to a lower lying molecular orbital than the latter, making reduction more exoenergic. In fact, the increase in reduction potential of an excited state relative to ground state can be calculated from the energy of the excited state. For the flavin radical of photolyase, the lack of any vibrational fine structure in the absorption spectrum precludes an accurate assessment of the position of the $0 \rightarrow 0$ vibronic band within the lowest electronic transition. However, the energy of E-^{2}FADH$^\bullet$ cannot be less than 170 kJ mol^{-1}, which corresponds to the red edge of the absorption spectrum (700 nm). Hence, a change in the reduction potential of E-^{2}FADH$^\bullet$ compared to the ground state of $>+1.77$ V can be calculated. While the increase in reduction potential of the quartet state (E-^{4}FADH$^\bullet$) would be slightly less than the doublet (due to the doublet - quartet splitting energy), this is still consistent with the minimum increase of +1.23 V for the proposed reaction scheme to be feasible.

The repair of oxidized tryptophan radicals by *n*-propyl gallate has previously been reported (Hoey & Butler, 1984). Bimolecular rate constants of 3.8×10^8 and 1.2×10^8 m^{-1} s^{-1} have been determined for repair of the oxidized free tryptophan and oxidized tryptophan residues of lysozyme, respectively. The much lower repair efficiency for *n*-propyl gallate determined in this work (3.8×10^6 M^{-1} s^{-1}) suggests that the active tryptophan residue of photolyase is more shielded from the solvent than the tryptophan residues of lysozyme.

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Active Site of *Escherichia coli* DNA Photolyase: Mutations at Trp277 Alter the Selectivity of the Enzyme without Affecting the Quantum Yield of Photorepair†

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ABSTRACT: *Escherichia coli* DNA photolyase repairs pyrimidine dimers by a photoinduced electron-transfer reaction. The enzyme binds to UV-damaged DNA independent of light (the dark reaction) and upon absorbing a 300–500-nm photon breaks the cyclobutane ring of the dimer (the light reaction) and thus restores the DNA. No structural information on the enzyme is available at present. However, comparison of the sequences of photolyases from five different organisms has identified highly conserved regions of homology. These regions are presumably involved in chromophore (flavin and folate) and substrate binding or catalysis. Trp277 (W277) in *E. coli* photolyase is conserved in all photolyases sequenced to date. We replaced this residue with Arg, Glu, Gln, His, and Phe by site-specific mutagenesis. Properties of the mutant proteins indicate that W277 is involved in binding to DNA but not in chromophore binding or catalysis. Of particular significance is the finding that compared to wild type W277R and W277E mutants have about 300- and 1000-fold lower affinity, respectively, for substrate but were indistinguishable from wild-type enzyme in their photochemical and photocatalytic properties.

Mechanisms and rate of electron transfer in biological systems are of much current interest (Closs & Miller, 1988). Of the various biological electron-transfer systems, those that involve photoinduced electron transfer are of particular significance because the transfer reactions are easily controlled by exposing the system to light flashes of desired intensity and duration. The most widely known and best characterized

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photoinduced electron-transfer system is the photosynthetic reaction center (Michel & Deisenhofer, 1988; Feher et al., 1989; Glazer, 1989). An equally interesting and relatively simpler system is DNA photolyase. Photolyases (EC 4.1.99.3) bind to DNA containing pyrimidine dimers and upon absorbing a near-UV photon (300–500 nm) break the cyclobutane ring joining the two pyrimidines of the dimer. The enzymes characterized to date fall into two classes (Sancar & Sancar, 1987, 1988). Both classes contain FAD (presumably in the form of $FADH_2$) as a chromophore-cofactor. In addition, the folate class contains methenyltetrahydrofolate (MTF)[1] (Johnson et al., 1988), while the deazaflavin class contains 8-hydroxy-5-deazaflavin (Eker et al., 1988) as the second chromophore. The enzymes from *Escherichia coli* and *Saccharomyces cerevisiae* are representatives of the folate class, while those of *Streptomyces griseus*, *Anacystis nidulans*, *Scendesmus acutus*, and *Methanobacterium thermoautotrophicum* belong in the deazaflavin class. Enzymes from both classes have been characterized biochemically in some detail (Sancar & Sancar, 1988; Sancar, 1990), but no structural information is available as yet. However, the genes of *E. coli*, *S. cerevisiae*, *A. nidulans*, *S. griseus*, and *Halobacterium halobium* photolyases have been cloned and sequenced (Takao et al., 1989). Sequence comparison reveals 25–30% homology between enzymes from these evolutionarily far removed species. We reasoned that regions of high homology might be involved in chromophore and/or substrate binding. Thus, the sequences that are uniquely conserved in the folate class enzymes may represent the folate binding site, while those that are uniquely conserved in the deazaflavin class may interact with this chromophore. By the same reasoning, regions that have been conserved in all photolyases may be in the flavin and/or DNA binding sites.

The region W277–Y281 in *E. coli* photolyase is highly homologous to the corresponding regions in all other photolyases (Takao et al., 1989) and in addition contains a Trp residue. Recent nanosecond flash photolysis studies have revealed that the FADH° (the semiquinone form observed in the purified enzyme; Jorns et al., 1984) cofactor is photoreduced by abstracting an electron from a Trp residue in close proximity (Heelis et al., 1990). Thus, it seemed possible to us that the W277–Y281 segment may be important for flavin and perhaps DNA binding. We tested this prediction by mutagenizing this region first by "doping" and then by site-specific mutagenesis. Our results indicate that Trp277 is at the DNA binding site of the enzyme but it does not make significant contact with either chromophore. As a result, replacement of this residue with amino acids of significantly different properties drastically alters the substrate affinity of the enzyme without having measurable effects on the photochemistry of the enzymatic reaction.

