Chromophore Function and Interaction in *Escherichia coli* DNA Photolyase: Reconstitution of the Apoenzyme with Pterin and/or Flavin Derivatives†

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ABSTRACT: Native DNA photolyase, as isolated from *Escherichia coli*, contains a neutral flavin radical ($FADH^{\bullet}$) plus a pterin chromophore (5,10-methenyltetrahydropteroylpolyglutamate) and can be converted to its physiologically significant form by reduction of $FADH^{\bullet}$ to fully reduced flavin ($FADH_2$) with dithionite or by photoreduction. Either $FADH_2$ or the pterin chromophore in dithionite-reduced native enzyme can function as a sensitizer in catalysis. Various enzyme forms ($EFAD_{ox}$, $EFADH^{\bullet}$, $EFADH_2$, $EPteFAD_{ox}$, $EPteFADH^{\bullet}$, $EPteFADH_2$, EPte) containing stoichiometric amounts of FAD in either of its three oxidation states and/or 5,10-methenyltetrahydrofolate (Pte) have been prepared in reconstitution experiments. Studies with $EFAD_{ox}$ and EPte showed that these preparations retained the ability to bind the missing chromophore. The results suggest that there could be considerable flexibility in the biological assembly of holoenzyme since the order of binding of the enzyme's chromophores is apparently unimportant, the binding of FAD is unaffected by its redox state, and enzyme preparations containing only one chromophore are reasonably stable. The same catalytic properties are observed with dithionite-reduced native enzyme or $EFADH_2$. These preparations do not exhibit a lag in catalytic assays whereas lags are observed with preparations containing FAD_{ox} or $FADH^{\bullet}$ in the presence or absence of pterin. Photochemical studies show that these lags can be attributed to enzyme activation under assay conditions in a reaction involving photoreduction of enzyme-bound FAD_{ox} or $FADH^{\bullet}$ to $FADH_2$. EPte is catalytically inactive, but catalytic activity is restored upon reconstitution of EPte with FAD_{ox}. The results show that pterin is not required for dimer repair when $FADH_2$ acts as the sensitizer but that $FADH_2$ is required when dimer repair is initiated by excitation of the pterin chromophore. The relative intensity of pterin fluorescence in EPte, $EPteFADH^{\bullet}$, $EPteFAD_{ox}$, or $EPteFADH_2$ has been used to estimate the efficiency of pterin singlet quenching by $FADH^{\bullet}$ (93%), FAD_{ox} (90%), or $FADH_2$ (58%). Energy transfer from the excited pterin to flavin is energetically feasible and may account for the observed quenching of pterin fluorescence and also explain why photoreduction of FAD_{ox} or $FADH^{\bullet}$ is accelerated by the pterin chromophore. An irreversible photobleaching of the pterin chromophore is accelerated by $FADH_2$ in a reaction that is accompanied by a transient oxidation of $FADH_2$ to $FADH^{\bullet}$. Both pterin bleaching and $FADH_2$ oxidation are inhibited by substrate.

Exposure of DNA to ultraviolet light results in the formation of cyclobutane dimers between adjacent pyrimidine residues. The presence of pyrimidine dimers in DNA has been associated with mutagenesis, carcinogenesis, and cell death. Pyrimidine dimers can be monomerized in a visible light requiring reaction catalyzed by DNA photolyase. *Escherichia coli* DNA photolyase contains pterin and flavin. The flavin chromophore in the isolated enzyme is present as a blue neutral FAD[1] radical ($FADH^{\bullet}$), but the physiologically significant form of the enzyme in *E. coli* contains fully reduced flavin (1,5-dihydro-FAD, $FADH_2$) (Jorns et al., 1984; Sancar et al., 1987; Payne et al., 1987). The flavin radical in the isolated enzyme can be reduced with dithionite or by photoreduction but is resistant toward oxidation (Jorns et al., 1987a,b). Formation of an enzyme–substrate complex stabilizes $FADH_2$ against air oxidation and also quenches the chromophore's fluorescence in a reaction that is fully reversible upon dimer repair (Jordan & Jorns, 1988). The pterin chromophore in native enzyme is present as 5,10-methenyltetrahydropterolypolyglutamate [5,10-CH^+-H_4Pte(Glu)$_n$] (Wang et al., 1988; Johnson et al., 1988; Wang & Jorns, 1989). The polyglutamate moiety in the native chromophore is not essential for binding. The isolated enzyme is depleted with respect to the pterin chromophore but can bind additional 5,10-CH^+-H_4Pte(Glu)$_n$ or 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) (Wang & Jorns, 1989). Reduction of the pterin chromophore with borohydride yields 5-methyltetrahydropterolypolyglutamate [5-CH_3-H_4Pte(Glu)$_n$] in a reaction that is accompanied by a complete loss of the chromophore's visible absorption and fluorescence (Jorns et al., 1987b; Wang & Jorns, 1989). A similar loss of pterin absorption is observed upon exposure of the enzyme to black light, but the product formed in this photobleaching reaction is not 5-CH_3-H_4Pte(Glu)$_n$ (Jorns et al., 1987b; Hamm-Alvarez et al., 1989).

Studies with the physiologically significant form of *E. coli* photolyase show that either the pterin chromophore or $FADH_2$ can function as a sensitizer in catalysis (Jorns, 1987; Sancar et al., 1987). It has been proposed that the enzyme can repair dimers via a pterin-independent pathway since turnover is not significantly affected by reduction of the pterin chromophore with borohydride (Jorns et al., 1987b). In contrast, preliminary studies have suggested that $FADH_2$ may be required when dimer repair is initiated by excitation of the pterin chromo-

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FAD_{ox}, oxidized FAD; $FADH^{\bullet}$, blue neutral FAD radical; $FADH_2$, 1,5-dihydro-FAD; DTT, dithiothreitol; 5,10-CH^+-H_4Pte(Glu)$_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; Pte, 5,10-CH^+-H_4folate or 5,10-CH^+-H_4Pte(Glu)$_n$; 5-CH_3-H_4Pte(Glu)$_n$, 5-methyltetrahydropteroylpolyglutamate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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phore (Jorns et al., 1987a). Evidence consistent with these proposals has been obtained in reconstitution experiments which have permitted us to prepare enzyme containing FAD in either of its three oxidation states ($FADH_2$, $FADH^•$, FAD_{ox}) in the presence or absence of pterin and also flavin-free enzyme containing 5,10-CH^+-H_4folate as the only chromophore. Comparison of the spectral properties observed for these various enzyme forms shows that energy transfer from the excited pterin singlet to FAD_{ox}, $FADH^•$, or $FADH_2$ is energetically feasible and might account for the observed quenching of pterin fluorescence by the flavin chromophore and also explain why photoreduction of FAD_{ox} or $FADH^•$ is accelerated by the pterin chromophore. The possible catalytic significance of energy transfer is discussed.

Experimental Procedures

Materials. Phenyl-Sepharose CL-4B was purchased from Pharmacia. Oligo$(dT)_{18}$ was obtained from P-L Biochemicals. 5-Formyltetrahydrofolate was purchased from Sigma. Coenzyme F_{420} from a *Methanobacterium* strain was a generous gift from Dr. Ralph S. Wolfe. TpTpTpT was a generous gift from Dr. James Alderfer.

Enzyme Purification and Assay. E. coli photolyase was purified as previously described (Jorns et al., 1987a). Unless otherwise specified, all handling of the enzyme was done under yellow light. Enzyme assays were performed at 21 °C with UV-irradiated oligo$(dT)_{18}$ as substrate, similar to those described by Jorns et al. (1985), except that the assay buffer (50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl, 1.6 mM DTT, and 1 mM EDTA) contained DTT. Substrate was prepared as previously described (Jorns et al., 1985). For some experiments, the flavin in the enzyme ($FADH^•$ or FAD_{ox}) was converted to $FADH_2$ with 5 mM dithionite and assayed immediately. Protein concentration, determined by the Bradford method (Bradford, 1976), was used in calculating specific activity values.

Preparation of Apoenzyme. All steps were done at 0 or 5 °C, according to a procedure similar to that described by Van Berkel et al. (1988), except that buffer conditions were varied to optimize the yield of apophotolyase. An aliquot (900 μL) of native enzyme ($\approx 10^{-4}$ M) in complete PRE buffer (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol) was diluted with 3.0 mL of 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). Solid ammonium sulfate was added to a final concentration of 1.7 M. The sample was applied to a 1.0-mL phenyl-Sepharose CL-4B column equilibrated with buffer A. The enzyme's chromophores were removed by washing the column with buffer B (buffer A adjusted to pH 3.5 and saturated with KBr). The column was brought back to neutral pH by washing with buffer A. The apoenzyme was then eluted with buffer C (100 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA, 10 mM DTT, and 50% ethylene glycol).

