DNA photolyase from the cyanobacterium *Anacystis nidulans* contains two chromophores, flavin adenine dinucleotide (FADH$_2$) and 8-hydroxy-5-deazaflavin (8-HDF) (Eker, A. P. M., Kooiman, P., Hessels, J. K. C., and Yasui, A. (1990) *J. Biol. Chem.* 265, 8009–8015). While evidence exists that the flavin chromophore (in FADH$_2$ form) can catalyze photorepair directly and that the 8-HDF chromophore is the major photosensitizer in photoreactivation it was not known whether 8-HDF splits pyrimidine dimer directly or indirectly through energy transfer to FADH$_2$ at the catalytic center. We constructed a plasmid which over-produces the *A. nidulans* photolyase in *Escherichia coli* and purified the enzyme from this organism. Apoenzyme was prepared and enzyme containing stoichiometric amounts of either or both chromophores was reconstituted. The substrate binding and catalytic activities of the apoenzyme (apoE), E-FADH$_2$, E-8-HDF, E-FADH$_{2*}$-8-HDF, and E-FADH$_2$-8-HDF were investigated. We found that FAD is required for substrate binding and catalysis and that 8-HDF is not essential for binding DNA, and participates in catalysis only through energy transfer to FADH$_2$. The quantum yields of energy transfer from 8-HDF to FADH$_2$ and of electron transfer from FADH$_2$ to thymine dimer are near unity.

DNA photolyses (DNA cyclobutane dipyrimidine photolyase, EC 4.1.99.3) repair cyclobutane pyrimidine dimers (Pyr<>Pyr)$^1$ in DNA by utilizing near UV visible light (300–500 nm) as a cofactor. Enzymes from several organisms have been purified and characterized in considerable detail. These studies have revealed that all photolyases identified to date contain FADH$_2$ and either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) as chromophore cofactors (Johnson et al., 1988; Eker et al., 1990). Accordingly, photolyases have been divided into two groups, the folate class and the deazaflavin class (Sancar and Sancar, 1988).

The functions of the two chromophores in the folate class are well-understood (Okamura et al., 1991; Kim et al., 1991): MTHF, which is the major chromophore because of its high extinction coefficient in near-UV ($\epsilon_{260} = 25,000$ M$^{-1}$ cm$^{-1}$), absorbs a photon transfers energy to FADH$_2$ ($\epsilon_{366} = 5600$ M$^{-1}$ cm$^{-1}$) by dipole-dipole interaction (Forster) which in turn transfers an electron to Pyr<>Pyr to initiate splitting of the cyclobutane ring by radical mechanism. The FADH$_2$ itself can be directly excited by absorbing a photon and thus enzyme containing FADH$_2$ alone is catalytically competent (Heclis et al., 1987), while enzyme containing MTHF alone cannot bind substrate (Payne et al., 1990), and enzyme with MTHF plus oxidized flavin (FAD$_{ox}$) binds to Pyr<>Pyr but is catalytically inert (Payne et al., 1990; Jorns et al., 1990). These studies suggested that the sole function of MTHF was to absorb light and transfer energy to FADH$_2$. This was directly demonstrated by time-resolved (picosecond) absorbance spectroscopy which revealed that MTHF excited singlet state decays to yield the first excited singlet state of FADH$_2$ (Kim et al., 1991). Similar high-resolution data regarding the roles of the chromophores in the deazaflavin class is not available.

Yasui and co-workers have cloned the photolyase apoenzyme gene, *phr*, of several deazaflavin class enzymes and expressed the genes in *Escherichia coli* (Yasui et al., 1988; Kobayashi et al., 1989; Takao et al., 1989a, 1989b). Functional complementation was achieved in this host which can provide the FADH$_2$ but not the deazaflavin cofactor. Thus, it could be concluded that FADH$_2$ can catalyze photorepair in the absence of the second chromophore. However, photoreactivation action spectrum *in vitro* of *E. coli* carrying the *Anacystis nidulans* *phr* gene revealed a 380-nm peak (Takao et al., 1989b), suggesting that MTHF might be bound to the 8-HDF-binding site in this heterologous system.