Materials and Methods

Bacterial Strain and Plasmids. The *E. coli* strain CSR603 F′ $laci^Q$ (*recA1 uvrA6 phr-1*) used for propagating the *tac-phr* plasmids and testing their complementing activity has been described before (Sancar et al., 1984). Uracil containing single-stranded DNA template was prepared in CJ236 (dut^- ung^-) as described by Kunkel (1985). The RF phage DNA synthesized in vitro was transformed into NM522 (ung^+ dut^+ $hsdR^-$ $hsdM^-$) to select for the mutated strand. Both strains were obtained from Bio-Rad. The plasmid used for site-specific mutagenesis, pIBI25, and the M13 phage derivative used in superinfection, M13K07 (Vierra & Messing, 1987), were obtained from International Biotechnology Inc. The source of the *phr* gene was pMS1310, which was generated from pMS969 by deleting the *Pst*I site (Sancar et al., 1984).

Mutagenesis. We mutagenized the W277–Y281 region of *phr* both by doping (Hutchison et al., 1985) and by site-specific mutagenesis (Zoller & Smith, 1983) methods and selected for the mutated genes as described by Kunkel (1985). The *Mlu*I fragment encompassing the coding region of the 441 carboxy-terminal amino acids of the 471 amino acid long protein was inserted into pIBI25 to obtain pUNC1989. This plasmid was inserted into CJ236, and single-stranded templates were obtained by superinfecting with M13K07 (Vierra & Messing, 1987). For doping mutagenesis of the 5 amino acid region, the single-stranded pUNC1989 template was annealed to a 30 nucleotide long oligomer (Operon Biotechnologies) that was doped over a 15-nucleotide region such that there was on average one mismatched base per oligomer. For site-specific mutagenesis of W277, 18 nucleotide long oligomers with the appropriate mismatches were annealed to the single-stranded template. The primed single-stranded template was then converted into RF with Klenow fragment (Bio-Rad), and the resulting plasmids were transformed into NM522. Ampicillin-resistant colonies were picked, and single-stranded DNA templates were prepared by superinfecting with M13K07. The mutant plasmids were identified by dideoxy sequencing using Sequenase (U.S. Biochemicals). Once mutant pUNC1989 plasmids were identified by this procedure, the *Mlu*I fragments carrying the Phr sequences were isolated from the plasmids and were substituted for the same *Mlu*I fragment in pMS1310 to obtain plasmids overexpressing the mutant proteins.

Enzyme Purification. Wild-type and two mutant photolyases (PL-W277E and PL-W277R) were purified by a modified form of previously published procedures (Sancar et al., 1984, 1987a). Briefly, cells containing the *tac-phr* plasmids were grown to A_{600} = 1.0, induced with 1 mM isopropyl β-D-thiogalactopyranoside for 12 h, and then collected, washed, and lysed by sonication. Photolyase was precipitated from cell-free extract with 55% saturated ammonium sulfate and then purified by successive chromatography on Blue Sepharose, ACA 44 gel filtration column, and hydroxylapatite column. The enzyme obtained by this purification procedure was better than 99% pure. Three of the mutant photolyases, PL-W277F, -W277H, and -W277Q, were not purified beyond the Blue Sepharose chromatography step, which routinely yields enzyme with about 95% purity. The concentration of the enzyme was estimated from absorption at 580 nm by using an extinction coefficient ϵ_{580} = 5000 M^{-1} cm^{-1} (Payne et al., 1987). The values obtained from these measurements were confirmed by running known amounts of protein on an SDS–polyacrylamide gel, staining with Coomassie blue, and then measuring the intensity of the photolyase band by scanning densitometry. The values obtained by the two methods were in agreement within the resolution of our measurements.

Substrates. The substrate was a synthetic 48-mer duplex that contained a T⟨⟩T dimer in the middle (Husain et al., 1987, 1988). The substrate was constructed by ligating an 11-mer with a T⟨⟩T dimer to five other oligomers. We used [γ-^{32}P]ATP to kinase the ligated 5′ termini (which become internal upon ligation) and therefore were able to estimate the concentration of substrate from the radioactivity of the 48-mer duplex. To measure the affinity of photolyase to nondamaged DNA, the 48-mer duplex was constructed in the same manner

[1] Abbreviations: MTF, methenyltetrahydrofolate; WTPL, wild-type photolyase; PL-W277R, etc., photolyase mutant with a Trp → Arg, etc., change at amino acid 277; E-$FADH_2$, enzyme with reduced flavin but no folate; E-MTF-$FADH_2$, holoenzyme with reduced flavin; T4 endo V, T4 phage endonuclease V which is specific for pyrimidine dimers.

by using an 11-mer without a thymine dimer. Alternatively, the dimer containing substrate was mixed with excess photolyase, and the dimer was repaired by photoreactivation; the DNA was then separated from photolyase by phenol/chloroform extraction followed by ethanol precipitation.

DNA Binding. Binding of photolyase to DNA was measured by gel retardation assay as described previously (Husain & Sancar, 1987). Photolyase–DNA complexes do not make tight bands in band-shift gels, and this problem is amplified in band-shift assays with mutant proteins of low affinity. Therefore, we estimate the ES concentration by cutting out the band corresponding to free (unbound) DNA and quantifying the radioactivity associated with this DNA by Cerenkov counting.

