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Direct Evidence for Singlet–Singlet Energy Transfer in *Escherichia coli* DNA Photolyase†

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ABSTRACT: The active form of native *Escherichia coli* DNA photolyase contains 1,5-dihydro-FAD ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4Pte$(Glu)_n$]. Enzyme containing $FADH_2$ and/or 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) can be prepared in reconstitution experiments. Fluorescence quantum yield measurements at various wavelengths with native or reconstituted enzyme provide a simple method for detecting singlet–singlet energy transfer from pterin to $FADH_2$, a key step in the proposed catalytic mechanism. The data satisfy the following criteria: (1) Wavelength-independent quantum yield values are observed for 5,10-CH^+-H_4folate in the absence (0.434) or presence (3.57×10^{-2}) of $FADH_2$, for 5,10-CH^+-H_4Pte$(Glu)_n$ in the presence of $FADH_2$ (5.58×10^{-2}) and for $FADH_2$ in the absence of pterin (5.34×10^{-3}); (2) The observed decrease in pterin fluorescence quantum yield in the presence of $FADH_2$ can be used to estimate the efficiency of pterin fluorescence quenching (E_Q = 0.918 or 0.871 with 5,10-CH^+-H_4folate or 5,10-CH^+-H_4Pte$(Glu)_n$, respectively); (3) The fluorescence quantum yield of $FADH_2$ is increased in the presence of pterin and varies depending on the excitation wavelength, in agreement with the predicted effect of energy transfer on acceptor fluorescence quantum yield [$\Phi_{\text{acceptor (+ donor)}}/\Phi_{\text{acceptor (alone)}} = 1 + E_{ET}(\epsilon_{donor}/\epsilon_{acceptor})$, where E_{ET} is the efficiency of the energy transfer process]. With 5,10-CH^+-H_4Pte$(Glu)_n$ in native enzyme the value obtained for E_{ET} (0.92) is similar to E_Q, whereas with 5,10-CH^+-H_4folate in reconstituted enzyme the value obtained for E_{ET} (0.46) is 2-fold smaller than E_Q. The results indicate that the observed quenching of pterin fluorescence in native enzyme is entirely due to energy transfer to $FADH_2$.

DNA photolyase repairs pyrimidine dimers in UV-damaged DNA in a reaction which requires visible light. The active form of the enzyme from *Escherichia coli* contains 1,5-dihydro-FAD[1] ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4Pte$(Glu)_n$, n = 3-6] (Wang et al., 1988; Wang & Jorns, 1989; Jorns et al., 1990; Johnson et al., 1988). [A reversible oxidation of $FADH_2$ to a blue neutral flavin radical ($FADH^\bullet$) occurs during enzyme

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^\bullet$, blue neutral FAD radical; $FADH_2$, 1,5-dihydro-FAD; 5,10-CH^+-H_4Pte$(Glu)_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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isolation (Jorns et al., 1984; Sancar et al., 1987a; Payne et al., 1987).] Either chromophore can act as a sensitizer in catalysis (Sancar et al., 1987a). However, 5,10-CH^+-H_4Pte(Glu)$_n$ is not required when $FADH_2$ acts as sensitizer, but $FADH_2$ is required when 5,10-CH^+-H_4Pte(Glu)$_n$ acts as sensitizer. These results and other studies indicate that $FADH_2$ is the chromophore that directly interacts with substrate whereas 5,10-CH^+-H_4Pte(Glu)$_n$ may act as an antenna, harvesting light energy which is then transferred to $FADH_2$ (Chanderkar & Jorns, 1991; Jorns et al., 1987a, 1990; Wang & Jorns, 1989; Jordan & Jorns, 1988). Formation of an enzyme–substrate complex causes a selective quenching of $FADH_2$ fluorescence in a reaction that is fully reversible upon dimer repair. This result and other data indicate that the excited singlet state of $FADH_2$ ($^1FADH_2$*) is likely to act as a catalytic intermediate (Jordan & Jorns, 1988; Chanderkar & Jorns, 1991; Okamura et al., 1991), suggesting that singlet–singlet energy transfer from excited pterin (^{1}Pte*) to ground-state $FADH_2$ may be catalytically significant.

Direct evidence for singlet–singlet energy transfer is most easily obtained when acceptor fluorescence can be observed upon selective excitation of the donor. The latter may not be feasible when donor and acceptor absorption spectra overlap, as observed with $FADH_2$ and 5,10-CH^+-H_4Pte(Glu)$_n$ bound to photolyase. In this case, fluorescence quantum yield measurements may provide evidence for singlet–singlet energy transfer since a decrease in donor quantum efficiency is predicted, accompanied by an increase in acceptor quantum efficiency. This approach requires that donor and acceptor fluorescence be distinguishable and, for protein-bound chromophores, an apoenzyme that can be reconstituted to yield preparations containing donor and/or acceptor. Both requirements can be satisfied in the case of *E. coli* photolyase.

