Effect of Flavin Structure and Redox State on Catalysis by and Flavin–Pterin Energy Transfer in *Escherichia coli* DNA Photolyase†

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ABSTRACT: 5-DeazaFAD bound to a hydrophobic site in apophotolyase and formed a stable reconstituted enzyme, similar to that observed with FAD. Although stoichiometric incorporation was observed, the flavin ring modification in 1-deazaFAD interfered with normal binding, decreased protein stability, and prevented formation of a stable flavin radical, unlike that observed with FAD. The results suggest that an important hydrogen bond is formed between the protein and N(1) in FAD, but not N(5), and that there is sufficient space at the normal flavin binding site near N(5) to accommodate an additional hydrogen but not near N(1). Catalytic activity was observed with enzyme containing 5-deazaFADH$_2$ (42% of native enzyme) or 1-deazaFADH$_2$ (11% of native enzyme) as its only chromophore, but no activity was observed with the corresponding oxidized flavins, similar to that observed with FAD and consistent with a mechanism where dimer cleavage is initiated by electron donation from excited reduced flavin to substrate. The protein environment in photolyase selectively enhanced photochemical reactivity in the fully reduced state, as evidenced by comparison with results obtained in model studies with the corresponding free flavins. Phosphorescence was observed with free or photolyase-bound 5-deazaFADH$_2$, providing the first example of a flavin that exhibits phosphorescence in the fully reduced state. Formation of an enzyme–substrate complex resulted in a nearly identical extent of quenching of 5-deazaFADH$_2$ phosphorescence (85.1%) and fluorescence (87.5%). The data are consistent with a mechanism involving exclusive reaction of substrate with the excited singlet state of 5-deazaFADH$_2$, analogous to that proposed for FADH$_2$ in native enzyme. Direct evidence for singlet–singlet energy transfer from enzyme-bound 5-deazaFADH$_2$ to 5,10-CH$^+$-H$_4$folate was provided by the fact that pterin fluorescence was observed upon excitation of 5-deazaFADH$_2$, accompanied by a decrease in 5-deazaFADH$_2$ fluorescence. On the other hand, the fluorescence of enzyme-bound pterin was quenched by 5-deazaFAD$_{ox}$, consistent with energy transfer from pterin to 5-deazaFAD$_{ox}$. In each case, the spectral properties of the chromophores were consistent with the observed direction of energy transfer and indicated that transfer in the opposite direction was energetically unlikely. Unlike 5-deazaFAD, energy transfer from pterin to FAD is energetically feasible with FADH$_2$ or FAD$_{ox}$. The results indicate that the direction of flavin–pterin energy transfer at the active site of photolyase can be manipulated by changes in the flavin ring or redox state which alter the energy level of the flavin singlet.

DNA photolyase repairs pyrimidine dimers in UV-damaged DNA in a rather unusual catalytic reaction which requires visible light. The active form of the enzyme from *Escherichia coli* contains 1,5-dihydroFAD (FADH$_2$)[1] plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH$^+$-H$_4$Pte-(Glu)$_n$] (Wang et al., 1988; Wang & Jorns, 1989; Johnson et al., 1988; Jorns et al., 1990). Either chromophore can act as a sensitizer in catalysis (Jorns, 1987a; Sancar et al., 1987). The pterin chromophore is not required when FADH$_2$ acts as sensitizer, but FADH$_2$ is required when the pterin chromophore acts as sensitizer (Jorns et al., 1987a, 1990; Wang & Jorns, 1989). FADH$_2$ is probably the chromophore that directly interacts with substrate since only the properties of FADH$_2$ are affected by substrate. For example, formation of an enzyme–substrate complex causes a selective quenching of FADH$_2$ fluorescence in a reaction that is fully reversible upon dimer repair, suggesting that the excited singlet state of FADH$_2$ (^{1}FADH$_2$*) can act as a catalytic intermediate (Jordan & Jorns, 1988). It has been suggested that the pterin chromophore may act as an antenna to harvest light energy which is then transferred to the FADH$_2$ reaction center (Jorns et al., 1990).

Although dimer repair is observed with enzyme containing FADH$_2$ as the only chromophore, the redox state of the flavin is critical since enzyme containing oxidized FAD (FAD$_{ox}$) or FAD radical (FADH•) is catalytically inactive (Jorns et al., 1990). [Oxidation of FADH$_2$ to FADH• occurs during isolation of the enzyme from *E. coli*, but the reaction is readily reversed by reduction with dithionite or photochemically (Jorns et al., 1987a,b, 1990; Payne et al., 1987).] The protein environment of FADH$_2$ in photolyase appears to exert a pronounced influence on the photochemical reactivity of the chromophore since model studies show that either fully oxidized or fully reduced flavin can act as a photosensitizer in the splitting of thymine dimers, although the reactions are less efficient as compared with the photolyase reaction (Jorns, 1987b; Rokita & Walsh, 1984). The photochemical properties of free flavins can be altered by modifications in the flavin ring. For example, unlike normal flavin, 1-deazaflavin is active as

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FAD$_{ox}$, oxidized FAD; FADH•, neutral FAD radical; FADH$_2$, 1,5-dihydroFAD; 1-deazaFAD$_{ox}$, oxidized 1-deazaFAD; 1-deazaFADH•, neutral 1-deazaFAD radical; 1-deazaFADH$_2$, 1,5-dihydro-1-deazaFAD; 5-deazaFAD$_{ox}$, oxidized 5-deazaFAD; 5-deazaFADH$_2$, 1,5-dihydro-5-deazaFAD; FMN, flavin mononucleotide; DTT, dithiothreitol; 5,10-CH$^+$-H$_4$Pte(Glu)$_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH$^+$-H$_4$folate, 5,10-methenyltetrahydrofolate; Pte, 5,10-CH$^+$-H$_4$folate or 5,10-CH$^+$-H$_4$Pte(Glu)$_n$; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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a sensitizer in the splitting of thymine dimers only in the fully reduced state whereas the converse is observed with 5-deazaflavin where only the oxidized chromophore is active (Jorns, 1987b). These observations prompted studies to characterize the properties of *E. coli* photolyase containing 5-deazaFAD or 1-deazaFAD in place of FAD.

Experimental Procedures

Materials. Phenyl-Sepharose CL-4B was purchased from Pharmacia. Oligo(dT)$_{18}$ was obtained from P-L Biochemicals. 1-Deazariboflavin was a generous gift from Dr. Wallace Ashton.

Enzyme Assay. Enzyme assays were generally performed at 21 °C with UV-irradiated oligo(dT)$_{18}$ as substrate, similar to those described by Jorns et al. (1985) except the assay buffer (50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl, 1.6 mM DTT, and 1 mM EDTA) contained DTT. For assays with 1-deazaFAD-containing enzyme, the coenzyme was reduced with 5 mM dithionite just prior to assay, and glycerol (50%) was added to the assay buffer. Substrate was prepared as previously described (Jorns et al., 1985). Protein concentration, determined by the Bradford (1976) method, was used in calculating specific activity values.

Holoenzyme and Apoenzyme Preparation. E. coli photolyase holoenzyme was purified as previously described (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). Unless otherwise specified, all handling of the enzyme was done under yellow light. Apophotolyase was prepared as described by Jorns et al. (1990). Briefly, native enzyme was bound to a small phenyl-Sepharose CL-4B column at neutral pH, the chromophores were removed by washing the column with buffer at pH 3.5, and then the apoenzyme was eluted with 100 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA, 10 mM DTT, and 50% ethylene glycol (buffer C).

Reconstitution Studies. 5-Deazariboflavin was synthesized according to the method of O'Brien et al. (1970). 5-Deazariboflavin and 1-deazariboflavin were converted to the corresponding FAD analogues according to the procedure of Spencer et al. (1976). Oxidized 5-deazaFAD (5-deazaFAD$_{ox}$) (0.25 mM) was converted to 1,5-dihydro-5-deazaFAD (5-deazaFADH$_2$) by reduction with sodium borohydride (31 mM) at 5 °C in buffer C. Excess borohydride was decomposed by storing the reaction mixture for 24 h at 5 °C before use. 1,5-Dihydro-1-deazaFAD (1-deazaFADH$_2$) was prepared immediately before use by reduction of the oxidized coenzyme with excess dithionite (0.03 mM) in buffer C at 5 °C. 5,10-Methenyltetrahydrofolate (5,10-CH$^+$-H$_4$folate) was prepared as described by Rabinowitz (1963). For reconstitution experiments, apophotolyase was incubated at 5 °C with a 10-fold excess of the desired chromophore(s) (5-deazaFAD$_{ox}$, 5-deazaFADH$_2$, 1-deazaFADH$_2$, 1-deazaFAD$_{ox}$, 5,10-CH$^+$-H$_4$-folate) in buffer C. After 20 h, the reconstituted enzyme was isolated according to a procedure similar to that described by Jorns et al. (1990). Briefly, the reconstituted enzyme was dialyzed against buffer A (50 mM potassium phosphate, pH 7.0, containing 1.7 M ammonium sulfate, 0.5 mM EDTA, 10 mM DTT, and 20% glycerol) and then applied to a phenyl-Sepharose CL-4B column which was washed with buffer A until elution of any unbound chromophores was complete, as judged by monitoring the absorbance or, as an extra precaution, by monitoring the fluorescence of the column eluate. The reconstituted enzyme was eluted in buffer C and then dialyzed against complete PRE buffer. For each reconstitution, a reaction mixture containing the same components, except that apoenzyme was omitted, was subjected to the same isolation procedure and used as a control buffer. In one experiment, enzyme isolated after reconstitution with 5-deazaFAD$_{ox}$ (*E*5-deazaFAD$_{ox}$) was incubated at 5 °C with a 10-fold excess of 5,10-CH$^+$-H$_4$folate in complete PRE buffer. After an overnight incubation, the reconstituted enzyme (*E*Pte5-deazaFAD$_{ox}$) was isolated as previously described (Jorns et al., 1990).