Additional information on the role of the two chromophores of the *A. nidulans* enzyme came from a study of the enzyme purified from its natural host. Eker et al. (1990) found that the purified enzyme contained 8-HDF and flavin in a semi-quinone state (FADH$^\ast$). Conversion of FADH$_2$ to FADH$^\ast$ is an *in vitro* purification artifact (Payne et al., 1987) which occurs with most photolyases (Jorns et al., 1984; Sancar et al., 1987b; Li and Sancar, 1991) and this form of flavin is catalytically inert (Payne et al., 1987; Kim et al., 1992). However, Eker et al. (1990) found that the E-FADH$_2$-8-HDF and the E-FADH$_{2*}$-8-HDF forms of the *A. nidulans* enzyme carried out photorepair with equal efficiencies at 440 nm (the 8-HDF absorption peak), where the absorption of FADH$_2$ is negligible. This observation raised the possibility that 8-HDF participates in photorepair by two mechanisms, energy transfer to FADH$_2$ and electron transfer to or from Pyr<>Pyr (Eker et al., 1990). Earlier work with free flavins in model systems had shown that 8-HDF catalyzed photodissociation of Pyr<>Pyr presumably by abstracting an electron from the photodimer (Eker et al., 1981; Rokita and Walsh, 1984).
To define the roles of the chromophores it is desirable to have enzyme with one or both chromophores and with the flavin chromophore in different redox states. The availability of the cloned A. nidulans gene (Yasui et al., 1988; Mayerl et al., 1990) enabled us to produce sufficient quantities of the enzyme, prepare apoenzyme, reconstitute with one or both chromophores, and thus directly assess the roles of each chromophore in catalysis. Our results show that the E-FADH<sub>2</sub> form of the enzyme is catalytically competent but that 8-HDF cannot catalyze photorepair in the absence of FADH<sub>2</sub> and thus the sole role of the deazaflavin is to function as a photoantenna in a manner analogous to MTHF in the folate class photolyases.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**E. coli CSH303 F<sup>lac</sup> (plh-1 ura4 recA) was the host for cloning of A. nidulans phr gene by complementation, propagation of the overproducing plasmid, and purification of the photolyase. NMS22 and C2326 were the hosts for site-specific mutagenesis by the method of Kunkel (1985) using the pBI125 phagemid (International Biotechnology, Inc.). The source of the A. nidulans phr gene was pUC18-phr(AN) described by Yasui et al. (1988).

**Photolyase Overproducing Plasmid—**A 2.06-kilobase fragment carrying the A. nidulans phr gene was excised from pUC18-phr(AN) by digesting with EcoRI and HindIII and inserted into pBI125. An Ncol site was created at the starting codon of the phr by site-specific mutagenesis (CTG77G—CCA77G). The mutant gene was digested with EcoRI, the termini were "blunted" by treating with Klenow fragment and then digested with Ncol, and the fragment carrying phr was isolated and inserted into pPK235-2 to obtain the overexpression plasmid pUNC1993.

**Materials—**Single-stranded DNA-cellulose, blue-Sepharose, and P-10 resins were purchased from Sigma. Restriction enzymes and T<sub>4</sub> DNA ligase were obtained from Bethesda Research Laboratories. Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Boehringer Mannheim. The following buffers were used in this study. Lysis buffer: 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% sucrose, and 20% saturated ammonium sulfate. Buffer A: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, and 20% (v/v) glycerol. Buffer B: 50 mM Tris-HCl, pH 7.4, 2 mM KCl, 1 mM EDTA, and 20% (v/v) glycerol. Buffer C: 67 mM potassium phosphate, pH 6.8, 1 mM MgCl<sub>2</sub>, and 10% (v/v) glycerol. Storage buffer: 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, and 50% glycerol. Reaction buffer: 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1-10 mM dithiothreitol, 1 mM EDTA.

**Purification of Photolyase—**All purification steps, unless stated otherwise, were carried out at 4 °C and the purification was monitored by SDS-PAGE, and the fractions containing photolyase were loaded onto SDS-PAGE, and the fractions containing photolyase were combined (10 ml) and concentrated in a Centricron C-30 filter to 2 ml (fraction 3).

Fraction 3 was loaded onto a P-100 column (45×1.6 cm) pre-equilibrated with buffer C. The column was developed with 180 ml of the same buffer, 1-ml fractions were collected, fractions containing enzyme were identified by SDS-PAGE, combined, and concentrated by ultrafiltration. The concentrated enzyme was then dialyzed against storage buffer for 6-10 h (fraction 4), aliquoted, frozen in a dry ice-ethanol bath, and stored at -80 °C.

