An Unnatural Folate Stereoisomer Is Catalytically Competent in DNA Photolyase†

Richard S. A. Lipman and Marilyn Schuman Jorns*

Department of Biochemistry, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19102

Received March 6, 1996; Revised Manuscript Received April 12, 1996⊗

ABSTRACT: The folate chromophore in native *Escherichia coli* DNA photolyase ([6*R*]-5,10-CH^+-H_4Pte$(Glu)_{n=3-6}$) serves as an antenna, transferring light energy to the fully reduced flavin ($FADH_2$) reaction center at high efficiency ($E_{ET} = 0.92$). Apophotolyase reconstituted after an overnight incubation with [6*R*,*S*]-5,10-CH^+-H_4folate (a monoglutamate analogue of the native cofactor) contains equimolar amounts of the [6*R*]- and [6*S*]-isomers, suggesting similar binding affinities. A rapid, biphasic increase in fluorescence (∼100-fold) is observed upon binding of 5,10-CH^+-H_4folate to apophotolyase at 5 °C; the [6*S*]-isomer binds about 25-fold faster than the [6*R*]-isomer. Although identical absorption and fluorescence emission maxima are observed for enzyme reconstituted with [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate, folate fluorescence quantum yield values vary depending on the stereochemical configuration at the 6 position ($\Phi = 0.18$, 0.82, or 0.46, respectively, at 5 °C), a feature not seen with free folate. The fluorescence of enzyme-bound folate is quenched upon flavin binding; the efficiency of quenching by flavin radical ($E_Q = 0.96$) or $FADH_2$ ($E_Q = 0.89$) is the same for both folate isomers. In contrast, energy transfer from folate to $FADH_2$ is sensitive to the stereochemical configuration at the 6 position. The efficiency of energy transfer observed for enzyme containing $FADH_2$ and [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate ($\Phi = 0.26$, 0.66, or 0.44, respectively) is directly proportional to the fluorescence quantum yield observed for folate in the absence of $FADH_2$, as expected for Förster-type energy transfer. Although less efficient, the unnatural [6*S*]-isomer is catalytically functional, a feature not previously observed with other folate-dependent enzymes. Fluorescence quantum yield studies at 77 K with free ($\Phi = 0.67$) and enzyme-bound ($\Phi = 1.0$) folate suggest that differences in solvent exposure may contribute to the fluorescence efficiency differences observed with the enzyme-bound folate isomers at 5 °C.

DNA photolyase repairs pyrimidine dimers in DNA damaged by ultraviolet light in a reaction which requires visible light. DNA photolyase from *Escherichia coli* contains folate ([6*R*]-5,10-CH^+-H_4Pte$(Glu)_{n=3-6}$) and reduced FAD.[1] The enzyme is isolated with the flavin present as a neutral radical ($FADH^•$), but the catalytically active form contains fully reduced FAD ($FADH_2$). DNA repair involves reaction of the pyrimidine dimer with the excited singlet state of $FADH_2$. The $FADH_2$ singlet state is generated by direct light absorption or by energy transfer from the singlet state of folate. The spectral properties of the two chromophores are such that the latter pathway predominates under natural (sunlight) light conditions (Jordan & Jorns, 1988; Jorns et al., 1987b, 1990; Lipman & Jorns, 1992; Ramsey et al., 1992; Rustandi & Jorns, 1995; Okamura et al., 1991; Kim et al., 1991).

Direct evidence for singlet−singlet energy transfer from folate to $FADH_2$ was obtained in fluorescence quantum yield studies (Lipman & Jorns, 1992). These studies showed that interchromophore energy transfer is a very efficient process in native enzyme ($E_{ET} = 0.92$). A 2-fold decrease in energy transfer efficiency was observed in enzyme reconstituted with [6*R*,*S*]-5,10-CH^+-H_4folate, a difference which might be due to the substitution of a single glutamate for the polyglutamate moiety found in the natural chromophore or because the synthetic folate was racemic.

Recent studies show that enzyme reconstituted with [6*R*,*S*]-5,10-CH^+-H_4folate contains nearly equimolar amounts of the [6*S*]- and [6*R*]-isomers (Lipman et al., 1995). Binding of unnatural folate isomers has been observed with other folate-dependent enzymes. The unnatural isomers are not catalytically functional, although binding is competitive with respect to the natural isomers (Smith et al., 1981; Leary et al., 1974). In the case of photolyase, the decreased energy transfer efficiency observed with enzyme reconstituted with racemic 5,10-CH^+-H_4folate might be due to the absence of energy transfer in 50% of the preparation containing the [6*S*]-isomer.

We sought to test this hypothesis by measuring energy transfer efficiency in enzyme reconstituted with *either* [6*S*]- or [6*R*]-5,10-CH^+-H_4folate. Surprisingly, the results show that the unnatural folate isomer is catalytically competent and able to transfer energy to $FADH_2$ at nearly 40% of the efficiency observed for the natural isomer.

