Effect of 5-Deazaflavin on Energy Transduction during Catalysis by *Escherichia coli* DNA Photolyase†

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ABSTRACT: DNA photolyase from *Escherichia coli* contains 1,5-dihydroFAD ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate. The action spectrum observed for apoenzyme reconstituted with 5-deaza$FADH_2$ (Ed$FADH_2$) matched its absorption spectrum after correction for the presence of a small amount of inactive 5-deazaFAD_{ox}. The quantum yield for dimer repair with Ed$FADH_2$ ($\Phi_{EdFADH2} = 0.110$) was 6-fold lower than that observed with apoenzyme reconstituted with $FADH_2$. Excited-state redox potential calculations indicate that 5-deaza$FADH_2$ singlet is a better one-electron donor ($E = -3.5$ V) than $FADH_2$ singlet ($E = -2.7$ V). Other studies indicate that the quantum yield for electron transfer from reduced flavin singlet to pyrimidine dimer (0.88) is unaffected when $FADH_2$ is replaced by 5-deaza$FADH_2$. Enhanced back electron transfer from pyrimidine dimer radical to flavin radical may account for the decreased quantum yield observed with Ed$FADH_2$ since, in the ground state, 5-deaza$FADH^{\bullet}$ is a better oxidant than $FADH^{\bullet}$. The action spectrum observed for apoenzyme reconstituted with 5-deaza$FADH_2$ plus 5,10-CH^+-H_4folate (EPtedFADH$_2$) matched the absorption spectrum determined for enzyme-bound 5-deaza$FADH_2$, indicating that the pterin chromophore was inactive as a sensitizer. This differs from results obtained with native enzyme, where pterin acts as a sensitizer via efficient singlet–singlet energy transfer to $FADH_2$. The quantum yield for dimer repair by 5-deaza$FADH_2$ bound to EPtedFADH$_2$ ($\Phi_{EPtedFADH2} = 0.0318$) was 28.9% of that observed for Ed$FADH_2$. Spectroscopic studies indicate that singlet–singlet energy transfer in EPtedFADH$_2$ is very efficient but only occurs in the "wrong" direction, i.e., from excited 5-deaza$FADH_2$ to pterin. The competition between substrate and pterin for reaction with 5-deaza$FADH_2$ singlet may account for the decreased quantum yield observed with EPtedFADH$_2$.

DNA photolyase binds UV-damaged DNA in a dark reaction and then repairs pyrimidine dimers when the complex is irradiated with visible light (Rupert, 1962). Visible light can be absorbed by either of the two chromophores found in all known photolyases (Jorns, 1990). DNA photolyase from *Escherichia coli* contains 1,5-dihydroFAD[1] ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4-Pte(Glu)$_n$, $n = 3$–6] (Wang et al., 1988; Wang & Jorns, 1989; Jorns et al., 1990; Johnson et al., 1988). Recent steady-state kinetic studies with *E. coli* photolyase (Ramsey et al., 1992) show that dimer repair proceeds via an ordered or equilibrium-ordered mechanism with light as the second substrate [eq 1;

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2[L]}{\rightleftarrows}} E^*S \xrightarrow{k_3} EP \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} E + P \qquad (1)$$

[L], light intensity (erg mm^{-2} s^{-1})]. For an (equilibrium) ordered mechanism, the velocity observed at saturating dimer and limiting light intensity can be used to determine the photolytic cross section (PC), as described by Ramsey et al. (1992) (eq 2). The action spectrum for dimer repair, obtained by plotting the photolytic cross section as a function of wavelength, provides information regarding chromophore function. Although the mechanism of dimer cleavage is not completely understood, it is known to involve reaction of the dimer with the excited singlet state of $FADH_2$ ($^1FADH_2^*$) (Jordan & Jorns, 1988; Okamura et al., 1991). $^1FADH_2^*$ can be generated by direct photon absorption, and enzyme containing only $FADH_2$ (E$FADH_2$) is catalytically active (Jorns et al., 1987a, 1990). The action spectrum observed for E$FADH_2$ exhibits the same shape as its absorption spectrum, and the quantum yield for dimer repair (Φ_{EFADH2}) is wavelength-independent, as expected for a reaction involving a single sensitizer (Ramsey et al., 1992; Payne & Sancar, 1990). Spectroscopic studies with native enzyme show that $^1FADH_2^*$ can also be generated via singlet–singlet energy transfer from 5,10-CH^+-H_4Pte(Glu)$_n$ (Lipman & Jorns, 1992; Kim et al., 1991). The action spectrum observed with native enzyme matched an action spectrum simulated for the case where dimer repair is initiated either by direct excitation of $FADH_2$ or by 5,10-CH^+-H_4Pte(Glu)$_n$ excitation followed by energy transfer to $FADH_2$ [eq 3; E_{ET}, efficiency of energy transfer;

