) \quad (8)$$

In this equation, R is the "critical distance" at which the transfer rate (k_{eT}) is equal to the decay rate of the donor in the absence of acceptor ($\phi_{eT} = 0.5$). The value of R can be calculated from

$$R\ (\text{Å}) = (K^2 n^{-4} \phi_d J)^{1/6} \times 9.79 \times 10^3 \quad (9)$$

In this equation, n is the refractive index of the medium (n = 1.39), ϕ_d is the fluorescence quantum yield of donor (ϕ_d = 0.32), K^2 is the orientation factor ($K^2 = {}^2/_3$), and J is the spectral overlap integral.

For systems showing rapid isotropic motion, $K^2 = {}^2/_3$. In contrast, the donor and acceptor may be fixed in a constant orientation to each other. If this were the case, then K^2 can take values ranging from 0 to 4. Hence, to access the applicability of the use of $K^2 = {}^2/_3$, experiments were carried out using fluorescence polarization. The results are summarized in Table II. These show that, for folate in the reduced enzyme, the rotational correlation τ_c is comparable to τ_f. Substantial rotation of the folate can thus occur during its decay. In fact, the angle through which the enzyme-bound folate rotates in one lifetime can be calculated as 34° using the Perrin equation (Perrin, 1926; Weber, 1966). Using the same approach, it can be shown that the flavin rotates 10° during one folate lifetime. Hence, even if the flavin and folate chromophores take up initially the most extreme orientation, i.e., K^2 = 4 or 0, we find that only a 20% error in the fo-

Table II: Fluorescence Polarization of MTHF and $FADH_2$[a]

fluorophore	P_o	P	τ_F (ns)	τ_c (ns)
MTHF	0.45	0.37	<0.03	<0.12
E-MTHF[b]	0.45	0.22	0.35	0.28
E-$FADH_2$-MTHF[b]	0.45	0.25	0.134	0.14
E-$FADH_2$[c]	0.45	0.23	1.4	1.2
E-FAD_{ox}[c]	0.45	0.1	3.5	0.9

[a] P_o = intrinsic fluorescence polarization in rigid media; P = observed fluorescence polarization; τ_F = fluorescence lifetime; τ_c = rotational correlation time. [b] MTHF fluorescence probed. [c] Flavin fluorescence probed.

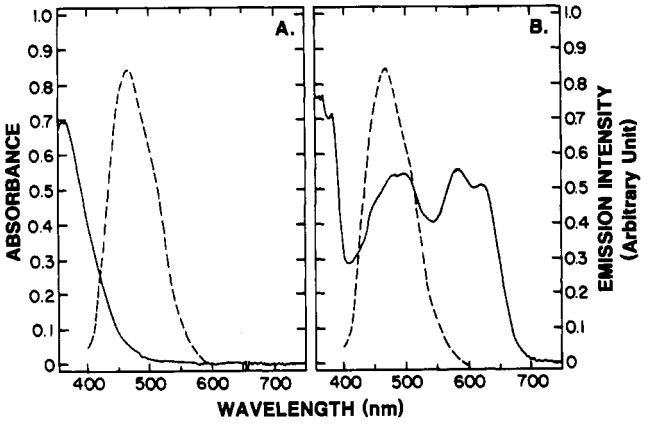

FIGURE 2: Spectral overlap of MTHF fluorescence emission spectrum (broken line) with absorbance spectrum (solid line) of enzyme-bound flavin. (A) Overlap with $FADH_2$ absorbance; (B) overlap with FADH° absorbance. The enzyme solution used for absorbance measurements was 1.1×10^{-4} M.

late–flavin distance would result. In reality, since the folate and flavin can rotate during fluorescence lifetime by 34° and 10°, respectively, the initial orientation of folate emission and flavin absorption vectors should be random, and hence the assumed value of $K^2 = {}^2/_3$ is not likely to result in significant error.