EXPERIMENTAL PROCEDURES

Materials. Phenyl Sepharose CL-4B was purchased from Pharmacia. Oligo$(dT)_9$ was purchased from Ransom Hill. [6*R*,*S*]-5-Formyltetrahydrofolate, FAD, and riboflavin were obtained from Sigma. [6*S*]- and [6*R*]-5-formyltetrahydrofolate were obtained from B. Schircks Laboratories (Jona, Switzerland). Quinine sulfate dihydrate (standard reference

† This work was supported in part by Grant GM 31704 (M.S.J.) from the National Institutes of Health.

* To whom correspondence and requests for reprints should be addressed. Phone: (215) 762-7946; FAX: (215) 246-5836; E-mail: jornsm@hal.hahnemann.edu.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^•$, 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.

S0006-2960(96)00554-5 CCC: $12.00

material 936) was obtained from The National Bureau of Standards. Anhydrous ethanol (U.S.P.) (The Warner-Graham Co.) was obtained from the Hahnemann University Hospital Pharmacy. All other organic solvents (Fisher Scientific) were HPLC grade except ethyl acetate and isobutyl alcohol (ACS grade).

Preparation of Native Enzyme and Substrate. *E. coli* photolyase was purified as described by Jorns et al. (1987a) and stored at −80 °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). Substrate was prepared by irradiating oligo(dT)$_9$ with germicidal light and then with black light, as previously described (Jorns et al., 1985; Chanderkar & Jorns, 1991).

Enzyme Assay. Enzyme assays were conducted similar to that previously described using UV-irradiated oligo(dT)$_9$ as substrate (Jorns et al., 1985, 1990). FADH$^\bullet$ in native or reconstituted enzyme is converted to $FADH_2$ under assay conditions (Jorns et al., 1990). Enzyme concentration was based on flavin content and used in calculating specific activity values which are expressed as μmol of dimer repaired/(μmol of enzyme·min).

Apoenzyme Preparation and Reconstitution. Apophotolyase was prepared and reconstituted with folate ([6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate) in the presence or absence of $FADH_2$ as described by Jorns et al. (1990). Anaerobic photoreduction with yellow light in complete PRE buffer (Jorns et al., 1990) was used to convert FADH$^\bullet$ back to $FADH_2$ in enzyme isolated after reconstitution with $FADH_2$. 5-Formyltetrahydrofolate ([6*S*], [6*R*], or [6*R*,*S*]) was converted to 5,10-CH^+-H_4folate as described by Rabinowitz (1963). A single peak was observed when [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate was subjected to HLPC analysis on a Rainin Microsorb C_{18} 80-225-C5 column using the following elution profile (flow rate = 0.5 mL/min) and detection at 290 nm: 10 min isocratic elution with 10% methanol adjusted to pH 2.2 with phosphoric acid, 10 min linear gradient to 30% methanol (pH 2.2), and 20 min isocratic elution with 30% methanol (pH 2.2). $FADH_2$ was prepared by reduction of FAD with excess sodium dithionite.

Absorption Spectroscopy. Unless otherwise noted, absorption spectra were recorded in complete PRE buffer at 5 °C with a Perkin Elmer Lambda 2S spectrophotometer. Chromophore content and protein concentration were determined as previously described (Jorns et al., 1990; Wang & Jorns, 1989). Enzyme reconstituted with [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate contained 0.66, 0.55, or 0.62 mol of folate/mol of enzyme, respectively. Enzyme reconstituted with [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate plus $FADH_2$ contained the following amounts of folate and flavin per mole of enzyme: 0.78 mol of [6*S*]-5,10-CH^+-H_4folate plus 0.75 mol of $FADH_2$; 0.77 mol of [6*R*]-5,10-CH^+-H_4folate plus 0.68 mol of $FADH_2$; or 0.74 mol of [6*R*,*S*]-5,10-CH^+-H_4folate plus 0.73 mol of $FADH_2$.