A certain ambiguity is introduced by comparison of fluorescence quantum yields for a given chromophore in different enzyme preparations since active site conformational changes, induced by chromophore binding, may affect rates of radiationless deactivation of excited chromophores. This ambiguity can be eliminated by measuring quantum yields at various excitation wavelengths. Donor quantum yield values are expected to behave normally, exhibiting wavelength independence in the presence or absence of acceptor. Similar behavior is expected for acceptor quantum yield values in the absence of donor ($\Phi_{\text{acceptor (alone)}}$). However, atypical wavelength-dependent behavior is predicted for acceptor quantum yield values in the presence of donor ($\Phi_{\text{acceptor (+ donor)}}$) because the increase in acceptor quantum yield due to energy transfer will depend on the relative extinction coefficients of donor (ϵ_{donor}) and acceptor ($\epsilon_{\text{acceptor}}$), which vary depending on the excitation wavelength, and also on the efficiency of energy transfer (E_{ET}), which should be wavelength-independent (eq 1).

$$\frac{\Phi_{\text{acceptor(+ donor)}}}{\Phi_{\text{acceptor(alone)}}} = 1 + E_{ET}(\epsilon_{\text{donor}}/\epsilon_{\text{acceptor}}) \qquad (1)$$

In this paper, we report quantum yield studies conducted with apophotolyase reconstituted with $FADH_2$ and/or 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate). Wavelength-independent quantum yield values were observed for all enzyme-bound chromophores except for $FADH_2$ in the presence of 5,10-CH^+-H_4folate where the observed quantum yield varied depending on the excitation wavelength, according to the relationship shown in eq 1. Similar results were obtained for $FADH_2$ and 5,10-CH^+-H_4Pte(Glu)$_n$ in studies with native enzyme. The data provide direct evidence for singlet–singlet energy transfer from pterin to $FADH_2$ in photolyase.

Experimental Procedures

Materials. Phenyl-Sepharose CL-4B was purchased from Pharmacia. Oligo(dT)$_{18}$ was purchased from the University of Pennsylvania DNA Synthesis Service. Quinine sulfate dihydrate (standard reference material 936) was obtained from The National Bureau of Standards. 5-Formyltetrahydrofolate, riboflavin, and FAD were purchased from Sigma.

Preparation of Native Enzyme and Substrate. E. coli photolyase was purified similarly to a previously described procedure (Jorns et al., 1987b) and stored at −70 °C in complete PRE buffer (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol). The flavin radical in the isolated enzyme was converted to $FADH_2$ by photoreduction with yellow light (Westinghouse, F15T8/GO) under anaerobic conditions as described by Jordan and Jorns (1988). The enzyme used for quantum yield studies contained 0.73 mol of 5,10-CH^+-H_4Pte(Glu)$_n$ plus 1.0 mol of $FADH_2$/mol of protein. Enzyme assays were performed at 21 °C with UV-irradiated oligo(dT)$_{18}$ as substrate, similarly to those described by Jorns et al. (1985) except that the assay buffer contained DTT (1.6 mM). For routine assays, substrate was prepared by irradiating oligo(dT)$_{18}$ with germicidal light, as previously described (Jorns et al., 1985). For use in fluorescence quenching experiments, UV-irradiated oligo(dT)$_{18}$ was exposed to black light, as described by Chanderkar and Jorns (1991), to eliminate the fluorescence of a minor photoproduct formed during irradiation with germicidal light (Mitchell & Clarkson, 1984). Protein concentration, determined by the Bradford method (Bradford, 1976), was used in calculating specific activity.

Apoenzyme Preparation and Reconstitution. Apophotolyase was prepared by a chromatographic procedure and then reconstituted with $FADH_2$ and/or 5,10-CH^+-H_4folate as described by Jorns et al. (1990). 5,10-CH^+-H_4folate was prepared as described by Rabinowitz (1963). $FADH_2$ was prepared by reduction of FAD with excess sodium dithionite (0.03 M). Reconstituted enzyme preparations were stored in complete PRE buffer at −20 °C. Enzyme reconstituted with 5,10-CH^+-H_4folate (0.61 mol of 5,10-CH^+-H_4folate/mol of protein) exhibited spectral properties (absorption λ_{max} = 387 nm, fluorescence λ_{max} = 470 nm) similarly to those previously reported (Chanderkar & Jorns, 1991). Enzyme containing $FADH^\bullet$ was isolated after reconstitution with $FADH_2$. The flavin radical was converted to $FADH_2$ by anaerobic photoreduction with yellow light in complete PRE buffer, similarly to that previously described (Jorns et al., 1990). The photoreduced enzyme ($EFADH_2$) exhibited absorption and fluorescence maxima (360 and 509 nm, respectively) similar to that previously described (Jorns et al., 1990). Enzyme containing $FADH^\bullet$ plus 5,10-CH^+-H_4folate was isolated after reconstitution with $FADH_2$ plus 5,10-CH^+-H_4folate. The flavin radical was converted to $FADH_2$ by photoreduction with yellow light in complete PRE buffer, as previously described (Jorns et al., 1990). The chromophore composition observed for two different preparations (0.65 mol of 5,10-CH^+-H_4folate plus 0.85 mol of $FADH_2$/mol of protein; 0.69 mol of 5,10-CH^+-H_4folate plus 0.85 mol of $FADH_2$/mol of protein) was similar to that previously reported (Jorns et al., 1990).