Reconstitution Studies. An aliquot (5 mL) of apoenzyme ($\approx 7 \times 10^{-6}$ M) was incubated at 6 °C in buffer C containing $\approx 10^{-4}$ M FAD and/or 5,10-CH^+-H_4folate. 5,10-CH^+-H_4folate was prepared as described by Rabinowitz (1963). In some experiments, excess sodium dithionite (0.03 M) was added to keep the flavin reduced during the incubation. After 20 h, the sample was dialyzed against buffer A and then applied to a phenyl-Sepharose CL-4B column (1.0 mL) equilibrated with buffer A. The column was washed with buffer A. The reconstituted enzyme was eluted with buffer C and then dialyzed against complete PRE buffer.

In some experiments, enzyme was isolated after reconstitution with oxidized FAD and then incubated at 6 °C with a 5-fold excess of coenzyme F_{420} or a 10-fold excess of 5,10-CH^+-H_4folate in complete PRE buffer containing 25% glycerol. After an overnight incubation, the sample (1.2 mL) was diluted with 2 mL of complete PRE buffer without glycerol. Solid ammonium sulfate was added to a final concentration of 1.7 M. The enzyme was applied to a phenyl-Sepharose CL-4B column and reisolated as described above. In other experiments, enzyme was isolated after reconstitution with 5,10-CH^+-H_4folate and then incubated at 6 °C with a 10-fold excess of FAD_{ox} in complete PRE buffer. After 20 h, the glycerol concentration was reduced to 20% by dilution with complete PRE buffer without glycerol, solid ammonium sulfate was added to 1.7 M, and the enzyme was reisolated as described above.

Spectroscopy. Absorption and fluorescence measurements were made with a Perkin-Elmer Lambda 3 spectrophotometer and a Perkin-Elmer Lambda 5 spectrophotofluorometer, respectively. Fluorescence measurements with free 5,10-CH^+-H_4folate, which is unstable at neutral pH, were made immediately after dilution of an acidic stock solution. Reduction of enzyme-bound flavin with dithionite and low-temperature fluorescence and phosphorescence measurements were conducted similar to those previously described (Jorns et al., 1987b). Unless otherwise specified, photoreactions were conducted under anaerobic conditions at 6 °C in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.6 mM DTT, plus 8.3% glycerol using the same light source (two black lights, Sylvania F15T8/BLB, 15 W) used for catalytic assays. In some experiments, the two black lights were replaced by two yellow lights (Westinghouse F15T8/GO, 15 W).

The concentration of enzyme-bound $FADH^•$ was calculated on the basis of its absorbance at 580 nm ($\epsilon_{580} = 4.8 \times 10^3$ M^{-1} cm^{-1}) (Wang & Jorns, 1989). The extinction coefficient of $FADH^•$ at 380 nm ($\epsilon_{380} = 6.0 \times 10^3$ M^{-1} cm^{-1}) was determined based on the absorption spectrum observed for reconstituted enzyme containing only $FADH^•$. Extinction coefficients for enzyme-bound FAD_{ox} ($\epsilon_{443} = 11.2 \times 10^3$ M^{-1} cm^{-1}; $\epsilon_{380} = 11.0 \times 10^3$ M^{-1} cm^{-1}) were determined on the basis of the amount of free FAD ($\epsilon_{450} = 11.3 \times 10^3$ M^{-1} cm^{-1}) released upon denaturation of enzyme reconstituted with FAD_{ox} alone (see Figure 3). The extinction coefficient of enzyme-bound pterin [5,10-CH^+-H_4Pte$(Glu)_n$ or 5,10-CH^+-H_4folate] ($\epsilon_{380} = 25.9 \times 10^3$ M^{-1} cm^{-1}) was determined in studies with native enzyme or enzyme reconstituted with 5,10-CH^+-H_4folate plus FAD_{ox} after correcting the observed absorbance of the enzyme at 380 nm for the contribution due to $FADH^•$ or FAD_{ox}, respectively. In these experiments, the pterin content of the enzyme was determined on the basis of the amount of free pterin [$\epsilon_{360} = 25.1 \times 10^3$ M^{-1} cm^{-1} for free 5,10-CH^+-H_4folate (Rabinowitz, 1963)] released upon denaturation of enzyme, according to the method described by Wang and Jorns (1989). To determine the extent of chromophore incorporation, protein concentration was determined on the basis of the absorbance of the reconstituted enzyme at 280 nm, as described by Wang and Jorns (1989), except that in some experiments absorbance measurements were made with intact rather than denatured enzyme since A_{280} was found to be unaffected by denaturation. (The ratio, $A_{280,\text{intact enzyme}}/A_{280,\text{denatured enzyme}}$ varied from 0.99 to 1.03 in studies with native enzyme or enzyme reconstituted with FAD_{ox} alone, $FADH_2$ alone, or FAD_{ox} plus 5,10-CH^+-H_4folate.)

Results

Effect of Thiols on Catalytic Assays with Native Enzyme.

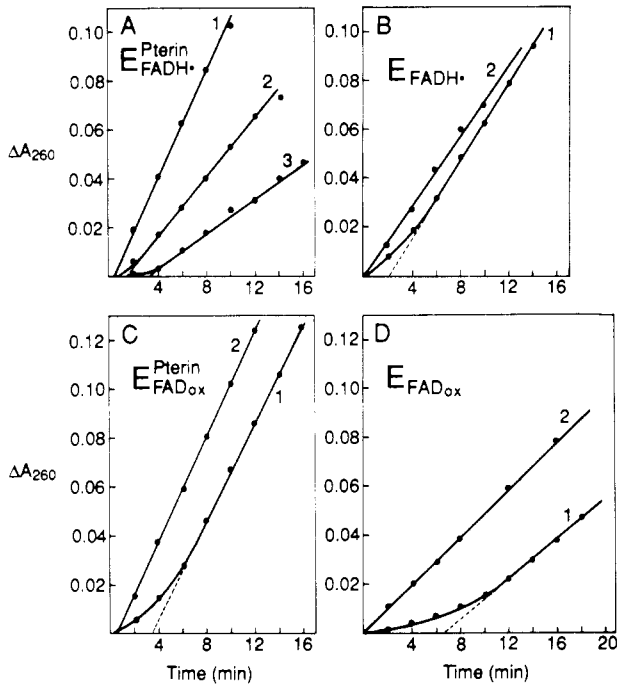

FIGURE 1: Catalytic activity with various photolyase preparations. The increase in absorbance at 260 nm, due to repair of dimers in UV-irradiated oligo(dT)$_{18}$, is plotted versus time of irradiation with photoreactivating light. Unless otherwise noted, assays were conducted under standard conditions (1.6 mM DTT). Data are normalized to the same protein concentration. Panel A: Lines 1–3 were obtained with native enzyme in the presence of 1.6 mM, 40 μM, or 5 μM DTT, respectively. Panel B: Line 1 was obtained with reconstituted enzyme containing only FADH$^{\bullet}$. Line 2 was obtained after reduction of FADH$^{\bullet}$ to FADH$_2$ with dithionite. Panel C: Line 1 was obtained with enzyme reconstituted with FAD$_{ox}$ plus 5,10-CH$^+$-H$_4$folate. Line 2 was obtained after conversion of FAD$_{ox}$ to FADH$^{\bullet}$ by reduction of the reconstituted enzyme with dithionite followed by air oxidation. Panel D: Line 1 was obtained with enzyme reconstituted with FAD$_{ox}$ alone. Line 2 was obtained after reduction of FAD$_{ox}$ to FADH$_2$ with dithionite.

FADH$_2$ in dithionite-reduced native enzyme acts as a sensitizer in catalysis whereas FADH$^{\bullet}$ in the isolated enzyme is photochemically inactive with respect to dimer repair (Jorns, 1987; Sancar et al., 1987). Nevertheless, dimer repair is readily detected in assays initiated with the isolated enzyme. Enzyme activity is typically measured at saturating light intensity in the presence of saturating substrate [UV-irradiated oligo(dT)$_{18}$] by following the increase in the absorbance at 260 nm that accompanies monomerization of thymine dimers (Jorns et al., 1985). The facile photoreduction of FADH$^{\bullet}$, observed with photolyase in the presence of thiols (Heelis & Sancar, 1986), suggested that a photochemical activation of the isolated enzyme might occur under standard assay conditions which include 1.6 mM dithiothreitol (DTT). Under standard conditions a plot of dimer repair versus time is linear. The presence of a lag is difficult to detect even when initial data points are collected at 1-min intervals. However, the existence of a small lag is suggested by the fact that plots of dimer repair versus time do not pass through the origin but rather intersect the x axis at about 0.4 min (Figure 1a, line 1). Although the rate of dimer repair was unaffected, plots that passed through the origin were obtained if the flavin radical was reduced with dithionite just prior to assay or if the DTT concentration in the assay buffer was increased to 17 mM (data not shown). At DTT concentrations lower than 1.6 mM, an initial lag was clearly discernible with untreated native enzyme and the rate observed after the lag decreased as the thiol concentration decreased (Figure 1a, lines 2 and 3). Assays with dithionite-reduced enzyme were unaffected by the DTT concentration in the assay buffer. The results suggest that native enzyme is activated under assay conditions by photoreduction of FADH$^{\bullet}$ to FADH$_2$ in a DTT-dependent reaction.