Fluorescence Quantum Yield Measurements at 5 °C. Fluorescence emission spectra were recorded using a Perkin Elmer LS 50 luminescence spectrometer. Fluorescence quantum yield values for photolyase-bound chromophores were determined as previously described (Lipman & Jorns, 1992; Sun et al., 1972). Briefly, fluorescence emission spectra were recorded in complete PRE buffer, a buffer blank was subtracted, and the spectra were then corrected using quinine sulfate dihydrate as an emission standard. Quantum yield values were determined using riboflavin in water (Φ = 0.25) (Moore et al., 1977) as standard. The fluorescence contributions of folate and $FADH_2$ in preparations containing both chromophores were estimated by selectively quenching $FADH_2$ fluorescence with substrate (UV-irradiated oligo(dT)$_9$). Samples exhibited absorbance values less than 0.1 at the excitation wavelengths used to record fluorescence emission spectra. Equation 1 was used to estimate the efficiency of energy transfer from folate to $FADH_2$ (E_{ET}). Quantum yield values obtained for $FADH_2$ at various excitation wavelengths in the presence of folate ($\Phi_{FADH_2\,(plus\ folate)} \equiv \Phi_{acceptor\ (plus\ donor)}$) were divided by the wavelength-independent quantum yield observed for $FADH_2$ in the absence of folate [$\Phi_{FADH_2\,(no\ folate)} \equiv \Phi_{acceptor\ (no\ donor)} = 5.34 \times 10^{-3}$ (Lipman & Jorns, 1992)]. Values for $\Phi_{acceptor\ (plus\ donor)}/\Phi_{acceptor\ (no\ donor)}$ were plotted *versus* the ratio of chromophore extinction coefficients at each excitation wavelength ($\epsilon_{folate}/\epsilon_{FADH_2} \equiv \epsilon_{donor}/\epsilon_{acceptor}$). Values for $\epsilon_{folate}/\epsilon_{FADH_2}$ were calculated using previously determined extinction coefficients for the enzyme-bound chromophores and were corrected for the somewhat less than stoichiometric amounts of $FADH_2$ and folate in the various reconstituted enzyme preparations, as described by Lipman and Jorns (1992).

Quantum yield values for free [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate were determined following a procedure similar to that described above for photolyase-bound chromophores. Fluorescence and absorption data were recorded for samples prepared by diluting stock solutions of free 5,10-CH^+-H_4folate into various solvents, as described in Table 2.

Low Temperature Fluorescence Quantum Yield Measurements. Measurements with enzyme-bound or free 5,10-CH^+-H_4folate were conducted in complete PRE buffer or ethanol, respectively, solvents which yield a clear glass at 77 K. Fluorescence emission spectra at 77 K were recorded in an EPR tube (3 mm i.d.) using a liquid nitrogen filled optical Dewar flask that was designed to fit into the normal cell holder of the luminescence spectrometer, as previously described (Jordan & Jorns, 1988). 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. Fluorescence emission spectra were corrected as described above. Quantum yield values were determined using riboflavin in water at 5 °C as standard. Quantum yield values determined in an EPR tube for riboflavin in ethanol at 5 °C (Φ = 0.31) or 77 K (Φ = 0.56) were in good agreement with values (Φ = 0.32 or 0.60, respectively) previously reported (Sun et al., 1972).

RESULTS AND DISCUSSION

Catalytic Activity of Reconstituted Enzyme. Reconstituted enzyme containing only folate is catalytically inactive. Activity can be restored by adding back $FADH_2$, the chromophore that directly interacts with substrate (Jorns et al., 1990). Similar catalytic activity was observed with native enzyme (SA = 56.9 min^{-1}) or reconstituted enzyme containing $FADH_2$ plus [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate ($SA_{average}$ = 50.8 ± 7.8 min^{-1}), in agreement with previous reconstitution studies with $FADH_2$ and [6*R*,*S*]-5,10-CH^+-H_4folate (Jorns et al., 1990). Differences in the efficiency

Table 1: Fluorescence Properties of 5,10-CH^+-H_4folate Bound to Photolyase[a]

isomer	quantum yield: flavin content of preparation: none	quantum yield: flavin content of preparation: $FADH^\bullet$	quantum yield: flavin content of preparation: $FADH_2$	E_Q: Quenching by $FADH^\bullet$	E_Q: Quenching by $FADH_2$	E_{ET}: Folate to $FADH_2$
[6*S*]	0.18 ± 0.01	$(0.85 \pm 0.02) \times 10^{-2}$	$(3.0 \pm 0.2) \times 10^{-2}$	0.95	0.83	0.26
[6*R*]	0.82 ± 0.05	$(2.1 \pm 0.3) \times 10^{-2}$	$(6.4 \pm 0.2) \times 10^{-2}$	0.97	0.92	0.66
av of [6*S*] and [6*R*]	0.50	1.5×10^{-2}	4.7×10^{-2}	0.96	0.88	0.46
[6*R*,*S*]	0.46 ± 0.02	$(1.2 \pm 0.03) \times 10^{-2}$	$(3.7 \pm 0.2) \times 10^{-2}$	0.97	0.92	0.44
[6*R*,*S*][b]	0.43 ± 0.01	$(0.68 \pm 0.01) \times 10^{-2}$	$(3.6 \pm 0.03) \times 10^{-2}$	0.98	0.92	0.46
native photolyase[c]		$(0.99 \pm 0.01) \times 10^{-2}$	$(5.6 \pm 0.04) \times 10^{-2}$	0.99[d]	0.94[d]	0.92

[a] Fluorescence was measured at 5 °C, as detailed in the methods section. [b] Data from Lipman and Jorns (1992). [c] Native enzyme contains [6*R*]-5,10-CH^+-H_4Pte(Glu)$_{n=3-6}$. Data from Lipman (1991) and Lipman and Jorns (1992). [d] Calculated as described in the text.

of energy transfer from folate to $FADH_2$ cannot be discriminated by activity measurements because assays are conducted under conditions where light absorption is not rate-limiting. Differences in energy transfer efficiency can be measured directly using a fluorescence quantum yield method or inferred based on the quantum yield determined for dimer repair (Jorns et al, 1990; Ramsey et al., 1992; Lipman & Jorns, 1992).