Predicted Effect of Energy Transfer on Acceptor Fluorescence Quantum Yield: Derivation of Equation 1. The fluorescence quantum yield of any molecule, including acceptor in the presence or absence of donor, can be defined as the fraction of total molecules which emit a photon of light (F_{emit}) divided by the fraction which *absorb* a photon of light (F_{absorb})

(eq 2). F_{absorb} can be expressed using the known relationship shown in eq 3 [I_0 = light fluence (mmol quanta/cm^2); Φ_S = quantum efficiency for formation of the excited singlet state, normally equal to 1]. In general, F_{emit} is equal to the probability that the excited acceptor (^{1}acceptor*) will emit light (P_{emit}) multiplied by the fraction of acceptor molecules that are converted to ^{1}acceptor* ($F_{ground\rightarrow singlet}$) (eq 4). P_{emit} is equivalent to the wavelength-independent quantum yield measured for the acceptor in the absence of donor ($\Phi_{acceptor\ (alone)}$) and is assumed to be unchanged in the presence of donor. In the absence of donor, $F_{ground\rightarrow singlet} = F_{absorb} = A\epsilon_{acceptor}$ and $F_{emit\ [acceptor\ (alone)]}$ can be expressed as shown in eq 5. In the presence of donor, $F_{ground\rightarrow singlet}$ is equal to the fraction of acceptor molecules converted to ^{1}acceptor* by direct excitation ($F_{absorb} = A\epsilon_{acceptor}$) plus the fraction formed via singlet–singlet energy transfer from ^{1}donor* ($F_{energy\ transfer} = A\epsilon_{donor}E_{ET}$, where E_{ET} is the efficiency of energy transfer). $F_{emit\ [acceptor\ (+\ donor)]}$ can therefore be expressed as shown in eq 6. Equation 7 is derived from eq 2 using the relationships in eq 3 and 6 and then rearranged to yield eq 1.

$$\Phi_{acceptor(alone\ or\ +\ donor)} = \frac{F_{emit}}{F_{absorb}} \quad (2)$$

$$F_{absorb\ [acceptor\ (alone\ or\ +\ donor)]} = 2.303 I_0 \Phi_S \epsilon_{acceptor} = A\epsilon_{acceptor} \quad (3)$$

$$F_{emit\ [acceptor\ (alone\ or\ +\ donor)]} = (P_{emit})(F_{ground\rightarrow singlet}) \quad (4)$$

$$F_{emit\ [acceptor\ (alone)]} = \Phi_{acceptor\ (alone)} A\epsilon_{acceptor} \quad (5)$$

$$F_{emit\ [acceptor\ (+\ donor)]} = \Phi_{acceptor\ (alone)}(A\epsilon_{acceptor} + A\epsilon_{donor}E_{ET}) \quad (6)$$

$$\Phi_{acceptor\ (+\ donor)} = \frac{\Phi_{acceptor\ (alone)}(A\epsilon_{acceptor} + A\epsilon_{donor}E_{ET})}{A\epsilon_{acceptor}} \quad (7)$$

$$\frac{\Phi_{acceptor\ (+\ donor)}}{\Phi_{acceptor\ (alone)}} = 1 + E_{ET}(\epsilon_{donor}/\epsilon_{acceptor}) \quad (1)$$

Absorption Spectroscopy. Unless otherwise indicated, spectra were recorded in complete PRE buffer at 5 °C with a Perkin-Elmer λ 3 spectrophotometer. For determination of chromophore content, protein concentration was determined on the basis of absorbance at 280 nm ($\epsilon_{280} = 1.00 \times 10^5$ M^{-1} cm^{-1}), after correcting for contributions from bound chromophores, as previously described (Jorns et al., 1990; Wang & Jorns, 1989). The concentration of enzyme-bound FADH$^\bullet$ was determined on the basis of its absorbance at 580 ($\epsilon_{580} = 4.8 \times 10^3$ M^{-1} cm^{-1}) (Wang & Jorns, 1989) and used to estimate the $FADH_2$ content after anaerobic photoreduction. In the absence of pterin, the concentration of enzyme-bound $FADH_2$ could also be determined on the basis of its absorbance at 360 nm ($\epsilon_{360} = 6.00 \times 10^3$ M^{-1} cm^{-1}) (Jorns et al., 1990). Enzyme-bound pterin [5,10-CH$^+$-H_4Pte(Glu)$_n$ or 5,10-CH$^+$-H_4folate] was determined on the basis of its absorption at 380 ($\epsilon_{380} = 25.9 \times 10^3$ M^{-1} cm^{-1}), corrected, when necessary, for contributions due to bound flavin, as previously described (Jorns et al., 1990).