The overlap integral (J), which represents the degree of spectral overlap betwen donor emission and acceptor absorption, was approximated by eq 10 using the data in Figure 2, where the summation was carried out over 5-nm intervals.

$$J = \sum F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda / \sum F_D(\lambda)\Delta\lambda \qquad (10)$$

In this equation, $F_D(\lambda)$ is the corrected fluorescence intensity of the donor and $\epsilon(\lambda)$ is the molar absorption coefficient of the acceptor at that wavelength. Using eqs 8, 9, and 10, the interchromophore distance r is calculated to be 21.1 Å for the MTHF–FADH° pair and 21.7 Å for the MTHF–$FADH_2$ pair. The energy transfer parameters are summarized in Table III.

(*D*) *Picosecond Flash Photolysis.* Direct evidence for energy transfer from folate to flavin was obtained from transient spectra. Figure 3A,B shows the transient absorption spectra of E-MTHF and E-$FADH_2$-MTHF. The E-MTHF spectra manifest two major bands: an intense one at ca. 480 nm and a weak band over the 600–900-nm region. This transient absorption band can be assigned to the first excited signlet state of enzyme-bound MTHF because its lifetime of 480 ps (the lifetime is calculated from the data at 40, 200, and 300 ps only, with a correlation coefficient of −0.9998) matches reasonably well with the fluorescence lifetime (354 ps) of E-MTHF. A longer (560 ps) transient lifetime is calculated with lower correlation coefficient if all time points from 40 ps to 2 ns are used; the discrepancy most likely arises from the contribution of oxidized MTHF impurity to the absorption spectra. We have found that this excited form shows a 1.6-ns fluorescence

Table III: Energy Transfer between Chromophores[a]

	MTHF to FADH°	MTHF to $FADH_2$
E (%)	92	62
R (Å)	31.8	23.5
$J \times 10^{14}$ ($cm^3\ M^{-1}$)	2.06	0.34
r (Å)	21.1	21.7

[a] E = efficiency of energy transfer; R = Förster critical distance assuming an orientation factor $k^2 = 0.67$; J = overlap integral; r = calculated distance. Fluorescence quantum yield of donor $\phi = 0.32$ and refractive index of solvent $n = 1.39$ were used for the calculation.