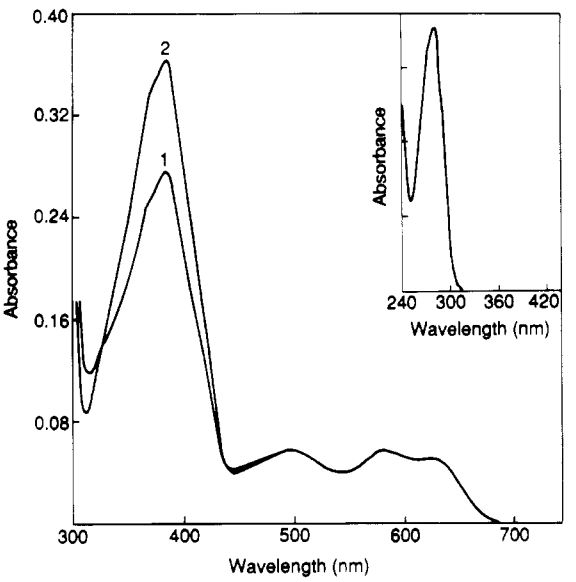

FIGURE 2: Absorption spectra of native enzyme and reconstituted enzyme containing FADH$^{\bullet}$ plus 5,10-CH$^+$-H$_4$folate. Spectra of native (curve 1) and reconstituted (curve 2) enzyme were recorded at 5 °C in complete PRE buffer containing 25% glycerol. The inset shows the absorption spectrum of apoenzyme in 100 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA, 10 mM DTT, and 50% ethylene glycol.

Apoenzyme Preparation. Native photolyase was bound to a small column of phenyl-Sepharose CL-4B at neutral pH in the presence of ammonium sulfate. Flavin and pterin were removed by washing the column with pH 3.5 buffer containing ammonium sulfate plus KBr. The apoprotein was recovered in 85–90% yield when the column was washed with neutral buffer containing 50% ethylene glycol. It exhibited a single absorption maximum at 280 nm (Figure 2, inset) and was catalytically inactive, but could be reactivated by adding back the removed chromophores (vide infra). The procedure used to prepare apoenzyme is similar to a method recently described by Van Berkel et al. (1988) for the reversible dissociation of flavoenzymes into apoprotein plus free flavin. We were unable to prepare reconstitutable apophotolyase using a variety of other published methods for the preparation of apoflavoproteins (e.g., acid ammonium sulfate precipitation, potassium bromide dialysis, treatment with guanidine hydrochloride, etc.). Apophotolyase could be prepared in 70% yield by passing a solution of the enzyme through a DEAE column at pH 3 in the presence of 50% glycerol. However, yields of reconstituted enzyme were poor (≈5%) although the properties of the recovered material were similar to that observed when apoenzyme was prepared by the hydrophobic column chromatography method (Chanderkar and Jorns, unpublished observations).

Reconstitution with FADH$_2$ plus 5,10-CH$^+$-H$_4$folate. The yield of the reconstituted enzyme was 67%, based on the amount of starting apoenzyme. Oxidation of FADH$_2$ to FADH$^{\bullet}$ occurred during isolation of the reconstituted enzyme, similar to that observed during purification of native enzyme (Sancar et al., 1987; Payne et al., 1987). The reconstituted enzyme (EPteFADH$^{\bullet}$) contained nearly equimolar amounts of FADH$^{\bullet}$ and 5,10-CH$^+$-H$_4$folate (Table I). The pterin content of the reconstituted enzyme was significantly greater than that observed for native enzyme which contains about 0.5 mol of pterin/mol of flavin (Table I). Native and re-

Table I: Properties of Various Photolyase Preparations

enzyme preparation	chromophore composition (mol of chromophore/mol of protein)				specific activity (%)	lag in assay (min)
	pterin[a]	FADH•	FAD_{OX}	$FADH_2$		
native enzyme	0.54	0.92			100	<1
reconstituted with						
$FADH_2$ plus pterin	0.84	0.85			104	<1
FAD_{OX} alone						
untreated			0.87		38	12–14
reduced[b]				0.87	47	0
$FADH_2$ alone						
untreated		0.88			70	6
reduced[b]				0.88	93	0
FAD_{OX} plus pterin						
untreated	0.81		0.93		89	6
reduced/reoxid[c]	0.81	0.93			98	<1
pterin alone						
untreated	0.94				0	
plus FAD_{OX}	0.50		0.89		82	6

[a] The pterin in native enzyme is 5,10-CH^+-H_4Pte(Glu)$_n$ whereas apoenzyme was reconstituted with 5,10-CH^+-H_4folate. [b] FAD_{OX} was reduced to $FADH_2$ with dithionite just prior to assay. [c] FAD_{OX} was converted to FADH• by reduction with dithionite, followed by air oxidation.

constituted enzyme exhibited identical visible absorption maxima (Figure 2), but the reconstituted enzyme showed enhanced absorption at 384 nm due to its higher pterin content. The pterin chromophore in both preparations exhibited the same fluorescence excitation (λ_{max} = 391 nm) and emission (λ_{max} = 470 nm) maxima. [FADH• is nonfluorescent (Jorns et al., 1984).] When corrected for its greater pterin content, the intensity of the fluorescence emission observed with the reconstituted enzyme was similar (90%) to that observed with native enzyme. Although the reconstituted enzyme contained more pterin, it exhibited the same specific activity (104%) as native enzyme. The results are consistent with previous studies (Wang & Jorns, 1989) which show that native enzyme is depleted with respect to the pterin chromophore but can bind additional pterin [5,10-CH^+-H_4Pte(Glu)$_n$ or 5,10-CH^+-H_4-folate] to yield enzyme containing equimolar amounts of pterin and flavin without affecting catalytic activity.

Reconstitution with FAD_{ox} Alone. The resistance of FADH• in native enzyme toward oxidation has hindered efforts to evaluate the properties of enzyme containing oxidized FAD. This obstacle is bypassed in reconstitution experiments which yield enzyme containing a nearly stoichiometric amount of FAD_{ox} (Table I). The reconstituted enzyme ($EFAD_{ox}$) exhibited absorption maxima at 378, 443 ($\epsilon_{443} = 11.2 \times 10^3$ M^{-1} cm^{-1}), and 467 nm with pronounced shoulders around 360 and 420 nm (Figure 3, curve 1). This should be contrasted with the two featureless absorption bands observed after treatment with SDS which released the flavin into solution (Figure 3, curve 2). An unresolved absorption band at 450 nm is characteristic of free flavins in hydrophilic solvents. Shoulders are observed in this band around 430 and 480 nm when the solvent polarity is decreased. In extreme cases (e.g., benzene) the shoulder near 480 nm is replaced by a distinct peak at 475 nm (Harbury et al., 1959), similar to that observed for photolyase-bound FAD_{ox}. The results are consistent with a hydrophobic binding site for FAD_{ox} in photolyase. $EFAD_{ox}$ exhibited a fluorescence emission maximum at 521 nm (Figure 4B, curve 1) and excitation maxima at 375 and 450 nm, accompanied by shoulders at 465 (pronounced), 425, and 360 nm (data not shown). Fluorescence intensity was 8-fold less than free FAD_{ox}.