Measuring Energy Transfer Efficiency Using the Fluorescence Quantum Yield Method. A fluorescence quantum yield method was developed in previous studies as a means to assess interchromophore, singlet−singlet energy transfer in DNA photolyase (Lipman & Jorns, 1992). This approach requires data that satisfy the following criteria. (1) The quantum yield of the donor (folate) must be wavelength-independent. (2) The quantum yield of the donor must decrease in the presence of the acceptor (flavin) but must remain wavelength-independent. (3) The quantum yield of the acceptor must be wavelength-independent in the absence of the donor. (4) The quantum yield of the acceptor must increase in the presence of the donor. The magnitude of this increase depends on the efficiency of energy transfer (E_{ET}) and on the relative extinction coefficients of the donor (ϵ_{donor}) and the acceptor ($\epsilon_{acceptor}$). Since the latter will vary depending on the excitation wavelength, the quantum yield of the acceptor should exhibit atypical wavelength-dependent behavior in the presence of the donor:

$$\frac{\Phi_{\text{acceptor (plus donor)}}}{\Phi_{\text{acceptor (no donor)}}} = 1 + E_{ET}(\epsilon_{donor}/\epsilon_{acceptor}) \qquad (1)$$

Quantum Yield Studies with Enzyme Containing Only Folate. Unless otherwise specified, fluorescence quantum yield measurements were conducted at 5 °C. Preparations isolated after reconstituting apophotolyase with [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate contained similar amounts of bound folate (0.66, 0.55, or 0.62 mol of folate/mol of enzyme, respectively). The reconstituted enzymes exhibited identical absorption (λ_{max} = 386 nm) and fluorescence emission (λ_{max} = 470 nm) spectra. The results obtained with the unnatural [6*S*]-isomer are consistent with the lack of binding stereospecificity deduced based on the analysis of the isomer composition in enzyme reconstituted with racemic 5,10-CH^+-H_4folate (Lipman et al., 1995).

Fluorescence emission spectra, obtained by excitation at seven different wavelengths (330, 340, 350, 360, 370, 380, and 390 nm), were used to determine quantum yield values. The observed values were wavelength-independent. The average value for each preparation is reported in Table 1. The quantum yield obtained for enzyme reconstituted with [6*R*,*S*]-5,10-CH^+-H_4folate (Φ = 0.46) is in good agreement with previous studies (Φ = 0.43) (Lipman & Jorns, 1992). A 1.8-fold higher quantum yield was obtained with enzyme reconstituted with the [6*R*]-isomer (Φ = 0.82). Enzyme reconstituted with the [6*S*]-isomer exhibited the lowest quantum yield. The observed value (Φ = 0.18) was 4.6-fold smaller than the [6*R*]-isomer. The average of the values for the [6*R*]- and [6*S*]-isomers ($\Phi_{average}$ = 0.50) is in good agreement with the quantum yield observed for enzyme reconstituted with the racemic compound.

The quantum yield values observed for photolyase-bound [6*R*]- and [6*S*]-5,10-CH^+-H_4folate are more than 100-fold larger than that of free folate in aqueous solution. Higher quantum yield values were observed with free folate in less polar solvents, but the maximum increase (40-fold) was less than seen upon binding to photolyase (Table 2). In each solvent, similar quantum yield values were obtained with [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate. The results show that the differences in the quantum yield observed with enzyme-bound [6*S*]- *versus* [6*R*]-5,10-CH^+-H_4folate must reflect differences in binding interactions of the isomers with the protein moiety.

Kinetics of Folate Binding to Apophotolyase. A rapid increase in fluorescence is observed upon mixing apoenzyme with either [6*R*]- or [6*S*]-5,10-CH^+-H_4folate (Figure 1), consistent with the increased quantum yield exhibited by the enzyme-bound chromophores. The binding kinetics with both isomers are clearly biphasic and gave a good fit to a double exponential expression. The fast phase of the reaction with the [6*R*]-isomer gave a rate constant of 0.092 s^{-1} and accounted for 75% of the fluorescence increase. With the [6*S*]-isomer, the fast phase accounts for a similar proportion of the total fluorescence increase (65%) but is more than 30-fold faster (k = 3.1 s^{-1}). The slow phase of the reaction is 20-fold faster with the [6*S*]-isomer (k = 0.069 s^{-1}) than with the [6*R*]-isomer (k = 0.0035 s^{-1}). Apophotolyase reconstituted after an overnight incubation (20 h at 5 °C) with [6*R*,*S*]-5,10-CH^+-H_4folate contains equimolar amounts of the [6*R*]- and [6*S*]-isomers (Lipman et al., 1995). The results suggest that, although the unnatural isomer binds more quickly, the enzyme exhibits similar binding affinity for both isomers.