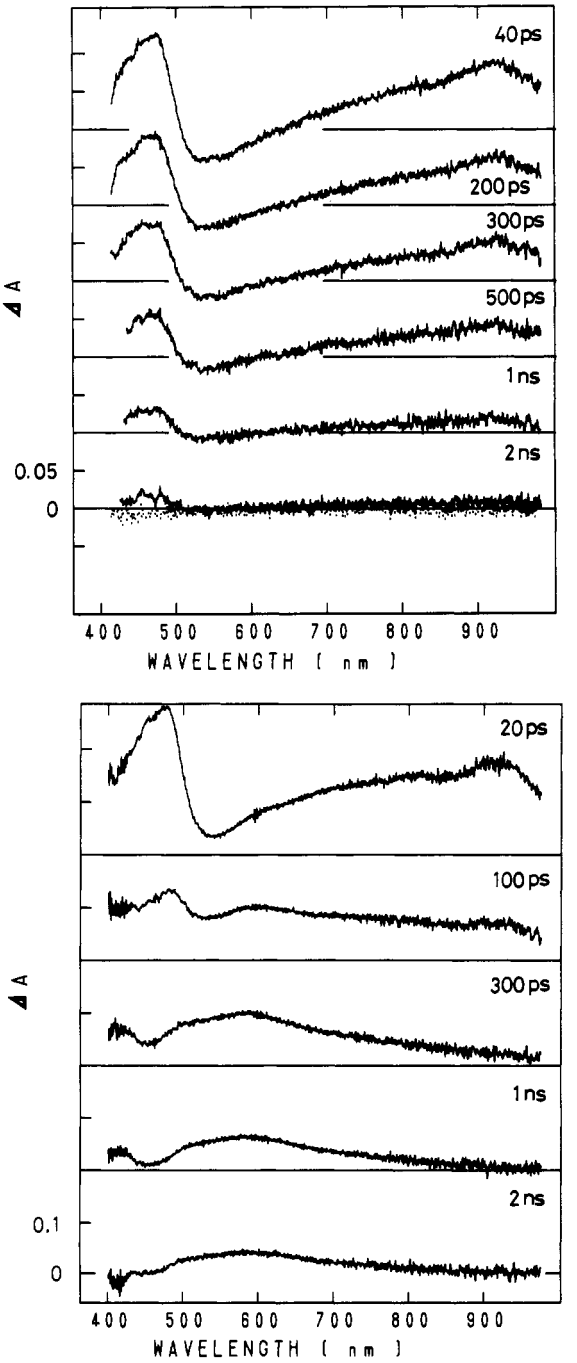

FIGURE 3: Transient absorption spectra of photolyase containing either or both chromophores. (A, top) E-MTHF (1.4×10^{-5} M); (B, bottom) E-$FADH_2$-MTHF (1.9×10^{-4} M with respect to flavin). Delay times after excitation are indicated in the figure. The dotted line shows a baseline (signal without excitation). The samples were excited with either a 336-nm picosecond laser pulse with a width of 8 ps (E-MTHF) or 340-nm pulse with a width of 12 ps (E-$FADH_2$-MTHF).

lifetime. With E-$FADH_2$-MTHF, at the earliest time (20 ps) the absorption spectrum is nearly identical to that of E-MTHF;

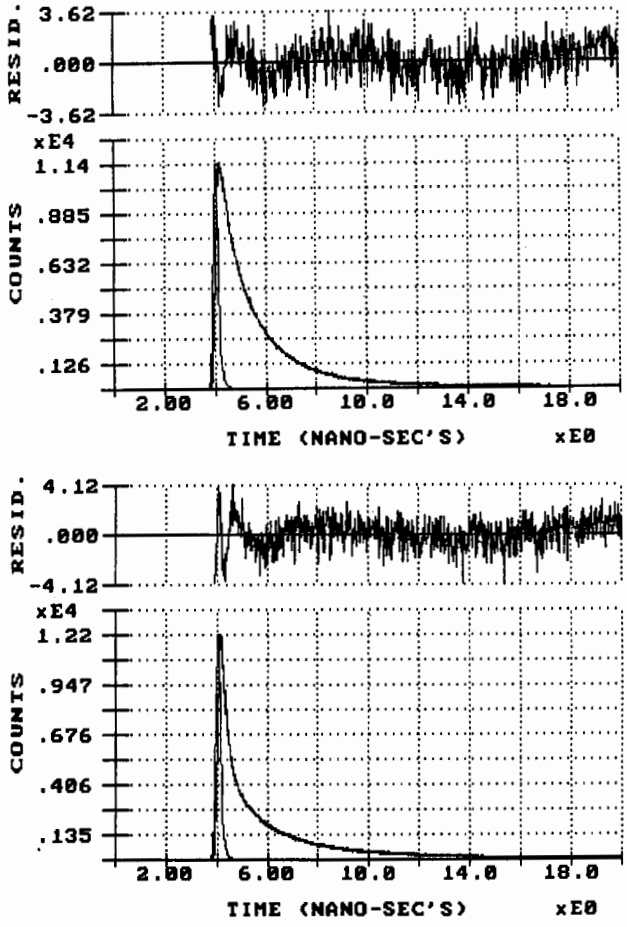

FIGURE 4: Fluorescence decay of E-$FADH_2$ in the presence and absence of substrate. Triexponential fits were used with $\chi^2 = 1.30 \pm 0.05$ (see Results for details). The excitation wavelength was 355 nm, and emission was monitored at $\lambda > 470$ nm. (A, top) E-$FADH_2$ (2×10^{-4} M); (B, bottom) E-$FADH_2$ (2×10^{-4} M) + T<>T (4×10^{-4} M).