$EFAD_{ox}$ can be reduced with dithionite (data not shown) or photochemically in the presence of DTT (Figure 3, inset). A radical intermediate was not detected in either reaction, but air oxidation of the reduced enzyme ($EFADH_2$) yielded EFADH• in 92% yield (data not shown). [Reduction of

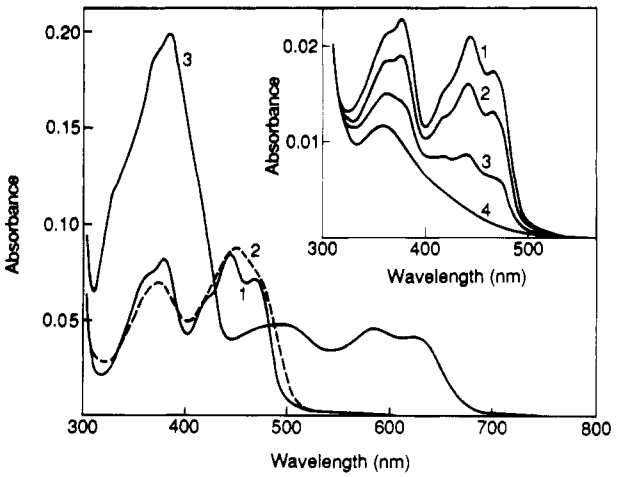

FIGURE 3: Spectral properties and photoreduction of enzyme reconstituted with FAD_{ox}. Curves 1 and 3 are absorption spectra of reconstituted (7.6 μM with respect to FAD_{ox}) and native (9.6 μM with respect to FADH•) enzyme, respectively, at 5 °C in complete PRE buffer containing 25% glycerol. Curve 2 was recorded after denaturing the reconstituted enzyme with 0.063% SDS at 25 °C. The inset shows the anaerobic photoreduction of the reconstituted enzyme observed upon irradiation with black light at 6 °C in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.6 mM DTT, plus 8.3% glycerol. Curves 1–4 were recorded after 0, 4, 12, and 42 min, respectively, of irradiation with same light source used for activity assays.

$EFAD_{ox}$ with excess dithionite was slow (3 h with 5.0 mM dithionite) as compared with the rapid reactions observed with FADH• bound to photolyase (Jorns et al., 1987a) or FAD_{ox} bound to other flavoproteins.] The reduced enzyme exhibited an absorption maximum at 360 nm (Figure 3, inset, curve 4) and a fluorescence emission maximum at 506 nm (Figure 4B, curve 2), similar to that observed for $FADH_2$ bound to native enzyme after eliminating the spectral contribution from its pterin chromophore (Jorns et al., 1987b).

$EFAD_{ox}$ retained the ability to bind substrate. Formation of an enzyme–substrate complex was accompanied by a perturbation of the visible absorption spectrum (Figure 4A, curve 2), but flavin fluorescence was unaffected. In contrast, the fluorescence observed for $EFADH_2$ was quenched by substrate (Figure 4B, curve 3) in a reaction that could be reversed upon exposure of the complex to photoreactivating light.

A pronounced lag (12–14 min) was observed when $EFAD_{ox}$ was assayed under standard conditions (i.e., 1.6 mM DTT) (Figure 1D, line 1). When the DTT concentration in the assay

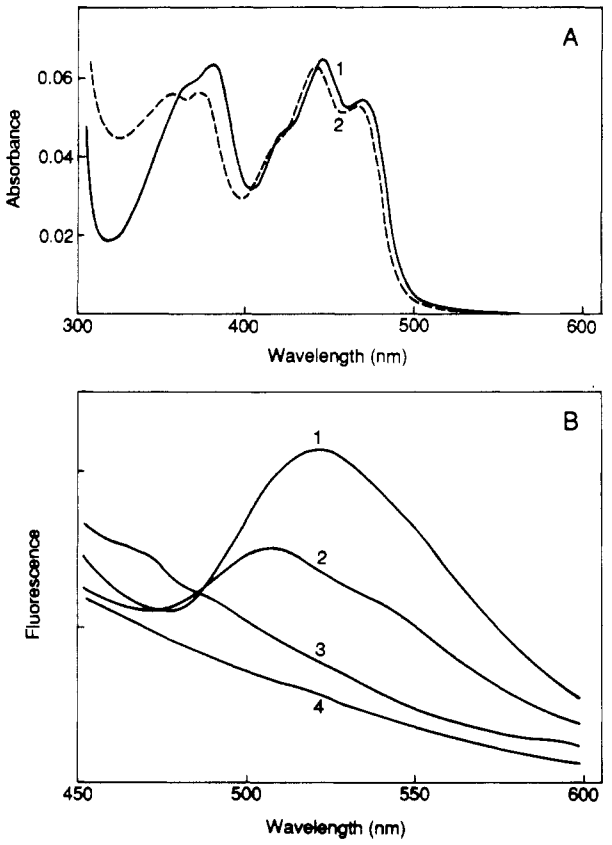

FIGURE 4: Effect of substrate and/or reduction on the absorption and fluorescence properties of enzyme reconstituted with FAD_{ox}. Curve 1 in panel A is the absorption spectrum of the reconstituted enzyme (5.5 μM with respect to FAD_{ox}) at 5 °C in complete PRE buffer containing 25% glycerol. Curve 1 in panel B shows the corresponding fluorescence emission spectrum (excitation λ = 390 nm). The fluorescence emission spectrum was unchanged upon addition of UV-irradiated (3.5 dimers/oligomer) oligo$(dT)_{18}$ (4.2 mol of oligomer/mol of enzyme) (data not shown) whereas curve 2 in panel A shows the effect of substrate on the absorption spectrum. Curve 2 in panel B is the fluorescence emission spectrum recorded after reduction of a separate aliquot of enzyme with excess dithionite (5 mM). Curve 3 was recorded after adding substrate to the reduced enzyme [5.0 mol of UV-oligo$(dT)_{18}$/mol of enzyme]. Curve 4 was recorded for buffer alone.

buffer was decreased to 27 μM, virtually no dimer repair was detected during the first 6 min of the assay and then a slow rate of repair was observed (data not shown). The specific activity observed for $EFAD_{ox}$ under standard assay conditions (estimated from the linear region after the lag) was 38% of that observed with native enzyme, which exhibits almost no lag under these conditions. No lag was detected when $EFAD_{ox}$ was converted to $EFADH_2$ by reduction with dithionite just prior to assay (Figure 1D, line 2). The specific activity observed with $EFADH_2$ was 125% of that observed with $EFAD_{ox}$. The results suggest that $EFAD_{ox}$ is catalytically inactive but can be activated in situ by photoreduction to $EFADH_2$ with DTT acting as the electron donor.

Reconstitution with $FADH_2$ Alone. Oxidation of $FADH_2$ occurred during isolation of the reconstituted enzyme which contained a nearly stoichiometric amount of FADH• (Table I). The reconstituted enzyme (EFADH•) exhibited absorption bands at wavelengths greater than 450 nm, similar to that observed for the flavin radical in native enzyme, plus three additional peaks at shorter wavelengths [λ_{max} = 335, 362, and 382 nm (preparation 2) (Figure 5, curve 1); λ_{max} = 328, 360, and 380 nm (preparation 1)] which are not detectable with native enzyme owing to interference from the pterin chromophore. These peaks can be observed with native enzyme

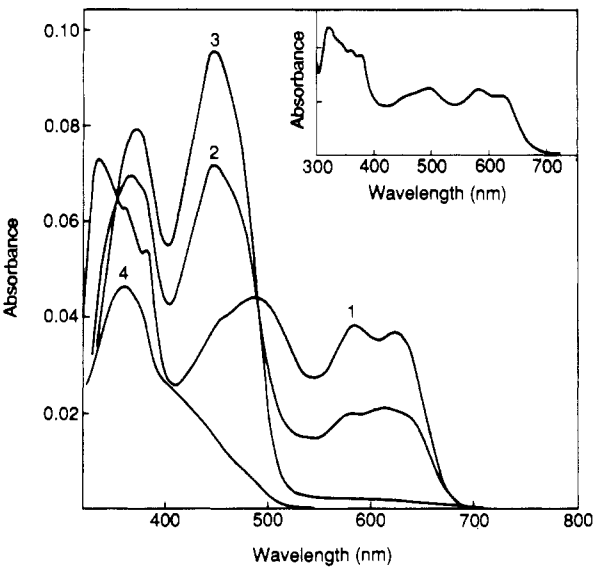

FIGURE 5: Effect of denaturation or reduction on the absorption spectrum of reconstituted enzyme containing FADH•. Curve 1 is the absorption spectrum of the reconstituted enzyme (8.1 μM with respect to FADH•) in complete PRE buffer containing 25% glycerol at 5 °C. The sample was warmed to 25 °C, and curve 2 was recorded immediately after addition of 0.08% SDS. Curve 3 was recorded 40 min later. Curve 4 is the spectrum recorded after photoreduction of a separate aliquot of reconstituted enzyme (8.1 μM with respect to FADH•) with black light in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.6 mM DTT, plus 8.3% glycerol. The inset shows the absorption spectrum of native enzyme in complete PRE buffer after reduction of the pterin chromophore with 67 mM sodium borohydride at 5 °C.

when the absorption of the pterin chromophore is eliminated by reduction with borohydride (Figure 5, inset). The band near 330 nm was not detected in earlier studies with borohydride-reduced native enzyme (Jorns et al., 1987b), possibly because of incomplete reduction of the pterin chromophore.