**Preparation of Photolyase with Various Chromophore Complements (apoenzyme, E-FADH<sub>2</sub>, E-FADH<sub>2</sub>, E-8-HDF, and E-FADH<sub>2</sub>-8-HDF)—**A. nidulans photolysase purified from E. coli contains only the flavin cofactor which is quantitatively in the semiquinone form (E-FADH<sub>2</sub>). Photoreduction of the radical to the fully reduced form (E-FADH<sub>2</sub>) was accomplished by irradiation at 580 nm (Heeis et al., 1986).

Apoenzyme was prepared from E-FADH<sub>2</sub> as described previously for E. coli photolyase (Payne et al., 1990; Kim et al., 1991). However, unlike the E. coli enzyme A. nidulans photolyase required extended dialysis to remove FAD. After a 30-day dialysis against 50 mM Tris-HCl, pH 4.0, 100 mM KCl, and 10 mM 2-mercaptoethanol the enzyme still contained 10-15% FAD. This residual flavin was removed by rechromatography. The apoenzyme preparation was dialyzed into a low-salt buffer (50 mM Tris-HCl, pH 4.0, 100 mM KCl) and then purified by chromatography on hydroxylapatite followed by DNA-cellulose (Kim et al., 1991). Following these steps the enzyme contained less than 0.5% FAD as determined by fluorescence spectroscopy.

E-FADH<sub>2</sub> and E-8-HDF were prepared by incubating apoenzyme with the respective chromophores. Typically, apoenzyme (1 eq) and chromophore (2 eq) were incubated in storage buffer on ice for 2 h, then dialyzed against storage buffer overnight and the residual cofactor was removed by passing the sample through a spin column as described previously (Payne and Sancar, 1990). E-FADH<sub>2</sub>-8-HDF was obtained by first reconstituting the enzyme with FAD and then binding with 8-HDF followed by removal of free chromophores by dialysis and spin columns.

The extinction coefficients of E-FADH<sub>2</sub> (ε<sub>280</sub> = 4.5 × 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup>) and E-8-HDF (ε<sub>280</sub> = 5.2 × 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup>) were determined on the basis of the amounts of FAD (ε<sub>280</sub> = 1.15 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup>) and 8-HDF (ε<sub>280</sub> = 3.75 × 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup>) released upon denaturation of the enzyme with 0.7% sodium dodecyl sulfate.

The stoichiometry of FAD to apoenzyme was unity as calculated from the known extinction coefficients of the apoenzyme at 280 nm (ε<sub>280</sub> = 3.75 × 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup>) and the E-FADH<sub>2</sub>-8-HDF (ε<sub>280</sub> = 1.15 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup>) was determined by microanalytical spectrophotometry at 580 nm. The stoichiometry of the 8-HDF was determined as follows: E-FADH<sub>2</sub> was supplemented with 8-HDF and the unbound 8-HDF was removed by dialysis and gel exclusion chromatography to obtain E-FADH<sub>2</sub>-8-HDF. From the absorption at 580 nm the concentration of the enzyme was estimated. This form was then denatured with 0.5% SDS to release FAD<sub>2</sub> (stoichiometric) and 8-HDF. Absorption spectrum of the mixture of FAD<sub>2</sub> plus 8-HDF was obtained from which the ratio of the two chromophores was estimated to be 1:1 based on the extinction coefficients of the two at 438 nm.

**Spectral Measurements—**Single-stranded DNA-20 mer with a centrally located T<sup>C</sup>&gt;T<sup>C</sup>, was prepared by the method of Taylor et al. (1987) as modified by Svoboda (1991) and was a kind gift of Drs. D. Svoboda and J. S. Taylor (Washington University, St. Louis). Oligo-(dT)1<sub>19</sub>, containing T<sup>C</sup>&gt;T<sup>C</sup> was prepared by acetone photosensitization (Kim and Sancar, 1991).