however, the folate singlet absorption decays much more rapidly, with a lifetime of ca. 180 ps, again in agreement with the fluorescence lifetime of folate in this form of the enzyme. More importantly, the decay of the band assigned to MTHF excited singlet is followed by the appearance of a new band at 500–600 nm. This band has been previously assigned to the first excited singlet state of enzyme-bound $FADH_2$ (Okamura et al., 1991). Therefore, we consider the spectra in Figure 3B direct evidence for singlet–singlet energy transfer from MTHF to $FADH_2$ in photolyase.

Electron Transfer

(*A*) *Time-Resolved Fluorescence.* The effect of *c-s*-T<>T on the decay curve of the singlet excited state of $FADH_2$ is shown in Figure 4. Analysis of the data shows that the singlet excited state of $FADH_2$ has a lifetime of $\tau_4 = 1.4$ ns (Figure 4A) which goes down to $\tau_5 = 0.16$ ns (Figure 4B) in the presence of T<>T. It should be pointed out that three-component fits were needed to analyze E-$FADH_2$ emission. The shortest component was the residual E-MTHF emission (the majority was removed by UV irradiation). The long-lived component ($\tau \sim 2.5$–3.5 ns) was previously identified with folate impurities. An intermediate lifetime ($\tau = 1.4$ ns) assigned to the $FADH_2$ emission was detected only when enzyme-bound flavin was fully reduced. In the presence of substrate, however, the intermediate lifetime (1.4 ns) is still present at 19% level, which reflects the amount of free enzyme under the experimental conditions employed. The E-$FADH_2$ fluorescence ($\tau = 1.4$ ns) gradually reappears when the enzyme–substrate solution is irradiated with photoreactivating light (data not shown). Use of irradiated oligo$(dT)_{15}$ instead of T<>T as a substrate causes the same effect as T<>T, that is, the reduction of fluorescence lifetime from 1.4 to 0.16 ns (data not shown). However, the amount of unbound enzyme, based on the ratio of the two components, is reduced to 9% under the same conditions (2 mol of photodimer/mole of enzyme).

While these results are consistent with deactivation of the excited state singlet flavin by electron transfer, they do not rule out the possibility of other deactivation pathways as well as deactivation by electron transfer when enzyme is bound to nonsubstrate DNA. To address this point, photolyase was mixed with oligo$(dT)_{15}$ at concentrations of 10^{-4} M photolyase and 3×10^{-1} M thymidine. Under these conditions, all the enzyme is bound nonspecifically as the nonspecific binding constant of photolyase is 2×10^3 M^{-1} (Husain & Sancar, 1987). In addition, the steady-state fluorescence measurements indicate that the decrease in lifetime is not accompanied by the occurrence of a charge-transfer emission at longer wavelengths. More importantly, the absolute action spectrum of E-$FADH_2$ form of *E. coli* photolyase provides evidence that the enzyme repairs thymine dimer with a quantum yield of $\phi_{spl} = 0.8$. These results suggest that the deactivation of singlet excited state of $FADH_2$ by pathways other than electron transfer is negligible in the presence of photodimer. Then the rate of electron transfer can be calculated from

$$k_{ET} = 1/\tau_5 - 1/\tau_4 = 5.5 \times 10^9 \text{ s}^{-1} \quad (11)$$

The quantum yield for electron transfer would then be given by

$$\phi_{ET} = k_{ET} \times \tau_5 = 0.88 \quad (12)$$

Using eqs 5 and 6 and ϕ_F ($FADH_2$) = 0.014, k_F and k_I for $FADH_2$ are calculated to be 1.0×10^7 and 7×10^8 s^{-1}, respectively.