Previously undetermined extinction coefficients in the 330–390-nm region were estimated using absorption spectra obtained for reconstituted enzyme containing only $FADH_2$ ($EFADH_2$) or 5,10-CH$^+$-H_4folate (EPte) to generate a set of extinction coefficients for each chromophore: ϵ_{EFADH_2} = 4.61, 4.87, 5.59, 6.00, 5.70, 4.98, and 3.84 mM^{-1} cm^{-1} at 330, 340, 350, 360, 370, 380, and 390 nm, respectively; ϵ_{EPte} = 8.99, 11.89, 15.86, 20.32, 24.15, 25.90, and 25.71 mM^{-1} cm^{-1} at 330, 340, 350, 360, 370, 380, and 390 nm, respectively. Values calculated for $\epsilon_{Pte}/\epsilon_{FADH_2}$ were used to plot quantum yield data according to eq 1, after corrections were made for the somewhat less than stoichiometric amounts of $FADH_2$ and pterin found in enzyme preparations containing both chromophores. Assuming a random chromophore distribution, a typical reconstituted enzyme preparation containing 0.69 mol of 5,10-CH$^+$-H_4folate plus 0.85 mol of $FADH_2$/mol of protein will consist of the following mixture of enzyme forms: $EPteFADH_2$, 58.7%; $EFADH_2$, 26.4%; EPte, 10.4%; E, 4.65%. The quantum yield observed for $FADH_2$ fluorescence with this preparation will be a weighted average of contributions due to the fraction of enzyme containing both chromophores [X = $EPteFADH_2/(EPteFADH_2 + EFADH_2)$ = 0.69] plus the fraction containing only $FADH_2$ (1 − X). The expression derived for the fluorescence quantum yield of $FADH_2$ under these conditions is identical to eq 1 provided the ratio $\epsilon_{Pte}/\epsilon_{FADH_2}$, calculated for 100% $EPteFADH_2$, is corrected by multiplying by X.

Quantum Yield Measurements. Unless otherwise noted, fluorescence emission spectra were recorded in complete PRE buffer at 5 °C using a Perkin-Elmer LS-50 luminescence spectrometer. A small blank due to buffer was subtracted, and the spectra were then corrected using quinine sulfate dihydrate as standard. No correction was made for possible polarization artifacts. Quantum yields were determined by a procedure similar to that described by Sun et al. (1972) using riboflavin (2.0 μM) in water as standard (Φ = 0.25) (Moore et al., 1977). Concentrations of enzyme-bound chromophores in experiments with various photolyase preparations are indicated in Table I and the legend to Figure 3. In all cases, the samples exhibited absorbance values less than 0.1 at the excitation wavelengths used to record fluorescence emission spectra.

Results and Discussion

Flavin-Free Reconstituted Enzyme. Enzyme containing only pterin (EPte) was prepared by reconstituting apophotolyase with 5,10-CH$^+$-H_4folate. 5,10-CH$^+$-H_4folate [5,10-CH$^+$-H_4Pte(Glu)$_n$, n = 1] lacks the polyglutamate moiety found in the less readily available native chromophore [5,10-CH$^+$-H_4Pte(Glu)$_n$, n = 3–6] and is less tightly bound, particularly in the absence of $FADH_2$ (R. S. A. Lipman and M. S. Jorns, unpublished results; Hamm-Alvarez et al., 1990). Slow dissociation of 5,10-CH$^+$-H_4folate, accompanied by a decrease in pterin fluorescence, is observed upon dilution of concentrated stock solutions. Errors due to pterin release were minimized by recording data immediately after sample preparation under conditions ([EPte] ≥ 0.8 μM, 50% glycerol, 5 °C) where the bound chromophore was stable during the time required for measurements. Neither pterin fluorescence intensity or stability was affected by addition of substrate (UV-irradiated oligo(dT)$_{18}$, 3-fold excess), but it is not known whether substrate binds to EPte. [Substrate is not bound to apoenzyme, but binding is observed with preparations containing only FAD in any redox state, suggesting that flavin may be essential for binding (Jorns et al., 1990; Payne et al., 1990; Sancar et al., 1987b).] Quantum yield values for EPte were determined on the basis of emission spectra obtained by excitation at various wavelengths in the range 330–390 nm. The results show that the quantum yield of enzyme-bound 5,10-CH$^+$-H_4folate is rather large (Φ_{EPte} = 0.434) and exhibits normal wavelength-independent behavior (Table I).