$$PC = \Phi\epsilon_{sensitizer} = (v_{obs}5.2 \times 10^9)/[E]_{total}[L]\lambda \qquad (2)$$

$$PC_{native} = \Phi_{EFADH2}[\epsilon_{FADH2} + E_{ET}\epsilon_{Pte}] \qquad (3)$$

Pte, 5,10-CH^+-H_4Pte(Glu)$_n$] (Ramsey et al., 1992). The action spectrum observed for native enzyme also closely matched its absorption spectrum, as predicted by eq 3, since interchromophore energy transfer is very efficient ($E_{ET} = 0.92$) (Lipman & Jorns, 1992).

In this paper we report quantum yield and action spectra studies conducted with enzyme containing 5-deazaFAD in place of FAD. This flavin analogue was selected because previous studies (Chanderkar & Jorns, 1991) showed that

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH_2$, 1,5-dihydro-FAD; 5-deaza$FADH_2$, 1,5-dihydro-5-deaza$FADH_2$; 5-deazaFAD_{ox}, oxidized 5-deazaFAD; 5,10-CH^+-H_4Pte(Glu)$_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; UV-oligo(dT)$_{18}$, dimer-containing oligothymidylate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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5-deazaFAD bound to a hydrophobic site in apophotolyase and formed a stable reconstituted enzyme, similar to that observed with FAD. Enzyme containing only 5-deazaFADH$_2$ (EdFADH$_2$) was catalytically active, whereas no activity was observed with enzyme containing 5-deazaFAD$_{ox}$, similar to that observed with FAD. Also, formation of a EdFADH$_2$–substrate complex quenched 5-deazaFADH$_2$ fluorescence, suggesting that the excited singlet state of 5-deazaFADH$_2$ acts as a catalytic intermediate. Our present studies show that replacing the nitrogen at position 5 in FADH$_2$ with carbon causes a 6-fold decrease in the quantum efficiency of dimer repair. More dramatic, the substitution eliminates the ability of 5,10-CH$^+$-H$_4$Pte(Glu)$_n$ to act as a sensitizer during catalysis. Furthermore, the presence of 5,10-CH$^+$-H$_4$Pte(Glu)$_n$ interferes with dimer splitting at the 5-deazaFADH$_2$ site.

EXPERIMENTAL PROCEDURES

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

Apoenzyme Preparation and Reconstitution with 5-DeazaFADH$_2$ and 5,10-CH$^+$-H$_4$folate. Native *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-HCl, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol). 5-Deazariboflavin was synthesized according to the method of O'Brien et al. (1970) and then converted to 5-deazaFAD as described by Spencer et al. (1976). Oxidized 5-deazaFAD (5-deazaFAD$_{ox}$) was converted to 1,5-dihydro-5-deazaFAD (5-deazaFADH$_2$) by reduction with sodium borohydride, as previously described (Chanderkar & Jorns, 1991), except that the reaction mixture also included 2.4 mM sodium dithionite. 5,10-Methenyltetrahydrofolate (5,10-CH$^+$-H$_4$folate) was prepared as described by Rabinowitz (1963). Apoenzyme was prepared using a chromatographic procedure described by Jorns et al. (1990) and then incubated for 10 h at 4 °C with 5-deazaFADH$_2$ (4-fold excess) in the presence or absence of 5,10-CH$^+$-H$_4$folate (10-fold excess). The reconstituted enzyme was separated from unbound chromophores and concentrated, as previously described (Jorns et al., 1990), and then stored in complete PRE buffer at −20 °C.