Spectroscopy. Absorption measurements were made with a Perkin-Elmer Lambda 3 spectrophotometer. Fluorescence and phosphorescence spectra were obtained by using a Perkin-Elmer Lambda 5 luminescence spectrometer. Unless otherwise indicated, all measurements were made in complete PRE buffer. This buffer freezes to yield a clear glass at 77 K. Luminescence measurements at 77 K were performed by using a liquid nitrogen filled optical Dewar flask as previously described (Jordan & Jorns, 1988). 10-Methyl-5-deazaisoalloxazine was synthesized according to the method of O'Brien et al. (1970). 5-DeazaFAD was reduced with 18 mM sodium borohydride in complete PRE buffer at 5 °C. Substrate used for luminescence quenching experiments was prepared by irradiating oligo(dT)$_{18}$ with germicidal light, as previously described (Jorns et al., 1985). Germicidal light produces mainly thymine dimers but also a 6–4 photoproduct whose fluorescence can be eliminated by exposure to black light without affecting the dimer content of the preparation (Mitchell & Clarkson, 1984). Since this fluorescence interfered with certain quenching experiments, substrate was exposed to black light (Sylvania F15T8/BLB, 5 cm from two lights) until the fluorescence of the 6–4 photoproduct was eliminated.

The concentration of free 5-deazaFAD$_{ox}$ was calculated on the basis of its absorbance at 399 ($\epsilon_{399} = 11.5 \times 10^3$ M^{-1} cm^{-1}) (Spencer et al., 1976). Extinction coefficients for enzyme-bound 5-deazaFAD$_{ox}$ ($\epsilon_{400} = 11.4 \times 10^3$ M^{-1} cm^{-1}, $\epsilon_{380} = 6.8 \times 10^3$ M^{-1} cm^{-1}, $\epsilon_{325} = 10.9 \times 10^3$ M^{-1} cm^{-1}) were determined on the basis of the amount of free 5-deazaFAD$_{ox}$ released upon denaturation of *E*5-deazaFAD$_{ox}$. The extinction coefficient of enzyme-bound 5,10-CH$^+$-H$_4$folate ($\epsilon_{380} = 25.9 \times 10^3$ M^{-1} cm^{-1}) (Jorns et al., 1990) was used to determine the pterin content in *E*Pte5-deazaFAD$_{ox}$ after correction of the observed absorbance of the enzyme at 380 nm for the contribution due to 5-deazaFAD$_{ox}$. The 5-deazaFADH$_2$ content in *E*5-deazaFADH$_2$ was estimated on the basis of the total 5-deazaflavin content after correction for the contribution due to 5-deazaFAD$_{ox}$. The total 5-deazaflavin content was determined on the basis of the amount of 5-deazaFAD$_{ox}$ recovered after SDS denaturation and oxidation of released 5-deazaFADH$_2$. The 5-deazaFAD$_{ox}$ content was determined on the basis of the absorbance of intact *E*5-deazaFADH$_2$ at 400 nm, where absorbance due to 5-deazaFADH$_2$ is negligible. The extinction coefficient of enzyme-bound 5-deazaFADH$_2$ at 325 ($\epsilon_{325} = 12.3 \times 10^3$ M^{-1} cm^{-1}) was determined after correction of the absorbance of *E*5-deazaFADH$_2$ for the contribution due to 5-deazaFAD$_{ox}$.

The concentration of free 1-deazaFAD$_{ox}$ was calculated on the basis of its absorbance at 540 nm ($\epsilon_{540} = 6.8 \times 10^3$ M^{-1} cm^{-1}) (Entsch et al., 1980). The extinction coefficient of enzyme-bound 1-deazaFAD was determined on the basis of the amount of free 1-deazaFAD$_{ox}$ released after SDS denaturation of reconstituted enzyme. The content of bound 5,10-CH$^+$-H$_4$folate ($\epsilon_{380} = 25.9 \times 10^3$ M^{-1} cm^{-1}) (Jorns et al., 1990) in enzyme containing both pterin and 1-deazaFAD was determined after correction of the absorption of the enzyme at 380 nm for the contribution due to 1-deazaFAD. The latter

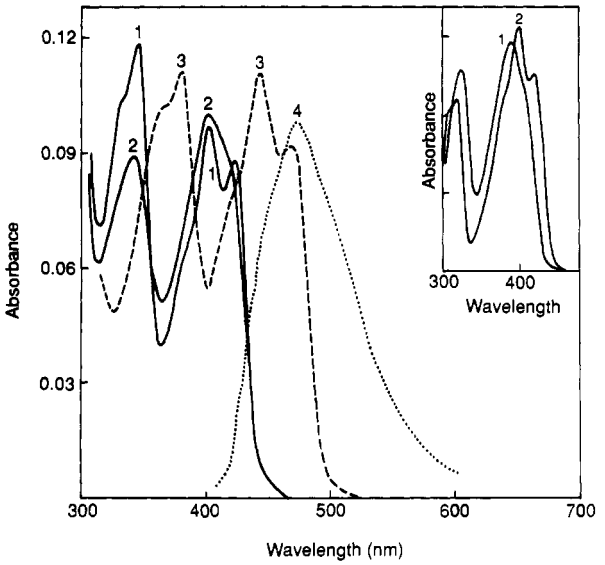

FIGURE 1: Spectral properties of enzyme reconstituted with 5-deazaFAD$_{ox}$. Curve 1 is the absorption spectrum of the reconstituted enzyme at 5 °C in complete PRE buffer containing 9% glycerol. Curve 2 was recorded after denaturation of the reconstituted enzyme with 0.08% SDS at 25 °C. The absorption spectrum of enzyme-bound FAD$_{ox}$ and the fluorescence emission spectrum of enzyme-bound 5,10-CH$^+$-H$_4$folate reported by Jorns et al. (1990) are shown in curves 3 and 4, respectively. The inset shows absorption spectra obtained with 10-methyl-5-deazaisoalloxazine (0.1 mM) in 0.2 M sodium phosphate, pH 7.5 (curve 1), and chloroform (curve 2).

was estimated on the basis of the absorbance observed at 380 nm after reaction of the enzyme with sodium borohydride (see Figure 8), which eliminates the spectral contribution of the pterin chromophore (Jorns et al., 1987a).

To determine the extent of chromophore incorporation in various reconstituted enzyme preparations, protein concentration was determined on the basis of the absorbance of the reconstituted enzyme at 280 nm after denaturation with SDS as described by Wang and Jorns (1989). For samples containing reduced flavin derivatives or 5,10-CH$^+$-H$_4$folate, absorbance measurements at 280 nm were made after complete oxidation of the released flavin chromophore or complete loss of the free pterin absorption band at 360 nm, respectively. The absorbance at 280 nm was corrected for the contribution expected from free 5-deazaFAD$_{ox}$ (ϵ_{280} = 16.8 × 10^3 M^{-1} cm^{-1}), 1-deazaFAD$_{ox}$ (ϵ_{280} = 14.3 × 10^3 M^{-1} cm^{-1}), or the pterin chromophore (ϵ_{280} = 16.0 × 10^3 M^{-1} cm^{-1}) (Wang & Jorns, 1989).

Results

Reconstitution of Apoenzyme with Oxidized 5-DeazaFAD. Oxidized 5-deazaFAD was readily bound to apophotolyase to yield a stable, reconstituted enzyme (*E*5-deazaFAD$_{ox}$) containing stoichiometric amounts of the modified flavin (0.93 mol of 5-deazaFAD$_{ox}$/mol of protein). *E*5-deazaFAD$_{ox}$ exhibited visible absorption maxima at 421, 400 (ϵ_{400} = 11 400 M^{-1} cm^{-1}), and 342 nm with shoulders at 380 and 330 nm (Figure 1, curve 1) whereas only two featureless absorption bands were observed after treatment of the enzyme with SDS, which released 5-deazaFAD$_{ox}$ into solution (Figure 1, curve 2). A resolved 400-nm band, similar to that observed with *E*5-deazaFAD$_{ox}$, was obtained with 10-methyl-5-deazaisoalloxazine in chloroform but not in aqueous solution (Figure 1, inset). This solvent effect is analogous to that observed with normal flavins, which exhibit a resolved 450-nm band only in nonpolar solvents (Harbury et al., 1959). *E*5-deazaFAD$_{ox}$ and enzyme containing oxidized normal FAD (*E*FAD$_{ox}$) (Figure 1, curve 3) exhibit strikingly similar absorption spectra, except for an expected 50-nm difference in absorption maxima. The results suggest that 5-deazaFAD$_{ox}$ binds at a hydrophobic site in photolyase, similar to that observed with FAD$_{ox}$.

