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# Reconstitution of *Escherichia coli* Photolyase with Flavins and Flavin Analogues†

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ABSTRACT: *Escherichia coli* DNA photolyase contains two chromophore cofactors, 1,5-dihydroflavin adenine dinucleotide ($FADH_2$) and (5,10-methenyltetrahydrofolyl)polyglutamate (5,10-MTHF). A procedure was developed for reversible resolution of apophotolyase and its chromophores. To investigate the structures important for the binding of FAD to apophotolyase and of photolyase to DNA, reconstitution experiments with FAD, FMN, riboflavin, 1-deazaFAD, 5-deazaFAD, and $F_{420}$ were attempted. Only FAD and 5-deazaFAD showed high-affinity binding to apophotolyase. The apoenzyme had no affinity to DNA but did regain its specific binding to thymine dimer containing DNA upon binding stoichiometrically to FAD or 5-deazaFAD. Successful reduction of enzyme-bound FAD with dithionite resulted in complete recovery of photocatalytic activity.

DNA photolyases convert light energy into chemical energy to break two bonds of the cyclobutane ring of pyrimidine dimers occurring in UV-irradiated DNA. The enzymes have been classified into two groups according to chromophore composition (Sancar & Sancar, 1987, 1988): the folate class enzymes exemplified by the *Escherichia coli* and *Saccharomyces cerevisiae* photolyases (Johnson et al., 1988) contain 5,10-MTHF and $FADH_2$; the deazaflavin class photolyases include enzymes from *Streptomyces griseus* (Eker et al., 1981), *Scenedesmus acutus* (Eker et al., 1988), and *Methanobacterium thermoautotrophicum* (Kiener et al., 1989), contain an oxidized 8-hydroxy-5-deazaflavin derivative (presumably $F_{420}$), and are thought to have an $FADH_2$ cofactor as well. However, $FADH_2$ has been identified unambigously in the *S. acutus* enzyme only.

The mechanism of photorepair cannot involve direct energy transfer from the chromophores to the cyclobutane ring because photoreactivation occurs in the near-UV and visible region, whereas pyrimidine dimers absorb at $\lambda < 250$ nm. In the absence of spectral overlap direct energy transfer cannot occur. Therefore, repair must be facilitated by a light-dependent redox reaction. However, at present the roles of the individual chromophores are uncertain. The redox reaction may involve the donation or abstraction of an electron to or from the pyrimidine dimer (Witmer et al., 1989). It is aesthetically unattractive but theoretically possible that the two classes of photolyases repair dimers by separate routes, the folate class by electron donation from $FADH_2$ (resulting in reduction of the cyclobutane ring) and the deazaflavin class by electron abstraction by 5-deazaflavin (resulting in oxidation of the cyclobutane ring). Alternatively, the deazaflavin class may catalyze dimer repair by a mechanism analogous to the folate class. In this case, the deazaflavin would act as a light harvester similar to folate transferring energy to the catalytically active chromophore, $FADH_2$.

In this paper we describe a procedure for high-yield preparation of apophotolyase and reconstitution with flavin analogues. We define the enzyme flavin cofactor binding requirements by comparing apoenzyme binding of FAD with that of other flavins and flavin analogues. In addition, specific DNA binding activity of apoenzyme and reconstituted enzyme is examined. Photoreactivation activity of $FAD_{ox}$- and 5-deaza$FAD_{ox}$-reconstituted enzyme and of dithionite-reduced $FAD_{ox}$-reconstituted enzyme is compared with that of active photolyase. The implications for the action mechanisms of

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deazaflavin and folate type photolyases are discussed.

## Materials and Methods

*Materials.* FAD, FMN, and riboflavin were purchased from Sigma Chemical Co. (St. Louis, MO). 5-DeazaFAD, 1-deazaFAD, and $F_{420}$ [the *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate] were prepared as described previously (Hausinger et al., 1986). Optical grade glycerol was purchased from Aldrich Chemical Co.

*Enzyme and Substrate. E. coli* photolyase was purified to apparent homogeneity by ammonium sulfate precipitation followed by chromatography on Blue Sepharose, ACA44 gel filtration, and hydroxylapatite columns (Sancar et al., 1984). The ptac*denV* overproducing strain and procedure for purification of T4 endonuclease V were kindly provided by Dr. Ann Ganesan (Stanford University). The substrate was a synthetic duplex 48-mer which contained a single thymine dimer (Husain et al., 1987, 1988). The duplex was constructed by ligating and hybridizing six oligomers of which the internal ones were radiolabeled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase (BRL). The concentration of the 48-mer duplex was estimated from the specific activity of $^{32}$P.