(*B*) *Time-Resolved Absorbance.* These measurements gave results in agreement with those obtained by time-resolved fluorescence. In the absence of substrate or in the presence of UpU dinucleotide, laser photolysis reveals an absorption spectrum with a major peak at ca. 550 nm which decays with a lifetime of 1.7 ns; in the presence of U<>U (Okamura et al., 1991) or T<>T (Figure 5), the lifetime of the transient is down to ca. 0.2 ns. From these values and using eqs 5 and 6, we calculate rate and quantum yields of electron transfer identical to those obtained from the fluorescence quenching experiments. The minimal kinetic data for energy and electron transfer in *E. coli* DNA photolyase are summarized in Figure 6.

(*C*) *Distance between $FADH_2$ and Pyr<>Pyr.* The distance dependence of electron transfer rate is given by (Marcus & Sutin, 1985; Closs & Miller, 1988; Fox, 1990)

$$k_{ET} = k_o \exp[-\beta(R - R_o)] \quad (13)$$

where k_o = the rate constant expected for a donor–acceptor pair in van der Waals contact and has a maximum value of 10^{13} s^{-1}. β is the range parameter and is usually in the range of 0.9–1.2 $Å^{-1}$. R is the center-to-center distance between the donor and acceptor, and R_o is the corresponding distance for a donor–acceptor pair at van der Waals contact (ca. 7 Å). Substituting $k_{ET} = 5.5 \times 10^9$ s^{-1} from eq 13, we obtain R ($FADH_2$–Pyr<>Pyr) $\sim$ 14 Å.

Discussion

The results presented in this study have enabled us to arrive at certain conclusions regarding the environments of the two

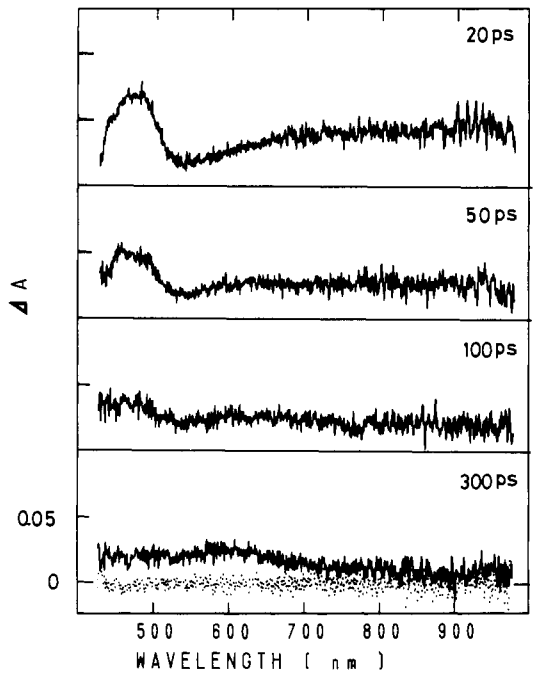

FIGURE 5: Transient absorption spectra of E-$FADH_2$-MTHF (1.9×10^{-4} M with respect to flavin) in the presence of T<>T (3×10^{-3} M). Delay times after excitation are indicated in the figure. The sample was excited with a 336-nm laser pulse with a width of 8 ps. The dotted line represents the baseline (signal without excitation).

chromophores of photolyase and the photophysical processes involved in interchromophore energy transfer and enzyme–substrate electron transfer.

Environments of the Chromophores. Photolyase containing FAD_{ox} either as a result of two-electron oxidation of the intrinsic flavin (Jorns et al., 1987) or as a result of reconstitution of holoenzyme with FAD_{ox} and apoenzyme (Jorns et al., 1990; Payne et al., 1990) reveals a characteristic absorption spectrum. Specifically, while free FAD_{ox} has a smooth absorption spectrum with peaks at 373 and 445 nm, in E-FAD_{ox} the relative heights of these two peaks are reversed and there are additional peaks at 355, 365, 430, and 468 nm. Similarly, enzyme reconstituted with 5-deaza-FAD shows considerable structure in its absorption spectrum compared with that of free 5-deaza-FAD with peaks at 330 and 420 nm superimposed onto the 342- and 402-nm peaks of free deaza-FAD (Payne et al., 1990). These absorption spectra with detailed "structures" are an indication for a nonpolar binding site which holds the chromophore in a unique configuration.