The bright blue fluorescence of free 5-deazaFAD$_{ox}$ (emission λ_{max} = 460 nm) was strongly quenched upon binding to photolyase. As normally isolated, the fluorescence intensity of *E*5-deazaFAD$_{ox}$ at 460 nm was approximately 5% of that observed with the free 5-deazaFAD$_{ox}$. However, this residual fluorescence was mainly due to contamination with a small amount of free 5-deazaFAD$_{ox}$ and could be largely (80%) eliminated by reagents [e.g., sulfite (Jorns & Hersh, 1976) or borohydride (vide infra)] which selectively reacted with the free chromophore or by taking extra precautions during the isolation of the reconstituted enzyme (see Experimental Procedures) to ensure quantitative removal of the 10-fold excess of free 5-deazaFAD$_{ox}$ used during the reconstitution procedure. The actual fluorescence due to enzyme-bound 5-deazaFAD$_{ox}$ was estimated to be about 1% or less as compared with that of the free 5-deazaFAD$_{ox}$.

*E*5-deazaFAD$_{ox}$ was catalytically inactive, similar to results obtained in previous studies with *E*FAD$_{ox}$ where full activity could be restored upon reduction to *E*FADH$_2$ (Jorns et al., 1990). However, *E*5-deazaFAD$_{ox}$ could not be reduced with borohydride, cyanoborohydride, or dithionite. The failure to observe reduction with borohydride was unexpected since free 5-deazaflavin is readily reducible by this reagent. Photochemical reduction of *E*5-deazaFAD$_{ox}$ was attempted under anaerobic conditions in the presence of EDTA and/or β-mercaptoethanol, but the reactions were very slow and could not be followed to completion owing to the onset of turbidity.

Reconstitution of E5-deazaFAD$_{ox}$ with Pterin. *E*5-deazaFAD$_{ox}$ was reconstituted with 5,10-CH$^+$-H$_4$folate to determine whether the active site of the enzyme was accessible to borohydride since enzyme-bound pterin is known to readily react with this reagent (Jorns et al., 1987a, 1990). Pterin incorporation was observed (0.75 mol of pterin/mol of protein) but, as expected, did not restore catalytic activity. The reisolated enzyme (*E*Pte5-deazaFAD$_{ox}$) exhibited a broad absorption band at 391 nm, reflecting contributions from both pterin (λ_{max} = 387 nm) and 5-deazaFAD$_{ox}$ (λ_{max} = 400 nm), plus a sharper band at 345 nm attributable to 5-deazaFAD$_{ox}$ (Figure 2, curve 1). As observed with native enzyme (Jorns et al., 1987a), the absorption due to the pterin chromophore in *E*Pte5-deazaFAD$_{ox}$ could be eliminated by reaction with borohydride (Figure 2, curve 2), but the absorption due to 5-deazaFAD$_{ox}$ was unaffected. The results show that at least the pterin subsite in *E*Pte5-deazaFAD$_{ox}$ is accessible to borohydride, suggesting that the inability of borohydride to reduce 5-deazaFAD$_{ox}$ may reflect a very low reduction potential of the bound chromophore.

*E*Pte5-deazaFAD$_{ox}$ exhibited fluorescence properties (excitation λ_{max} = 390 nm, emission λ_{max} = 470 nm) similar to those observed for the pterin chromophore in flavin-free enzyme (*E*Pte) (Jorns et al., 1990). The latter was not surprising since 5-deazaFAD$_{ox}$ fluorescence is strongly quenched when 5-deazaFAD$_{ox}$ is bound to photolyase. However, the fluorescence intensity of the pterin chromophore in *E*Pte5-deazaFAD$_{ox}$ was 3.9-fold weaker than that in *E*Pte. The extent of quenching of pterin fluorescence by 5-deazaFAD$_{ox}$ was less than that previously observed for enzyme containing normal oxidized flavin (*E*PteFAD$_{ox}$) where pterin fluorescence is 9.6-fold weaker than that of *E*Pte (Jorns et al., 1990). This difference is expected if pterin fluorescence quenching is due to energy transfer to flavin since the pterin fluorescence emission spectrum (Figure 1, curve 4) exhibits greater overlap

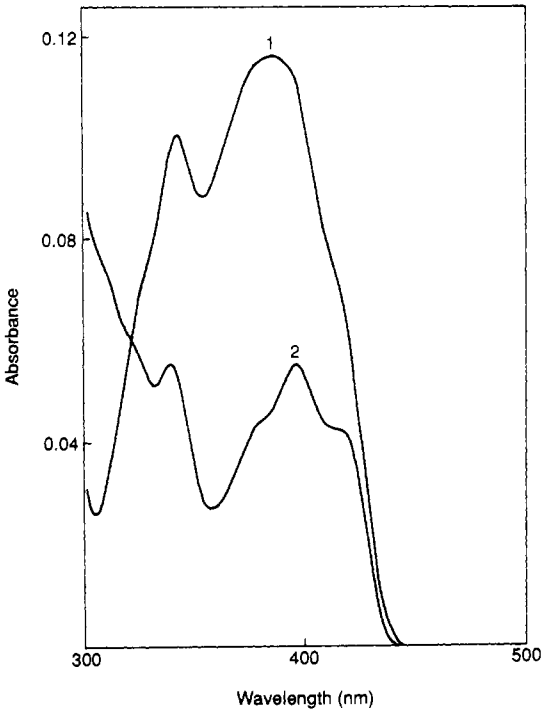

FIGURE 2: Spectral properties of enzyme reconstituted with 5-deazaFAD$_{ox}$ plus 5,10-CH$^+$-H$_4$folate. Curve 1 is the absorption spectrum of the reconstituted enzyme in complete PRE buffer at 5 °C. Curve 2 was recorded after reaction with 8.3 mM sodium borohydride.

with the absorption spectrum of *E*FAD$_{ox}$ (Figure 1, curve 3) as compared with that of *E*5-deazaFAD$_{ox}$ (Figure 1, curve 1).

Reconstitution of Apoenzyme with Reduced 5-DeazaFAD. The reconstituted enzyme (*E*5-deazaFADH$_2$) exhibited a prominent absorption band at 325 nm plus a weaker band near 400 nm (Figure 3A, curve 1). The preparation contained a stoichiometric amount of 5-deazaFAD (1.1 mol of 5-deazaFAD/mol of enzyme), as judged by the amount of 5-deazaFAD$_{ox}$ recovered after enzyme denaturation and air oxidation of the released 5-deazaFADH$_2$ (Figure 3, curve 2). Studies with three preparations of *E*5-deazaFADH$_2$ showed that about 70% of the bound coenzyme was in the fully reduced state with the remainder (27–32%) present as 5-deazaFAD$_{ox}$. After subtraction of the contribution due to 5-deazaFAD$_{ox}$, the corrected absorption spectrum of *E*5-deazaFADH$_2$ exhibited a single band at 325 nm (ϵ_{325} = 12.3 × 10^3 M^{-1} cm^{-1}) (Figure 3, curve 3), similar to that observed for free 5-deazaFADH$_2$ (ϵ_{325} = 11.5 × 10^3 M^{-1} cm^{-1}, in complete PRE buffer containing 18 mM borate). Although FADH$_2$ is oxidized to a stable, neutral radical (FADH$^\bullet$) during isolation of enzyme reconstituted with normal reduced flavin (Jorns et al., 1990), reconstitution with 5-deazaFADH$_2$ was expected to result in the quantitative incorporation of the fully reduced coenzyme since, unlike FADH$_2$, 5-deazaFADH$_2$ is fairly stable against air oxidation but 5-deazaflavin radicals are very unstable and difficult to form (Hemmerich et al., 1977). No oxidation of bound 5-deazaFADH$_2$ was observed during storage of the reconstituted enzyme at −20 °C, but it is possible that the reduced coenzyme was less stable during the isolation procedure. Alternatively, if the apoprotein preferentially binds 5-deazaFAD$_{ox}$, the observed incorporation might be explained by contamination of the 5-deazaFADH$_2$ used for reconstitution reactions (10-fold excess with respect to apoprotein) with a small amount (3%) of oxidized coenzyme. Preferential binding of 5-deazaFAD$_{ox}$ would result in a low reduction potential, as suggested by the observed resistance of enzyme-bound 5-deazaFAD$_{ox}$ against reduction.