*Spectral Measurements.* Absorption spectra were recorded with a Hewlett-Packard 8451A diode array spectrophotometer. The fluorescence of the enzyme was quantified with a SLM48000 spectrofluorometer. Activity measurements were performed by using a Quantacount (Photon Technology, Inc.).

*Preparation of Apoenzyme.* The method used to make photolyase apoenzyme is essentially the procedure described by Husain and Massey (1978). Native photolyase at 45.4 μM was dialyzed against 50 mM Tris·HCl, pH 4.0, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 2 M KBr at 4 °C for 5–9 days. The buffer was changed several times over the course of the experiment. The spectrum was monitored every other day, and when no absorption was detectable in the near-UV–visible region (340–700 nm), the apoenzyme was dialyzed into photolyase storage buffer: 50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 50% glycerol. The apoenzyme was kept in this buffer at −20 °C until further use.

*Reconstitution.* Reconstitution experiments were done by incubating the apoenzyme with the indicated flavin on ice for at least 90 h to ensure binding equilibrium. Unbound flavin was removed from protein by passage through a Penefsky column (Hamm-Alvarez et al., 1989; Penefsky, 1977) containing Sephadex G-50 fine resin (Pharmacia LKB Biotechnology, Inc.) equilibrated with photolyase storage buffer.

Reconstitution experiments with FAD, 5-deazaFAD, and 1-deazaFAD contained apoenzyme at 19.1 μM and either FAD at 810 μM, 5-deazaFAD at 406 μM, or 1-deazaFAD at 405 μM. Reconstitution experiments with riboflavin, FMN, and $F_{420}$ contained apoenzyme at 32.9 μM and a greater than 20-fold molar excess of the indicated flavin. The concentration of photolyase apoenzyme was determined by Bradford assay, and the concentrations of the cofactors were based on the literature values for extinction coefficients in the near-UV–visible range. To investigate the affinity of the binding of $F_{420}$ for the 5,10-MTHF binding site of *E. coli* photolyase, the residual 5,10-MTHF was released from the enzyme by treatment with $NaBH_4$ (Hamm-Alvarez et al., 1989). The defolated enzyme (45.4 μM) was then mixed with a greater than 20-fold molar excess of F420.

*Assays.* The binding of photolyase to DNA was measured by the gel retardation assay (Husain et al., 1987). A 50-μL reaction mixture contained 50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 20 mM dithiothreitol, 1 mM EDTA, 100 μg/mL bovine serum albumin, 4% glycerol, 2.46 nM substrate, and the indicated amounts of enzyme. The mixture was incubated in the dark for 20 min and then loaded on a 5% polyacrylamide gel. The gel was electrophoresed for 90 min. Following electrophoresis, the free and enzyme-bound DNAs were located by autoradiography, excised from the gel, and quantified by Cerenkov counting.

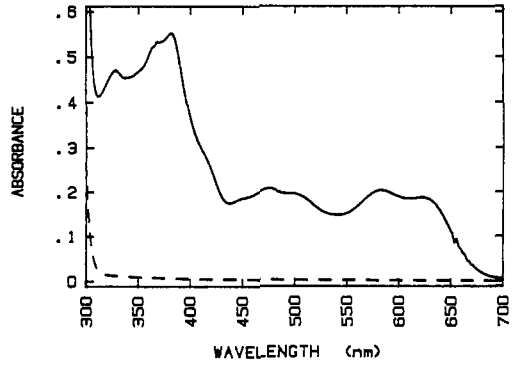


FIGURE 1: Near-UV–visible absorption spectra of photolyase holo- and apoenzyme. The spectra of photolyase at 45.4 μM before dialysis (—) and after dialysis (---) (38.2 μM, 84% recovery) against 50 mM Tris·HCl, pH4, 100 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 2 M KBr are shown. Both the holoenzyme and apoenzyme were in photolyase storage buffer during spectral measurements. The holoenzyme used in this experiment was about 80% depleted in 5,10-MTHF.

