Active Site of DNA Photolyase: Tryptophan-306 Is the Intrinsic Hydrogen Atom Donor Essential for Flavin Radical Photoreduction and DNA Repair in Vitro†

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ABSTRACT: DNA photolyases repair cyclobutadipyrimidines (Pyr〈〉Pyr) in DNA by photoinduced electron transfer. The enzyme isolated from *Escherichia coli* contains methenyltetrahydrofolate (MTHF), which functions as photoantenna, and $FADH_2$, which is the redox-active cofactor. During purification, $FADH_2$ is oxidized to the blue neutral radical form, $FADH^•$, which has greatly diminished activity. Previous nanosecond flash photolysis studies [Heelis, P. F., Okamura, T., & Sancar, A. (1990) *Biochemistry 29*, 5694-5698] indicated that excitation of $FADH^•$ either directly by absorbing a photon or indirectly by electronic energy transfer from MTHF excited singlet state yielded an $FADH^•$ quartet which abstracted a hydrogen atom from a nearby tryptophan to generate the catalytically competent $FADH_2$ from of the enzyme. Using site-directed mutagenesis, we replaced all 15 photolyase tryptophan residues by phenylalanine, individually, in order to identify the internal hydrogen atom donor responsible for photoreduction. We found that W306F mutation abolished photoreduction of $FADH^•$ without affecting the excited-state properties of $FADH^•$ or the substrate binding ($K_A \sim 10^9$ M^{-1}) of the enzyme. The specificity constant (k_{cat}/K_m) was ~ 0 for the mutant enzyme in the absence of reducing agents in the reaction mixture, indicating that photoreduction of $FADH^•$ is an essential step for photorepair by photolyase in vitro. Chemical reduction of $FADH^•$ of the mutant enzyme restored the specificity constant to the wild-type level.

Photolyases catalyze cycloreversion of pyrimidine dimers (Pyr〈〉Pyr)[1] generated in DNA by ultraviolet light and thus prevent the harmful effects of UV light. Catalysis is accomplished through photoinduced electron transfer between the enzyme's cofactor and the substrate (Sancar & Sancar, 1988; Sancar, 1991). All photolyases characterized to date contain a flavin adenine dinucleotide (FAD) cofactor and either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin as the major light-gathering chromophore (Johnson et al., 1988; Eker et al., 1988). The active form of the flavin cofactor is thought to be $FADH_2$ (Payne et al., 1987; Sancar et al., 1987a). However, this cofactor is quantitatively oxidized to the semiquinone form in the *Escherichia coli* (Jorns et al., 1984; Payne et al., 1987) and *Streptomyces griseus* (Eker et al., 1990) enzymes during routine purification procedures. The same occurs in *Saccharomyces cerevisiae* (Sancar et al., 1987b) and *Methanobacterium thermoautotrophicum* (Kiener et al., 1989; Payne & Sancar, 1990) photolyases under somewhat harsher purification and storage conditions.

In *E. coli* photolyase, the E-$FADH^•$-MTHF form of the enzyme repairs DNA as efficiently as the E-$FADH_2$-MTHF form under saturating light and enzyme turnover conditions (Jorns et al., 1987; Sancar et al., 1987a) when 366 nm is used as the light source and the reaction is carried out in buffers containing reducing agents such as DTT. However, the quantum yield of repair by the radical form of the enzyme is 1/10th-1/15th of that achieved in vivo (Sancar et al., 1987a) and is drastically improved when $FADH^•$ is converted to $FADH_2$ (Payne et al., 1987; Payne & Sancar, 1990). Furthermore, when $\lambda > 425$ nm was used as the photoreactivating light to excite the flavin radical selectively (as neither MTHF nor $FADH_2$ absorbs at these wavelengths), only borderline activity was observed (Payne et al., 1987) which could have been caused by stray light of shorter wavelength. In addition, it was observed that illumination of E-$FADH^•$-MTHF resulted in photoreduction of E-$FADH^•$ to E-$FADH_2$ by an internal electron donor; photoreduction occurred even in the absence of MTHF, and therefore it was concluded that MTHF was not the electron donor (Heelis & Sancar, 1986; Heelis et al., 1987).