Fluorescence characteristics of MTHF in various solvent systems as well as fluorescence properties of E-MTHF and E-$FADH_2$-MTHF suggest that the folate chromophore too must be in a nonpolar environment in photolyase. However, in contrast to $FADH_2$, MTHF appears to be relatively mobile. Steady-state and time-resolved fluorescence on MTHF in various solvents (Table I) showed that both nonpolar and viscous solvents caused a bathochromic shift in MTHF fluorescence emission from 463 (aqueous) to 469 nm and increased the lifetime from <30 ps to 300–400 ps with a concomitant increase in quantum yield of fluorescence from $\phi_F = 0.012$ to ~ 0.3. Fluorescence polarization studies on E-$FADH_2$-MTHF revealed much higher rotational flexibility for MTHF ($\tau_c = 0.14$ ns) compared to $FADH_2$ ($\tau_c = 1.2$ ns). Thus it appears that the bathochromic shift and increased lifetime of photolyase-bound MTHF is due to a nonpolar environment rather than "high viscosity" of the binding site. However, additional factors must also contribute to the spectroscopic and functional properties of enzyme-bound

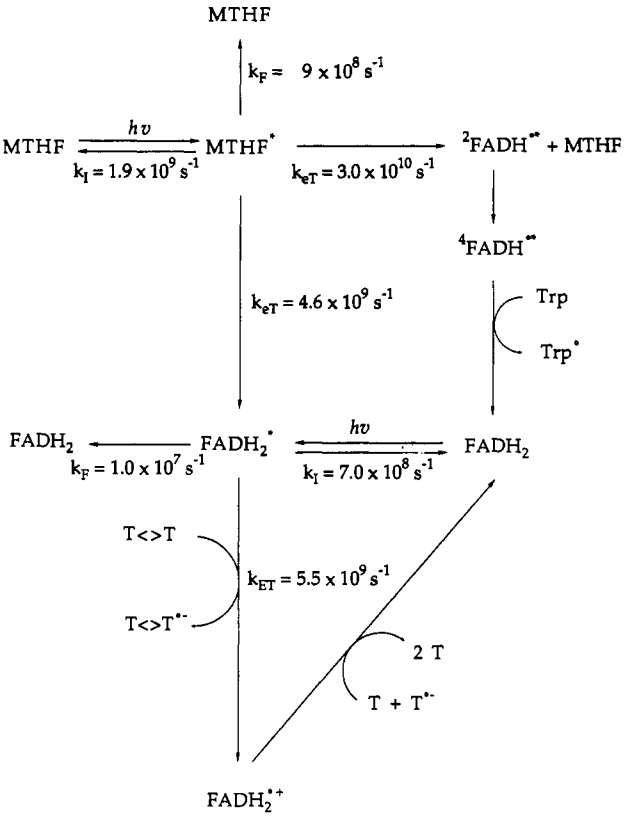

FIGURE 6: Photoexcited state decay kinetics of the two chromophores (MTHF and $FADH_2$) in *E. coli* DNA photolyase. The native enzyme contains $FADH_2$ which is oxidized to the catalytically inert FADH° during purification. The excited singlet state of MTHF transfers energy to FADH°, which following intersystem crossing to excited quartet state abstracts a hydrogen atom from a nearby tryptophan on the apoenzyme. An absorption of a photon by MTHF in the E-$FADH_2$-MTHF results in singlet–singlet energy transfer from MTHF to $FADH_2$ by dipole–dipole interaction. The excited singlet state of $FADH_2$ donates an electron to the Pyr<>Pyr substrate, which is split rapidly with concomitant generation of $FADH_2$. Note that even though electron transfer between $FADH_2$ and substrate is unquestionable, the direction of electron transfer has not been established experimentally. Electron transfer from $FADH_2$ excited state singlet to Pyr<>Pyr appears to be the only thermodynamically feasible mechanism, and hence it is shown as such in this diagram.