Spectroscopy. The absorption spectra of wild-type and mutant photolyases were obtained with a Hewlett-Packard 8451A spectrophotometer. Fluorescence studies were conducted with an SLM48000 spectrofluorometer. The fluorescence of both the folate and the flavin chromophores was measured. Both measurements necessitated special handling of enzyme samples. In comparing the folate fluorescence of two enzyme preparations, it is essential that enzyme samples contain the same amount of folate (and the same amount of reduced flavin). The folate comes off the enzyme easily during purification (Johnson et al., 1988; Hamm-Alvarez et al., 1989), and therefore different enzyme preparations contain different amounts of folate. Second, the fluorescence intensity of folate is strongly affected by the redox state of the flavin chromophore; the intensity increases 4-fold when the flavin radical is converted to fully reduced form (P. F. Heelis and A. Sancar, unpublished results; Jordan & Jorns, 1988), and different enzyme preparations are known to contain different amounts of reduced flavin. We overcame these problems by supplementing the enzyme with folate as described by Hamm-Alvarez et al. (1989) and converting all the flavin to fully reduced form by photoreduction in an anaerobic cuvette (Payne et al., 1987) using light from a camera flash filtered through a >600-nm cutoff filter. The majority of flavin (80–90%) in a typical photolyase preparation is in neutral radical form (FADH°), which is nonfluorescent. Upon reduction it fluoresces weakly at 505 nm, and this is observed as a shoulder on the folate fluorescence peak at 480 nm (Jordan & Jorns, 1988). The flavin fluorescence becomes easily quantifiable when the folate is eliminated by photodecomposition (Heelis et al., 1987) or treatment with sodium borohydride (Jorns et al., 1987) which converts the fluorescent methenyltetrahydrofolate to nonfluorescent 5-methyltetrahydrofolate (Hamm-Alvarez et al., 1989). Therefore, to measure flavin fluorescence we first treated the enzyme with $NaBH_4$, removed the reductant by size exclusion through a Sephadex G-50 column, and placed the enzyme in an anaerobic cuvette containing photolyase storage buffer (50 mM Tris·HCl, H 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM dithiothreitol, and 50% glycerol) supplemented with 0.86 unit/mL of Oxyrase (Oxyrase, Inc.) and 150 mM lactate. The enzyme was photoreduced to completeness as monitored by the disappearance of the 580-nm FADH° absorption peak. Both folate and flavin fluorescence measurements were conducted at 15 °C, and the measurements of wild-type and mutant enzymes were conducted on the same day to prevent artifactual differences that may be introduced by instrumental idiosyncrasies. The precaution was especially important for flavin fluorescence because this relatively weak fluorescence shows greater day-to-day variations. The enzyme concentrations used for folate and flavin fluorescence measurements were 6.8×10^{-6} and 8.8×10^{-6} M, respectively.

Photoreduction and Photodecomposition. The photoreduction of FADH° and photodecomposition of MTF were achieved by exposing the enzyme to successive camera flashes. Photolyase in storage buffer was placed in an anaerobic cuvette and exposed to light flashes from the Vivitar 2500 flash units at room temperature. To prevent heating and enzyme denaturation, the samples were cooled in ice water at 5–10 flash intervals. Photoreduction of the flavin radical was minitored by the decrease in absorption at 580 nm, and photodecomposition of folate was monitored by the decrease in absorption at 384 nm. The amount of photodecomposition after 120 flashes was taken to be complete, and the extent of this process at various levels of illumination was expressed relative to the 120-flash level.

Quantum Yield of DNA Repair. The photolytic cross section ($\epsilon\phi$) of WT and mutant photolyases was measured in vivo as described previously (Sancar et al., 1987b). The quantum yield for repairing a thymine dimer by wild-type and mutant photolyases was measured with both photolyase holoenzyme and photolyase depleted of folate but containing fully reduced flavin. By conducting the experiments with these two forms, we were able to compare the wild-type and mutant enzymes with regard to two photochemical processes: energy transfer from folate to flavin and photoinduced electron transfer to or from $FADH_2$ to or from a pyrimidine dimer. The 50-μL reaction mixtures contained 50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM dithiothreitol, 100 μg/mL bovine serum albumin (photolyase reaction buffer), 0.86 unit/mL Oxyrase (Oxyrase, Inc.), 150 mM lactate, 1 nM substrate, and 4–5 μM photolyase. The mixture was placed in an anaerobic cuvette and was flushed with argon for 30 min. The flavin radical was then reduced with filtered ($\lambda > 600$ nm) flash light. It was possible to photoreduce the enzyme in the presence of substrate without repair because the flavin radical is inert in photorepair (Payne et al., 1987). Following photoreduction, the mixture was exposed to photoreactivating light of 384 (holoenzyme) or 366 nm (E-$FADH_2$) at fluence rates of 20–40 ergs mm^{-2} s^{-1} by using a Quantacount monochromator (Photon Technology International). Samples were withdrawn anaerobically after the delivery of the desired fluences. Following photoreactivation, the DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 20 μL of buffer containing 50 mM Tris·HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol, and 100 μg/mL bovine serum albumin (T4 endo V buffer). Then, approximately 10 units of T4 endonuclease V (T4 endo V) was added to the mixture, and the mixture was incubated at 37 °C for 30 min to digest the remaining nonphotoreactivated substrate. The DNA was then lyophilized and the repaired (full-length) and unrepaired (cut in half) forms were separated on 12% sequencing gels. Following autoradiography, the bands were cut out and quantified by Cerenkov counting. The data were analyzed by the method of Rupert (1962) in the form of a pseudo-first-order reaction (Rupert plot).

Results

Construction of Photolyase Mutants. The sequences of five photolyases from all three kingdoms are known (Takao et al., 1989): *E. coli*, *A. nidulans*, and *S. griseus* (prokaryotes), *H. halobium* (archaebacteria), and *S. cerevisiae* (eukaryotes). Enzymes from all these organisms contain or are presumed to contain $FADH_2$ (Sancar & Sancar, 1988) and bind to DNA by very similar mechanisms (Husain et al., 1987); Kiener et al., 1989; Baer & Sancar, 1989). It is therefore likely that the most conserved regions would be in the DNA and/or