To our knowledge, a peak near 330 nm has not previously been observed for neutral flavin radicals bound to other flavoproteins which typically exhibit a broad absorption maximum near 350 nm plus additional bands in the 500–700-nm region (Muller et al., 1972). An similar absorption maximum near 350 nm is observed with a protein-free neutral flavin radicals in aqueous solution, but two bands are observed in the 300–400-nm region in less polar solvents. The more intense band exhibits a maximum near 330 nm whereas the position of the weaker band is variable (365–390 nm), depending on solvent polarity (Muller et al., 1972). Several observations suggest that the 330-nm band observed with the reconstituted photolyase preparation is due to FADH•. Firstly, the 330-nm band was lost upon aerobic denaturation of the reconstituted enzyme (8.1 μM with respect to FADH•), accompanied by the release of a stoichiometric amount of FAD_{ox} (8.1 μM) (Figure 5, curve 3). The 330 nm band was also lost upon photoreduction of EFADH•. The photoreduced enzyme ($EFADH_2$) exhibited an absorption maximum at 360 nm (ϵ_{360} = 6.0 × 10^3) (Figure 5, curve 4), similar to that observed after photoreduction of $EFAD_{ox}$ (λ_{max} = 360 nm, ϵ_{360} = 6.1 × 10^3) (Figure 3, inset, curve 4). The results are consistent with a hydrophobic binding site for FADH• in photolyase.

The specific activity of EFADH• was 70% of that observed with native enzyme, but unlike native enzyme, the reconstituted enzyme exhibited a pronounced lag (6 min) under standard assay conditions (Figure 1B, line 1). Reduction of EFADH• with dithionite, just prior to assay, eliminated the lag and increased the specific activity to a value that was 93% of native enzyme (Figure 1B, line 2). The results suggest that photo-

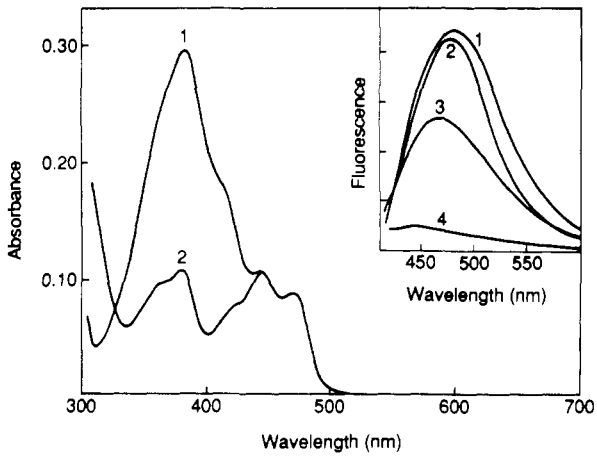

FIGURE 6: Spectral properties of enzyme reconstituted with FAD_{ox} plus 5,10-CH^+-H_4folate. Curve 1 is the absorption spectrum of the reconstituted enzyme in complete PRE buffer containing 25% glycerol at 5 °C. Curve 2 was recorded after reaction with 63 mM borohydride for 30 min. Inset: Curves 1 and 2 show fluorescence emission spectra of the reconstituted enzyme (7.4 μM with respect to 5,10-CH^+-H_4-folate) before and after correction for the contribution from FAD_{ox}, respectively. Curve 3 is the emission spectrum observed with native enzyme (6.6 μM with respect to 5,10-CH^+-H_4folate). Curve 4 was recorded for buffer alone. All spectra were recorded at 5 °C in complete PRE buffer containing 25% glycerol with excitation at 390 nm.

reduction of the flavin radical in EFADH• is slower than in native enzyme.

Reconstitution with FAD_{ox} plus 5,10-CH^+-H_4folate. The reconstituted enzyme (EPteFAD_{ox}) contained nearly stoichiometric amounts of FAD_{ox} and 5,10-CH^+-H_4folate (Table I). EPteFAD_{ox} exhibited absorption maxima at 443 (ϵ_{443} = 11.0 × 10^3) and 467 nm due to FAD_{ox}, plus a more intense band at 382 nm where both chromophores absorb (Figure 6, curve 1). A spectrum due only to FAD_{ox} was observed after reaction of the enzyme with borohydride (Figure 6, curve 2) which eliminated the absorption due to the pterin chromophore, similar to that observed in studies with native enzyme (Jorns et al., 1987b).

The fluorescence properties observed with EPteFAD_{ox} reflect contributions from both chromophores. Excitation spectra, recorded by monitoring emission at 470 or 520 nm, were similar in shape to that obsered for the pterin chromophore in native enzyme or the flavin chromophore in EFAD_{ox}, respectively (data not shown). EPteFAD_{ox} exhibited an emission maximum at 482 nm (Figure 6, inset, curve 1) which shifted to 475 nm after correcting for the contribution from FAD_{ox} (Figure 6, inset, curve 2). The corrected emission maximum was still shifted somewhat as compared with native enzyme (λ_{max} = 470 nm) (Figure 6, inset, curve 3). The intensity of the fluorescence emission due to the pterin chromophore in the reconstituted enzyme was 1.5-fold higher than that observed for native enzyme.

The specific activity of EPteFAD_{ox} was 89% of that observed for native enzyme, but the reconstituted enzyme exhibited an initial lag (6 min) under standard assay conditions (Figure 1C, line 1). The lag observed with EPteFAD_{ox} was 2-fold shorter than that observed with EFAD_{ox}. Reduction of EPteFAD_{ox} with dithionite followed by air oxidation converted EPteFAD_{ox} to EPteFADH• (data not shown). This material exhibited almost no lag, and its specific activity was 98% of that observed with native enzyme (Figure 1C, line 2).

Reconstitution with 5,10-CH^+-H_4folate Alone. The absorption spectrum of the reconstituted enzyme (EPte) exhibited a single band in the visible region at 387 nm (Figure 7A, curve

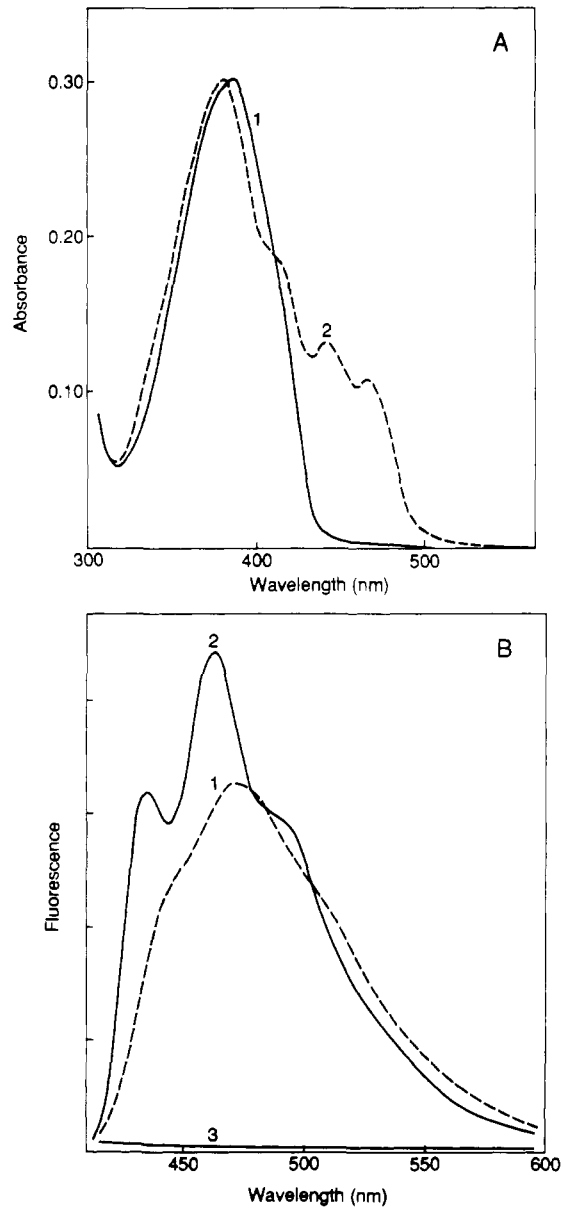

FIGURE 7: Spectral properties of enzyme reconstituted with 5,10-CH^+-H_4folate alone. Panel A: Curve 1 is the absorption spectrum of the reconstituted enzyme in complete PRE buffer at 5 °C. Curve 2 shows the absorption spectrum obtained when the reconstituted enzyme was reisolated after incubation with excess FAD_{ox}. The spectrum was recorded in complete PRE buffer containing 25% glycerol at 5 °C and normalized to the same protein concentration as shown for the original reconstituted enzyme. Panel B: Curves 1 and 2 show fluorescence emission spectra (excitation λ_{max} = 390 nm) of the reconstituted enzyme, as originally isolated, in complete PRE buffer at 5 °C and 77 K, respectively. Curve 1 is shown expanded 2-fold relative to curve 2. Curve 3 was recorded for buffer alone.