**Assays—**The equilibrium binding constant of photolyase to T<sup>C</sup>&gt;T<sup>C</sup> in 20-mer was determined by the gel retardation method described previously (Husain and Sancar, 1987). The photolytic cross-sections (σ<sub>o</sub>) of repair at 360 and 440 nm for E-FADH<sub>2</sub> and E-FADH<sub>2</sub>-8-HDF were determined as described previously (Husain et al., 1987a; Payne and Sancar, 1990). To obtain the absolute action spectra, photoreactivation with these forms of enzyme was
RESULTS

Overproduction and Purification of A. nidulans Photolyase—
A plasmid which overproduces the A. nidulans photolyase was
constructed by genetic engineering. An NcoI site at the A.
A plasmid which overproduces the A. nidulans photolyase was
were calculated by comparison to the values obtained at the two
reference wavelengths, 360 and 440 nm (Kim and Sancar, 1991).

Photolyase was purified from a 5-liter culture of CSR603
F'IacIq/pUNC1993. Fig. 2 shows an SDS-PAGE analysis of
the purification steps. Cell-free extract (lane 1) was loaded
onto a DNA-cellulose column. A. nidulans photolyase, in contrast to E. coli photolyase (Sancar et al., 1984), bound
ont to this column and was eluted with 0.5 M KCl. At this
stage the enzyme was 60% pure (lane F3) and exhibited a
blue-purple color typical of flavin neutral radical (Jorns et al.,
1984; Li and Sancar, 1991) found in some other photolyases.
The enzyme was purified to >90% with 3.4% overall yield by
applying this fraction to a P-100 gel permeation column. The purification steps are summarized in Table I.

Spectral Properties of A. nidulans Photolyase of Various
Chromophore Compositions and Redox States—The purified
enzyme has a blue color with visible absorption peaks at 480,
588, and 630 nm typical of neutral flavin semiquinone (E-
FADH²; curve 1 in Fig. 3A). Upon illumination with 580 nm
in the presence of dithiothreitol the radical is bleached yielding
in a new form of the enzyme with 355 nm near-UV
absorption peak (curve 2 in Fig. 3A). We interpret this change
to photoreduction of FADH² to generate the E-FADH₂ form of
the enzyme as was previously observed with the E-FADH⁺
form of E. coli photolyase (Heelis et al., 1987). However, the
absorption peak of enzyme bound FADH₂ is 11 nm blue-
shifted in the A. nidulans enzyme compared to the two folate
class photolyases investigated, the E. coli and Salmonella
typhimurium enzymes (Heelis et al., 1987; Li and Sancar,
1991). Whether this difference, which reflects the degree of
nonpolarity in the flavin-binding site, is a common difference
between the folate and deazaflavin class enzymes remains to
be seen. In contrast to the difference in the absorption spectra,
the E-FADH₂ form of A. nidulans enzyme has a fluorescence
emission spectrum indistinguishable from those of E-FADH⁺
forms of the folate class with an emission maximum at 505
nm (Fig. 3B). Perhaps more significantly, neither the E-
FADH⁺ nor the E-FADH₂ forms of the enzyme contained
MTHF as evidenced by the lack of an absorption peak at 384
nm and a fluorescence peak at 465 nm. Repeated attempts to
supplement the E-FADH⁺ form with MTHF (Hamm-Alvarez

<table>
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<th>Yield</th>
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Fig. 3. A, absorption spectra of A. nidulans photolyase expressed in E. coli. The purified photolyase is blue in color with absorption
maxima at 588 and 630 nm (curve 1). The radical form of the enzyme
(E-FADH⁺) was photoreduced (E-FADH₂) by illuminating with 580
nm in the presence of dithiothreitol under anaerobic conditions (curve
2). B, emission spectrum of the E-FADH₂ form of the enzyme. The
excitation wavelength was 355 nm with the bandwidth of 3.0 nm.
et al., 1989) were unsuccessful (data not shown). We conclude that A. nidulans photolyase does not bind MTHF.

To investigate the spectral properties of enzyme containing either flavin in various oxidation states, or 8-HDF, or both, we prepared apoenzyme and reconstituted photolyase with one or both chromophores. Fig. 4A shows the absorption spectrum of our apoenzyme preparation. As apparent from absorption spectrum and from the more sensitive fluorescence emission data (not shown), the preparation contains <1% FADom, the major form of flavin contaminant in apoenzyme preparations. Fig. 4B shows the absorption spectrum of enzyme reconstituted with FAD. As is the case in most other flavoproteins the bound flavin has an absorption spectrum distinct from that of free FADom. While free FAD has peaks at 373 and 445 nm, E-FADom, in addition to these, has peaks at 365 and 445 nm and a shoulder at 420 nm. This spectrum, again, is similar but not identical to that of the E-FADom form of the E. coli photolyase (Payne et al., 1990). This also indicates that although both enzymes contain a hydrophobic flavin-binding pocket resulting in absorption fine structure, there are subtle differences between the two binding sites in the two enzymes, in agreement with the difference observed between the spectra of the E-FADH2 forms of the two enzymes.