Difference spectra obtained by nanosecond flash photolysis revealed an absorption peak at 510 nm with $\Delta\epsilon \sim 2000$ M^{-1} cm^{-1} at 4 μs after the flash which was ascribed to a Trp neutral radical on the enzyme, and it was proposed that the immediate electron donor in photoreduction was a Trp residue which following photooxidation was reduced by the reducing agents in the medium (Heelis et al., 1990). The quantum yield for photoreduction of $FADH^•$ by Trp was estimated to be about 0.1. This is a relatively high quantum yield for H-atom transfer and would indicate that the donor(s) is (are) in close proximity to $FADH^•$ at the active site of the enzyme. *E. coli* photolyase contains 15 Trp residues. We replaced each of these with Phe individually, in the hope of identifying the residue(s) acting as electron donor as Phe is incapable of functioning as such while it is isosteric with Trp and is expected to cause minor (or no) conformational change in the enzyme. We found that PL-W306F was incapable of photoreduction.

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[1] Abbreviations: MTHF, methenyltetrahydrofolate; WTPL, wild-type photolyase; PL-W306F etc., photolyase mutant with a Trp → Phe etc. change at amino acid 306; E-$FADH_2$-MTHF, photolyase holoenzyme; E-$FADH_2$ and E-$FADH^•$, photolyase containing reduced or radical flavin but not folate; respectively; Pyr〈〉Pyr and T〈〉T, pyrimidine dimer and thymine dimer, respectively; DTT, dithiothreitol.

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These studies provide independent and unambiguous evidence that a tryptophan is indeed the electron donor in photoreduction and that of the 15 investigated only 1 is involved in the process. Furthermore, we found PL-W306F to be inactive under standard assay conditions. We conclude that Trp-306 is the electron donor in photoreduction and that E-FADH$^{\bullet}$ is totally inactive in photorepair while it has normal conformation and substrate binding properties. These studies also show that photolyase is one of the rare biological systems where a tryptophan radical is generated during enzymatic catalysis.

Materials and Methods

Bacterial Strains and Plasmids. The *E. coli* strains CSR603Flaci^{Q}(*recA1 uvrA6 phr-1*), CJ236(*dut^{-} ung^{-}*), and DH5α(F′ *hsdR17 recA1*) used in this study have been described previously (Sancar et al., 1984; Kunkel, 1985). The source of the *phr* gene was plasmids pMS725 (Sancar et al., 1984) and pMS1310 (Li & Sancar, 1990). The plasmids used for site-specific mutagenesis were pIBI25 (International Biotechnology Inc.), and M13 phage and its derivative M13mp18 (Vierra & Messing, 1987).

Mutagenesis. The Trp residues of photolyase were replaced by Phe or other amino acids as required, by the site-specific mutagenesis method of Zoller and Smith (1983), and the mutant genes were selected for by the method of Kunkel (1985). These procedures as applied to site-specific mutagenesis in *phr* have been described elsewhere (Li & Sancar, 1990). In addition, we constructed a second plasmid, pUNC1991, for mutating the first Trp (W6) in the *phr* gene. This plasmid was obtained by inserting the *Pvu*II fragment encompassing the 369 amino-terminal amino acid encoding region of the *phr* gene into M13mp18. Once the W6F substitution was made in pUNC1991, the *Pvu*II fragment carrying the mutant *phr* sequence was isolated and exchanged with the wild-type fragment of pMS725 to obtain a plasmid overexpressing PL-W6F protein.