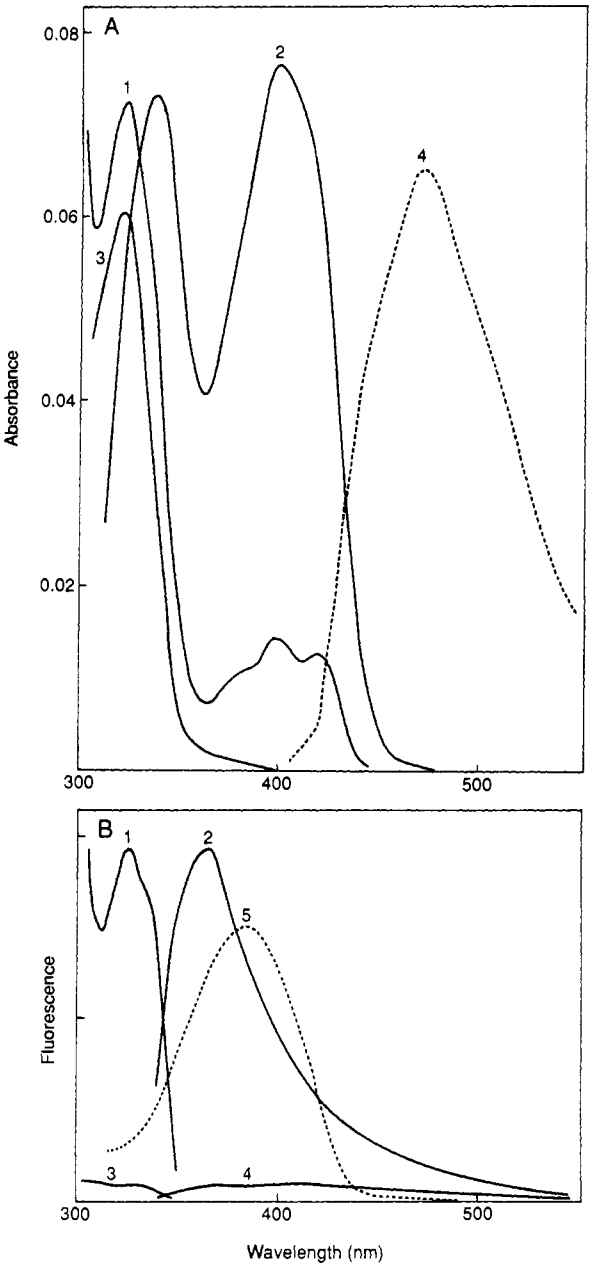

FIGURE 3: Spectral properties of enzyme reconstituted with 5-deazaFADH$_2$. Panel A: Curve 1 is the absorption spectrum of the isolated enzyme (*E*5-deazaFADH$_2$) in complete PRE buffer at 5 °C. *E*5-deazaFADH$_2$ was denatured with 0.08% SDS at 25 °C, and curve 2 was recorded after 5 days when the reoxidation of released 5-deazaFADH$_2$ was complete. Curve 3 is the absorption spectrum calculated for enzyme-bound 5-deazaFADH$_2$ by correction of the absorption spectrum of the reconstituted enzyme for the contribution due to 5-deazaFAD$_{ox}$. Curve 4 is the fluorescence emission spectrum of enzyme-bound 5,10-CH$^+$-H$_4$folate reported by Jorns et al. (1990). Panel B: Curve 1 is the fluorescence excitation spectrum (emission λ = 365 nm) of *E*5-deazaFADH$_2$ in complete PRE buffer at 5 °C. Interference from scattered excitation light occurred at 350 nm < λ < 380 nm but the excitation spectrum returned to baseline at 380 nm and remained flat at longer wavelengths (data not shown). Curve 2 is the fluorescence emission spectrum (excitation λ = 325 nm) of *E*5-deazaFADH$_2$ in complete PRE buffer at 5 °C. Curves 3 and 4 are the corresponding excitation and emission spectra, respectively, recorded for buffer alone. Curve 5 is the absorption spectrum of enzyme-bound 5,10-CH$^+$-H$_4$folate reported by Jorns et al. (1990).

A fluorescence emission spectrum with a maximum at 365 nm was obtained when *E*5-deazaFADH$_2$ was excited at 325 nm (Figure 3B, curve 2). (Excitation at 400 nm, an absorption maximum for 5-deazaFAD$_{ox}$ where 5-deazaFADH$_2$ exhibits negligible absorption, yielded baseline fluorescence identical

with that obtained for a buffer control.) The fluorescence excitation spectrum of *E*5-deazaFADH$_2$ obtained by monitoring emission at 365 nm exhibited a single maximum at 325 nm (Figure 3B, curve 1), similar to the absorption spectrum calculated for enzyme-bound 5-deazaFADH$_2$ (Figure 3A, curve 3). The results indicate that the fluorescence observed with *E*5-deazaFADH$_2$ is due to 5-deazaFADH$_2$ and reflects a negligible contribution from 5-deazaFAD$_{ox}$ (30% of the coenzyme in the preparation) whose fluorescence is strongly quenched (≥99%) when bound to photolyase. The maximum fluorescence intensity observed for enzyme-bound 5-deazaFADH$_2$ was 50-fold greater than that observed for bound FADH$_2$ under the same conditions (complete PRE buffer at 5 °C) or 60% of that obtained for free 5-deazaFADH$_2$ under similar conditions (complete PRE buffer containing 18 mM borate at 5 °C). Fluorescence intensity increases were observed for both bound and free 5-deazaFADH$_2$ when the temperature was decreased from 5 °C to 77 K, but the effect was greater for the bound chromophore. As a consequence, bound 5-deazaFADH$_2$, although less fluorescent at 5 °C, was actually 1.6-fold more fluorescent than free 5-deazaFADH$_2$ at 77 K. The same fluorescence excitation maximum (excitation λ_{max} = 325 nm) was observed for free or enzyme-bound 5-deazaFADH$_2$ at either 5 °C or 77 K. The fluorescence emission maximum of bound 5-deazaFADH$_2$ (emission λ_{max} = 365 nm) was also temperature independent. However, with free 5-deazaFADH$_2$ when the temperature was lowered from 5 °C to 77 K, the emission maximum shifted from 410 nm to a value (emission λ_{max} = 373 nm) (Figure 4B, curve 1) closer to that observed for the bound chromophore.

The fluorescence of free reduced normal flavin has been observed only in a rigid glass at 77 K (Ghisla et al., 1974). In contrast, as described above and in previous studies (Stankovich & Massey, 1976), the fluorescence of free reduced 5-deazaflavin is readily detectable at ambient temperature. At 77 K the fluorescence of free reduced 5-deazaflavin may actually exceed that observed for the oxidized species (e.g., 5-deazaFADH$_2$ is 1.2-fold more fluorescent than 5-deazaFAD$_{ox}$ at 77 K but 4-fold less fluorescent at 5 °C in complete PRE buffer containing 18 mM borate). This difference prompted studies to determine whether other differences might exist in the luminescence properties of reduced normal versus 5-deazaflavin. Phosphorescence has been observed for normal flavin in the oxidized state (Bowd et al., 1968), but attempts to detect phosphorescence with either free (Jordan and Jorns, unpublished observations) or photolyase-bound (Jordan & Jorns, 1988) reduced normal flavin at 77 K proved unsuccessful. In contrast, phosphorescence was readily detected at 77 K in studies with enzyme-bound 5-deazaFADH$_2$ (emission λ_{max} = 442 nm) (Figure 4A, curve 2) and free 5-deazaFADH$_2$ (emission λ_{max} = 430 nm) (Figure 4B, curve 2). In each case, phosphorescence excitation spectra exhibited a maximum at 325 nm (data not shown), indicating that the observed phosphorescence was due to the reduced chromophore. The maximum phosphorescence intensity observed for bound 5-deazaFADH$_2$ was 1.2-fold greater than that for free 5-deazaFADH$_2$. The phosphorescence of free 5-deazaFADH$_2$ was 50% of that observed for the free 5-deazaFAD$_{ox}$ (emission λ_{max} = 503 nm in complete PRE buffer at 77 K).

*E*5-deazaFADH$_2$ was catalytically active. Linear rates of dimer repair were observed when reactions were monitored for at least two turnovers in the presence of excess substrate (data not shown). The activity observed with three preparations of *E*5-deazaFADH$_2$ was about 29% (25–32%) of that observed with native enzyme or 42% when corrected for the presence of 30% inactive enzyme containing 5-deazaFAD$_{ox}$. The results show that only reduced 5-deazaFAD can act as a sensitizer when bound to photolyase, similar to results obtained with normal flavin but opposite to results obtained with aqueous solutions of free 5-deazaflavin where photosensitized cleavage of thymine dimers was observed only with the oxidized chromophore (Jorns, 1987b).