The chromophore content of reconstituted enzyme was determined as described by Chanderkar and Jorns (1991). Briefly, protein concentration was determined on the basis of absorbance at 280 nm (ϵ_{280} = 1.00 × 10^5 M^{-1} cm^{-1}), after correction for contributions from bound chromophores (Chanderkar & Jorns, 1991; Wang & Jorns, 1989). In pterin-free enzyme, 5-deazaFADH$_2$ was estimated on the basis of its absorbance at 325 nm (ϵ_{325} = 12.3 × 10^3 M^{-1} cm^{-1}) (Chanderkar & Jorns, 1991) after correction for the contribution from a small amount of 5-deazaFAD$_{ox}$. The latter was estimated on the basis of its absorbance at 400 nm (ϵ_{400} = 11.4 × 10^3 M^{-1} cm^{-1}) (Chanderkar & Jorns, 1991), a wavelength where 5-deazaFADH$_2$ does not absorb. The preparation of pterin-free enzyme used for these studies contained 0.634 mol of 5-deazaFADH$_2$ plus 0.152 mol of 5-deazaFAD$_{ox}$ per mole of protein. In pterin-containing enzyme, 5,10-CH$^+$-H$_4$folate was determined on the basis of its absorbance at 380 nm (ϵ_{380} = 25.9 × 10^3 M^{-1} cm^{-1}), as estimated by the decrease in absorbance observed at this wavelength upon reduction of the chromophore with sodium borohydride (Jorns et al., 1987a, 1990). 5-DeazaFAD$_{ox}$ was estimated on the basis of the absorbance observed at 400 nm after borohydride treatment.

5-DeazaFADH$_2$ was estimated on the basis of total 5-deazaFAD content. To estimate the latter, borohydride-treated enzyme was denatured with SDS and incubated until air oxidation of released 5-deazaFADH$_2$ was complete. Total 5-deazaFAD content was determined on the basis of the absorbance of free 5-deazaFAD$_{ox}$ at 399 nm (ϵ_{399} = 11.5 × 10^3 M^{-1} cm^{-1}) (Spencer et al., 1976). The preparation of pterin-containing enzyme used for these studies contained 0.758 mol of 5-deazaFADH$_2$ and 0.147 mol of 5-deazaFAD$_{ox}$ plus 0.640 mol of 5,10-CH$^+$-H$_4$folate per mole of protein. Assuming a random chromophore distribution, the preparation will consist of the following mixture of enzyme forms: EPtedFADH$_2$ (49.0%); EdFADH$_2$ (27.3%); EPtedFAD$_{ox}$ (9.41%); EdFAD$_{ox}$ (5.29%); EPte (6.14%); E (3.46%).

Unless otherwise noted, with both reconstituted preparations, enzyme concentration was estimated on the basis of 5-deazaFADH$_2$ content.

Substrate Preparation. UV-irradiated oligo(dT)$_{18}$ [UV-oligo(dT)$_{18}$, 4.0–4.2 dimers/mol of oligomer] was used as the substrate for action spectra and quantum yield studies. Dimers were introduced by irradiating oligo(dT)$_{18}$ with germicidal light, as previously described (Jorns et al., 1985). Samples were then treated with black light, as described by Chanderkar and Jorns (1991), to eliminate the near-UV absorbance and fluorescence of a minor photoproduct formed during irradiation with germicidal light (Mitchell & Clarkson, 1984).

Spectroscopy. Absorption spectra were recorded with a Perkin-Elmer Lambda 3 spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer Lambda 5 luminescence spectrometer.