Enzyme Purification. Wild-type photolyase and PL-W306F and PL-W306Y were purified by successive chromatography on Blue Sepharose, AcA44 gel filtration, and hydroxylapatite resins to apparent homogeneity (Sancar et al., 1984, 1987c). The other purifiable mutant photolyases were not purified beyond the Blue Sepharose column step, which routinely yields enzyme of 95% purity. The concentration of the enzyme was estimated from the extinction coefficient at 580 nm, $\epsilon = 4444$ M^{-1} cm^{-1} (Payne & Sancar, 1990). The purification and storage buffers did not contain reducing agents; these were later added when necessary.

Spectroscopy. The absorption spectra were obtained with a Hewlett-Packard 8451A spectrophotometer and the fluorescence spectra with a Shimadzu RF5000U spectrofluorometer. Folate-supplemented photolyase (Hamm-Alvarez et al., 1989) and defolated photolyase (Payne et al., 1987) were used for absorption and fluorescence studies, respectively. The enzyme concentrations in fluorescence experiments were 2×10^{-5} M.

Photoreduction and Photodecomposition. Photoreduction of FADH$^{\bullet}$ and photodecomposition of MTHF were conducted as described previously using a Vivitar 2500 flash unit as the light source (Li & Sancar, 1990). The enzyme was in storage buffer (50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 50% glycerol, and DTT when indicated).

DNA Binding. The substrate binding properties of wild-type and mutant photolyases were investigated by the gel retardation assay using a 48-mer duplex with a centrally located T⟨⟩T as substrate (Husain & Sancar, 1987; Husain et al., 1988). The binding constants were obtained from Scatchard analysis of binding isotherms.

Quantum Yield of DNA Repair. We investigated the repair of T⟨⟩T with photolyase containing either one-electron-reduced (radical) or two-electron-reduced forms of the flavin cofactor.

For photorepair with the radical form of the enzyme, E-FADH$^{\bullet}$-MTHF was mixed with substrate at 5–10 μM enzyme and 1.0 nM substrate in reaction buffer containing 50 mM Tris·HCl, pH 7.4 (or 20 mM potassium phosphate buffer, pH 7.4), 100 mM NaCl, 1 mM EDTA, 100 μg/mL bovine serum albumin, and 0, 10, or 20 mM DTT as indicated. The 200-μL reaction mixture was placed into a cuvette and exposed to photoreactivating light of 366 nm at fluence rates of 20–40 erg mm^{-2} s^{-1} using a Quantacount monochromator (Photon Technology International). Samples were withdrawn after various fluences and processed as follows. The DNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended in 20 μL of buffer containing 50 mM Tris·HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, and 100 μg/mL bovine serum albumin, digested with T4 endonuclease V, and separated on a DNA sequencing gel as described previously (Li & Sancar, 1990). The fraction of undigested DNA was a measure of the extent of photorepair. The quantum yield for the E-FADH$^{\bullet}$-MTHF form of the enzyme was calculated by assuming ϵ(366 nm) = 29 149 M^{-1} cm^{-1} (Payne & Sancar, 1990).

For photorepair with reduced photolyase, defolated enzyme was placed in an anaerobic cuvette, reduced with dithionite, mixed with substrate, and exposed to photoreactivating light anaerobically as described in detail elsewhere (Payne & Sancar, 1990). The extent of repair was quantified by digestion of deproteinized DNA with T4 endonuclease V. The extinction coefficient of E-$FADH_2$ at 366 nm was taken to be $\epsilon = 5680$ M^{-1} cm^{-1} for E-$FADH_2$ (Payne & Sancar, 1990).

Transient Absorption Spectroscopy. The transient spectra of wild-type and mutant photolyases were obtained with a nanosecond flash photolysis system based on a JK Lasers System 2000 Nd^{3+}YAG laser emitting 10–300-mJ pulses of 532 nm and 20-ns duration. The transient spectra were recorded on a Phillips PM3111 oscilloscope and then transferred to a microcomputer for storage and analysis (Heelis & Sancar, 1986).