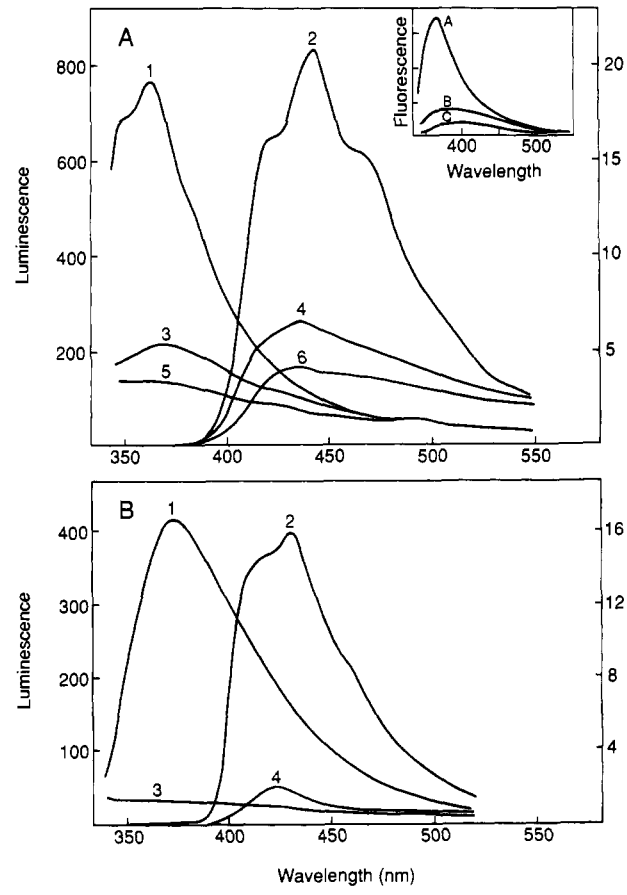

FIGURE 4: Luminescence properties of enzyme-bound 5-deazaFADH$_2$ in the presence and absence of substrate and comparison with free 5-deazaFADH$_2$. Unless otherwise noted, all spectra were recorded in complete PRE buffer at 77 K. In each panel, fluorescence intensity is plotted according to the scale on the left whereas the scale on the right is used for phosphorescence data. (Spectra reported for enzyme–substrate complexes are corrected for a small contribution due to a luminescent impurity in the substrate preparation.) Panel A: Fluorescence and phosphorescence emission spectra (excitation λ = 325 nm) of *E*5-deazaFADH$_2$ (2.28 μM with respect to enzyme-bound 5-deazaFADH$_2$) in the absence of substrate are shown in curves 1 and 2, respectively. Fluorescence and phosphorescence emission spectra of *E*5-deazaFADH$_2$ in the presence of substrate [UV-irradiated oligo(dT)$_{18}$, 5 mol of dimer/mol of enzyme-bound 5-deazaFADH$_2$] are shown in curves 3 and 4, respectively. Fluorescence emission and phosphorescence emission spectra recorded for buffer alone are shown in curves 5 and 6, respectively. The inset shows the effect of substrate on *E*5-deazaFADH$_2$ fluorescence at 5 °C. Fluorescence emission spectra recorded in the absence and presence of substrate [UV-irradiated oligo(dT)$_{18}$, 5 mol of dimer/mol of enzyme-bound 5-deazaFADH$_2$] are shown in curves A and B, respectively. Curve C was recorded for buffer alone. Panel B: Fluorescence and phosphorescence emission spectra (excitation λ = 325 nm) of free 5-deazaFADH$_2$ are shown in curves 1 and 2, respectively. The spectra were normalized to the same 5-deazaFADH$_2$ concentration as shown for the enzyme-bound chromophore in panel A. Fluorescence emission and phosphorescence emission spectra recorded for buffer alone are shown in curves 3 and 4, respectively.

Previous studies with native enzyme suggested that the excited singlet state of FADH$_2$ (^{1}FADH$_2$*) might act as a catalytic intermediate, but attempts to study possible interaction of substrate with the triplet state (^{3}FADH$_2$*) were not

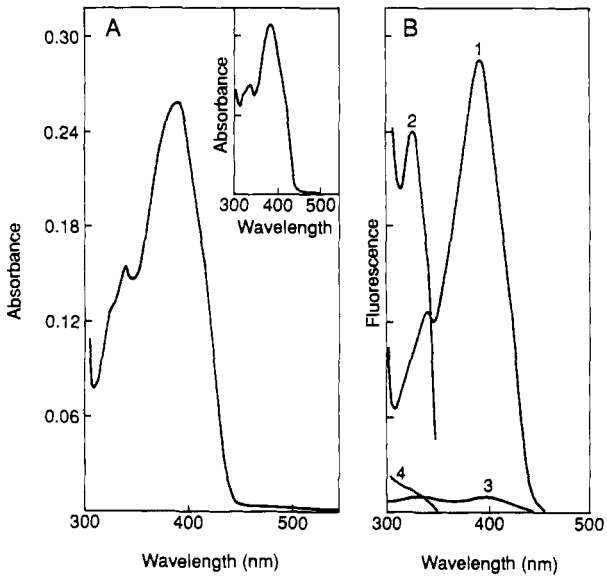

FIGURE 5: Comparison of absorption and fluorescence excitation spectra of enzyme reconstituted with 5-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate. All spectra were recorded in complete PRE buffer at 5 °C. Panel A: The absorption spectrum shown was obtained with the isolated enzyme (*E*Pte5-deazaFADH$_2$). Inset: The absorption spectrum shown was calculated on the basis of absorption spectra observed for enzyme reconstituted with 5-deazaFADH$_2$ alone (*E*5-deazaFADH$_2$) or 5,10-CH$^+$-H$_4$folate alone (*E*Pte) (Jorns et al., 1990) and corresponds to an equimolar mixture of *E*5-deazaFADH$_2$ and *E*Pte. Panel B: Curve 1 is the fluorescence excitation spectrum of *E*Pte5-deazaFADH$_2$ obtained by monitoring emission 470 nm. Curve 2 was obtained by monitoring emission at 365 nm. Excitation spectra recorded for buffer alone with emission at 470 or 365 nm are shown in curves 3 and 4, respectively. Curves 2 and 4 are shown expanded 10.1-fold relative to curves 1 and 3.

feasible since ^{3}FADH$_2$* was not detected in phosphorescence or microsecond flash photolysis experiments (Jordan & Jorns, 1988). That *E*5-deazaFADH$_2$ was catalytically active and also exhibited detectable fluorescence and phosphorescence prompted studies to determine the effect of substrate on the luminescence properties of *E*5-deazaFADH$_2$. Formation of an enzyme–substrate complex quenched the fluorescence emission of enzyme-bound 5-deazaFADH$_2$ at 365 nm and also the phosphorescence emission at 442 nm. A similar extent of quenching was observed for fluorescence at 5 °C (86.7%) [Figure 4A (inset), curve B] or 77 K (87.5%) (Figure 4A, curve 3) and for phosphorescence at 77 K (85.1%) (Figure 4A, curve 4). In each case, the observed luminescence quenching was fully reversible upon exposure of the enzyme–substrate complex to photoreactivating light at 21 °C. As will be discussed, the results are fully compatible with and most plausibly explained by a mechanism involving exclusive reaction of substrate with ^{1}FADH$_2$*.

Reconstitution of Apoenzyme with Reduced 5-DeazaFAD plus 5,10-CH$^+$-H$_4$folate. The reconstituted enzyme (*E*Pte5-deazaFADH$_2$) exhibited absorption maxima at 387 and 339 nm (Figure 5A). The band at 387 nm coincided with the absorption maximum observed for enzyme reconstituted with 5,10-CH$^+$-H$_4$folate alone (*E*Pte) (Jorns et al., 1990), but assignment of the band at 339 nm was less obvious since enzyme reconstituted with 5-deazaFADH$_2$ alone (*E*5-deazaFADH$_2$) exhibited a peak at 325 nm. However, the absorption spectrum calculated for an equimolar mixture of *E*5-deazaFADH$_2$ and *E*Pte (Figure 5A, inset) exhibited maxima at 387 and 339 nm, similar to that observed for *E*Pte5-deazaFADH$_2$. Furthermore, reaction of *E*Pte5-deazaFADH$_2$ with borohydride eliminated the absorption due to the pterin chromophore and yielded an absorption spectrum similar to that observed for *E*5-deazaFADH$_2$ (data not shown). Most of the 5-deazaFADH$_2$ in *E*Pte5-deazaFADH$_2$ was present as 5-deazaFADH$_2$ (70%). The latter was estimated after reaction with borohydride by comparing the amount of bound 5-deazaFAD$_{ox}$ present in intact enzyme with the amount of free 5-deazaFAD$_{ox}$ observed after SDS denaturation of borohydride-treated enzyme and air oxidation of released 5-deazaFADH$_2$.

*E*Pte5-deazaFADH$_2$ was catalytically active. The observed activity was 32% of that obtained with native enzyme or 46% when corrected for the presence of 30% inactive enzyme containing 5-deazaFAD$_{ox}$, very similar to results obtained with *E*5-deazaFADH$_2$ where the corrected activity was 42% of that of native enzyme. The activity observed with borohydride-treated *E*Pte5-deazaFADH$_2$ was similar (88%) to that observed with untreated enzyme. The results show that the presence of the pterin chromophore does not affect the rate of dimer repair with 5-deazaFADH$_2$-containing enzyme as measured under standard assay conditions. Similar results have been obtained previously with enzyme containing FADH$_2$ (Jorns et al., 1987a, 1990).