Quantum Yield Studies with Enzyme Containing Folate plus Flavin. Enzyme containing 5,10-CH^+-H_4folate plus flavin radical is obtained after reconstitution with folate plus fully reduced flavin because partial flavin oxidation occurs during enzyme isolation. The fluorescence observed for these preparations is entirely due to folate since the flavin

Table 2: Fluorescence Properties of Free 5,10-CH^+-H_4folate[a]

solvent	quantum yield ($\times 10^3$) [6*S*]-5,10-CH^+-H_4folate	[6*R*]-5,10-CH^+-H_4folate	[6*R*,*S*]-5,10-CH^+-H_4folate	dielectric constant
H_2O, pH 4 citrate	1.9	1.7	1.6	80.4
	1.4 ± 0.06[b]	1.6 ± 0.06[b]	1.5 ± 0.02[b]	
H_2O, 0.01 N HCl	1.7	1.8	1.6	80.4
methanol	1.5	1.4	1.3	33.6
	1.6 ± 0.06[b]	1.2 ± 0.09[b]	1.4 ± 0.09[b]	
ethanol	nd[c]	3.7	4.7	24.3
2-propanol	10	12	9.0	18.3
isobutyl alcohol	13	14	11	17.7
ethyl acetate	51	65	65	6.02
chloroform	62	43	40	4.8

[a] Unless otherwise indicated, quantum yield values were calculated based on emission spectra obtained by excitation at 370 nm at 5 °C. Samples were prepared by diluting a methanol stock solution (1000-fold) into the indicated solvent. [b] Quantum yield values are the average of values that were determined based on fluorescence emission spectra obtained by excitation at 330, 340, 350, 360, 370, 380, and 390 nm. Stock solutions for studies in citrate buffer or methanol were prepared in 0.01 N HCl or methanol, respectively. [c] Not determined.

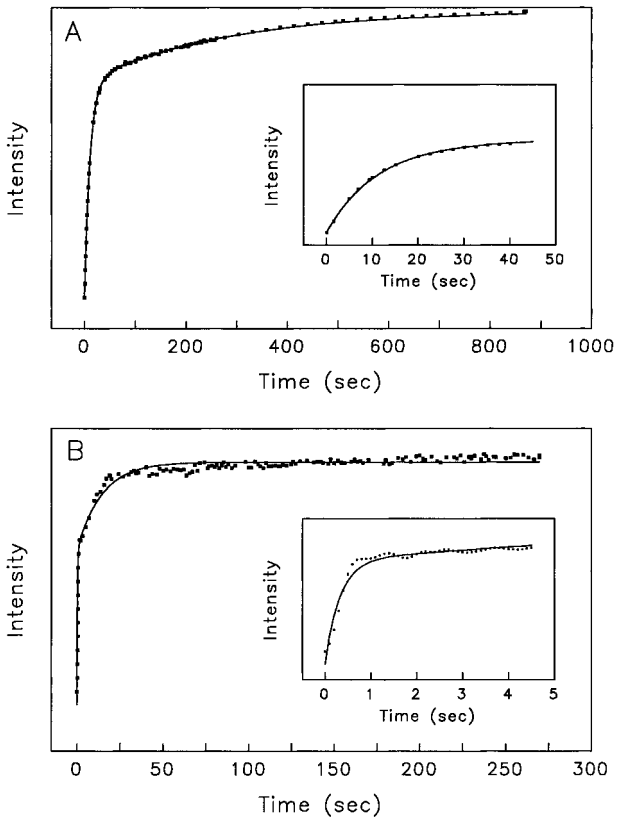

FIGURE 1: Kinetics of folate binding to apophotolyase. A sample of apoenzyme (0.7 μM) in 1 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA, 10 mM DTT, and 5% (v/v) ethylene glycol at 5 °C was mixed with a 20-fold excess of [6*R*]- or [6*S*]-5,10-CH^+-H_4folate. The time courses for the increase in fluorescence intensity ($\lambda_{emission} = 470$ nm, $\lambda_{excitation} = 380$ nm) with the [6*R*]- and the [6*S*]-isomer are shown in panels A and B, respectively. Data point (dots) are the average of four independent trials. The solid lines show the fit of the data to a double exponential expression. The initial fast phase of each reaction is shown expanded in the insets. The data were fitted using an iterative nonlinear least squares Marquardt−Levenberg algorithm (Bevington, 1969).

radical is nonfluorescent. The flavin radical is easily photoreduced under anaerobic conditions to yield enzyme containing folate plus fully reduced flavin. Emission spectra observed for enzyme-bound folate ($\lambda_{max} = 470$ nm) and fully reduced flavin ($\lambda_{max} = 505$ nm) overlap. However, substrate selectively quenches reduced flavin fluorescence, a feature which can be used to estimate the fluorescence contributions of each chromophore in preparations containing both chromophores (Jordan & Jorns, 1988; Lipman & Jorns, 1992).