The repair of DNA was measured by a coupled enzyme assay: photolyase was incubated with excess substrate, and the mixture was irradiated with 344-nm monochromatic light at an average fluence rate of 192 ergs mm$^{-2}$ s$^{-1}$ for various time intervals. The photoreactivated samples were then treated with >10 units of T4 endonuclease V for 30 min at 37 °C. T4 endonuclease V cuts the DNA strand containing the thymine dimer in half. The decrease in cleavage products as a function of photoreactivation was analyzed by separating the cleaved and intact DNA on a 12% sequencing gel followed by excision of the DNA and quantification by Cerenkov counting.

## Results

*Preparation and Properties of Photolyase Apoenzyme.* The 5,10-methenyltetrahydrofolate (5,10-MTHF) cofactor of *E. coli* photolyase is readily lost from the enzyme during purification such that after a three-column purification procedure only about 25% of the enzyme molecules contain 5,10-MTHF as is estimated by the 385-nm/580-nm absorption ratio of the pure enzyme. The remaining 5,10-MTHF and FAD cofactor are removed by dialysis against 2 M KBr at pH 4. As shown in Figure 1, enzyme-bound 5,10-MTHF and FAD are not detectable by absorption spectrum measurements. The small amount of residual FAD remaining associated with the enzyme was quantified by fluorescence spectroscopy. Samples of holoenzyme and apoenzyme were heat denatured, resulting in the release of FAD from the enzyme. Precipitated protein was removed by centrifugation. FAD fluorescence measurements were then conducted on the supernatant (excitation 370 nm, emission 535 nm). It was found that the apoenzyme preparation shown in Figure 1 contained 3.3% of the starting photolyase holoenzyme FAD content.

In contrast to other methods of apoenzyme preparation, the procedure used to make apophotolyase is considered to be relatively mild, relying on a decreased affinity of flavin for apoprotein in the presence of high concentrations of KBr (Husain & Massey, 1978). Protein loss was routinely found

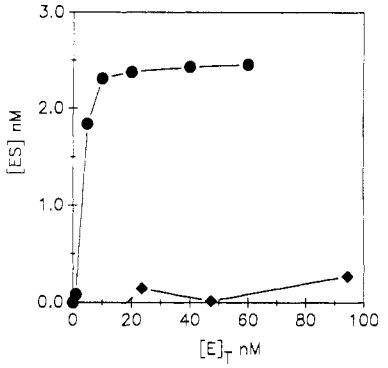


FIGURE 2: Substrate binding properties of photolyase apoenzyme (◆) and holoenzyme (●). Reaction mixtures containing 50 μL of photolyase reaction buffer, 2.46 mM of 48-mer substrate (internally labeled), and the indicated amounts of enzyme were incubated at 23 °C for 30 min, and then the enzyme-bound and unbound fractions were separated by gel retardation and quantified.

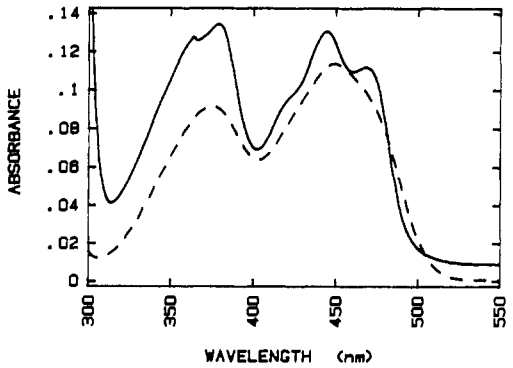


FIGURE 3: Reconstitution of photolyase with FAD. Apophotolyase at 19.1 μM was incubated at 0 °C with 810 μM of FAD in 300 μL of photolyase storage buffer for 91 h. Unincorporated FAD was removed by centrifugation through a Penefsky column. The spectrum of photolyase (—) is superimposed onto that of FAD (- - -) at approximately the same concentration for comparison.

to be no greater than 15% in the preparation of apophotolyase. Thus, it seemed likely that after removal of KBr, apophotolyase would be in a conformation very close to active state. Therefore, we wished to determine whether apophotolyase retained substrate binding activity. We found that the apoenzyme had no DNA binding activity within the detection limit of our assay (Figure 2). It must be pointed out, however, that because of the 3.3% holoenzyme contamination in our apoenzyme preparation, we would have been unable to detect a substrate affinity of less than $^1/_{30}$ of that of the holoenzyme. This reservation notwithstanding, we conclude that apoenzyme cannot bind to thymine dimer containing DNA substrate and, therefore, the flavin may help create the high-affinity DNA binding site.