MTHF. The bathochromic shift in the absorption maximum of enzyme-bound MTHF (from 354 to 382 nm) is more pronounced than that achievable either by high viscosity or low polarity (from 354 to 368 nm). The significant bathochromic shift in the absorption maximum of the enzyme-bound MTHF might, therefore, be taken as evidence for specific interaction of the MTHF with the polypeptide enzyme.

Electronic Energy Transfer. Previous studies on photoreduction of FADH° (Heelis et al., 1987), fluorescence quenching of MTHF (Jorns et al., 1990), and absolute action spectrum of repair (Payne & Sancar, 1990) with enzyme containing one or both chromophores provided experimental evidence for energy transfer from MTHF to FADH° or $FADH_2$. Further evidence was obtained by picosecond and nanosecond flash photolysis which indicated that the excited state singlet MTHF gave rise, perhaps by an excited state intermediate, to a flavin radical doublet and then quartet (Okamura et al., 1989; Heelis et al., 1990). While the combined data from these various experimental approaches made a strong case for interchromophore electronic energy transfer, the evidence was, nevertheless, indirect. The work reported in this paper provides direct evidence for energy transfer from MTHF to FADH° or $FADH_2$. We have found that the lifetime of MTHF excited singlet state is drastically reduced

from 354 ps (480 ps by absorbance) to less than 30 ps in the presence of FADH° (>90% quenching) and to 134 ps in the presence of $FADH_2$ (62% quenching). This reduction is mainly due to efficient energy transfer: our picosecond laser photolysis experiments with enzyme containing only folate (E-MTHF) and with enzyme containing both chromophores (E-$FADH_2$-MTHF) positively demonstrate that light energy absorbed by MTHF is used to produce the first excited singlet state of $FADH_2$. Upon excitation at 340 nm, MTHF in E-MTHF shows a strong absorption band at ca. 480 nm which decays with a lifetime which agrees reasonably with the fluorescence lifetime and therefore is assigned to MTHF excited singlet state. In E-$FADH_2$-MTHF form, again the earliest observable species is the 480-nm band at 20 ps; however, in this form of the enzyme the 480-nm species decays rapidly, and the decay is followed by the appearance of a new band at 500–600 nm (Figure 3). The absorption band at 500–600 nm has previously been assigned to the first excited singlet state of enzyme bound $FADH_2$ (Okamura et al., 1991). Hence, we conclude that the first excited singlet state of MTHF transfers energy to flavin to give rise to excited singlet state of $FADH_2$.

Having thus established singlet–singlet energy transfer from folate to flavin, we can now consider the mechanism of the transfer process. There are mainly two mechanisms for intermolecular energy transfer (Turro, 1978), dipole–dipole interaction (Förster type) and exchange interaction mechanisms. For Förster-type energy transfer to take place several conditions must be fulfilled. (i) The energy donor must be fluorescent, the higher its fluorescent quantum yield (in the absence of acceptor) the more efficient the energy transfer. (ii) There must be an overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor; efficiency of energy transfer increases with increase in spectral overlap. (iii) For efficient energy transfer, the donor–acceptor separation must not exceed 50–100 Å. The first two conditions are met satisfactorily in photolyase, and, therefore, electronic energy transfer by Förster's mechanism is quite likely.