Folate fluorescence quantum yield values were measured for reconstituted enzyme containing [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate plus $FADH^\bullet$ or $FADH_2$. In each case, folate fluorescence was quenched in the presence of flavin. Folate fluorescence quantum yield values remained wavelength-independent, and an average value is reported for each preparation in Table 1. The data were used to estimate the efficiency of folate fluorescence quenching by flavin ($E_Q = 1 - [\Phi_{folate\ (plus\ flavin)}/\Phi_{folate\ (no\ flavin)}]$). Similar quenching efficiency by $FADH^\bullet$ was observed with preparations containing [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate ($E_{Q(average)} = 0.96 \pm 0.01$). The quenching of folate fluorescence by $FADH_2$ is somewhat less efficient, but similar values for E_Q were obtained with [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate ($E_{Q(average)} = 0.89 \pm 0.03$). The results compare favorably with values obtained previously for reconstituted enzyme containing [6*R*,*S*]-5,10-CH^+-H_4folate plus $FADH^\bullet$ ($E_Q = 0.98$) or $FADH_2$ ($E_Q = 0.92$) (Lipman & Jorns, 1992). The efficiency of folate quenching in native photolyase containing $FADH^\bullet$ ($E_Q = 0.99$) or $FADH_2$ ($E_Q = 0.94$) was estimated using values reported for $\Phi_{folate\ (plus\ flavin)}$ with native enzyme (Lipman & Jorns, 1992; Lipman, 1991) and assuming that $\Phi_{folate\ (no\ flavin)}$ is ~1 (*vide infra*). The results suggest that the efficiency of folate quenching by flavin is not sensitive to the stereochemical configuration at the 6 position of the pterin ring or the number of glutamate residues in the side chain.

In the absence of folate, photolyase-bound $FADH_2$ exhibits a wavelength-independent quantum yield (Lipman & Jorns, 1992). Quantum yield values observed for $FADH_2$ in preparations containing [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate varied depending on the excitation wavelength; the observed quantum yield increased 3.3-, 13.2-, or 5.0-fold, respectively, as the excitation wavelength was increased from 330 to 390 nm. In each case, a straight line was obtained when the data were plotted according to eq 1 (regression coefficient = 0.87, 0.97, or 0.94, respectively). The efficiency of energy transfer was estimated from the slope of these plots. Energy transfer with the natural [6*R*]-isomer ($E_{ET} = 0.66$) was found to be 2.5-fold more efficient than with the unnatural [6*S*]-isomer ($E_{ET} = 0.26$). The average of the values for the [6*R*]- and [6*S*]-isomers ($E_{ET(average)} = 0.46$) is in good agreement with values observed for enzyme

containing [6*R*,*S*]-5,10-CH^+-H_4folate (E_{ET} = 0.44 (this study); E_{ET} = 0.46 (Lipman & Jorns, 1992)). Although less efficient, the results show that the unnatural [6*S*]-isomer is catalytically functional, a feature not previously observed with other folate-dependent enzymes.

Energy transfer with native photolyase (E_{ET} = 0.92) is 1.4-fold more efficient than in reconstituted enzyme containing [6*R*]-5,10-CH^+-H_4folate, a difference presumably due to the substitution of the polyglutamate moiety found in the native folate ([6*R*]-5,10-CH^+-H_4Pte(Glu)$_{n=3-6}$) with a single glutamate residue. The observed quenching of folate fluorescence by $FADH_2$ in native enzyme is entirely due to energy transfer. In reconstituted enzyme containing [6*R*]- or [6*S*]-5,10-CH^+-H_4folate, energy transfer accounts for 70% or 30%, respectively, of the observed quenching of folate fluorescence.