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A. nidulans     A-------SIRVWQQELAWREFYQHALYHFPSL-------S
S. griseus      S-------GGEAFYRQLAWRDFHHQYLADRPDA-------D
H. halobium     Q-------NVAAFIGQLAWREFYAZVLYFNQNV-------E
S. cerevisiae   M-------STQNFIKEVAWRDFYRHCMCNWPYT-------M
E. coli         T-------AGSVWLNELIWREFYRHLITYHPSL-------K
                1                                       471
                          277
                        E,F,H,Q,R
```

FIGURE 1: Sequence homology between DNA photolyases. The amino acid sequence of *E. coli* photolyase includes the 5 residues, W277–Y281, that were mutagenized by doping and 10 amino acids on either side of this segment. The top three enzymes are of deazaflavin class, and the bottom two are folate enzymes. The numbers 1, 277, and 471 refer to position of amino acids in the *E. coli* enzyme. The residues that are conserved in all five enzymes are boxed. The five *E. coli* photolyase mutants at position 277 that were investigated in this study are indicated.

$FADH_2$ binding sites. Although several highly conserved regions have been identified (Sancar, 1985; Yasui & Longeveld, 1985; Yasui et al., 1988; Takao et al., 1989), we were particularly interested in the W277–Y281 segment of *E. coli* DNA photolyase because, in addition to being conserved, it contains a Trp residue. Recent nanosecond flash photolysis studies revealed that there is a Trp residue in close proximity of FADH° in *E. coli* photolyase (Heelis et al., 1990). For these reasons we decided to mutagenize this segment, hoping to isolate mutants with altered catalytic properties.

Figure 1 shows the amino acid sequences of five photolyases at the region corresponding to W277–Y281 of *E. coli* photolyase. We originally mutagenized the W277–Y281 segment by doping and identified 14 mutations in this region. Most of the mutant proteins were either not overproduced or not soluble and therefore not amenable to in vitro analysis. However, PL-W277R was overproduced at a level comparable to wild type, was soluble, and showed some interesting properties in vivo. Therefore, we decided to concentrate our efforts on W277. The PL-W277R mutant protein has about 300-fold lower affinity for dimer containing DNA and 5-fold higher affinity to undamaged DNA compared to wild-type protein (see below). We interpreted these results to mean that W277 is in the DNA binding site of photolyase. How photolyase binds to DNA is not known. We only know that ionic interactions with the phosphodiester backbone contribute only 10–20% of free energy of binding (Sancar et al., 1987c; Husain et al., 1987). The W277 residue could contribute to binding by making H bonds with the dimer, by van der Waals interactions, or by intercalation. To test the hypothesis that W277 is at the DNA binding site and to differentiate between these different forms of interactions, we generated the following mutants by site-directed (single amino acid change) mutagenesis (Figure 1):

(*a*) *W277F*. If W277 is contributing to specificity by H bonding to DNA, then this mutant should have lower affinity because Phe is not a conventional H-bond donor or acceptor. However, if stacking or van der Waals interactions are important, then W277F should behave similar to WT-PL.

(*b*) *W277H and W277Q*. Both His and Gln are H-bond donors, and these mutants would be expected to behave somewhat similarly to WT-PL should H bonding be the important factor in Trp function (ignoring steric considerations).

(*c*) *W277E*. If W277 is at the DNA–protein interface and if the increased nonspecific binding accompanying the decreased specific binding of W277R is the result of introducing a positive charge capable of making an ionic bond with the phosphodiester backbone, then W277E should behave similarly to W277R regarding specific binding but it should have an opposite effect on nonspecific binding.

DNA Binding of Mutant Proteins. All five mutant proteins were overproduced to levels comparable to that of WT photolyase and showed the same chromatographic properties (data not shown). PL-W277R and PL-W277E were purified to homogeneity, and PL-W277H, W277Q, and W277F were approximately 95% pure after one chromatographic step. Further purification was unnecessary for our studies in the latter three cases.

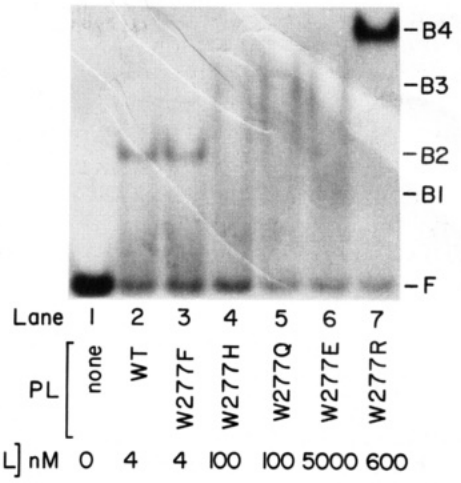

FIGURE 2: Analysis of mutant photolyases by gel retardation. DNA (1 nM) substrate was mixed with the indicated amounts of photolyases (to obtain about 70% binding) in 50 μL of reaction buffer, and the mixtures were incubated at 23 °C for 30 min in the dark. Then the samples were loaded on a 5% polyacrylamide gel. After electrophoresis for 2 h, the gel was autoradiographed. F, free DNA; B1–B4 indicate the location of bound DNA bands observed with wild-type and mutant photolyases.