The fluorescence excitation spectrum observed with *E*Pte5-deazaFADH$_2$ by *monitoring emission at 365 nm* exhibited a peak at 325 nm (Figure 5B, curve 2), identical with that observed with *E*5-deazaFADH$_2$ (emission λ_{max} = 365 nm), and was not eliminated by reduction of the pterin with borohydride (vide infra). As expected, the excitation spectrum observed under these conditions was clearly attributable to emission from 5-deazaFADH$_2$. However, the maximum fluorescence intensity observed for 5-deazaFADH$_2$ in *E*Pte5-deazaFADH$_2$ was, unexpectedly, 2.3-fold weaker than that observed with *E*5-deazaFADH$_2$ under the same conditions (complete PRE buffer at 5 °C).

The fluorescence excitation spectrum observed with *E*Pte5-deazaFADH$_2$ by *monitoring emission at 470 nm* exhibited peaks at 390 and 340 nm (Figure 5B, curve 1), similar to the absorption spectrum of the enzyme (Figure 5A) but significantly different from that observed with *E*Pte (emission λ_{max} = 470 nm) which exhibits a single excitation maximum at 390 nm (Jorns et al., 1990). On the other hand, the excitation spectrum observed with *E*Pte5-deazaFADH$_2$ under these conditions was eliminated by reduction of the pterin chromophore with borohydride, indicating that the excitation spectrum observed for untreated enzyme was entirely attributable to *emission* from the pterin chromophore. The difference between excitation spectra observed for *E*Pte5-deazaFADH$_2$ versus *E*Pte suggested that emission from the pterin chromophore in *E*Pte5-deazaFADH$_2$ might, in part, be initiated by excitation of 5-deazaFADH$_2$ followed by energy transfer from excited 5-deazaFADH$_2$ singlet to the pterin chromophore.

The fluorescence emission spectrum observed with *E*Pte5-deazaFADH$_2$ by exciting at 390 nm exhibited a maximum at 470 nm (Figure 6A, curve 1), was eliminated by reaction with borohydride, and could therefore be attributed entirely to emission from the pterin chromophore. The emission spectrum observed with *E*Pte5-deazaFADH$_2$ by exciting at 325 nm (Figure 6A, curve 2) also exhibited a maximum at 470 nm attributable to emission from the pterin chromophore. However, the intensity of the band at 470 nm was 30% of that observed upon excitation at 390 nm, a value 3-fold greater than expected on the basis of results obtained with *E*Pte. This discrepancy is consistent with the proposal that pterin emission in *E*Pte5-deazaFADH$_2$ may be initiated by energy transfer

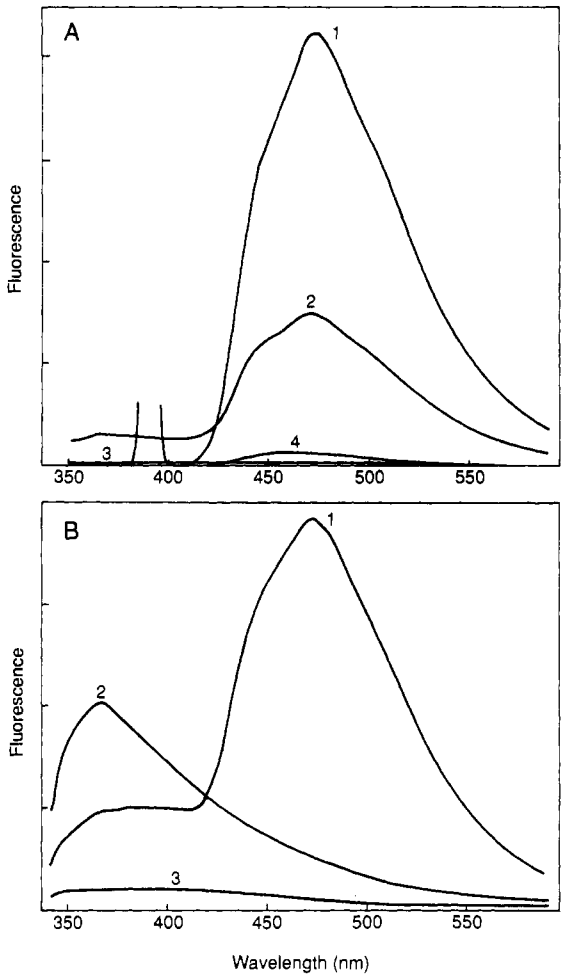

FIGURE 6: Effect of excitation wavelength or borohydride on the fluorescence emission spectrum of enzyme reconstituted with 5-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate (*E*Pte5-deazaFADH$_2$). All spectra were recorded in complete PRE buffer at 5 °C Panel A: Curves 1 and 2 are fluorescence emission spectra of *E*Pte5-deazaFADH$_2$ recorded by exciting at 390 or 325 nm, respectively. Emission spectra recorded for buffer alone with excitation at 390 or 325 nm are shown in curves 3 and 4, respectively. Panel B: Fluorescence emission spectra obtained with excitation at 325 nm before and after reaction of *E*Pte5-deazaFADH$_2$ with borohydride (8.3 mM) are shown in curves 1 and 2, respectively. Curve 3 was recorded for buffer alone.

from excited 5-deazaFADH$_2$. Although only a single maximum was observed, the emission spectrum observed with *E*Pte5-deazaFADH$_2$ by exciting at 325 nm showed significant emission in the 350–380-nm region where emission from 5-deazaFADH$_2$ would be expected. The latter was not observed for the emission spectrum obtained by exciting *E*Pte5-deazaFADH$_2$ at 390 nm which returned to baseline in the 350–380-nm region. The emission spectrum of *E*Pte5-deazaFADH$_2$ obtained by exciting at 325 nm was dramatically altered by reaction with borohydride. Borohydride eliminated the 470-nm band, as expected, but the reaction was also accompanied by a 2.4-fold increase in fluorescence intensity at 365 nm. Borohydride-treated *E*Pte5-deazaFADH$_2$ exhibited a single emission maximum at 365 nm (Figure 6B, curve 2), similar to that observed for *E*5-deazaFADH$_2$. The maximum fluorescence intensity observed for 5-deazaFADH$_2$ in borohydride-treated *E*Pte5-deazaFADH$_2$ was comparable to that observed for *E*5-deazaFADH$_2$. The results provide clear evidence for energy transfer from the excited singlet state of 5-deazaFADH$_2$ to the pterin in *E*Pte5-deazaFADH$_2$. Although the direction is opposite to that which has been proposed for native enzyme (Jorns et al., 1990), the direction of energy transfer observed with *E*Pte5-deazaFADH$_2$ is consistent with the spectral properties observed for 5-deazaFADH$_2$ and pterin bound to photolyase. In particular, the pterin absorption spectrum (Figure 3B, curve 5) overlaps with the fluorescence emission spectrum of 5-deazaFADH$_2$ (Figure 3B, curve 2) whereas there is almost no overlap of the absorption spectrum of 5-deazaFADH$_2$ (Figure 3A, curve 3) with the pterin emission spectrum (Figure 3A, curve 4).

Fluorescence measurements were conducted at 5 °C in the studies described above. When measurements were made at 77 K, it was found that reaction of *E*Pte5-deazaFADH$_2$ with borohydride was accompanied by an increase in 5-deazaFADH$_2$ fluorescence (2.2-fold), similar to that observed at 5 °C (2.4-fold). Phosphorescence studies at 77 K showed that reaction of *E*Pte5-deazaFADH$_2$ with borohydride was accompanied by a 1.4-fold increase in 5-deazaFADH$_2$ phosphorescence (excitation λ = 325 nm, emission λ = 440 nm) (data not shown). The latter suggests that, in addition to singlet–singlet energy transfer, energy transfer may also occur from 5-deazaFADH$_2$ triplet to the pterin chromophore. However, unlike the evidence obtained for singlet–singlet energy transfer, it could not be determined whether the observed quenching of 5-deazaFADH$_2$ phosphorescence was accompanied by a corresponding increase in the yield of pterin triplet since the pterin in *E*Pte5-deazaFADH$_2$ is nonphosphorescent, as observed with native enzyme.

Formation of an enzyme–substrate complex with *E*Pte5-deazaFADH$_2$ did not appear to affect energy transfer from 5-deazaFADH$_2$ singlet to pterin since there was no change in the fluorescence excitation spectrum obtained by monitoring emission at 470 nm. However, formation of an enzyme–substrate complex with *E*Pte5-deazaFADH$_2$ was accompanied by a quenching of 5-deazaFADH$_2$ fluorescence (58.0% at 5 °C, 59.4% at 77 K) and phosphorescence (62.0% at 77 K) which could be reversed upon exposure to photoreactivating light, similar to that observed with *E*5-deazaFADH$_2$, except that a greater extent of luminescence quenching was observed with *E*5deazaFADH$_2$ (≈85%). However, the *residual* luminescence observed for the enzyme–substrate complex with *E*Pte5-deazaFADH$_2$ was comparable to that observed for the complex formed with borohydride-treated *E*Pte5-deazaFADH$_2$ where the observed extent of fluorescence (80.3% at 5 °C; 80.5% at 77 K) and phosphorescence (85.1% at 77 K) quenching was similar to that observed with *E*5-deazaFADH$_2$. The difference in the extent of quenching observed for untreated versus borohydride-treated *E*Pte5-deazaFADH$_2$ is clearly attributable to the enhanced 5-deazaFADH$_2$ luminescence in uncomplexed, borohydride-treated enzyme.