Fluorescence and absorption spectra observed for enzyme-bound 5-deazaFADH$_2$ (this paper; Chanderkar & Jorns, 1991) and FADH$_2$ (Lipman & Jorns, 1992; Ramsey et al., 1992) were used to estimate the one-electron potential corresponding to the zero spectroscopic energy of the reduced flavin in the excited state [E_{00}(red/red*) (V) = 1240.62/λ_{00} (nm), where λ_{00} is the wavelength corresponding to the 0,0 transition]. In the ideal case, λ_{00} is determined on the basis of the position of the highest energy vibrational band in the fluorescence emission spectrum but can also be estimated on the basis of the position of the lowest energy vibrational band in the absorption spectrum. In the absence of vibrational structure, as observed with EdFADH$_2$ and EFADH$_2$, λ_{00} can be estimated from the blue edge of the emission spectrum (λ_F) or the red edge of the absorption spectrum (λ_A). These estimates typically bracket the true value for λ_{00} ($\lambda_F < \lambda_{00} < \lambda_A$) (Turro, 1978). A value near the middle of this bracket is obtained from the point of intersection of absorption and fluorescence spectra, normalized to the same intensity at their respective maxima, and was used to estimate λ_{00}. The value for E_{00}(red/red*) calculated for EdFADH$_2$ using this approach (3.63 V) is not very different from values calculated using λ_A (3.49 V) or λ_F (3.76 V). Similar results were obtained with EFADH$_2$ [E_{00}(red/red*) = 2.58 vs 2.39 or 2.64 V using λ_A or λ_F, respectively].

In studies with enzyme reconstituted with both 5-deazaFADH$_2$ and 5,10-CH$^+$-H$_4$folate (EPtedFADH$_2$), fluorescence due to enzyme-bound 5-deazaFADH$_2$ was estimated by measuring emission at 365 nm (excitation λ = 325 nm), a wavelength where pterin fluorescence is negligible. Measurements were made in 50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 2 mM DTT, and 20% glycerol at 5 °C before and after addition of 16 mM sodium borohydride.

Action Spectra Measurements. Action spectra were determined by following the procedure described by Ramsey et al. (1992). Briefly, reaction mixtures [50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl, 1 mM EDTA, 1.6 mM DTT, 13.2 μM UV-oligo(dT)$_{18}$ plus apoenzyme reconstituted with only 5-deazaFADH$_2$ (2.3 μM) or with both 5-deazaFADH$_2$ and 5,10-CH$^+$-H$_4$folate (7.5 μM)] were exposed to light at 9.5 °C under aerobic conditions using the irradiation apparatus previously described (Ramsey et al., 1992). Light intensity (erg mm^{-2} s^{-1}) was determined by ferrioxalate actinometry (Hatchard & Parker, 1956; Bowman & Demas, 1976) and was corrected for sample absorbance as described by Morowitz (1950). Dimer repair was monitored spectrophotometrically (Jorns et al., 1985) by following the increase in absorbance at 260 nm ($\Delta\epsilon_{260} = 16.6 \times 10^3$ M^{-1} cm^{-1}) (Naylor & Gilham, 1966) as a function of the light dose. Absorbance changes at 260 nm were corrected for a small blank rate observed when reaction mixtures were irradiated at 460 nm, a wavelength where reconstituted enzyme preparations did not absorb. Values for the photolytic cross section (PC = $\Phi\epsilon$) at each wavelength were determined on the basis of the observed velocity and measured values for enzyme concentration and light intensity (see eq 2). Quantum yield values were calculated using observed photolytic cross section values and extinction coefficients determined for enzyme-bound 5-deazaFADH$_2$, as described in the text.

RESULTS AND DISCUSSION

Action Spectrum of Enzyme Reconstituted with 5-DeazaFADH$_2$. The absorption spectrum observed for enzyme isolated after reconstitution with 5-deazaFADH$_2$ (EdFADH$_2$) (Figure 1 inset, curve 1) exhibited a prominent band at 325 nm plus a weaker band near 400 nm due to a small amount of 5-deazaFAD$_{ox}$ (19.1% of total enzyme-bound 5-deazaFAD). After the contribution due to 5-deazaFAD$_{ox}$ was subtracted, the corrected absorption spectrum of EdFADH$_2$ (Figure 1 inset, curve 2) exhibited a single band at 325 nm, similar to that previously reported for enzyme-bound 5-deazaFADH$_2$ (Chanderkar & Jorns, 1991).

Photolytic cross-section values were determined for EdFADH$_2$ at six different wavelengths in the range from 300 to 400 nm (Table I). The action spectrum observed for EdFADH$_2$ (Figure 1, open circles) matches the absorption spectrum calculated for enzyme-bound 5-deazaFADH$_2$. The results show that 5-deazaFADH$_2$, but not 5-deazaFAD$_{ox}$, can act as a sensitizer, consistent with previous studies where no activity was observed with enzyme containing only 5-deazaFAD$_{ox}$ (Chanderkar & Jorns, 1991). Similar quantum yield values were obtained with EdFADH$_2$ at several different wavelengths (Table I). The average quantum yield value ($\Phi_{EdFADH2} = 0.110$) is 6-fold smaller than previously observed for enzyme containing FADH$_2$ ($\Phi_{EFADH2} = 0.655$) (Ramsey et al., 1992).