The binding of mutant photolyases to DNA was investigated by gel retardation. In Figure 2 we present the results of a gel retardation experiment with all five mutants. In addition to quantitative differences to be discussed below, the complexes of PL-W277H, -W277Q, -W277E, and -W277R show qualitative differences in their migration patterns. At comparable levels of binding WT and PL-W277F yield a distinctive "retarded" DNA–protein band. The PL-W277H and PL-W277Q proteins yield bands of slower mobility (B3), and W277E gives a band of higher mobility (B1) compared to wild-type proteins (B2), although the majority of ES complexes of these three mutants are in the form of "streaks" throughout the lanes. The W277R mutant does not streak much and yields the slowest migrating DNA–protein band (B4). There is no comprehensive theory or empirical relationship which explains the mobility of protein–DNA complexes in band-shift assays. In general, larger proteins (or multiple complexes of the same protein) cause greater band shift (Fried & Crothers, 1984), and bends introduced into DNA upon binding to a protein cause additional retardation compared to binding modes which do not cause or amplify DNA bending (Gartenberg & Crothers, 1988). It is likely, therefore, that the slower migrating ES complexes of PL-W277H and PL-W277Q and the faster migrating ES complex of PL-W277E are the results of different rigidity of DNA in the complexes. On the other hand, the slowest migrating and sharp PL-W277R–DNA band most likely resulted from binding of several photolyase molecules because of the increased nonspecific and decreased specific binding of this mutant.

The binding curves for WT-PL and five mutant proteins are shown in Figure 3. Analysis of these curves by Scatchard plots gave the following specific binding constants (M^{-1}): WT-PL (2.2×10^9), PL-W277F (1.7×10^9), PL-W277Q (2.8×10^8), PL-W277H (1.6×10^8), PL-W277R (8.2×10^6), and W277E (1.3×10^6). The nonspecific binding constants (M^{-1})

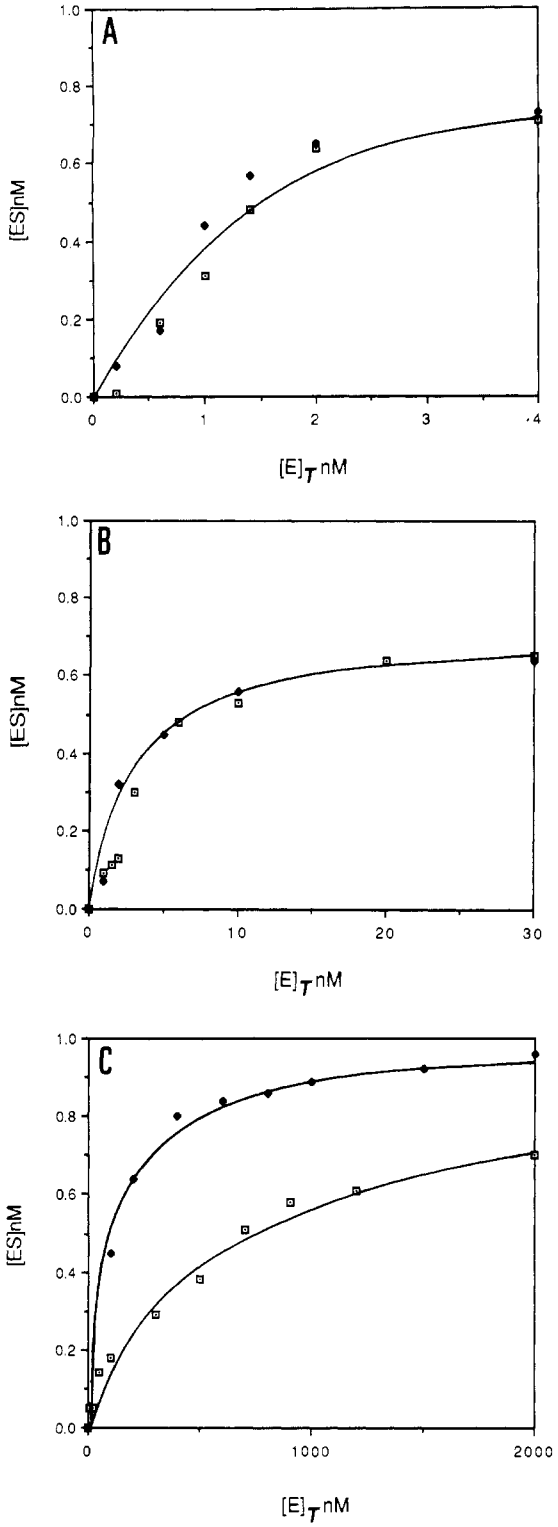

FIGURE 3: Binding of mutant photolyases to DNA. The enzymes are grouped according to their affinities. The binding data were generated from binding mixtures that contained 1 nM substrate and the indicated amounts of enzyme which were then analyzed by gel retardation. (A) High-affinity enzymes: (dotted square) WT-PL; (solid diamond) PL-W277F. (B) Medium-affinity enzymes: (dotted square) PL-W277H; (solid diamond) PL-W277Q. (C) Low-affinity enzymes: (dotted square) PL-W277E; (solid diamond) PL-277R.

were determined in the same-manner for WT (2.8×10^5), PL-W277R (1.5×10^6), and PL-W277E (2.4×10^5). Thus, it appears that W277 is at the DNA binding site of photolyase and contributes to specific binding by stacking or van der Waals interactions. (The binding constant of PL-W277F is identical with that of wild type within experimental error of our measurements.) Introduction of a positive charge at this site increases nonspecific binding while reducing the specific affinity of the enzyme; in contrast, a negative charge at this location reduces both specific and nonspecific affinity of photolyase to DNA. Since PL-W277R and PL-W277E show the greatest differences in affinity compared to WT, we concentrated most of our efforts on these two mutants.

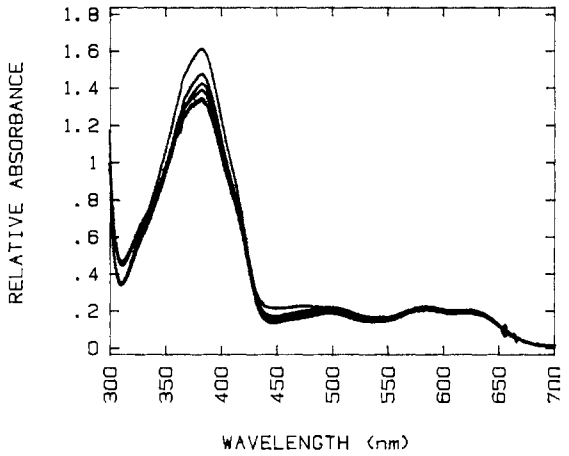

FIGURE 4: Abgsorption spectra of WT and PL-W277E, F, H, Q, and R. The enzyme preparations were supplemented with folate in storage buffer and passed through a Sephadex G-50 column to remove free folate. The spectra were then recorded and normalized to the same magnitude at 580 nm. The actual absorbances at 580 nm ranged from 0.095 (W277E) to 0.204 (W277Q). The spectra according to absorbance at 384 nm from top to bottom are for W277E, WTPL, W277R, W277H, and W277F (W277Q).

Spectroscopic Properties of Mutant Photolyases. It was conceivable that the differences in DNA binding of wild-type and mutant photolyases may have resulted from partial denaturation of mutant proteins (Lim & Sauer, 1989). To address this possibility, we compared the absorption and fluorescence spectra of mutant and wild-type enzymes. As is apparent from Figure 4, the visible absorption spectra of the folate-supplemented enzymes are all identical. Since the 450–700-nm absorption is exclusively due to FADH°, which is easily oxidized under suboptimal conditions for the enzyme (e.g., low pH, long-term storage at 4 °C), we regard the absorption spectra as a strong indicator of native conformation. Further evidence for native conformation was obtained from fluorescence spectra. Photolyase contains two fluorophores (in addition to aromatic amino acids), flavin and folate. Both of these chromophores were used to probe the conformation of mutant proteins. Figure 5A shows the fluorescence spectra of WT and two mutant photolyases. There is no significant difference in either the shape or intensity of folate fluorescence between the wild-type and mutant enzymes (Figure 5A). Similarly, defolated-photoreduced (E-$FADH_2$ form) wild-type and mutant enzymes have very similar $FADH_2$ fluorescence spectra (Figure 5B). Thus, by these two criteria we conclude that mutant photolyases have native conformation.