1), similar to a spectrum calculated for the pterin chromophore in native enzyme (λ_{max} = 390 nm) (Jorns et al., 1987b). The intensity of the fluorescence due to the pterin chromophore in EPte was 67-fold greater than free 5,10-CH^+-H_4folate at neutral pH, 14.4-fold greater than native enzyme, or 2.4-fold greater than native enzyme after reduction of the flavin radical which is known (Jorns et al., 1987a) to quench pterin fluorescence. At 5 °C EPte exhibited fluorescence emission (λ_{max} = 470 nm) (Figure 7B, curve 1) and excitation (λ_{max} = 390 nm) maxima similar to that observed with native enzyme at this temperature. When the temperature was lowered from 5 °C to 77 K, a 2.7-fold increase in fluorescence intensity was observed, accompanied by a pronounced change in the shape of the fluorescence emission spectrum which exhibited

peaks at 462 and 435 nm plus a pronounced shoulder at 490 nm (Figure 7B, curve 2). The spectrum observed with EPte at 77 K is similar to that attributed to the pterin chromophore in low-temperature studies with native enzyme (Jordan & Jorns, 1988). No phosphorescence was detectable with EPte at 77 K. A complete loss of the visible absorption and fluorescence of the pterin chromophore in EPte was observed upon reduction with borohydride or photobleaching with black light (data not shown), similar to results obtained with native enzyme (Jorns et al., 1987b).

EPte was catalytically inactive. To determine whether this was due to an irreversible denaturation of the protein moiety, the reconstituted enzyme was incubated with excess FAD_{ox} and reisolated. The reisolated enzyme exhibited absorption maxima at 380, 443, and 469 nm (Figure 7A, curve 2) and contained a stoichiometric amount of FAD_{ox} (0.89 mol/mol of protein) but the additional isolation procedure reduced the pterin content to a value (0.50 mol/mol of protein) similar to that observed with native enzyme. The reisolated enzyme was catalytically active. An initial lag (6 min) was observed with the reisolated enzyme, which exhibited a specific activity that was 82% of that observed with native enzyme. The catalytic properties of the reisolated enzyme were virtually identical with those observed when the apoenzyme was reconstituted with 5,10-CH^+-H_4folate plus FAD_{ox} in a single step. The results show that flavin is essential for catalytic activity.

Attempted Reconstitution with Coenzyme F_{420}. A high degree of amino acid sequence homology (40.7% identical residues) has been observed for *E. coli* and *Anacystis nidulans* photolyases, suggesting that these enzymes have very similar structures (Yasui et al., 1988). However, the pterin chromophore in *E. coli* photolyase is replaced by coenzyme F_{420} in the *A. nidulans* enzyme (Eker, 1983). Coenzyme F_{420} is an 8-hydroxy-5-deazaflavin derivative. The substituent at position N(10) in the 5-deazaflavin ring contains a polyglutamate moiety [R = ribityl-phosphate-lactyl-$(Glu)_n$, n = 2–4] (Eker et al., 1980), similar to the polyglutamate moiety found in the natural pterin chromophore in *E. coli* photolyase. These observations prompted studies to determine whether the pterin chromophore in *E. coli* photolyase could be replaced by coenzyme F_{420}. In these experiments, the flavin binding site in the apoenzyme was first blocked with FAD_{ox}. The isolated, FAD_{ox}-reconstituted enzyme was then incubated with excess coenzyme F_{420}. However, no bound coenzyme F_{420} was detected when the enzyme was reisolated. A control experiment was conducted using 5,10-CH^+-H_4folate in place of coenzyme F_{420}. In this case, the reisolated enzyme contained nearly equimolar amounts of 5,10-CH^+-H_4folate (0.70 mol/mol of protein) and FAD_{ox} (0.83 mol/mol of protein). The results show that the pterin site in FAD_{ox}-reconstituted enzyme is available but that coenzyme F_{420} does not bind to this site or else forms a weak complex that does not survive the isolation procedure.

Kinetics of Chromophore Photoreactions in Various Enzyme Preparations. Comparison of the length of the lag observed in assays with various reconstituted enzyme preparations ($EFAD_{ox}$ > $EFADH^{\bullet} \approx EPteFAD_{ox}$ > $EPteFADH^{\bullet}$) (see Figure 1 and Table I) suggested that photoreduction of $FADH^{\bullet}$ might be faster than FAD_{ox} and that both reactions might be accelerated by the pterin chromophore. Evidence to evaluate this hypothesis was sought by measuring the rate of flavin photoreduction under reaction conditions as close as possible to a standard assay. However, photoreactions were conducted under anaerobic conditions at 6 °C, substrate was

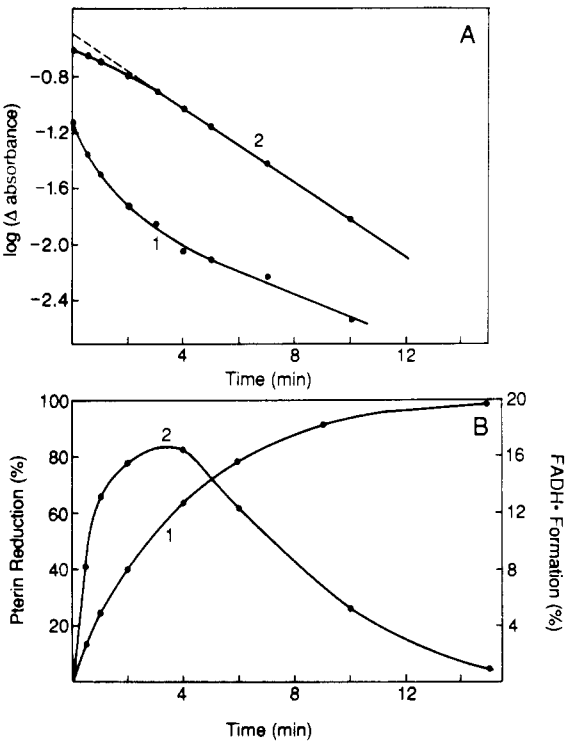

FIGURE 8: Photoreactions with native enzyme. Reactions were conducted under anaerobic conditions at 6 °C in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.6 mM DTT, plus 8.3% glycerol. Panel A: The sample was irradiated with black light. Flavin radical reduction was monitored at 580 nm. Pterin photobleaching was estimated from absorbance changes at 380 nm after correcting for the contribution due to flavin reduction. Lines 1 and 2 show first-order plots of absorbance changes at 580 nm and corrected absorbance changes at 380 nm, respectively. Panel B: The flavin radical was reduced with yellow light, and then the sample was irradiated with black light. Absorbance changes observed during black light irradiation at 380 and 580 nm are plotted in lines 1 and 2, respectively. Absorbance changes at 380 nm were corrected for the contribution due to $FADH_2$ oxidation.

omitted, and the photoreaction buffer included a small amount of residual glycerol (8.3%) from the enzyme storage buffer which had no effect on assays. Anaerobic conditions were chosen, even though assays are conducted aerobically, because enzyme-bound $FADH_2$ is stable against air oxidation in the presence of substrate (Jordan & Jorns, 1988). Photoreactions were measured at 6 °C owing to enzyme instability in the absence of substrate or substantial amounts of glycerol at the normal assay temperature (21 °C). A lag lasting about 1 min was detected when assays were conducted with native enzyme in standard assay buffer at 6 °C, but surprisingly, the rate of dimer repair was unaffected by the decrease in temperature.