The quenching of the flavin excited singlet state by substrate, observed with enzyme-bound 5-deazaFADH$_2$ (Chanderkar & Jorns, 1991) and FADH$_2$ (Jordan & Jorns, 1988; Okamura et al., 1991), has been attributed to electron transfer from (5-deaza)^{1}FADH$_2$* to pyrimidine dimer. Although direct evidence is lacking, this proposal is supported by the fact that pyrimidine dimer radical anions are extremely unstable, as shown in model studies where splitting is observed on a nanosecond time scale (Yeh & Falvey, 1991). Also, alternate modes of singlet quenching appear unlikely. For example, energy transfer from 5-deaza^{1}FADH$_2$* (emission λ_{max} = 365 nm) or ^{1}FADH$_2$* (emission λ_{max} = 509 nm) to pyrimidine dimer ($\epsilon \approx 0$ at $\lambda > 260$ nm) is energetically unfeasible. Singlet quenching via enhanced intersystem crossing to the triplet can be ruled out on the basis of studies with EdFADH$_2$ since formation of an enzyme–substrate complex results in comparable quenching of 5-deazaFADH$_2$ fluorescence and phosphorescence (Chanderkar & Jorns, 1991). Singlet quenching via enhanced internal conversion is incompatible with the high quantum efficiency of dimer repair observed with EFADH$_2$.

If the electron-transfer mechanism is correct, the decreased quantum yield observed with EdFADH$_2$ may reflect differences in the ground- and/or excited-state redox properties of 5-deazaFADH$_2$ vs FADH$_2$. In the ground state, FADH$_2$ is

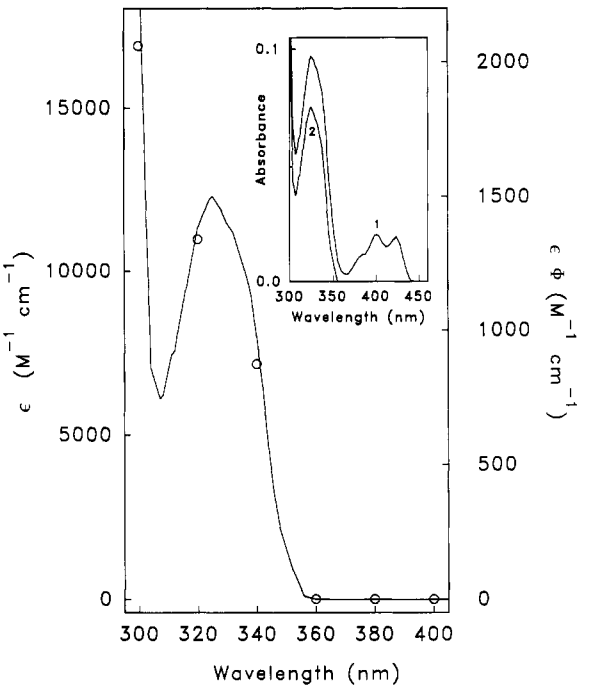

FIGURE 1: Comparison of absorption and action spectra obtained for enzyme containing only 5-deazaFADH$_2$ (EdFADH$_2$). The solid curve is the absorption spectrum calculated (see inset) for EdFADH$_2$ in 50 mM Tris-HCl, pH 7.4, containing 18 mM NaCl, 1 mM EDTA, 10 mM DTT, and 20% glycerol at 5 °C. Photolytic cross-section values are indicated by open circles. (Inset) Curve 1 is the absorption spectrum observed for enzyme isolated after reconstituting apoenzyme with 5-deazaFADH$_2$. The preparation contained 0.634 mol of 5-deazaFADH$_2$ plus 0.152 mol of 5-deazaFAD$_{ox}$ per mole of protein. Curve 2 is the absorption spectrum calculated for enzyme-bound 5-deazaFADH$_2$ by correcting the spectrum of the reconstituted enzyme for the contribution due to 5-deazaFAD$_{ox}$. The latter was estimated on the basis of spectral properties previously reported for enzyme-bound 5-deazaFAD$_{ox}$ (Chanderkar & Jorns, 1991). The known extinction coefficient for enzyme-bound 5-deazaFADH$_2$ at its absorption maximum ($\epsilon_{325} = 12.3 \times 10^3$ M^{-1} cm^{-1}) (Chanderkar & Jorns, 1991) was used to calculate extinction coefficients at other wavelengths, as indicated in the main panel.