The spectrum of the E-8-HDF form of the enzyme is shown in Fig. 4C. As apparent, the absorption maximum (438 nm) of enzyme-bound 8-HDF is red-shifted by 20 nm compared with free 8-HDF, again implying nonpolar interactions with the apoenzyme. Perhaps, from a mechanistic point of view, more significantly the intensity and shape of the 438-nm peak does not change whether the enzyme also contains FAD or not (Fig. 4D). Enzyme containing both chromophores shown in Fig. 4D was obtained by supplementing the native enzyme purified from E. coli with 8-HDF. This procedure yields the E-FADH2-8-HDF form (curve 1) which upon exposure to 580 nm is photoreduced to E-FADH2-8-HDF (curve 2). Note the identical shape and position of the 438-nm peak in Fig. 4, C and D, indicating no electronic interactions between the two chromophores.

Fluorescence of Enzyme-bound 8-HDF—Although deazaflavin class photolyases are weakly fluorescent Eker and co-workers (Eker et al., 1981; 1986; 1988; 1990) have presented strong evidence that this weak fluorescence is due to free 8-HDF or Fo which are invariably present in small quantities in photolyase preparations. Since these enzymes contained both chromophores it was not possible to tell whether the lack of fluorescence was due to nonradiative decay of excited state of enzyme bound 8-HDF or to very efficient energy transfer to nonfluorescent FADH2 or very weakly fluorescent FADH2. The availability of E-8-HDF made it possible to answer this question.

Fig. 5A shows that even though upon binding to the apoenzyme the absorbance of 8-HDF is red-shifted by 20 nm (cf. Fig. 4C) the fluorescence emission spectrum is nearly identical to that of free 8-HDF with a peak at 465 nm (Fig. 5A, curve 1). That is this is not due to unbound 8-HDF present in our preparation is apparent from the fluorescence of E-FADH2-8-HDF (curve 2) and E-FADH2-8-HDF (curve 3) forms of the enzyme. In both forms the deazaflavin fluorescence is quenched >90. However, a measurable fluorescence does remain which is nearly equal to the very weak fluorescence of the enzyme bound FADH2 (Fig. 5B, curve 2). This observation combined with the finding that 440 nm of light photoreduces

![Fig. 4. Absorption spectra of the photolyase reconstituted with FAD and 8-HDF. A, absorption spectrum of apophotolyase; B, E-FADom; C, E-8-HDF; D, E-FADH2-8-HDF. The purified photolyase (1 eq) was incubated at 0 °C with 8-HDF (2 eq) in photolyase storage buffer for 1 h. Unincorporated HDF was removed by dialysis followed by centrifugation through a Penefsky column. Curve 1, absorption spectrum of E-FADH2-8-HDF. Curve 2, absorption spectrum of E-FADH2-8-HDF. The enzyme containing E-FADH2 was photoreduced with 580 nm of light in the presence of dithiothreitol under anaerobic conditions. The chromophores (either or both) were present in stoichiometric amounts with regard to apoenzyme in panels B, C, and D.](image)

![Fig. 5. Effect of flavin (FADH2 or FADH2) on the fluorescence of enzyme bound 8-HDF. A, curve 1, E-8-HDF; curve 2, E-8-HDF-FADH2; curve 3, E-8-HDF-FADH2. B, curve 2 and 3 were expanded 29-fold relative to those in A.](image)
FADH\(^+\) with about the same quantum yield as 580 nm of light (Eker et al., 1990; data not shown) leads us to conclude that singlet excited state 8-HDF transfers energy to the flavin cofactor with a quantum yield close to unity in deazaflavin class photolyases.