*Reconstitution of Photolyase with FAD and 5-DeazaFAD.* Photolyase apoenzyme readily bound FAD and 5-deazaFAD. By use of the Bradford assay to measure protein content and the published extinction coefficients of unbound chromophore to estimate the enzyme bound chromophore content, the binding of flavin to apophotolyase appears to be approximately stoichiometric (14.3 μM FAD/12.0 μM apoenzyme and 21.6 μM 5-deaza FAD/19.2 μM apoenzyme). The spectra of the reconstituted enzymes and the spectra of the corresponding free chromophores are shown in Figures 3 and 4, respectively. It is apparent that the absorption spectra of enzyme-bound cofactors have more "structure" than those of free flavins. Thus, while free $FAD_{ox}$ has a smooth absorption spectrum with near-UV peaks at 373 and 445 nm, the enzyme-bound $FAD_{ox}$ has additional peaks at 365 and 468 nm and a well-resolved

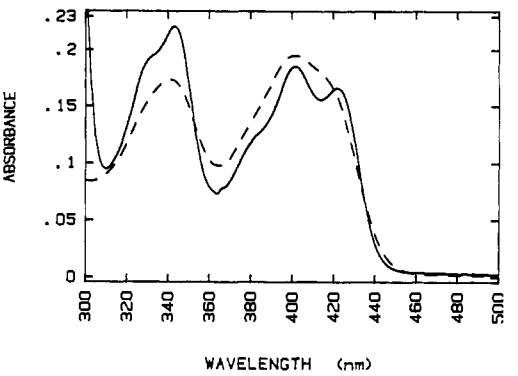


FIGURE 4: Reconstitution of photolyase with 5-deazaFAD. Photolyase apoenzyme at 19.1 μM was incubated with a 21-fold molar excess of 5-deazaFAD. Following removal of excess cofactor, the absorption spectrum of the reconstituted enzyme (—) was recorded and compared to that of free 5-deazaFAD (- - -).

shoulder at 430 nm. In addition, the relative heights of the 377- and 445-nm peaks are reversed by comparison with the free $FAD_{ox}$ spectrum (Figure 3). Similarly, the absorption spectrum of 5-deazaFAD undergoes significant change upon binding to apophotolyase. The free 5-deazaFAD has a smooth near-UV spectrum with peaks at 342 and 402 nm, while the bound form has a well-defined shoulder at 330 nm and a new peak at 422 nm in addition to the two peaks at 342 and 402 nm (Figure 4). These results indicate that the binding of either flavin to apophotolyase occurs in a nonpolar environment as is the case with yeast glutathione reductase [see Ghisla and Massey (1986) and Jacobson and Walsh (1984)].

*Substrate Binding of Reconstituted Photolyase.* Even though apophotolyase has no measurable affinity for DNA, enzyme reconstituted with either FAD or 5-deazaFAD binds specifically and with high affinity to the DNA substrate, in this case a 48-mer duplex containing a thymine–thymine dimer. Figure 5A shows the binding isotherms for photolyase holoenzyme as well as the reconstituted forms, and in parts B and C of Figure 5 are the Scatchard plots for the reconstituted enzymes. Binding constants of $3.6 \times 10^8$ and $1.1 \times 10^9$ $M^{-1}$ are obtained for enzyme reconstituted with FAD and 5-deazaFAD, respectively. These values are not significantly different from the values of $1-10 \times 10^8$ $M^{-1}$ published for holoenzyme containing a full complement of FAD in radical form and 30–50% of 5,10-MTHF (Sancar et al., 1987; Husain & Sancar, 1987). These results indicate that 5,10-MTHF depleted enzyme binds to substrate with the same affinity as holoenzyme. The recovery of DNA binding activity of apophotolyase reconstituted with FAD is further indication that this cofactor is structurally important in forming the DNA binding site and may, in fact, be in proximity to or in physical contact with the thymine dimer. The fact that the 5-deazaFAD is able to mimic FAD in both binding to apophotolyase and reforming the DNA binding site indicates that the $N_5$ is not required for either function. In contrast to FAD and its 5-deaza analogue, enzyme reconstituted with 5,10-MTHF has no specific affinity to thymine dimer containing DNA, suggesting that this chromophore does not participate in making the DNA binding pocket of photolyase (unpublished observation).