Table I: Efficiency of Dimer Repair with Enzyme Containing Only 5-DeazaFADH$_2$

wavelength (nm)	intensity (erg mm^{-2} s^{-1})	$\epsilon\Phi$ (M^{-1} cm^{-1})	ϵ_{EdFADH_2} (M^{-1} cm^{-1})	Φ^a
300	9.96	2060	20200	0.102
320	22.0	1340	11300	0.118
340	21.9	874	7980	0.110
360	32.3	0	0	
380	49.9	0	0	
400	71.7	0	0	
				av 0.110 ± 0.0046

[a] Quantum yield values were estimated using extinction coefficients determined for enzyme-bound 5-deazaFADH$_2$ (ϵ_{EdFADH_2}), as described in the legend to Figure 1.

Table II: Comparison of Flavin Redox Properties and Quantum Yield Values for Dimer Repair by Photolyase

flavin	Φ	E(rad/red)[a] (V)	E(rad/red*)[b] (V)
5-deazaflavin	0.110	+0.130	−3.5
normal flavin	0.655[c]	−0.124	−2.7

[a] Ground-state potentials for one-electron reduction of flavin radical at pH 7.0 as reported by Blankenhorn (1977) and Anderson (1983) for free 5-deazaflavin and normal flavin, respectively. [b] Excited-state reduction potentials were calculated, as described by Julliard and Chanon (1983), using the following equation: E(rad/red*) = E(rad/red) − E_{00}(red/red*). Values for the one-electron potential corresponding to the zero spectroscopic energy of fully reduced flavin in the excited state [E_{00}(red/red*)] were calculated using the absorption and fluorescence properties observed for enzyme-bound 5-deazaFADH$_2$ (this paper; Chanderkar & Jorns, 1991) and FADH$_2$ (Lipman & Jorns, 1992; Ramsey et al., 1992), as described under Experimental Procedures. [c] Value reported for EFADH$_2$ by Ramsey et al. (1992).

a better one-electron reductant than 5-deazaFADH$_2$ (Table II). However, light absorption results in the excitation of an electron to a higher level where it is more weakly bound. Ground-state one-electron reduction potentials are therefore shifted to more negative values in the excited state (Table II), by an amount corresponding to the zero spectroscopic energy of the excited state [E_{00}(red/red*)]. The excited-state energy calculated for 5-deazaFADH$_2$ [E_{00}(red/red*) = 3.63 V] is considerably larger than the value obtained for FADH$_2$ [E_{00}(red/red*) = 2.58 V], as expected owing to the hypsochromic shift in the spectral properties of 5-deazaFADH$_2$ as compared with FADH$_2$. As a consequence, in the excited state, 5-deazaFADH$_2$ is actually a better reductant [E(rad/red*) = −3.5 V] than FADH$_2$ [E(rad/red*) = −2.7 V]. In fact, as judged by the efficiency of quenching of reduced flavin fluorescence by substrate, the quantum yield for electron transfer from excited reduced flavin to pyrimidine dimer is not affected when FADH$_2$ (Φ_E = 0.88) (Kim et al., 1991) is replaced by 5-deazaFADH$_2$ (Φ_E = 0.88) (Chanderkar & Jorns, 1991). The efficiency of conversion of the resulting flavin/dimer radical pair [(5-deaza)FADH$^\bullet$/T$\hat{}$T$^{\bullet-}$] to products will depend on the rate of back electron transfer from dimer radical to flavin radical. Back electron transfer is likely to be more favorable with EdFADH$_2$ since 5-deazaFADH$^\bullet$ is a better oxidant than FADH$^\bullet$ (Table II). The results suggest that the decreased efficiency of dimer repair observed with EdFADH$_2$ may stem from differences in the ground-state redox properties of 5-deazaflavin vs normal flavin.