Reconstitution Studies with 1-DeazaFAD. Enzyme isolated after reconstitution of the apoenzyme with fully reduced 1-deazaFAD (1-deazaFADH$_2$) contained a stoichiometric amount of oxidized 1-deazaFAD (1.05 mol of 1-deazaFAD$_{ox}$/mol of protein) and exhibited an absorption spectrum [λ_{max} = 540 (ϵ_{540} = 7.0 × 10^3 M^{-1} cm^{-1}) and 365 nm] (Figure 7, curve 1) similar to that observed with free 1-deazaFAD$_{ox}$ in aqueous solution. The reconstituted enzyme (*E*1-deazaFAD$_{ox}$) did not contain any neutral 1-deazaFAD radical (1-deazaFADH$^\bullet$), as evidenced by the absence of absorbance at λ > 700 nm (Entsch et al., 1980; Spencer et al., 1977b), and only small spectral shifts were observed upon aerobic denaturation of the enzyme with SDS (Figure 7, curve 2). The absence of 1-deazaFADH$^\bullet$ in the reconstituted enzyme was unexpected since 1-deazaflavin and normal flavin exhibit similar properties with respect to stability of the fully reduced and semiquinoid states (Spencer et al., 1977b), enzyme-bound

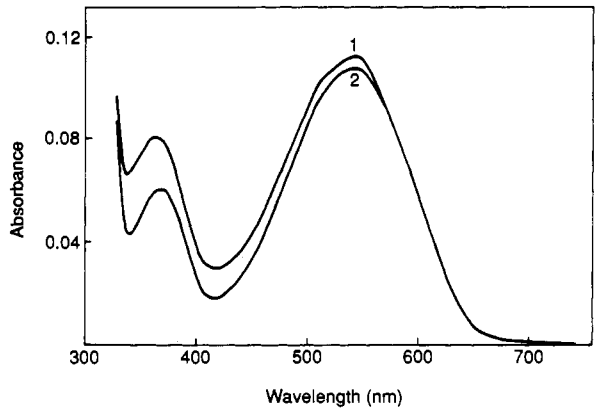

FIGURE 7: Comparison of spectral properties of enzyme-bound versus free 1-deazaFAD$_{ox}$. Curve 1 is the absorption spectrum of *E*1-deazaFAD$_{ox}$ in complete PRE buffer at 5 °C. Curve 2 was recorded 2 h after denaturation with 0.08% SDS at 25 °C.

FADH$^{\bullet}$ is stable against air oxidation or disproportionation (Jorns et al., 1984), and preparations isolated after reconstitution of apoenzyme with FADH$_2$ contain stoichiometric amounts of FADH$^{\bullet}$ (Jorns et al., 1990). The lack of vibronic resolution in the 540-nm band of *E*1-deazaFAD$_{ox}$ is quite different from results obtained with *E*FAD$_{ox}$ or *E*5-deazaFAD$_{ox}$ which exhibit highly resolved bands at 450 or 400 nm, respectively. In contrast, the vibronic resolution observed for the 540-nm band of 1-deazaFAD$_{ox}$ bound to other flavoenzymes has generally been found to parallel that observed for the 450-nm band of FAD$_{ox}$ in the corresponding native enzymes (Entsch et al., 1980; Spencer et al., 1977a; O'Donnell & Williams, 1984). The results suggest that replacement of N(1) in FAD by CH interferes with the normal mode of flavin binding to photolyase.

In other studies, apophotolyase was reconstituted with 1-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate in order to determine whether the redox and/or spectral properties of enzyme-bound 1-deazaFAD might be altered in the presence of the pterin chromophore. The reconstituted enzyme prepared in these studies (*E*Ptel-deazaFAD) contained stoichiometric amounts of pterin (0.94 mol of 5,10-CH$^+$-H$_4$folate/mol of protein) and 1-deazaflavin (1.0 mol of 1-deazaFAD/mol of protein). The preparation exhibited an unresolved band at 540 nm attributable to 1-deazaFAD$_{ox}$ plus an intense band at 380 nm due largely to the pterin chromophore but showed no absorption at $\lambda > 700$ nm which might be due to 1-deazaFADH$^{\bullet}$ (Figure 8, curve 1). Although the 1-deazaFAD in *E*Ptel-deazaFAD appeared to be mainly in the oxidized state, its apparent extinction coefficient at 540 nm ($\epsilon_{540} = 4.8 \times 10^3$ M^{-1} cm^{-1}) (see Figure 8, curves 1 and 3) was significantly lower than that determined for the fully oxidized chromophore in *E*1-deazaFAD$_{ox}$ ($\epsilon_{540} = 7.0 \times 10^3$ M^{-1} cm^{-1}). The low value observed for the extinction coefficient of *E*Ptel-deazaFAD at 540 nm raised the possibility that 30% of the 1-deazaflavin in the preparation was present as 1-deazaFADH$_2$, which does not absorb at 540 nm. Alternatively, the presence of pterin at the active site of *E*Ptel-deazaFAD might cause a significant perturbation of the absorption spectrum of bound 1-deazaFAD$_{ox}$. The latter possibility could be ruled out since the absorbance of *E*Ptel-deazaFAD at 540 nm was unaffected by reduction of the pterin with borohydride (Figure 8, curve 2), even though this reaction is accompanied by a release of the reduced pterin from the enzyme (Hamm-Alvarez et al., 1989). In other studies, apoenzyme was reconstituted with 1-deazaFAD$_{ox}$ plus 5,10-CH$^+$-H$_4$folate since in this case the resulting preparation (*E*Ptel-deazaFAD$_{ox}$) could not contain

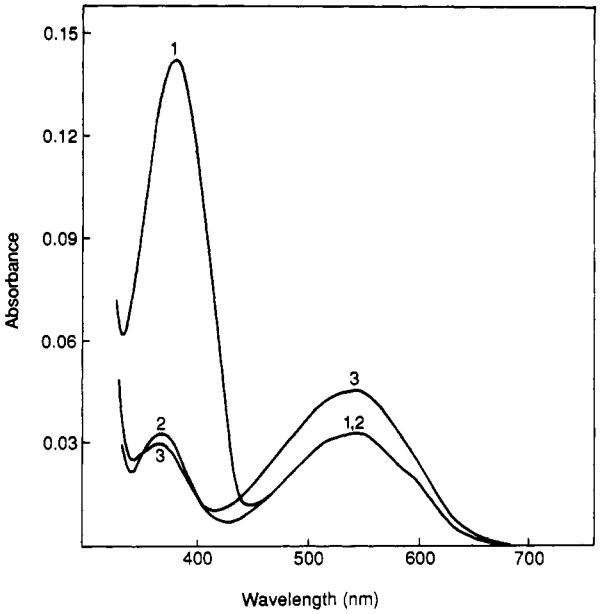

FIGURE 8: Spectral properties of enzyme isolated after reconstitution with 1-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate. Curve 1 is the absorption spectrum of the isolated enzyme (*E*Ptel-deazaFAD) in complete PRE buffer at 5 °C. Curve 2 was recorded after reaction with 20.8 mM sodium borohydride at 5 °C. Curve 3 was recorded 2 h after denaturation of borohydride-treated enzyme with 0.08% SDS at 25 °C.

any 1-deazaFADH$_2$. *E*Ptel-deazaFAD$_{ox}$ exhibited an extinction coefficient at 540 nm ($\epsilon_{540} = 7.5 \times 10^3$ M^{-1} cm^{-1}) similar to that observed for *E*1-deazaFAD$_{ox}$. The results indicate that the low extinction coefficient at 540 nm observed for enzyme reconstituted with 1-deazaFADH$_2$ plus 5,10-CH$^+$-H$_4$folate is attributable to the presence of 1-deazaFADH$_2$ in the isolated enzyme and suggest that the stability of bound 1-deazaFADH$_2$, but not 1-deazaFADH$^{\bullet}$, is enhanced by the pterin chromophore.

The differences observed for enzyme-bound 1-deazaFAD versus FAD prompted studies to determine whether enzyme stability was affected by the modified flavin. These studies were conducted with *E*Ptel-deazaFAD since the presence of 1-deazaFADH$_2$ in the preparation meant that release of 1-deazaFAD would be accompanied by a substantial increase in absorbance at 540 nm (see Figure 8) whereas pterin release would be detectable by a loss of absorbance at 380 nm (Jorns et al., 1984). In these studies it was found that *E*Ptel-deazaFAD was stable in complete PRE buffer, but chromophore release was observed when the glycerol concentration in the buffer was reduced from 50% to 8%. 1-DeazaFAD release was faster and complete in 50 min whereas about 25% of the pterin was released during the same period (data not shown). In contrast, the pterin and flavin chromophores in native enzyme are stable under the same conditions. The results indicate the stability of photolyase is decreased when FAD is replaced with 1-deazaFAD.