*Catalytic Properties of Reconstituted Enzyme.* The physiological form of *E. coli* photolyase is thought to contain the electron-rich, fully reduced FAD ($FADH_2$), suggesting a mechanism of electron donation to the dimer. It is, therefore, of no surprise that enzyme reconstituted with $FAD_{ox}$ shows no detectable activity. However, when the same enzyme is treated with dithionite to reduce the $FAD_{ox}$ to $FADH_2$ and

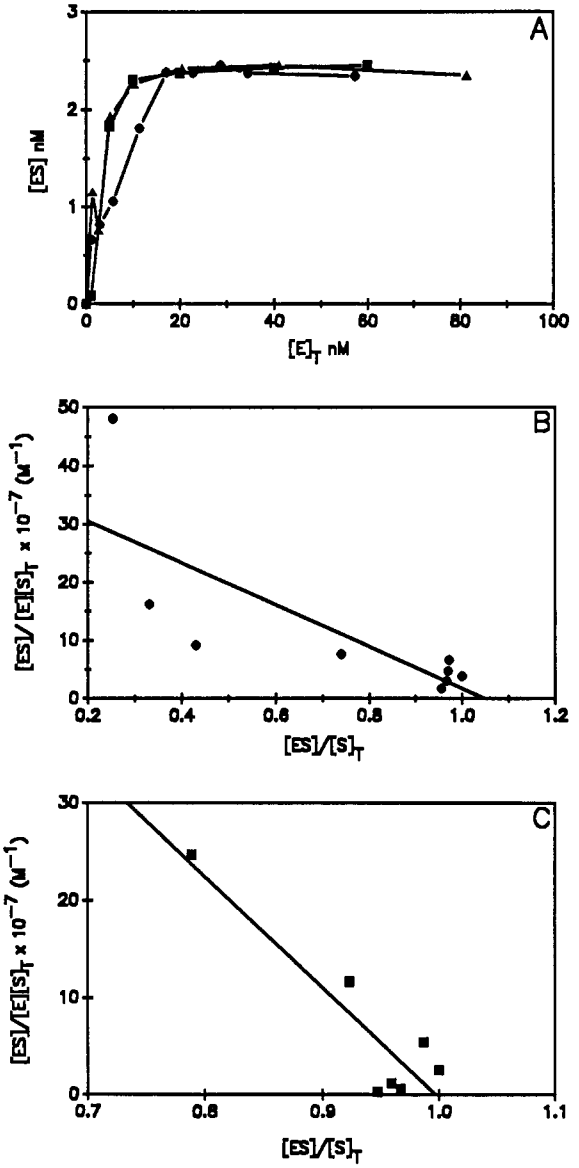


FIGURE 5: Binding of native and reconstituted photolyase to substrate. The 48-mer duplex at 2.46 nM was mixed with the indicated amounts of photolyase, and the enzyme-bound DNA was quantified by gel retardation. (A) Binding isotherms; (▲) native photolyase; (●) enzyme reconstituted with FAD; (■) enzyme reconstituted with 5-deazaFAD. (B and C) Scatchard analysis of binding of FAD and 5-deazaFAD reconstituted enzymes, respectively.

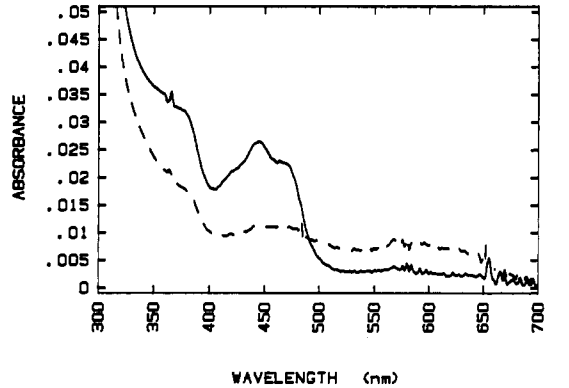


FIGURE 6: Near-UV–visible absorption spectra of $FAD_{ox}$-reconstituted photolyase and dithionite-reduced $FAD_{ox}$-reconstituted photolyase. $FAD_{ox}$-reconstituted photolyase (—) was incubated with 11 mM dithionite under anaerobic conditions at 4 °C for 1 h. The enzyme was passed through a Penefsky column equilibrated with photolyase storage buffer and allowed to oxidize to the semiquinone, E-FADH• (- - -).