Action Spectrum of Enzyme Reconstituted with 5-DeazaFADH$_2$ and 5,10-CH$^+$-H$_4$folate. The absorption spectrum observed for enzyme isolated after reconstitution with 5-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate [5,10-CH$^+$-H$_4$Pte(Glu)$_n$, n = 1] (EPtedFADH$_2$) exhibited maxima at 387 and 338 nm plus a shoulder near 325 nm (Figure 2 inset, curve 1), similar to that previously reported (Chanderkar & Jorns, 1991). Similar spectral properties (λ_{max} = 387, 336, and 324 nm) were observed after the observed spectrum was corrected for a small contribution due to 5-deazaFAD$_{ox}$ (16.2% of total flavin), except that the shoulder near 325 nm emerges as a weak peak (Figure 2 inset, curve 2). The maximum at 387 nm is due to 5,10-CH$^+$-H$_4$folate and is identical to that observed for enzyme reconstituted with only 5,10-CH$^+$-H$_4$folate (Jorns et al., 1990). The bands at shorter wavelengths reflect contributions from both 5,10-CH$^+$-H$_4$folate and 5-deazaFADH$_2$.

Photolytic cross-section values were determined with EPtedFADH$_2$ at various wavelengths in the 300–400-nm range (Table III). The action spectrum (Figure 2, open circles) does not match the corrected absorption spectrum for

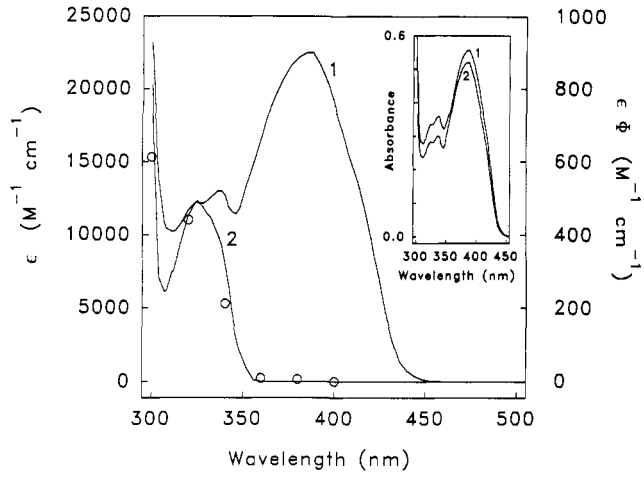

FIGURE 2: Comparison of absorption and action spectra obtained for enzyme containing 5-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate (EPtedFADH$_2$). Curve 1 is the absorption spectrum calculated (see inset) for EPtedFADH$_2$ in 50 mM Tris-HCl, pH 7.4, containing 18 mM NaCl, 1 mM EDTA, 10 mM DTT, and 20% glycerol at 5 °C. Curve 2 is the absorption spectrum of enzyme containing only 5-deazaFADH$_2$, as described in Figure 1. Photolytic cross-section values are indicated by open circles. (Inset) Curve 1 is the absorption spectrum observed for enzyme isolated after reconstituting apoenzyme with 5-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate. The preparation contained 0.758 mol of 5-deazaFADH$_2$, 0.147 mol of 5-deazaFAD$_{ox}$, plus 0.640 mol of 5,10-CH$^+$-H$_4$folate per mole of protein. Curve 2 is the absorption spectrum calculated for EPtedFADH$_2$ by correcting the absorption spectrum of the reconstituted enzyme for the contribution due to 5-deazaFAD$_{ox}$. The extinction coefficients indicated in the main panel were calculated using curve 2 with enzyme concentration estimated on the basis of 5-deazaFADH$_2$ content. The calculated values therefore correspond to a preparation containing a stoichiometric amount of 5-deazaFADH$_2$ plus 0.844 mol of 5,10-CH$^+$-H$_4$folate per mole of protein.