**Binding of Photolyase to DNA Substrate**—The equilibrium binding constants of photolyases are 10\(^8\)-10\(^9\) M\(^{-1}\) for the folate class enzymes and 10\(^10\)-10\(^11\) M\(^{-1}\) for the *Methanobacterium thermoautotrophicum* photolyase which is a deazaflavin class enzyme (Kiener et al., 1989; see Sancar, 1992). To find out whether high substrate affinity was a general property of the deazaflavin class and also to investigate the roles of chromophore composition and oxidation state on binding we conducted gel retardation experiments with the 20-mer (T\(^\rightarrow\)T) substrate as described previously. We obtained equilibrium binding constants 1 \times 10^6 M\(^{-1}\) for E-FAD\(_{iso}\), 3 \times 10^6 M\(^{-1}\) for E-FADH\(^+\), 2.4 \times 10^6 M\(^{-1}\) for E-FAD\(_{iso}\)-8-HDF, and 3.1 \times 10^6 M\(^{-1}\) for E-FADH\(^+\)-8-HDF. We ascribe the marginally lower binding affinity of enzyme containing FAD\(_{iso}\) to the fact that these preparations were reconstituted from apoenzyme and may have had a small fraction of partially denatured enzyme. With this caveat we conclude that the oxidation state of FAD and the presence of 8-HDF do not affect binding to substrate. In contrast, the presence of FAD is absolutely required for binding as no substrate binding at K\(_d\) > 10\(^6\) M\(^{-1}\) could be detected with either apoenzyme or E-8-HDF. Thus, as is the case in folate class photolyases (Payne et al., 1990) FAD makes part of the substrate-binding pocket either directly or by inducing a conformational change, while the second chromophore has no effect on binding, and therefore is likely to be more removed from the substrate-binding site compared to FAD.

**Catalysis**—The data presented so far are consistent with 8-HDF functioning as a photoantenna which transfers the light energy by a singlet mechanism to the flavin at the catalytic center which then repairs T\(!\rightarrow\!\)T by electron transfer. This model was tested directly by attempting photorepair with various forms of photolyase and by obtaining absolute action spectra. Fig. 6 shows that E-8-HDF is inactive as expected since this form of the enzyme does not bind DNA substrate. The E-FAD\(_{iso}\) form binds but does not repair, in agreement with the results obtained with the *E. coli* photolyase (Payne et al., 1990). However, long exposure to photoreactivating light in the presence of dithiothreitol causes photoreduction to the E-FADH\(^2\) form and eventual repair following a lag period. Similar results are obtained with the E-FAD\(_{iso}\)-8-HDF form; however, the lag period is shorter in this form because energy transfer from 8-HDF to FADH\(^2\) accelerates the photoreduction reaction and following photoreduction there is still strong absorption at 440 nm in contrast to E-FADH\(_{iso}\) which absorbs only weakly at this wavelength. With the E-FADH\(^2\)-8-HDF form, photoreactivation proceeds without delay and with maximum rate which corresponds to repair with a quantum yield of about unity as is shown below.

The absolute action spectra shown in Fig. 7, combined with the photoreduction data (Eker et al., 1990), fluorescence quenching of 8-HDF by FADH\(^2\), and the rates of repair shown in Fig. 6 clearly define the roles of the two chromophores. To obtain the absolute action spectrum of the E-FADH\(^2\) form, enzyme purified from *E. coli* (E-FADH\(^+\)) was mixed with substrate and the mixture was irradiated with 580 nm of light under an oxygen-free argon atmosphere. This treatment photoreduces flavin but does not cause any repair. The mixture was then irradiated with the indicated wavelengths to obtain the photolytic cross-section (\(\phi\)) which is plotted superimposed on the absorption spectrum (Fig. 7A). As apparent, the data points fall onto the absorption spectrum very closely indicating that within experimental error FADH\(^2\) repairs T\(!\rightarrow\!\)T with a quantum yield of unity.

Fig. 7B shows the results of the same type of experiments conducted with the E-FADH\(^2\)-8-HDF form of the enzyme. Again \(\phi\) values fall onto \(\epsilon\) values over the 350-600 nm range indicating that every photon absorbed by 8-HDF leads to repair of a T\(!\rightarrow\!\)T. These results together with those on energy transfer between the two chromophores leads us to conclude that 8-hydroxy-5-deazaflavin transfers energy to FADH\(^2\) with a quantum yield of unity, and that FADH\(^2\) repairs T\(!\rightarrow\!\)T (most likely by electron transfer) by the same quantum yield with the consequence of overall quantum yield of repair of unity whether the photon is absorbed by the flavin cofactor or the deazaflavin chromophore.