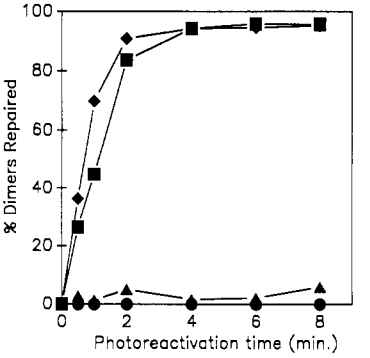


FIGURE 7: Catalytic activities of reconstituted photolyases. Photolyase [(◆) native; reconstituted with (●) FAD or with (▲) 5-deazaFAD; (■) reconstituted with FAD, reduced with dithionite, and oxidized to FADH•] was mixed with excess substrate in 100 μL of reaction buffer under aerobic conditions and irradiated with 344-nm (192 ergs $mm^{-2}$ $s^{-1}$) monochromatic light for the indicated time periods. The amount of DNA repair was quantified by incision with T4 endonuclease V followed by electrophoresis on a 12% denaturing polyacrylamide gel and autoradiography.

Table I: Affinities of Flavins in *E. coli* Photolyase

| flavin | concn (μM) cofactor | concn (μM) apoenzyme | binding constant ($M^{-1}$) |
|---|---|---|---|
| FAD | 810 | 19 | $\sim 1.5 \times 10^{6}$ [a] |
| 5-deazaFAD | 406 | 19 | $>10^{5}$ [b] |
| 1-deazaFAD | 406 | 20 | <294 [c] |
| riboflavin | >658 | 33 | <67 [c] |
| FMN | >658 | 33 | <67 [c] |
| $F_{420}$ | >658 | 33 | <17 [c] |
| | 910 [d] | 45 [d] | <9 [c,d] |

[a] $K_A$ for FAD binding to apophotolyase was estimated by titrating 250 μL of 6.8 μM FAD with aliquots of 130.5 μM apophotolyase and monitoring the spectral change of FAD as binding to apophotolyase occurred. The concentration of bound FAD was taken to be the percent of total spectral change (occurring at saturation of FAD with apophotolyase) at 374 nm. $K_A$ was determined by Scatchard analysis of the data. [b] A lower limit for $K_A$ for the binding of 5-deazaFAD to apophotolyase was estimated from the concentration of apophotolyase and 5-deazaFAD used in a reconstitution experiment which resulted in stoichiometric binding of 5-deazaFAD to apophotolyase (see Reconstitution of Photolyase with FAD and 5-DeazaFAD under Results). [c] Upper limits for the binding constants of various flavins to apophotolyase were estimated from the concentrations of apophotolyase and cofactor used in reconstitution experiments that resulted in no detectable binding. The minimum spectrophotometrically detectable concentration of enzyme-bound chromophore was calculated on the basis of the published extinction coefficients of the free chromophore. The lowest detectable absorption was taken to be 0.015. [d] In this reconstitution experiment defolated but fully FAD-saturated enzyme was used, and therefore these numbers apply to the affinity of $F_{420}$ to the 5,10-MTHF site of photolyase.

then allowed to oxidize to the flavin seminiquinone form in which it is purified (see Figure 6), it becomes as active as 5,10-MTHF-depleted, native enzyme (see Figure 7).

It is of interest that photolyase containing the oxidized form of 5-deazaFAD was also unable to catalyze photorepair (see Figure 7). This suggests, by analogy, that the deaza-type photolyases which contain the oxidized form of 8-hydroxy-5-deazaflavin as a natural cofactor use this chromophore as a light harvester rather than as an acceptor of electrons in a thymine dimer radical cation generating mechanism.

*Other Flavins.* To identify the structurally important features of FAD necessary for binding to apophotolyase, we attempted reconstitution of apophotolyase with other flavin cofactors. The results are shown in Table I. Riboflavin and FMN failed to bind to apoenzyme, suggesting that the phosphodiester linkage and the adenine ring are necessary for

specific binding to apophotolyase. It appears that the N1 of the flavin ring is also important for binding because 1-deazaFAD, in contrast to 5-deazaFAD, failed to associate with apoenzyme. This suggests that the N1 of the flavin ring is buried and makes contact (e.g., by H bonding) within the protein while the N5 may be exposed to the solvent in the substrate binding pocket. $F_{420}$, which is a lactylpolyglutamyl-8-hydroxy-7-desmethyl-5-deazariboflavin, may be considered either a flavin analogue because of its tricyclic ring structure or a 5,10-MTHF analogue because of its polyglutamate tail. Therefore, we tested the binding of this cofactor to both the 5,10-MTHF and the FAD binding sites by using either defolated enzyme (by $NaBH_4$ treatment) or apoenzyme. In both cases we failed to detect any binding. Thus, we conclude that the polyglutamate tail is insufficient to direct $F_{420}$ to the folate binding site and may also prevent the binding of this modified 5-deazaFAD moiety to the FAD pocket.