Reaction of *E*1-deazaFAD$_{ox}$ or *E*Ptel-deazaFAD$_{ox}$ with excess dithionite resulted in reduction of 1-deazaFAD$_{ox}$, as evidenced by the loss of its absorption band at 540 nm and the loss of a weak fluorescence emission band at 670 nm (excitation $\lambda_{max} = 540$ nm) attributable to the oxidized chromophore (data not shown). The untreated preparations exhibited no catalytic activity, but dimer repair was detectable if the enzymes were reduced with dithionite just prior to assay. The rates observed with *E*1-deazaFADH$_2$ and *E*Ptel-deazaFADH$_2$ were low as compared with that of native enzyme (11% and 16%, respectively) even though reaction rates were

measured in assay buffer containing 50% glycerol in order to improve the stability of the 1-deazaFAD-containing enzymes.

Discussion

Photolyase containing 1-deazaFAD or 5-deazaFAD as its only chromophore or enzyme containing modified flavin plus 5,10-CH^+-H_4folate was prepared in reconstitution studies with apoenzyme. 5-DeazaFAD bound to a hydrophobic site in apophotolyase and formed a stable reconstituted enzyme, similar to that observed with FAD. Although stoichiometric incorporation was observed, the flavin ring modification in 1-deazaFAD interfered with normal binding, decreased protein stability, and prevented formation of a stable flavin radical, unlike that observed with FAD. The results suggest that an important hydrogen bond is formed between the protein and N(1) in FAD, but not N(5), and that there is sufficient space at the normal flavin binding site near N(5) to accommodate an additional hydrogen but not near N(1). A marked difference in binding ability of 5-deaza- versus 1-deazaflavin has previously been observed in studies with flavocytochrome b_2 where it was found that FMN-free enzyme would readily bind 5-deazaFMN but not 1-deazaFMN (Pompon & Lederer, 1979).

Catalytic activity was observed with enzyme containing 5-deaza$FADH_2$ (42% of native enzyme) or 1-deaza$FADH_2$ (11% of native enzyme) as its only chromophore, but no activity was observed with the corresponding oxidized flavins, similar to that observed with FAD. Previous model studies (Jorns, 1987b) on the photosensitized cleavage of thymine dimers in aqueous solution showed that 1-deazaflavin could act as a sensitizer only in the fully reduced state, similar to photolyase, but 5-deazaflavin was active only in the oxidized state, behavior opposite to that observed with the enzyme-bound chromophore. The results suggest that the protein environment in photolyase selectively enhanced the photochemical reactivity of 5-deazaflavin in the fully reduced state. The requirement of fully reduced flavin for photolyase activity is consistent with a proposed catalytic mechanism (Jordan & Jorns, 1988) where electron donation from excited reduced flavin to substrate generates an unstable dimer radical anion which might spontaneously monomerize. In this regard, it relevant to note that photoejection of an electron from excited fully reduced FMN has been observed in recent model studies (Lindqvist, 1990). On the other hand, it is difficult to account for the fully reduced flavin requirement if catalysis by photolyase involves abstraction of an electron by excited fully reduced flavin from substrate to generate an unstable dimer radical cation, as postulated by Witmer et al. (1989), since photochemical electron abstraction reactions are readily observed with excited oxidized flavins (Hemmerich, 1976) and substrate binding to photolyase is unaffected when $FADH_2$ is replaced by FAD_{ox} (Jorns et al., 1990).

In addition to fluorescence, phosphorescence was observed with free or photolyase-bound 5-deaza$FADH_2$, providing the first example of a flavin which exhibits phosphorescence in the fully reduced state. Formation of an enzyme–substrate complex was accompanied by a nearly identical extent of quenching of 5-deaza$FADH_2$ phosphorescence (85.1%) and fluorescence (87.5%). The observed luminescence quenching is consistent with a catalytic mechanism involving exclusive reaction of substrate with the excited singlet state of 5-deaza$FADH_2$. In this case, formation of an enzyme–substrate complex is expected to decrease the fluorescence lifetime of 5-deaza$FADH_2$. This will cause an equivalent decrease in fluorescence and phosphorescence intensity because fluorescence and phosphorescence quantum yields are directly proportional to the fluorescence lifetime. Alternatively, it may be argued that 5-deaza$FADH_2$ phosphorescence is quenched because the triplet state acts as a catalytic intermediate whereas 5-deaza$FADH_2$ fluorescence is quenched because a substrate-induced conformational change enhances radiationless deactivation of the singlet state. However, in this case it would appear rather fortuitous that the same extent of quenching is observed for both 5-deaza$FADH_2$ fluorescence and phosphorescence. Also, previous studies show that formation of a catalytically inactive enzyme–substrate complex with $E$$FAD_{ox}$ has no effect on FAD_{ox} fluorescence whereas formation of a catalytically competent complex with $E$$FADH_2$ causes quenching of $FADH_2$ fluorescence (Jorns et al., 1990).

The first direct evidence of singlet–singlet energy transfer between chromophores at the active site of photolyase was obtained with enzyme containing 5-deaza$FADH_2$ plus 5,10-CH^+-H_4folate. Energy transfer from 5-deaza$FADH_2$ to pterin was classically demonstrated by the fact that pterin fluorescence was observed upon excitation of 5-deaza$FADH_2$, accompanied by a decrease in 5-deaza$FADH_2$ fluorescence. On the other hand, pterin fluorescence was quenched when 5-deaza$FADH_2$ was replaced with 5-deazaFAD_{ox}, consistent with energy transfer in the opposite direction from pterin to 5-deazaFAD_{ox}. In each case, the spectral properties of the chromophores were consistent with the observed direction of energy transfer and indicated that transfer in the opposite direction was energetically unlikely. Unlike 5-deazaFAD, energy transfer from pterin to FAD is energetically feasible with $FADH_2$ or FAD_{ox}, and quenching of pterin fluorescence is observed with FAD in either redox state (Jorns et al., 1990). The results indicate that the direction of pterin–flavin energy transfer at the active site of photolyase can be manipulated by changes in the flavin ring or redox state which alter the energy level of the flavin singlet. It has been proposed that the pterin chromophore in native enzyme acts as an antenna to harvest light energy which is transferred to the $FADH_2$ reaction center where dimer repair can also be initiated via direct absorption of light by $FADH_2$ (Jorns et al., 1990). Since energy is transferred in the wrong direction, pterin is not likely to act as an antenna when $FADH_2$ is replaced by 5-deaza-$FADH_2$ and is actually expected to decrease the quantum efficiency of dimer repair at the 5-deaza$FADH_2$ reaction center. That pterin incorporation did not inhibit activity with 5-deaza$FADH_2$-containing enzyme suggests that the light-harvesting step may not be rate determining under standard assay conditions. Studies to evaluate these predictions are in progress and will be reported elsewhere.

While the manuscript was in preparation, Payne et al. (1990) briefly reported that reconstitution of apophotolyase with 5-deazaFAD_{ox} yielded inactive enzyme whereas reconstitution attempts with 1-deazaFAD_{ox} were unsuccessful.

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Registry No. FAD, 146-14-5; 5-deazaFAD, 57818-88-9; 1-deazaFAD, 64183-67-1; 1-deazaFADH, 104466-00-4; 5-deazaFADH, 58534-71-7; 5,10-CH^+-H_4folate, 10360-12-0; DNA photolyase, 37290-70-3.

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^{1}H NMR Study of the Base-Pairing Reactions of d(GGAATTCC): Salt Effects on the Equilibria and Kinetics of Strand Association†

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ABSTRACT: Previously, we examined the imino proton relaxation of d(GGAATTCC) in order to characterize salt and polyamine effects on the base-pair opening kinetics of this oligonucleotide [Braunlin, W. H., & Bloomfield, V. A. (1988) *Biochemistry 27*, 1184-1191]. Here, we report salt-dependent measurements of the NMR behavior of the nonexchangeable base proton resonances of d(GGAATTCC). From chemical shift measurements, we find an unexpectedly large salt dependence of K_a, the equilibrium constant for helix association. A total of 1.8 ± 0.3 sodium ions are thermodynamically released upon dissociation of the octamer duplex. Most of the salt dependence of the equilibrium constant can be traced to a large salt dependence of the association rate. Thus, 1.4 ± 0.2 sodium ions associate during the rate-limiting step of helix association. In agreement with our previous imino proton results, we also find a significant salt dependence of the duplex dissociation rate. Activation energies for helix association are very small, and possibly negative; most of the temperature dependence of the association equilibrium can be traced to a large activation energy (~50 kcal/mol) for duplex dissociation.

Convenient access to milligram quantities of highly pure, synthetic oligonucleotides now offers unprecedented opportunities for the biophysical chemist concerned with understanding the details of sequence-dependent effects on DNA structural transitions and ligand-binding interactions. However, some caution must be exercised; oligomeric DNAs do not appear to be ideal models for some aspects of the behavior of polymeric DNA. For example, a large difference has been predicted in the ion-binding behavior of oligomeric and

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