Pterin-Free Reconstituted Enzyme. Enzyme containing $FADH_2$ ($EFADH_2$) is formed during reconstitution of apophotolyase with $FADH_2$ but oxidation to a blue neutral flavin radical (EFADH$^\bullet$) occurs during isolation, a process which can be reversed by anaerobic photoreduction of the isolated

Table I: Fluorescence Quantum Yield of Chromophores Bound to Photolyase

excitation wavelength (nm)	fluorescence quantum yield[a] (Φ)			
	Φ($EFADH_2$) ($\times 10^3$)	Φ(EPte)	Φ(Pte in EPteFADH•) ($\times 10^3$)	Φ(Pte in $EPteFADH_2$) ($\times 10^2$)
330	4.97	0.407	6.70	3.56
340	5.48	0.445	6.45	3.47
350	5.60	0.436	6.60	3.55
360	5.69	0.436	6.88	3.73
370	5.61	0.438	6.93	3.63
380	5.12	0.434	6.84	3.55
390	4.92	0.439	6.86	3.53
av	5.34 ± 0.12	0.434 ± 0.005	6.75 ± 0.07	3.57 ± 0.03

[a] Quantum yield values were determined as detailed under Experimental Procedures using various reconstituted enzyme preparations: $EFADH_2$ (1.06 μM $FADH_2$); EPte (1.02 μM pterin); EPteFADH• (2.29 μM pterin, 2.58 μM FADH•); $EPteFADH_2$ (2.93 μM pterin, 3.85 μM $FADH_2$).

enzyme (Jorns et al., 1990). The fluorescence of $EFADH_2$ (emission λ_{max} = 509 nm) was quenched upon addition of substrate (UV-irradiated oligo(dT)$_{18}$, 3-fold excess), similarly to that previously reported. Fluorescence quantum yield values were determined on the basis of the fluorescence quenched by substrate and were found to be wavelength-independent ($\Phi_{EFADH_2} = 5.34 \times 10^{-3}$, see Table I), similar to that observed for EPte, but nearly 2 orders of magnitude smaller.

Pterin Fluorescence in Reconstituted Enzyme Containing both Flavin and Pterin. Enzyme containing 5,10-CH^+-H_4-folate plus FADH• (EPteFADH•) was prepared by reconstitution of apoenzyme with $FADH_2$ plus 5,10-CH^+H_4folate, followed by partial air oxidation of $FADH_2$ during isolation. The fluorescence observed for EPteFADH• (emission λ_{max} = 470 nm) is due to 5,10-CH^+-H_4folate since FADH• is nonfluorescent (Jorns et al., 1984). The fluorescence quantum yield of the pterin in EPteFADH• [Φ(Pte in EPteFADH•) = 6.75×10^{-3}] is wavelength-independent but 64.3-fold smaller than that observed with EPte. This difference can be used to determine the efficiency of pterin fluorescence quenching by FADH• [E_Q = 1 − [Φ(Pte in EPteFADH•)/Φ(EPte)] = 0.984]. The observed value for E_Q is in good agreement with a value estimated in previous studies based on fluorescence intensity measurements (E_Q = 0.931) (Jorns et al., 1990) and a value recently determined on the basis of pterin fluorescence lifetime data (E_Q = 0.915) (Kim et al., 1991). The observed effects of FADH• on pterin fluorescence are consistent with singlet–singlet energy transfer from pterin to FADH•. Direct evidence has been obtained in picosecond laser flash photolysis studies (Heelis et al., 1990).

Anaerobic photoreduction of EPteFADH• was used to generate enzyme containing $FADH_2$ plus 5,10-CH^+-H_4folate ($EPteFADH_2$). The fluorescence of $EPteFADH_2$ (Figure 1, curve 1) is dominated by pterin emission (emission λ_{max} = 470 nm), but a pronounced shoulder around 510 nm is contributed by $FADH_2$. The shoulder is virtually eliminated upon addition of substrate which selectively quenches $FADH_2$ fluorescence (Jordan & Jorns, 1988). The emission due to pterin in free $EPteFADH_2$ (Figure 1, curve 2) was estimated on the basis of the emission spectrum observed for the enzyme–substrate complex (not shown), after correction was made for a small (5.8%) increase in pterin fluorescence caused by substrate binding. A similar effect of substrate was seen on pterin fluorescence in EPteFADH• but not in EPte which may not bind substrate (vide supra). The pterin fluorescence quantum yield in $EPteFADH_2$ [Φ(Pte in $EPteFADH_2$) = 3.57×10^{-2}] was found to be wavelength-independent (Table I) and 5.3-fold larger than in the presence of FADH•. The latter is consistent with previous studies with the isolated form of native enzyme