Photoreduction and Photodecomposition. We used two additional photochemical reactions to probe the conformation of PL-W277R and PL-W277E: photoreduction and photodecomposition. Photoreduction of FADH° occurs by abstraction of an electron from a nearby Trp residue by the quartet state of FADH° followed by repair of the resulting Trp° radical by an electron donor (typically dithiothreitol) in solution (Heelis et al., 1987, 1990; Okamura et al., 1989). Figure 6A shows that the rates of photoreduction for WT and mutant proteins are superimposable. This not only indicates that the mutant proteins have native conformations but also

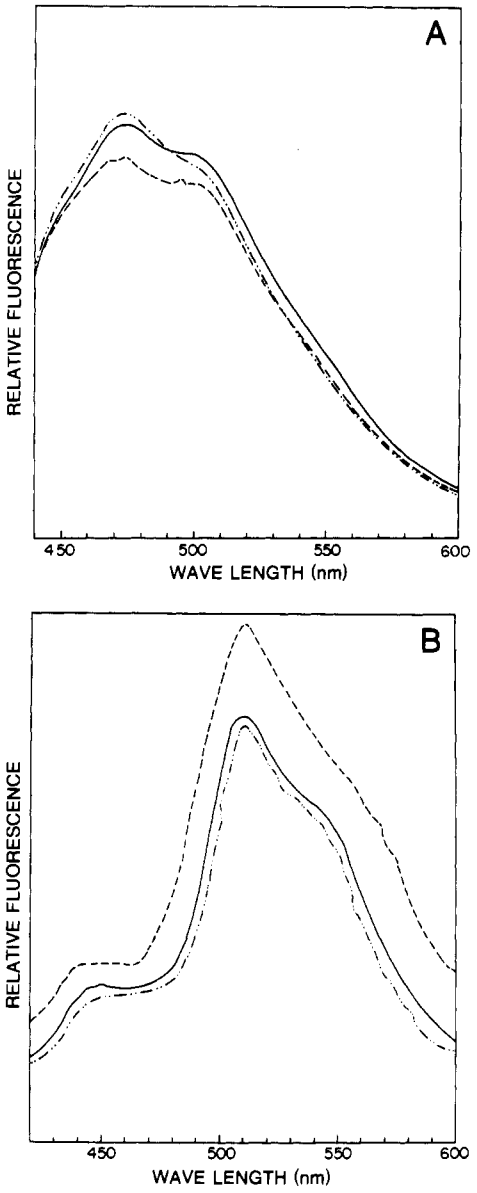

FIGURE 5: Folate and flavin fluorescence of mutant photolyases. (A) Folate fluorescence. The enzymes (6.8 μM) were supplemented with folate in storage buffer and photoreduced with $\lambda > 600$ nm under anaerobic conditions, and fluorescence emission spectra were recorded at excitation wavelength of 390 nm. The main peak at 470–480 nm is due to folate emission, and the shoulder at 503 nm is caused by fluorescence of $FADH_2$. (B) Flavin fluorescence. Defolated enzymes in storage buffer (8.8 μM) were photoreduced and excited at 390 nm, and the fluorescence spectra were recorded under anaerobic conditions. (dashed line) WT-PL; (dot-dashed line) PL-W277R; (solid line) PL-W277E.

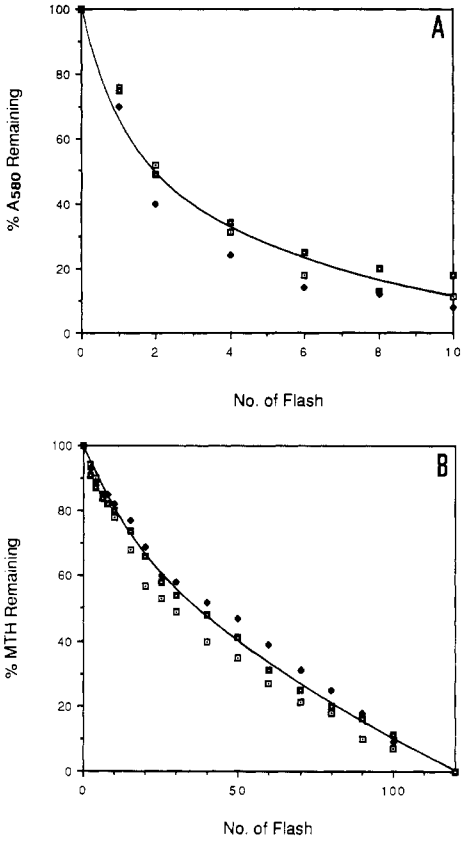

FIGURE 6: Photoreduction and photodecomposition. The samples in storage buffer were exposed to camera flashes at 4–5-s intervals, and the absorption spectra were measured to monitor (A) photoreduction by the decrease at 580 nm and (B) photodecomposition by the decrease at 384 nm. The initial absorbance at 580 was 0.12–0.14 for all three enzymes, and the final absorbance was 0.01–0.02. In photodecomposition experiments even after complete elimination of folate, about 20% of the absorbance at 384 nm remains because of the absorbance of $FADH_2$ in this region. After 120 flashes, no further decrease is observed at 384 nm, and therefore this decrease is taken to represent 100% photodecomposition. (dotted square) WT-PL; (solid square with white dot) PL-W277R; (solid diamond) PL-W277E.

shows that W277 is not the Trp residue involved in photoreduction. The conformation of mutant enzymes as well as the chromophore binding site was probed by photodecomposition experiments as well. The enzyme-bound MTF is photodecomposed to as yet unidentified species with a quantum yield of about 0.01 (Heelis et al., 1987; Hamm-Alvarez et al., 1989). The protein matrix plays an important role in this photochemical reaction because MTF is not photodecomposed perceptibly when free in solution or when bound to yeast DNA photolyase (Sancar et al., 1987). Therefore, photodecomposition may be considered an important probe for conformational integrity as well as folate binding site. Figure 6B shows that MTF is degraded with the same quantum yield in the wild-type and mutant enzymes. Thus, on the basis of absorption (flavin) and fluorescence (flavin and folate) spectra as well as the rate of flavin photoreduction and folate photodecomposition, we conclude that there is no significant conformational change in PL-W277R and PL-W277E and that the reduced specific binding affinity of these enzymes is the direct result of changing an amino acid involved in specific binding.

Quantum Yield of Dimer Repair. The experiments described so far indicate that mutant photolyases have native conformation but bind to substrate with reduced affinity. Do this reduced affinity and the presence of a positive (W277R) or a negative (W277E) charge at the DNA–protein interface affect the rate of electron transfer between $FADH_2$ and the thymine dimer? We addressed this question by measuring the photolytic cross section ($\epsilon\phi$) in vivo and in vitro. The results of in vivo experiments are shown in Figure 7. Both PL-W277E and PL-W277R have apparent photolytic cross sections (directly proportional to the slope) about half that of the wild type. However, the slower repair kinetics in these mutants is not due to slower photochemical reactions. Using flash photolysis, we found that in both mutants only half of the dimers were bound as opposed to wild type, in which all dimers are bound under our experimental conditions (data not shown). When the repair kinetics was normalized to the fraction of dimers bound at any given moment in vivo, the repair kinetics of mutants were superimposable with that of wild type. When we conducted the same type of experiments in vitro with