**DISCUSSION**

The pioneering work of Eker and Yasui (Eker et al., 1981; 1986; 1988; 1990; Yasui et al., 1988; Takao et al., 1989a, 1989b) has laid the foundation of biochemical and genetic research on deazaflavin class DNA photolyases. However, the photo-
chemical and photophysical properties of these enzymes are still ill defined. In particular, investigations into the roles of the two chromophores (8-HDF and FADH₂) in the photosensitized splitting of Pyr<>Pyr have suggested that they might catalyze photorepair independently as well as through interacting with one another.

First, upon expression of deazaflavin class phr genes in E. coli (which does not synthesize deazaflavin), functional complementation was achieved providing evidence that catalysis could be carried out by enzyme containing solely FADH₂ cofactor; however, the in vivo photoreactivation action spectra of E. coli strains carrying these genes did not match the predicted absorption spectra of E-FADH₂ raising the possibility that these enzyme contained a nondeazaflavin second chromophore in E. coli (possibly MTHF) which could be responsible for the photoreactivation (Takao et al., 1989b).

Second, Eker et al. (1990) obtained evidence for energy transfer from 8-HDF to flavin as manifested by photoreduction of FADH⁺ upon excitation of 8-HDF. However, no difference in activity could be detected between the E-FADH⁺-8-HDF and E-FADH₂-8-HDF forms of the enzyme leading to the suggestion that splitting of T<>T may occur by direct energy or electron transfer mediated by 8-HDF. Finally, in model reactions it has been shown that free 8-HDF can catalyze the photosplitting of T<>T (Eker et al., 1981), providing independent evidence for the possibility of direct catalysis by 8-HDF in deazaflavin class photolyases.

Therefore, a main goal of the research reported in this paper was to find out whether 8-HDF in deazaflavin class photolyases could repair T<>T directly or, as is the case for MTHF in folate class enzymes, did so indirectly by transferring energy to FADH₂. Our results recapitulated below indicate that the sole function of 8-HDF is to gather light and transfer excitation energy to flavin and that FADH₂ is the catalytic co-factor. Our work and the preliminary report of Eker and Yasui (1991) show that A. nidulans photolyase purified from E. coli contains flavin (FADH⁺) only and does not bind MTHF. We show that the A. nidulans enzyme in the form of E-FADH₂ repairs T<>T with a quantum yield of 100% efficiency. The enzyme containing 8-HDF and flavin binds substrate with equal affinity as E-FADH⁺ alone and the affinity is not influenced by the redox state of flavin. However, the redox state greatly affects the repair rate. The E-FADH₂-8-HDF repair T<>T with a linear rate, while the E-FADH⁺-8-HDF and E-FADH₂-8-HDF forms repair T<>T only after a lag period during which the enzyme is converted into the E-FADH₂-8-HDF form. Thus, the sole role of 8-HDF, like MTHF in the folate class, is to act as a photoantenna.

The following, based on the extensive work conducted by Eker and co-workers (e.g. Eker et al., 1990) and on this reconstitution study, is a likely scenario for the photophysical events during T<>T repair by deazaflavin class photolyases.

The 8-HDF absorbs a photon and transfers energy from excited singlet state to FADH₂ by dipole-dipole interaction (Forster mechanism). The excited singlet state of FADH₂ resulting from energy transfer from deazaflavin, or by direct absorption of a photon, transfers an electron to T<>T generating a dimer radical which collapses to two pyrimidines concomitant with regeneration of FADH₂. The scheme is similar to that of the folate class with the notable exception of much higher efficiency of the deazaflavin class enzymes due to three factors (see Payne and Sancar (1990) and Eker et al. (1990)): greater abundance of λ = 430–450 nm photons compared to λ = 350–400 nm photons in solar spectrum; higher extinction coefficient of deazaflavin compared to folate; and higher efficiency of energy transfer from 8-HDF to FADH₂ compared to that from MTHF to FADH₂. As a consequence organisms containing deazaflavin class enzymes are able to eliminate T<>T by photoreactivation more rapidly than those containing the folate class enzymes. Whether this has any evolutionary significance is beyond the scope of our study.

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REFERENCES