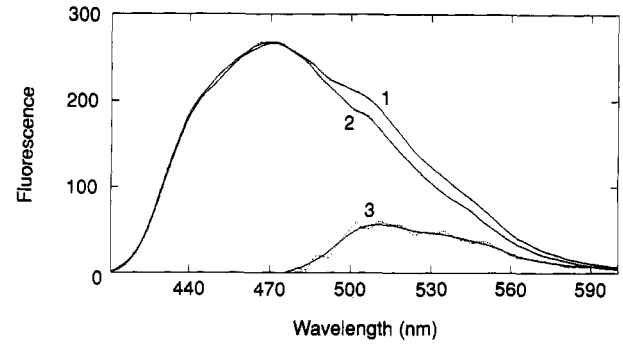

FIGURE 1: Fluorescence properties of reconstituted enzyme containing 5,10-CH^+-H_4folate plus $FADH_2$ ($EPteFADH_2$). Curve 1 is the fluorescence emission spectrum of $EPteFADH_2$ recorded under anaerobic conditions in complete PRE buffer at 5 °C (excitation λ = 390 nm). Substrate (UV-irradiated oligo(dT)$_{18}$, 3-fold excess) was added from a side arm to quench $FADH_2$ fluorescence. The emission spectrum of the pterin component in free $EPteFADH_2$ (curve 2) was calculated by correcting the emission spectrum of the enzyme–substrate complex (not shown) for a small (5.8%) increase in pterin fluorescence caused by substrate. The emission spectrum of $FADH_2$ bound to $EPteFADH_2$ (curve 3) was calculated by subtracting curve 2 from curve 1 and is shown expanded 2.5-fold relative to curves 1 and 2. The solid line in curve 3 was generated using an unweighted running average procedure. The actual data points (noisy trace) were used for quantum yield calculations.

where a 6-fold increase in pterin fluorescence intensity was observed upon reduction of FADH• to $FADH_2$ (Jorns et al., 1987a).

The fluorescence quantum yield observed for 5,10-CH^+-H_4folate in the presence of $FADH_2$ is 12.2-fold smaller than the value observed with EPte, corresponding to an estimated 91.8% efficiency of pterin fluorescence quenching by $FADH_2$. This value is considerably higher than values estimated in other studies based on pterin fluorescence intensity measurements (E_Q = 0.583) (Jorns et al., 1990) or pterin fluorescence lifetime data (E_Q = 0.621) (Kim et al., 1991). To directly estimate the effect of $FADH_2$ on pterin fluorescence in a single enzyme preparation, EPte was mixed with a 10-fold excess of $FADH_2$ and the decrease in pterin emission was monitored at 470 nm, a wavelength where emission due to enzyme-bound $FADH_2$ is negligible. (Free $FADH_2$ is nonfluorescent under the experimental conditions.) Binding of $FADH_2$ to EPte was complete within 70 min (Figure 2, curve 1) and resulted in a 16.2-fold quenching of pterin fluorescence or 14.9-fold when corrected for a small fluorescence decrease (7.6%) observed with a control sample over the same time period (Figure 2, curve 2). The decrease in pterin fluorescence intensity upon binding $FADH_2$ corresponds to an estimated 93.3% efficiency of pterin fluorescence quenching, in good agreement with the quantum yield data. The reason for the discrepancy between these results and previous studies is unclear, but it is noteworthy that EPte fluorescence intensity can be underestimated unless care is taken to prevent pterin dissociation.

$FADH_2$ Fluorescence in Reconstituted Enzyme Containing both Flavin and Pterin. The emission due to $FADH_2$ in $EPteFADH_2$ was estimated from the difference spectrum obtained after subtraction of the contribution due to the pterin chromophore (Figure 1, curve 3). Difference spectra generated from data obtained at various excitation wavelengths exhibited emission maxima at 509 nm, similar to that observed for $EFADH_2$. However, unlike $EFADH_2$, quantum yield values determined for $FADH_2$ in $EPteFADH_2$ varied depending in the excitation wavelength, increasing by more than 200% as the excitation wavelength was increased from 330 nm [Φ($FADH_2$ in $EPteFADH_2$) = 6.96×10^{-3}] to 390 nm [Φ($FADH_2$ in $EPteFADH_2$) = 1.50×10^{-2}]. If the observed

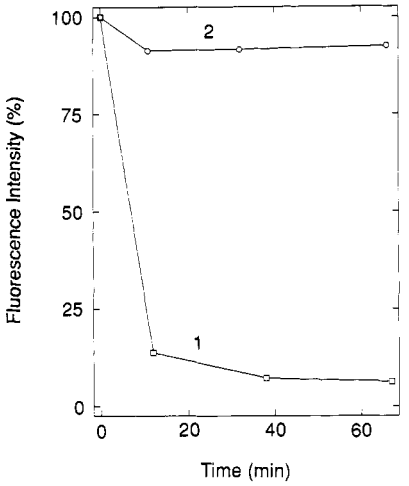

FIGURE 2: Binding of $FADH_2$ to enzyme containing 5,10-CH^+-H_4-folate (EPte). EPte (1.39×10^{-6} M) in anaerobic complete PRE buffer at 5 °C was mixed with 1.39×10^{-5} M $FADH_2$ containing 10 mM sodium dithionite. Binding of $FADH_2$ was monitored by following the decrease in pterin fluorescence intensity at 470 nm (excitation λ = 380 nm) (curve 1). Curve 2 is a control experiment where EPte was incubated with 10 mM sodium dithionite. The initial sharp decrease in fluorescence intensity is not seen in the absence of dithionite and may reflect a small quenching effect of dithionite.