Table I: Binding, Spectroscopic, and Catalytic Properties of Mutant Photolyases

photolyase	K_A (M^{-1})	K_N[a] (M^{-1})	rel fluorescence at[b] 480 nm	rel fluorescence at[b] 505 nm	rel repair ϕ at[c] 366 nm	rel repair ϕ at[c] 384 nm
WT	2.2×10^9	2.8×10^5	1.0	0.78–1.0	1.0	1.0
W277R	8.2×10^6	1.5×10^6	1.03	0.97	0.9	0.9
W277E	1.3×10^6	2.4×10^5	0.98	1.24	0.9	1.0
W277F	1.7×10^9	ND	1.04	ND	0.9	ND
W277H	1.6×10^9	ND	0.76	ND	0.8	ND
W277Q	2.8×10^8	ND	1.01	0.72	ND	ND

[a] Expressed per base pair. [b] The relative fluorescence is expressed relative to wild type. Fluorescence measurements were conducted twice with the wild-type enzyme on two different preparations. The two values are given. The fluorescence of the mutants is expressed relative to the higher of the two values obtained for the wild-type enzyme. ND, not determined. [c] The quantum yields are expressed relative to wild type.

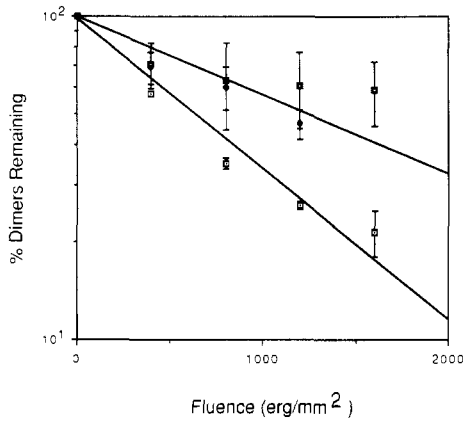

FIGURE 7: Rate of DNA repair by PL-WT and PL-W277R and PL-W277E in vivo. Cells irradiated with 2 ergs mm^{-2} of 254 nm (13 dimers per genome) were exposed to photoreactivating light doses indicated in the figure; samples were taken and plated. From the increase in survival the fraction of dimers repaired was calculated by assuming Poisson distribution of dimers and 1 dimer per cell to be lethal. Note that a single flash repaired 100% of the dimers in WT and only 50% of the dimers in both mutants (data not shown). (dotted square) WT-PL; (solid square with white dot) PL-W277R; (solid diamond) PL-W277E.

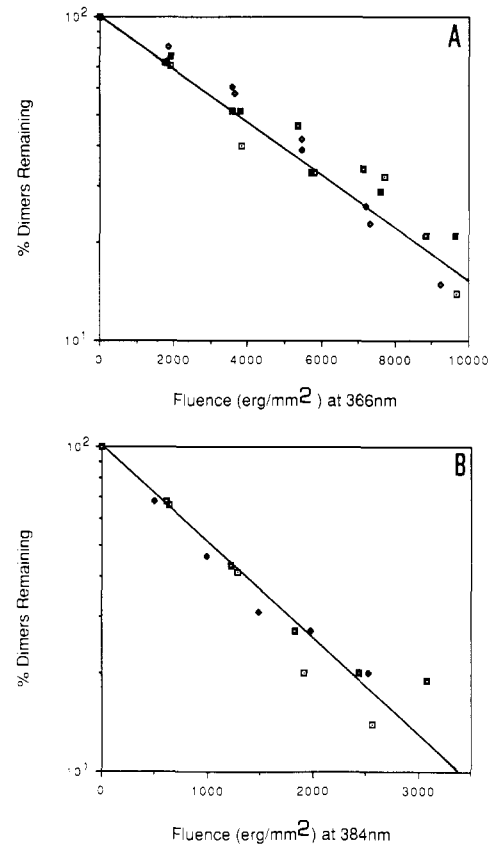

FIGURE 8: Rate of DNA repair by WT and mutant photolyases in vitro. Reaction mixtures containing 1 nM substrate and 5 μM photolyase (reduced form) were exposed to the indicated fluences at a fluence rate of about 20–40 ergs mm^{-2} s^{-1}, and at the indicated fluences samples were taken and the fraction of unrepaired dimer quantified by digesting with T4 endo V. (A) E-$FADH_2$ form of the enzymes; (B) holoenzyme (E-MTF-$FADH_2$). In all cases (mutant or WT) enzymes were in vast excess such that a single flash repaired 70% of all dimers both in WT and mutant enzymes. (dotted square) WT-PL; (solid diamond) PL-W277E; (solid square with white dot) PL-W277F; (solid diamond with white dot) PL-W277H; (solid square) PL-W277R.

E-$FADH_2$ and E-MTH-$FADH_2$ forms of the enzymes, the results shown in Figure 8 were obtained. There is no difference between the wild type and the mutants. Thus, we conclude that none of the mutations investigated affect the efficiency of energy transfer from folate to flavin or the rate of electron transfer between flavin and DNA substrate.

The DNA binding, photophysical, and photochemical properties of wild-type photolyase and of W277 mutants are summarized in Table I. It is clear from this table that W277 is at the DNA binding site and that this residue has little, if any, effect on binding of the flavin cofactor, the folate chromophore, or on energy transfer from folate to flavin and on electron transfer between flavin and DNA.

Discussion

Photolyases are relatively simple systems for studying energy and electron transfer in biomolecules. All photolyases characterized to date are proteins of M_r = 50000–60000 and contain two chromophores (Sancar & Sancar, 1988; Sancar, 1990). One of the chromophores (folate or deazaflavin) appears to function as an antenna, while the other ($FADH_2$) is thought to be responsible for electron transfer. Thus, photolyases offer a convenient system for investigating factors that influence energy and electron transfer in a protein milieu and electron transfer from protein to DNA. Furthermore, the quantum yield of electron transfer (repair of DNA), contrary to earlier claims (Eker et al., 1986; Sancar et al., 1987c), is not 1.0. It appears that the deazaflavin class repairs DNA with a quantum yield of 0.2 (Kiener et al., 1989) and the folate class repairs with a quantum yield of 0.5 (Payne et al., 1987). Therefore, in principle at least, it should be possible to obtain mutant proteins with either increased or decreased efficiency of electron transfer. This is in contrast to the photosynthetic reaction center where various electron-transfer reactions occur with a quantum yield of 1.0, and as a consequence only mutants with lowered quantum efficiency can be obtained (Bylina & Youvan, 1988).