Efficiency of Energy Transfer. Three mechanisms for singlet−singlet energy transfer have been described (Turro, 1978; Lamola, 1969). In radiative transfer, the excited donor emits light ($D^* \rightarrow D + h\nu$) which is absorbed by the acceptor ($A + h\nu \rightarrow A^*$). The acceptor does not affect the fluorescence lifetime of the donor, a feature not observed with photolyase where the fluorescence lifetime of folate is decreased in the presence of $FADH_2$ (Kim et al., 1991). Radiationless energy transfer may occur via dipole−dipole interaction (Förster-type) or by an electron exchange mechanism. The electron exchange mechanism requires physical contact between donor and acceptor and is unlikely in photolyase since folate and $FADH_2$ are separated by 16.8 Å (Park et al., 1995). Förster-type energy transfer is applicable in cases where the donor and acceptor are well-separated, the donor is fluorescent in the absence of acceptor, and the fluorescence emission spectrum of the donor overlaps with the absorption spectrum of the acceptor. All three criteria are satisfied in the case of photolyase (Park et al., 1995; Jorns et al., 1990).

The efficiency of Förster-type energy transfer will depend on the following: (1) the magnitude of the donor fluorescence quantum yield in the absence of the acceptor; (2) the extent of spectral overlap; (3) the distance between donor and acceptor; (4) the orientation of donor and acceptor (Turro, 1978; Lamola, 1969). The efficiency of energy transfer observed for photolyase preparations containing $FADH_2$ and [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate is found to be directly proportional to the fluorescence quantum yield observed for folate in the absence of $FADH_2$ (Figure 2), as expected for Förster-type energy transfer. The fluorescence quantum yield for the native folate ([6*R*]-5,10-CH^+-H_4Pte(Glu)$_{n=3-6}$) in the absence of $FADH_2$ is not known, but a value near unity is estimated based the data in Figure 2 and the measured efficiency of energy transfer in native photolyase. Folate fluorescence emission spectra are not affected by changing the stereochemical configuration at the 6 position of the pterin ring, suggesting a similar extent of spectral overlap in preparations containing $FADH_2$ and [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate. The configuration at the 6 position clearly affects the interaction of folate with photolyase and may cause changes in the separation and/or orientation of folate and $FADH_2$. However, the observed differences in energy transfer efficiency appear to be mainly attributable to differences in the quantum efficiency of folate fluorescence.

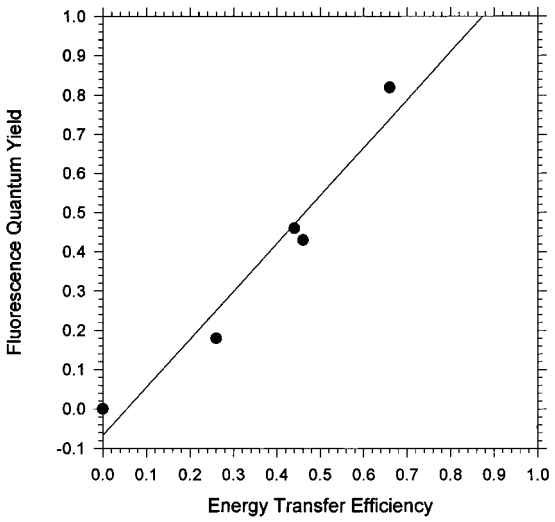

FIGURE 2: Folate fluorescence quantum yield and energy transfer efficiency. The fluorescence quantum yield observed for photolyase-bound folate ([6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate) at 5 °C in the absence of $FADH_2$ is plotted *versus* the efficiency of energy transfer observed for preparations containing both folate and $FADH_2$. The data were analyzed by linear regression analysis (regression coefficient = 0.95).

Table 3: Fluorescence Efficiency of Free and Enzyme-Bound Folate at Low Temperature[a]

isomer	quantum yield	
	photolyase-bound[b]	free[c]
[6*S*]-5,10-CH^+-H_4folate	1.0 ± 0.01	0.67
[6*R*]-5,10-CH^+-H_4folate	1.2 ± 0.01 (1.1±0.01)	0.64
[6*R*,*S*]-5,10-CH^+-H_4folate	0.9 ± 0.01	0.69

[a] Fluorescence was measured at 77 K, as detailed in the methods section. [b] Quantum yield values are the average of values determined from fluorescence emission spectra obtained by excitation at 360, 370, 380, and 390 nm. The value for the [6*R*]-isomer shown in parentheses was determined in a separate experiment. [c] Quantum yield values were calculated based on emission spectra obtained by excitation at 370 nm.

Efficiency of Folate Fluorescence. Differences in the interaction of the folate isomers with photolyase that affect folate fluorescence efficiency are eliminated when the temperature is decreased from 5 °C to 77 K. Quantum yield values near unity are observed with photolyase-bound [6*S*]-, [6*R*]-, or [6*R*,*S*]-5,10-CH^+-H_4folate at low temperature (Table 3). The high fluorescence quantum efficiency at 77 K means that other processes, such as intersystem crossing, are not competitive with fluorescence emission. This is consistent with the fact that no phosphorescence could be detected in low temperature studies with enzyme containing [6*R*,*S*]-5,10-CH^+-H_4folate (Jordan & Jorns, 1988).