Table II: Efficiency of Interchromophore Energy Transfer (E_{ET}) versus Pterin Fluorescence Quenching Efficiency (E_Q)

enzyme preparation	chromophores	E_{ET}	E_Q
reconstituted	5,10-CH^+-H_4folate, $FADH_2$	0.46	0.918
native	5,10-CH^+-H_4Pte(Glu)$_n$, $FADH_2$	0.92	0.918 (0.871)[a]

[a] Values estimated as described in the text.

variation in quantum yield is due to singlet–singlet energy transfer from 5,10-CH^+-H_4folate to $FADH_2$, the data should obey the relationship shown in eq 1 (see Experimental Procedures for derivation) and a plot of $\Phi(FADH_2$ in $EPteFADH_2)/\Phi(EFADH_2)$ versus $\epsilon_{Pte}/\epsilon_{FADH_2}$ should be linear with a y-intercept equal to 1 and a slope equal to the efficiency of energy transfer (E_{ET}). A straight line was obtained (correlation coefficient = 0.96) when data from two separate experiments with different preparations of $EPteFADH_2$ were pooled and plotted according to eq 1 (Figure 3A, line 1). The efficiency of energy transfer, estimated from the slope (E_{ET} = 0.46 ± 0.01), is 2-fold smaller than the efficiency of pterin fluorescence quenching by $FADH_2$ (Table II). In the derivation of eq 1, it is assumed that the probability that excited $FADH_2$ will emit fluorescence (P_{emit}) is unaffected by the presence of pterin. The small discrepancy between the observed y-intercept (0.71 ± 0.03) and the expected value suggests that this assumption may be an approximation. A plot of quantum yield values obtained for pterin in $EPteFADH_2$ versus $\epsilon_{Pte}/\epsilon_{FADH_2}$ (Figure 3B, line 1) illustrates the dramatic difference in results obtained for the energy donor versus acceptor at the same active site.

Studies with Native Enzyme. Native enzyme containing $FADH_2$ plus 5,10-CH^+-H_4Pte(Glu)$_n$ (n = 3–6) was prepared by anaerobic photoreduction of the flavin radical in the isolated enzyme and quantum yields were determined as described for reconstituted enzyme. The quantum yield of 5,10-CH^+-H_4Pte(Glu)$_n$ in native enzyme was found to be wavelength-independent [Φ(Pte(native) in EPte(native)$FADH_2$) = (5.58 ± 0.04) $\times 10^{-2}$] (Figure 3B, line 2) but was 1.56-fold larger than observed for 5,10-CH^+-H_4folate in reconstituted enzyme containing $FADH_2$ ($EPteFADH_2$). [A similar difference (1.63-fold) is found when the quantum yield reported for