By sequence comparison among photolyases, a region of high homology, W277–Y281, was identified as a likely active-site region in *E. coli* photolyase. We isolated mutations affecting four of five residues by doping mutagenesis, and some of the mutant proteins had interesting in vivo properties. For ex-

ample, PL-R278L appeared to have normal DNA binding (determined by flash photolysis) but had a 5-fold lower $\epsilon\phi$ compared to wild type. However, the protein was not overproduced and therefore was not amenable to in vitro characterization. In contrast, W277R mutant was reproducibly overproduced at levels comparable to wild type and was soluble and easily purifiable. The mutant protein had 270-fold less affinity for thymine dimer and 5-fold higher affinity to nondimer DNA compared to wild-type protein. These figures result in a more than 3 orders of magnitude decrease in selectivity, which is defined as the ratio of specific-to-nonspecific binding (Von Hippel & Berg, 1986). Replacement of Trp with an acidic amino acid in PL-W277E had the same effect of the specific binding affinity (1700-fold lower compared to wild type), but it also marginally reduced nonspecific binding. Thus, the overall changes in selectivities of the two mutants are comparable. The simplest explanation of these results is that W277 is in the DNA binding site of photolyase and it contributes to binding by van der Waals interactions or intercalation, most likely the former. Introducing a positive charge at this location decreases specific binding by loss of the aromatic ring and increases nonspecific binding by providing a charge that would favorably interact with the phosphodiester backbone. Introducing a negative charge, on the other hand, should decrease specific binding by the same mechanism while reducing nonspecific binding as well, by its unfavorable interaction with the phosphodiester backbone. The fact that PL-W277F has the same properties as the wild type and that PL-W277H and PL-W277Q have moderately lower affinity than the wild type is entirely consistent with this conclusion.

Hélène and co-workers (Hélène & Maurizot, 1981), using model peptides of the sort Lys-Trp-Lys, have shown a two-step binding to DNA whereby the tripeptide initially binds by ionic interactions of Lys residues followed by a tighter binding which involves intercalation of the Trp indole ring. Intercalation of aromatic residues is also the main driving force in the binding of single-stranded DNA binding proteins, M13 gene 5, *E. coli* SSB, and T4 gene 32 protein (Chase & Williams, 1986). More recently, evidence has been obtained that T4 endonuclease V, which like photolyase binds to pyrimidine dimers, binds to DNA by intercalation superimposed on ionic interaction. Site-specific mutagenesis studies indicate that the Trp-Tyr-Lys-Tyr-Tyr pentapeptide is important for recognition of substrate and catalysis of a β-elimination reaction by this enzyme (Dodson & Lloyd, 1989). Thus, it is quite likely that W277 contributes to specificity by intercalating into DNA. It is worth pointing out, however, that ionic interactions play only a minor role in photolyase binding to DNA and therefore the binding cannot be approximated by Hélène's tripeptide model. Indeed, R278C mutation had no detectable effect on the binding or the photochemistry of photolyase (data not shown), even though this residue is conserved in all photolyases sequenced to date. Clearly, more studies are needed to define the exact mechanism of photolyase binding.

We find it quite remarkable that in all the mutants investigated the quantum yield of interchromophore energy transfer as well as of intermolecular electron transfer did not change. In W277R and W277E mutants there are positive and negative charges, respectively, at the protein DNA interface, yet the dimer is repaired with the same efficiency. It is well established that the rate of electron transfer is very sensitive to polarity and charge of the transfer pathway (Closs & Miller, 1988), yet obviously these charges do not affect the rate of transfer in photolyase. It is worth noting that the main binding determinants of DNA are confirmed to two bases on either side of the dimer (Husain et al., 1987), and therefore the binding and catalytic centers (if they can be called such) cannot be more than 10–15 Å apart. Clearly, isolation of additional mutants that affect DNA binding as well as of mutants which alter the photochemical step(s) without affecting DNA binding will help define more precisely how photolyase binds to and repairs DNA.

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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-deazaFAD_{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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