However, we previously suggested that energy transfer from MTHF to FADH° occurred through an exchange interaction mechanism (Heelis et al., 1990). This conclusion was made primarily on the basis of depletion (minus absorbance) at the 500–600-nm region observed at 40 ps in the transient spectrum of E-FADH°-MTHF. As FADH° is the only absorbing species at this wavelength, it was concluded that an intermediate species involving interaction of ^{1}MTHF with FADH° (encounter complex) was present 40 ps after excitation. However, with the availability of sufficient quantities of E-MTHF, we have now obtained the transient spectra of MTHF in the absence of FADH° (Figure 3). The absorbance decrease in the 500–600-nm region is observed for this species as well. Indeed, we have even observed the same absorbance decrease with free MTHF in acidic 2-propanol (data not shown). We have no definitive explanation for this phenomenon at present. It is conceivable that this arose from excitation of MTHF with an intense picosecond laser pulse which has very high photon density with the consequent secondary luminescence reactions. It must be noted though that the absence of a new transient intermediate in the absorption spectra does not rule out energy transfer by an exchange mechanism. However, paramagnetic NMR studies with [^{13}C]MTHF-FADH° ($FADH_2$) forms of the enzyme showed that FADH° does not cause any significant line broadening in resonance of the methenyl carbon of MTHF, which suggests that the two chromophores are more than 10 Å apart (S.-T. Kim, K. V. Rajagopalan, and A. Sancar, unpublished observation). Thus, we conclude that MTHF transfers energy to $FADH_2$ by dipole–dipole interaction. Such a mechanism might also explain the puzzling observation that, while the photolyase holoenzyme has a near-UV absorption peak at 382 nm, the absolute action spectrum peak is at 366 nm and coincides with the absorption peak of $FADH_2$ (Payne & Sancar, 1990).

Assuming energy transfer by dipole–dipole interaction, the interchromophore distance was calculated using the Förster equation (Förster, 1965) and values of 21.1 and 21.7 Å were found for MTHF-FADH° and MTHF-$FADH_2$ distances, respectively. In general, the most uncertain quantity in calculating the interchomophore distance by the Förster equation is the orientation factor (K^2). By conducting fluorescence polarization, we have established that $FADH_2$ is rotationally restrained while MTHF is relatively mobile, which validated the assumed value of $K^2 = {}^2/_3$ (see Results). Thus, a value of ca. 22 Å for interchromophore distance is quite reasonable and consistent with all available data.

Electron Transfer. The quenching of $FADH_2$ fluorescence (Jorns et al., 1990) and of $FADH_2$ excited singlet state absorbance (Okamura et al., 1991) by Pyr<>Pyr, and the close match between E-$FADH_2$ absorbance and the absolute action spectrum (Payne & Sancar, 1990), provided unambiguous evidence for the occurrence of the photochemical reaction from the first excited singlet state of $FADH_2$. The photochemical reaction apparently involves electron transfer, as evidenced by appearance of a radical intermediate in picosecond laser photolysis reaction (Okamura et al., 1991). From the quenching of $FADH_2$ fluorescence and $FADH_2$ excited singlet state absorbance, we calculate an electron transfer rate of 5.5×10^9 s^{-1}; and from this value, and making certain assumptions regarding the electron transfer milieu, we obtain a value of ~14 Å for the distance between $FADH_2$ and Pyr<>Pyr. The quantum yield for electron transfer at 355 nm is calculated to be $\phi_{ET} = 0.88$. At this wavelength, the quantum yield of repair by E-$FADH_2$ is $\phi_R = 0.72$. It thus appears that the Pyr<>Pyr radical decays with very high yield ($\phi_{spl} = 0.82$) via splitting of the cyclobutane ring. Indeed, considering the technical difficulties in obtaining an accurate extinction coefficient for E-$FADH_2$, it is quite conceivable that ϕ_R (355 nm) is the same as ϕ_R (366 nm) = 0.84 (Payne & Sancar, 1990), which would imply that the Pyr<>Pyr radical splits with a quantum yield of near unity and that the back electron transfer or other deactivating pathways are negligible.

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