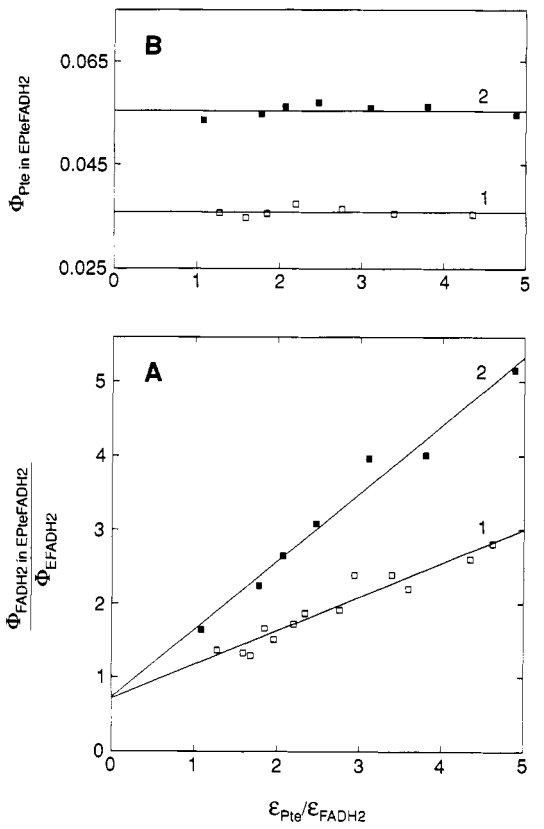

FIGURE 3: Effect of excitation wavelength on the fluorescence quantum yield of $FADH_2$ and 5,10-CH^+-H_4folate in reconstituted enzyme containing both chromophores and on the fluorescence quantum yield of $FADH_2$ and 5,10-CH^+-H_4Pte(Glu)$_n$ in native photolyase. Panel A: Quantum yield values determined for $FADH_2$ in reconstituted enzyme (line 1) or native enzyme (line 2) at various excitation wavelengths were divided by the wavelength-independent quantum yield determined for $FADH_2$ in $EFADH_2$ and the ratio [$\Phi(FADH_2$ in $EPteFADH_2)/\Phi(EFADH_2)$] was plotted versus $\epsilon_{Pte}/\epsilon_{FADH_2}$, corrected for the observed chromophore content of each preparation, as described under Experimental Procedures. Results obtained with one preparation of reconstituted enzyme (1.97 mM pterin, 2.58 μM $FADH_2$) were pooled with those obtained in a separate experiment using a different preparation of reconstituted enzyme (2.93 μM pterin, 3.85 μM $FADH_2$). The native enzyme data were collected under similar conditions (2.55 μM pterin, 3.90 μM $FADH_2$). In each case, the data were analyzed by linear regression analysis. Panel B: Quantum yield values determined for 5,10-CH^+-H_4folate in reconstituted enzyme (line 1) or for 5,10-CH^+-H_4Pte(Glu)$_n$ in native enzyme (line 2) were plotted versus $\epsilon_{Pte}/\epsilon_{FADH_2}$, corrected as described above. The data with reconstituted (2.93 μM pterin, 3.85 μM $FADH_2$) or native (2.55 μM pterin, 3.90 μM $FADH_2$) enzyme were collected under similar conditions.

5,10-CH^+-H_4Pte(Glu)$_n$ in native enzyme containing $FADH^\bullet$ (1.1×10^{-2}) (Jordan & Jorns, 1988) is compared with the corresponding value obtained for 5,10-CH^+-H_4folate in reconstituted enzyme [Φ(Pte in $EPteFADH^\bullet$) = 6.75×10^{-3}].] Values for the efficiency of pterin fluorescence quenching by $FADH_2$ in native enzyme (E_Q = 0.918 or 0.871) were estimated by assuming that the quantum yield of 5,10-CH^+-H_4Pte(Glu)$_n$ in flavin-free enzyme is 1.56-fold larger than or equal to, respectively, the value determined for 5,10-CH^+-H_4folate in EPte.

Quantum yield values determined for $FADH_2$ in native enzyme varied depending on the excitation wavelength, increasing by more than 300% as the excitation wavelength was increased from 330 nm [$\Phi(FADH_2$ in EPte(native)$FADH_2$) = 8.76×10^{-3}] to 390 nm ($\Phi(FADH_2$ in EPte(native)$FADH_2$) = 2.75×10^{-2}). A straight line (correlation coefficient = 0.99, y-intercept = 0.73 ± 0.08) was obtained when the data was plotted according to eq 1 (Figure 3A, line 2). The value

obtained for the efficiency of energy transfer (E_{ET} = 0.92 ± 0.03) is similar to the estimated efficiency of pterin fluorescence quenching by $FADH_2$ (Table II).

Conclusions

Fluorescence quantum yield measurements at various wavelengths provide a simple method for detecting singlet–singlet energy transfer from pterin [5,10-CH^+-H_4folate or 5,10-CH^+-H_4Pte(Glu)$_n$] to $FADH_2$ bound to *E. coli* photolyase. The data satisfy the following criteria: (1) Wavelength-independent quantum yield values are observed for pterin in the presence or absence of $FADH_2$ and for $FADH_2$ in the absence of pterin; (2) A decrease in pterin fluorescence quantum yield is observed in the presence of $FADH_2$, and the data can be used to calculate the efficiency of pterin fluorescence quenching (E_Q); (3) The fluorescence quantum yield of $FADH_2$ is increased in the presence of pterin and varies depending on the excitation wavelength, in agreement with the predicted effect of energy transfer on acceptor fluorescence quantum yield (eq 1). The data were used to estimate the efficiency of the energy transfer process (E_{ET}).

E_{ET} will be equal to E_Q only if the observed quenching of pterin fluorescence is entirely due to energy transfer to $FADH_2$. This appears to be the case for 5,10-CH^+-H_4Pte(Glu)$_n$ in native enzyme but not for 5,10-CH^+-H_4folate in reconstituted enzyme where E_{ET} is 2-fold smaller than E_Q, although similar values are obtained for E_Q with both preparations (Table II). E_{ET} has been estimated previously by comparing the quantum efficiency of dimer repair observed for enzyme containing only $FADH_2$ versus enzyme containing both $FADH_2$ and pterin. However, these studies were conducted with enzyme containing a mixture of native pterin plus 5,10-CH^+-H_4folate and values estimated for E_{ET} at different excitation wavelengths exhibited a puzzling variability, ranging from 0.649 to 0.968 (Payne & Sancar, 1990).

Direct evidence for singlet–singlet energy transfer between pterin and fully reduced flavin bound to photolyase was previously obtained with enzyme containing a modified flavin, 1,5-dihydro-5-deaza-FAD, in place of $FADH_2$ (Chanderkar & Jorns, 1991). In this case, pterin fluorescence was observed upon excitation of the reduced flavin, providing classic evidence for energy transfer, but the direction was opposite to what is believed to occur during catalysis with native enzyme. While this manuscript was in preparation, Kim et al. (1991) briefly noted that evidence for singlet–singlet energy transfer between $FADH_2$ and pterin had been obtained in time-resolved absorption spectroscopy studies, but no details were reported.

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