## Discussion

This paper describes an efficient and reproducible method for the reversible resolution of apophotolyase and chromophores. Apophotolyase has been used to study the DNA binding activity, cofactor binding, and photocatalytic activity of *E. coli* DNA photolyase.

In the DNA binding studies it was found that although apophotolyase was likely to be in near native conformation, it was unable to bind to thymine dimer containing DNA. Reconstitution with FAD and 5-deazaFAD restored substrate binding activity, suggesting that FAD is required to form the substrate binding pocket. There are two possible ways that FAD may assist in forming the substrate binding site. Contacts that FAD makes with the protein may dictate the overall conformation of photolyase, thus forming the substrate binding pocket. Alternatively, FAD may be in actual contact with the DNA substrate, forming, for example, essential hydrogen bonds necessary to maintain stable ($t_{1/2}$ = 1–5 min, in the dark) enzyme–substrate complexes (Sancar & Sancar, 1988). In the latter case, the N5 position of the isoalloxazine ring system clearly is not involved as 5-deazaFAD is able to restore substrate binding affinity to the same extent as FAD.

The ability of 5-deazaFAD (and FAD) to convert apoenzyme to holoprotein, with concomitant recovery of high-affinity binding to UV-damaged DNA, is clear proof of a very structural role for the FAD/5-deazaFAD cofactor, separate from its redox competence and electron-transfer role in catalysis. The 5-deazaFAD–photolyase reconstitution system is ideal for physical studies, e.g., circular dichroism, Raman spectroscopy, and NMR, to assess induced effects of coenzyme binding on global enzyme structure and/or in the region of the damaged DNA substrate recognition site. It is clear that binding of the folate cofactor to apophotolyase is not sufficient to induce the creation of the substrate binding pocket and, thus, will not be useful for studying this phenomenon.

From the reconstitution studies described in this paper it is evident that apophotolyase has specific structural requirements for cofactor binding. First, the failure of riboflavin or FMN to bind to apophotolyase demonstrates the importance of the phosphoribosyl adenine moiety. These results are in agreement with other flavin protein studies which indicate that the N10 substituent of the isoalloxazine ring system is of primary importance in determining specific flavin–protein interactions (Massey & Hemmerich, 1980). Of the greatest interest is that 1-deazaFAD is also unable to bind to apophotolyase while 5-deazaFAD binds quite readily. This result is quite unexpected as most other flavin binding enzymes bind 1- and 5-deazaflavins with almost equal affinity (Spencer et al., 1977a,b). It is therefore likely that in the case of photolyase the N1 position is in close contact with the protein moiety, forming one or more important hydrogen bond(s) with the apoenzyme. Lastly, the failure of $F_{420}$ to bind to either the 5,10-MTHF or the FAD site indicates that the polyglutamate tail does not sufficiently mimic the 5,10-MTHF polyglutamate tail to promote binding to the 5,10-MTHF site and that it interferes with the binding of this 5-deazaflavin derivative to the FAD site. If the bound deazaflavin of deazaflavin-type photolyases is, in fact, $F_{420}$, these results demonstrate a clear divergence of the two types of photolyases in their "second" chromophore binding pockets.

The photorepair studies have enabled us to confirm that enzyme containing FAD in the oxidized state is unable to catalyze photorepair. However, treatment of reconstituted FAD photolyase with dithionite followed by air oxidation to yield $FADH^{\bullet}$– photolyase results in complete restoration of photocatalytic activity, confirming that removal of the chromophore is fully reversible. The failure of *E. coli* photolyase containing 5-deazaFAD to show any photocatalytic activity suggests two conclusions. First, it demonstrates that 5-deazaFAD cannot function as both light harvester and dimer repair cofactor in this class of photolyases, analogous to the behavior observed with apophotolyase reconstituted with oxidized FAD. In the oxidized, electron-deficient redox state 5-deazaFAD cannot initiate electron transfer to or from the thymine dimer to initiate fragmentation and repair of the cyclobutane adduct. It will be of interest to see if 5-deaza-$FADH_2$–photolyase, when prepared, will be active in dimer repair. If the enzyme mechanism involves one electron transfer and $FADH^{\bullet}$ participation, the 5-deaza$FADH_2$ species should be inactive as it is appreciably restricted to hydride transfer pathways in ground-state redox chemistry (Hemmerich & Massey, 1980; Walsh, 1986).