Table III: Efficiency of Dimer Repair with Enzyme Containing 5-DeazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate

wavelength (nm)	intensity (erg mm^{-2} s^{-1})	$\epsilon\Phi$ (M^{-1} cm^{-1})	ϵ_{EdFADH_2} (M^{-1} cm^{-1})	Φ[a]
300	7.42	613	20200	0.0303
320	19.5	440	11300	0.0388
340	18.7	211	7980	0.0264
360	26.7	9	0	
380	40.9	6	0	
400	59.3	0	0	
				av 0.03180 ± 0.0063

[a] Values were calculated using extinction coefficients determined for enzyme-bound 5-deazaFADH$_2$, as described in the text.

EPtedFADH$_2$ (Figure 2, curve 1) but instead coincides with the absorption spectrum determined for enzyme-bound 5-deazaFADH$_2$ (Figure 2, curve 2). The results are quite different from that obtained with native enzyme, where the observed action spectrum matched the absorption spectrum. This difference is not due to the fact that EPtedFADH$_2$ contained 5,10-CH$^+$-H$_4$folate instead of the natural pterin because similar action and absorption spectra are observed with pterin-depleted native enzyme supplemented with 5,10-CH$^+$-H$_4$folate (70% of total pterin) (Ramsey et al., 1992). The results show that the pterin chromophore cannot act as a sensitizer when FADH$_2$ is replaced by 5-deazaFADH$_2$. The action spectrum observed with EPtedFADH$_2$ can be simulated using eq 3 in the extreme case where energy transfer does not occur from excited pterin to 5-deazaFADH$_2$ (E_{ET} = 0). This is consistent with spectroscopic studies which show that energy transfer in this direction is not energetically feasible with 5-deazaFADH$_2$ (Chanderkar & Jorns, 1991).

Since only 5-deazaFADH$_2$ acts as a sensitizer, quantum yield values were calculated using photolytic cross-section data observed with EPtedFADH$_2$ and extinction coefficients determined for enzyme-bound 5-deazaFADH$_2$ (Table III). The average quantum yield ($\Phi_{EPtedFADH2}$ = 0.0318) is only 28.9% of that observed with EdFADH$_2$ (Φ = 0.110). Although "normal" interchromophore energy transfer is not feasible with EPtedFADH$_2$, previous spectroscopic studies indicate that energy transfer in the "wrong" direction does occur, i.e., from 5-deaza^{1}FADH$_2$* to 5,10-CH$^+$-H$_4$folate (Chanderkar & Jorns, 1991). This means that both 5,10-CH$^+$-H$_4$folate and substrate will compete for 5-deaza^{1}FADH$_2$*, a feature which could account for the decreased quantum yield observed with EPtedFADH$_2$. However, it should be noted that the reconstituted enzyme preparation consists of a mixture of enzyme forms. Using the observed chromophore content of the preparation and assuming a random chromophore distribution (see Experimental Procedures), it can be estimated that 65.4% of total 5-deazaFADH$_2$ is bound to protein which contains 5,10-CH$^+$-H$_4$folate but 35.8% is bound to protein that lacks pterin. It is therefore conceivable that enzyme containing both chromophores is inactive and that the observed dimer repair is due to contamination with pterin-free enzyme. If quantum yield calculations using EPtedFADH$_2$ photolytic cross-section data are based on 5-deazaFADH$_2$ bound to pterin-free enzyme, instead of total 5-deazaFADH$_2$, the average quantum yield (Φ = 0.089) approximates that observed with EdFADH$_2$ ($\Phi_{EdFADH2}$ = 0.110). 5-DeazaFADH$_2$ fluorescence in EPtedFADH$_2$ is quenched by 5,10-CH$^+$-H$_4$folate and increases when the pterin chromophore is released from the enzyme by reduction with sodium borohydride (Chanderkar & Jorns, 1991). In the untreated preparation of EPtedFADH$_2$ used for quantum yield measurements, 5-deazaFADH$_2$ fluorescence was 30.6% of that observed after treatment with borohydride. This means that essentially all of the 5-deazaFADH$_2$ fluorescence emission from untreated enzyme is attributable to 5-deazaFADH$_2$ bound to pterin-free enzyme. The data indicate that energy transfer from 5-deaza^{1}FADH$_2$* to 5,10-CH$^+$-H$_4$folate is extremely efficient and is likely to strongly interfere with dimer splitting at the 5-deazaFADH$_2$ site.

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