Linear first-order plots were observed for photoreduction of $EFADH^{\bullet}$ or $EFAD_{ox}$. The reaction with $EFADH^{\bullet}$ (k = 0.22 min^{-1}) was 2-fold faster than $EFAD_{ox}$ (k = 0.11 min^{-1}), consistent with the 2-fold difference in the length of the lag observed in catalytic assays (Table I). On the basis of initial rates, photoreduction of $FADH^{\bullet}$ in native enzyme (k = 1.1 min^{-1}) was 5-fold faster than $EFADH^{\bullet}$ whereas photoreduction of FAD_{ox} in $EPteFAD_{ox}$ (k = 0.22 min^{-1}) was 2-fold faster than $EFAD_{ox}$. The results are consistent with the proposal that flavin photoreduction is accelerated by the pterin chromophore. However, linear first-order plots were not observed for flavin photoreduction with native enzyme (Figure 8A, line 1) or with $EPteFAD_{ox}$ (data not shown). Both reactions were accompanied by a slower photobleaching of the pterin chromophore. Pterin photobleaching results in a large decrease in absorption at 380 nm ($\Delta\epsilon_{380}$ = 25.9 × 10^3 M^{-1} cm^{-1}), as

compared with smaller decreases due to $FADH_2$ formation from FADH• ($\Delta\epsilon_{380} = 1.1 \times 10^3$ M^{-1} cm^{-1}) or FAD_{ox} ($\Delta\epsilon_{380} = 6.1 \times 10^3$ M^{-1} cm^{-1}). Except for an initial lag, linear first-order plots were obtained for pterin photobleaching with native enzyme ($k = 0.29$ min^{-1}) (Figure 8A, line 2). Qualitatively similar results were obtained with EPteFAD_{ox}, except that pterin photobleaching was slower ($k = 0.077$ min^{-1}) and the initial lag was more pronounced (data not shown).

Although flavin photoreduction in native enzyme or EPteFAD_{ox} is accelerated by the pterin chromophore, this rate acceleration is expected to diminish over the course of the reaction, owing to the accompanying photobleaching of the pterin chromophore. This suggested that linear first-order plots might be observed for flavin photoreduction in these preparations if the effect of the pterin chromophore could be eliminated. To test this hypothesis, FADH• photoreduction in native enzyme was measured under yellow light (500–750 nm, $\lambda_{max} \approx 585$), which excites only FADH•, instead of the normal black light (300–450 nm, $\lambda_{max} \approx 355$ nm), which excites both chromophores. In agreement with the predicted behavior, linear first-order kinetics were observed with yellow light ($k = 0.99$ min^{-1}). [No attempt was made to correct for differences in light intensity or spectral overlap of the light source with the absorption spectrum of the FADH•. However, the efficacy of yellow versus black light mediated FADH• reduction can be estimated by comparing the rate observed for the yellow light photoreduction of FADH• in native enzyme with that observed for black light photoreduction of EFADH• ($k = 0.22$ min^{-1}).]

In a separate experiment, native enzyme was irradiated with yellow light and then exposed to black light to determine whether pterin photobleaching might be affected by prior reduction of FADH• to $FADH_2$. Prior treatment with yellow light eliminated the initial lag in first-order plots but did not otherwise affect the rate of pterin photobleaching ($k = 0.26$ min^{-1}). However, pterin photobleaching was accompanied by an unexpected transient formation of FADH• which reached a maximum level after 3 min of irradiation, corresponding to a 17% reoxidation of $FADH_2$ formed in the yellow light reaction (Figure 8B). The results suggest that when native enzyme is irradiated with black light, without prior exposure to yellow light, the actual initial rate of FADH• photoreduction is probably faster than the observed rate, owing to the competing reoxidation of $FADH_2$ that accompanies pterin photobleaching. Pterin photobleaching is observed with flavin-free enzyme, but the reaction with EPte ($k = 0.050$ min^{-1}) is 5-fold slower than native enzyme. The results indicate that $FADH_2$ is not an obligatory electron donor but that it does accelerate the photobleaching of the pterin chromophore.

The rate of dimer repair observed with EFAD_{ox} was 80% of the rate observed when the flavin was reduced with dithionite just prior to assay (Table I), suggesting that 80% of EFAD_{ox} must be photoreduced to E$FADH_2$ during the initial 12–14-min lag. This is consistent with photochemical studies where 75% of EFAD_{ox} was reduced in 14 min. In the case of EFADH•, the catalytic data suggested that 75% of EFADH• would be photoreduced during the initial 6-min lag (Table I), in good agreement with photochemical experiments where 73% reduction was observed in 6 min. In contrast, discrepancies between photochemical and catalytic studies were found for pterin-containing enzyme preparations. In the case of native enzyme, it was expected that photoreduction of FADH• would be complete in less than 1 min since almost no lag is detected under standard assay conditions and reaction rates are unaffected by reduction with dithionite just prior to assay. However, photochemical studies with native enzyme showed that only 56% reduction of FADH• occurred in 1 min. Similarly, on the basis of catalytic data obtained with EPte-FAD_{ox} (Table I), it was expected that 91% reduction of FAD_{ox} would occur in 6 min whereas only 60% reduction was observed in photochemical studies. The discrepancy between the catalytic and the photochemical data might be explained if photobleaching of the pterin chromophore was prevented by substrate since flavin photoreduction is slower in the absence of the pterin chromophore and is also decreased by the competing reoxidation of $FADH_2$ which accompanies pterin photobleaching. To test this hypothesis, the flavin radical in native enzyme (1.8×10^{-5} M) was photoreduced with yellow light, oligonucleotide substrate (1.1 mM T$\hat{p}$TpT$\hat{p}$T) was added, and then the mixture was irradiated with black light. Formation of FADH• was completely suppressed during the first 4 min of irradiation, and only a small amount of pterin photobleaching (6%) was detected. Substrate was apparently consumed during this period since further irradiation resulted in reaction rates similar to that observed in a control experiment without substrate. When the experiment was repeated with a 2-fold smaller amount of substrate, formation of FADH• was suppressed during the first 2 min of irradiation and pterin photobleaching exhibited a pronounced lag lasting about 1.5 min. The results show that pterin photobleaching and reoxidation of $FADH_2$ are inhibited by substrate.

Discussion

The hydrophobic column chromatography method recently developed by Van Berkel et al. (1988) was easily adapted to give good yields of reconstitutable apophotolyase. Enzyme containing equimolar amounts of pterin and flavin could be prepared by reconstituting the apoprotein with 5,10-CH^+-H_4folate plus FAD_{ox} or $FADH_2$. Stoichiometric flavin or pterin incorporation was also observed upon reconstitution of the apoprotein with flavin (FAD_{ox} or $FADH_2$) in the absence of pterin or with pterin (5,10-CH^+-H_4folate) in the absence of flavin, respectively. Enzyme reconstituted with FAD_{ox} or 5,10-CH^+-H_4folate alone retained the ability to bind the missing chromophore. The results show that the order of binding of the enzyme's chromophores is unimportant, that the binding of the flavin chromophore is unaffected by its redox state, and that it is possible to prepare reasonably stable preparations of reconstituted enzyme that contain only one chromophore. The results suggest that there could be considerable flexibility in the biological assembly of holoenzyme and that neither the pterin nor the flavin chromophore has a major structural and/or stabilizing function in native enzyme. The pterin chromophore in *E. coli* photolyase is replaced by coenzyme F_{420} in photolyase from *A. nidulans*. Although these enzymes show a high degree of amino acid sequence homology (Yasui et al., 1988), an attempt to bind coenzyme F_{420} to the pterin site in *E. coli* photolyase was unsuccessful.

Pterin-Free Enzyme. Reconstitution experiments have permitted us to prepare pterin-free enzyme containing flavin in either of its three oxidation states. E$FADH_2$ is oxygen-sensitive but can be generated by reduction of EFADH• or EFAD_{ox}. EFADH• is isolated after reconstitution with $FADH_2$ and can also be prepared by reduction of EFAD_{ox} followed by air oxidation. The spectral properties observed for EFADH• or E$FADH_2$ are similar to that observed in studies with native enzyme when the visible absorption of the pterin chromophore was eliminated by borohydride reduction (Jorns et al., 1987b). Absorption spectra observed for EFAD_{ox} or EFADH• exhibit features similar to those observed for the corresponding free flavins in nonpolar solvents, consistent with

a hydrophobic binding site for flavin in photolyase.

$EFADH_2$, prepared via reduction of EFADH• or $EFAD_{ox}$, did not exhibit an initial lag in catalytic assays whereas pronounced lags were observed with EFADH• or $EFAD_{ox}$. Photochemical studies showed that these lags could be attributed to enzyme activation under assay conditions in a reaction involving photoreduction of EFADH• or $EFAD_{ox}$ to $EFADH_2$. That $EFAD_{ox}$ cannot repair dimers unless reduced to $EFADH_2$ is not due to an inability to bind substrate since formation of an $EFAD_{ox}$–substrate complex was readily detected by the perturbation of the enzyme's visible absorption spectrum.