Second, the failure of *E. coli* photolyase containing 5-deazaFAD to show any photocatalytic activity suggests that the oxidized form of 8-hydroxy-5-deazariboflavin, which is the natural chromophore of the deazaflavin class of photolyases, functions as the photoantenna and not the cofactor in direct catalytic repair of the cyclobutane-containing thymine dimer adduct. This observation implies that the deaza class of photolyases functions in a manner analogous to the folate class. Our attempt, and most recently an independent effort by Jorns et al. (1990), to assess the role of the 8-hydroxy-5-deazaflavin cofactor by functional insertion into either the folate binding site (to assess antenna function) or the FAD site (to assess initiation of dimer fragmentation) was foiled due to the failure of $F_{420}$ to bind to either apoenzyme or semiapoenzyme (containing FAD but not folate).

While the manuscript was in preparation, Jorns et al. (1990) reported the reconstitution of photolyase with FAD and restoration of catalytic activity; however, the binding affinity to the DNA substrate of either the apoenzyme or reconstituted enzyme was not addressed in that study.

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# Substrate Overlap and Functional Competition between Human Nucleotide Excision Repair and *Escherichia coli* Photolyase and (A)BC Excision Nuclease†

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ABSTRACT: Human cell free extract prepared by the method of Manley et al. (1980) carries out repair synthesis on UV-irradiated DNA. Removal of pyrimidine dimers by photoreactivation with DNA photolyase reduces repair synthesis by about 50%. With excess enzyme in the reaction mixture photolyase reduced the repair signal by the same amount even in the absence of photoreactivating light, presumably by binding to pyrimidine dimers and interfering with the binding of human damage recognition protein. Similarly, the UvrB subunit of *Escherichia coli* (A)BC excinuclease when loaded onto UV-irradiated or psoralen-adducted DNA inhibited repair synthesis by cell-free extract by 75–80%. The opposite was true also as HeLa cell free extract specifically inhibited the photorepair of a thymine dimer by DNA photolyase and its removal by (A)BC excinuclease. Cell-free extracts from xeroderma pigmentosum (XP) complementation groups A and C were equally effective in blocking the *E. coli* repair proteins, while extracts from complementation groups D and E were ineffective in blocking the *E. coli* enzyme. These results suggest that XP-D and XP-E cells are defective in the damage recognition subunit(s) of human excision nuclease.

Wood et al. (1988, 1989) and Sibghat-Ullah et al. (1989) have recently shown that cell-free extract of human cells prepared by the method of Manley et al. (1980) carries out repair synthesis on DNA damaged by UV light, psoralen, and cisplatinum. The repair synthesis activity was absent in all XP cell lines tested, and therefore it was concluded that the DNA adducts were removed by nucleotide excision repair. Efforts in our laboratory to characterize this activity further by fractionation of extracts or by using defined substrates were of limited success. Therefore, we have resorted to alternative methods to define the substrate specificity of the human repair enzyme and its interaction with DNA substrate. In this study we have used *Escherichia coli* and *Saccharomyces cerevisiae* photolyase and *E. coli* (A)BC excinuclease, whose interaction with DNA is well understood (Husain et al., 1987; Baer & Sancar, 1989; Van Houten et al., 1987), to probe the interaction of the human nucleotide excision repair enzyme(s) with DNA. Our findings suggest that about 50% of the repair synthesis by HeLa cell free extract on UV-irradiated DNA is blocked by excess photolyase in the reaction mixture and therefore must be due to the repair of pyrimidine dimers. Similarly, 60–80% of repair synthesis on UV-irradiated or psoralen-adducted DNA can be blocked by UvrB[1] loaded onto

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[1] Abbreviations: UV-DNA, UV-irradiated DNA; UvrA, UvrB, and UvrC, gene products of *uvrA*, *uvrB*, and *uvrC*; CFE, cell-free extract; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; UDS, unscheduled DNA synthesis.

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