The fluorescence quantum yield observed with free folate in ethanol increased by more than 2 orders of magnitude when the temperature was decreased from 5 °C to 77 K (Table 3). The value observed with free folate at low temperature ($\Phi_{average}$ = 0.67 ± 0.01) is close to that obtained for the enzyme-bound natural isomer at 5 °C and not much lower than that observed with either enzyme-bound isomer at 77 K.

Fluorescence quantum efficiency will depend on the relative rates of deactivation of the singlet (S_1) by fluorescence emission, intersystem crossing from S_1 to the triplet,

and nonradiative interconversion from S_1 to S_0 by various quenching mechanisms, including bimolecular diffusional quenching processes. The rate of fluorescence emission is generally temperature-independent. Intersystem crossing may exhibit temperature dependence if upper vibrational levels of S_1 have a different mechanism for the transition than the lowest vibrational level (Turro, 1978).

With free folate, the large increase in fluorescence efficiency at 77 K is probably attributable to the rigidity of the glassy solvent which eliminates bimolecular quenching processes. Reduction of bimolecular quenching processes may also be an important factor contributing to the increase in folate fluorescence observed upon binding to photolyase at 5 °C. However, the crystal stucture reported for enzyme containing [6*R*]-5,10-CH^+-H_4folate shows that the cofactor is accessible to solvent (Park et al., 1995). Therefore, bimolecular quenching processes with enzyme-bound folate may not be totally abolished unless the temperature is lowered from 5 °C to 77 K. This suggests that differences in solvent exposure may account, at least in part, for the differences in fluorescence quantum yield observed at 5 °C with [6*R*]- *versus* [6*S*]-5,10-CH^+H_4folate bound to photolyase.

ACKNOWLEDGMENT

We thank Dr. Steven Bailey and Dr. Annie Willie for helpful discussions.

REFERENCES

Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, McGraw Hill, New York.
Chanderkar, L. P., & Jorns, M. S. (1991) *Biochemistry 30*, 745−754.
Jordan, S. P., & Jorns, M. S. (1988) *Biochemistry 27*, 8915−8923.
Jorns, M. S., Sancar, G. B., & Sancar, A. (1985) *Biochemistry 24*, 1856−1861.
Jorns, M. S., Baldwin, E. T., Sancar, G. B., & Sancar, A. (1987a) *J. Biol. Chem. 262*, 486−491.
Jorns, M. S., Wang, B., & Jordan, S. P. (1987b) *Biochemistry 26*, 6810−6816.
Jorns, M. S., Wang, B. Y., Jordan, S. P., & Chanderkar, L. P. (1990) *Biochemistry 29*, 552−561.
Kim, S. T., Heelis, P. F., Okamura, T., Hirata, Y., Mataga, N., & Sancar, A. (1991) *Biochemistry 30*, 11262−11270.
Lamola, A. A. (1969) in *Energy Transfer and Organic Photochemistry* (Lamola, A. A., Ed.) pp 17−133, Interscience, New York.
Leary, R. P., Gaumont, Y., & Kisliuk, R. L. (1974) *Biochem. Biophys. Res. Commun. 56*, 484−488.
Lipman, R. S. A. (1991) M.S. Thesis, Hahnemann University.
Lipman, R. S. A., & Jorns, M. S. (1992) *Biochemistry 31*, 786−791.
Lipman, R. S. A., Bailey, S. W., Jarrett, J. T., Matthews, R. G., & Jorns, M. S. (1995) *Biochemistry 34*, 11217−11220.
Moore, W. M., McDaniels, J. C., & Hen, J. A. (1977) *Photochem. Photobiol. 25*, 505−512.
Okamura, T., Sancar, A., Heelis, P. F., Begley, T. P., Hirata, Y., & Mataga, N. (1991) *J. Am. Chem. Soc. 113*, 3143−3145.
Park, H. W., Kim, S. T., Sancar, A., & Deisenhofer, J. (1995) *Science 268*, 1866−1872.
Rabinowitz, J. C. (1963) *Methods Enzymol. 6*, 814−815.
Ramsey, A. J., Alderfer, J. L., & Jorns, M. S. (1992) *Biochemistry 31*, 7134−7142.
Rustandi, R. R., & Jorns, M. S. (1995) *Biochemistry 34*, 2284−2288.
Smith, G. K., Benkovic, P. A., & Benkovic, S. J. (1981) *Biochemistry 20*, 4034−4036.
Sun, M., Moore, T. A., & Song, P. S. (1972) *J. Am. Chem. Soc. 94*, 1730−1740.
Turro, N. J. (1978) in *Modern Molecular Photochemistry*, The Benjamin Cummings Publishing Co., Menlo Park.
Wang, B., & Jorns, M. S. (1989) *Biochemistry 28*, 1148−1152.

BI9605548