The same turnover rate was observed with native enzyme or with $EFADH_2$ prepared via reduction of EFADH•. A 2-fold slower rate was observed when $EFADH_2$ was prepared via reduction of $EFAD_{ox}$, suggesting that better recovery of enzyme activity is achieved via reconstitution with $FADH_2$ versus FAD_{ox}. The results provide strong support for the proposal that enzyme containing only $FADH_2$ is fully competent catalytically. Formation of an enzyme–substrate complex with $EFADH_2$ quenched the fluorescence of the reduced flavin in a reaction that was fully reversible upon exposure of the complex to photoreactivating light, similar to that observed in studies with dithionite-reduced native enzyme (Jordan & Jorns, 1988). On the other hand, formation of an enzyme–substrate complex with $EFAD_{ox}$ did not affect FAD_{ox} fluorescence. The results suggest that the observed quenching of $FADH_2$ fluorescence is not an "artifact" caused by substrate binding (e.g., a substrate-induced conformational change that affects flavin fluorescence) but is rather an indication that the singlet state of $FADH_2$ ($^1FADH_2$*) functions as an intermediate in catalysis. It has been proposed (Jordan & Jorns, 1988) that $^1FADH_2$* donates an electron to the pyrimidine dimer, generating FADH• plus an unstable dimer radical anion ($T\hat{p}T^{\bullet -}$) which rapidly monomerizes to yield $T^{\bullet -}$ + T. The catalytic cycle is completed by electron return from $T^{\bullet -}$ to FADH•, regenerating $FADH_2$.

Flavin-Free Enzyme. The binding of 5,10-CH^+-H_4folate to apophotolyase causes several changes in the properties of the chromophore. Free 5,10-CH^+-H_4folate is unstable in aqueous solution at neutral pH owing to a ring opening reaction to yield 10-formyltetrahydrofolate (Kay et al., 1960; May et al., 1951). This hydrolytic reaction is suppressed in EPte, similar to that observed for native enzyme. A pronounced bathochromic shift of the absorption maximum of 5,10-CH^+-H_4folate (from 360 to 387 nm) is observed upon binding to apophotolyase, accompanied by a 67-fold increase in chromophore fluorescence. Although borohydride reduction converts either enzyme-bound or free 5,10-CH^+-H_4folate to 5-CH_3-H_4folate (Jorns et al., 1987b; Wang & Jorns, 1989), attempts to observe a comparable photobleaching reaction with free 5,10-CH^+-H_4folate have not been successful (Wang and Jorns, unpublished observations).

No dimer repair was detected with EPte whereas reconstitution of EPte with FAD_{ox} yielded an enzyme preparation, $EPteFAD_{ox}$, with catalytic properties similar to that observed when the apoprotein was reconstituted with pterin plus FAD_{ox} in a single step. In each case, an initial lag was observed in assays with $EPteFAD_{ox}$ which could be eliminated by prior reduction with dithionite to generate $EPteFADH_2$. The results show that flavin is essential for catalytic activity and provide the first direct evidence in support of a proposal (Jorns et al., 1987a) that light absorbed by the pterin chromophore does not result in dimer repair unless $FADH_2$ is also present.

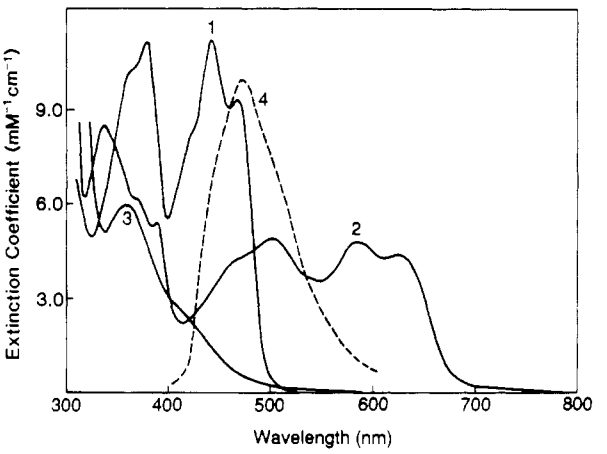

FIGURE 9: Spectral overlap between flavin absorption versus pterin fluorescence as a function of the flavin redox state. Absorption spectra observed for enzyme-bound FAD_{ox}, FADH•, and $FADH_2$ are shown in curves 1–3, respectively. Curve 4 is the emission spectrum of the enzyme-bound pterin chromophore.

Evidence for Pterin–Flavin Interaction. Photobleaching of the pterin chromophore is accelerated by $FADH_2$ in a reaction that is accompanied by a transient oxidation of $FADH_2$ to FADH•. The latter observation suggests that photobleaching may involve abstraction of an electron from $FADH_2$ by the excited pterin. $FADH_2$ is not an obligatory electron donor since pterin photobleaching can occur in flavin-free enzyme, although the rate is 5-fold slower. When $EPteFADH_2$ is irradiated in the presence of substrate, pterin bleaching and oxidation of $FADH_2$ are inhibited until the substrate is consumed. The effect of substrate may be related to the fact that oxidation of ground state $FADH_2$ to FADH• is suppressed upon formation of an enzyme–substrate complex (Jordan & Jorns, 1988) or may reflect formation of a common intermediate during catalysis and photobleaching.

Photoreduction of FADH• or FAD_{ox} is accelerated by the pterin chromophore (5- or 2-fold, respectively), as evidenved by comparison of initial rates of flavin photoreduction in the presence ($EPteFADH^{\bullet}$, $EPteFAD_{ox}$) or absence ($EFADH^{\bullet}$, $EFAD_{ox}$) of pterin. The fluorescence intensity of the pterin chromophore varies depending on the redox state of the flavin. The fluorescence intensity observed with flavin-free enzyme (EPte) was 14.4-fold greater than native enzyme ($EPteFADH^{\bullet}$), 9.6-fold greater than $EPteFAD_{ox}$, and 2.4-fold greater than dithionite-reduced native enzyme ($EPteFADH_2$). Since a similar shape was observed for the pterin fluorescence emission band in these preparations, the relative intensity of pterin fluorescence should be comparable to the relative fluorescence quantum yield. In this case, the data can be used to estimate the efficiency of pterin singlet ($^1Pte^*$) quenching by FADH• (93%), FAD_{ox} (90%), or $FADH_2$ (58%) (efficiency = 1 − $\text{fluorescence}_{EPteFlavin}/\text{fluorescence}_{EPte}$), assuming that the presence of flavin introduces a new path for $^1Pte^*$ deactivation but does not affect the rate of other deactivation reactions (e.g., emission, internal conversion). The observed quenching of pterin fluorescence by flavin and the accelerated rate of flavin photoreduction observed with pterin-containing enzyme might be due to energy transfer ($^1Pte^*$ + flavin → Pte + ^{1}flavin*). Energy transfer, by either a dipole–dipole or an electron exchange mechanism, is unlikely unless the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor (Turro, 1978). Figure 9 compares the fluorescence emission spectrum of EPte (curve 4) with absorption spectra observed for $EFAD_{ox}$ (curve 1), EFADH• (curve 2), and $EFADH_2$ (curve 3). Spectral overlap is observed in each case,

although the extent varies depending on the redox state of the flavin ($EFADH^{\bullet} > EFAD_{ox} > EFADH_2$). The results show that energy transfer from $^1Pte^*$ to flavin is energetically feasible.

Energy transfer from $^1Pte^*$ to $FADH_2$ may be important during catalysis. Previous studies with $EPteFADH_2$ show that dimer repair can be initiated by excitation of the pterin chromophore (Jorns, 1987; Sancar et al., 1987) whereas our current studies show that $FADH_2$ is required for this reaction. Formation of an enzyme–substrate complex with $EPteFADH_2$ affects only the properties of $FADH_2$ (Jordan & Jorns, 1988), suggesting that $FADH_2$ is the chromophore that directly interacts with substrate. The observed quenching of $FADH_2$ fluorescence by substrate suggests that $^1FADH_2^*$ is a catalytic intermediate. Energy transfer from $^1Pte^*$ would generate $^1FADH_2^*$ and provide a mechanism for utilization of light energy harvested by the pterin chromophore. The quantum yield determined for dimer repair with $EPteFADH_2$ ($\Phi = 1$) (Jorns, 1987; Sancar et al., 1987) is higher than the estimated efficiency of $^1Pte^*$ quenching by $FADH_2$ (58%). However, quantum yield calculations were made using an extinction coefficient for $FADH^{\bullet}$ in the isolated enzyme ($\epsilon_{580} = 3.6 \times 10^3\ M^{-1}\ cm^{-1}$) that has since been revised ($\epsilon_{580} = 4.8 \times 10^3\ M^{-1}\ cm^{-1}$) (Wang & Jorns, 1989). A lower value for the quantum yield ($\Phi = 0.75$) is estimated using the new extinction coefficient. The major path for dimer repair in $EPteFADH_2$ is likely to involve the pterin chromophore as sensitizer since the pterin chromophore absorbs most of the photoreactivating light. Pterin can be removed without affecting activity because activity measurements are made at saturating light and the light-harvesting step is not rate-determining under these conditions.

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