Results

Flash photolysis studies have shown that E-FADH$^{\bullet}$ is reduced to E-$FADH_2$ by abstraction of an electron by the flavin excited-state quartet from an internal electron donor (Heelis & Sancar, 1986). Difference spectroscopy revealed a transient species with $\lambda_{max} = 510$ nm which was ascribed to the Trp neutral radical (Heelis et al., 1990). While the difference spectrum is quite reliable, it did not rigorously exclude other possible electron donors nor did it reveal any information as to which of the 15 photolyase Trp residues was the electron donor. To provide further evidence for the proposed scheme and to identify the specific Trp residue, we generated mutant photolyases in which 1 of the 15 tryptophans was substituted with Phe and the properties of the mutant enzymes were investigated in vivo and when possible in vitro.

Preparation of Mutant Photolyases. The sequences of photolyases from five different species have been determined (Takao et al., 1989; Sancar, 1990). Sequence comparison reveals 30–40% amino acid sequence homology between these widely divergent species. A simplified diagram of regions of homology is shown in Figure 1. There is a region of high homology in the carboxy-terminal half among all five proteins, which might be involved in DNA and/or flavin binding because enzymes from all these species contain $FADH_2$ (Sancar

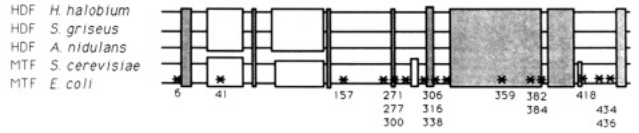

FIGURE 1: Amino acid sequence homology among DNA photolyases. The open boxes indicate regions conserved within each class; the shaded boxes represent areas conserved in both classes. The top three enzymes are of the 8-OH-deazaflavin class (HDF), and the bottom two belong in the folate class (MTHF). The positions of the Trp residues in *E. coli* photolyase are marked. The Trp residues conserved in all five photolyases are those at positions 277, 306, 316, 359, and 382 of the *E. coli* enzyme.

Table I: Properties of W → F Mutants of *E. coli* Photolyase

amino acid position photolyase	over-production	in vivo relative $\epsilon\phi$[a]	in vitro relative photoreduction rate[b]
PL-WT	+	1.0	1 (0.7–1.4)
W6F	–	0	ND[c]
W41F	+	0.98	1.1
W157F	+	0.98	0.7
W271F	–	1.10	ND
W277F	+	0.96	1.0
W300F	–	1.00	ND
W306F	+	1.00	0.0
W316F	–	1.05	ND
W338F	–	1.03	ND
W359F	–	0.89	ND
W382F	–	1.14	ND
W384F	+	1.10	1.3
W418F	+	1.00	1.1
W434F	+	1.00	1.4
W436F	+	1.00	0.9

[a] The $\epsilon\phi$ measurements at 384 nm were conducted in parallel with a strain containing the wild-type enzyme. [b] Photoreduction was conducted in the presence of 25 mM DTT. The two values given for the wild type are the rates obtained in two separate experiments. The rates of the mutants are relative to the average value for the wild type. [c] Not determined.

& Sancar, 1984; Eker et al., 1990) and appear to bind to DNA by the same mechanism (Husain et al., 1987; Kiener et al., 1989; Baer & Sancar, 1989). In contrast, the amino-terminal half appears to be conserved within each class of photolyase, the folate class (*E. coli* and yeast) and the deazaflavin class (*Anacystis nidulans*, *Streptomyces griseus*, and *Halobacterium halobium*) raising the possibility that this region might be involved mainly—but not exclusively—in second chromophore (folate or deazaflavin) binding.

The locations of the 15 Trp residues in *E. coli* photolyase are marked in Figure 1 to indicate their conservation across the spectrum. It is expected that a Trp residue important for maintaining the flavin in its reduced-functional state may be conserved in both classes of photolyases. Initially, we concentrated our efforts on Trp residues conserved in all five enzymes. This work resulted in the identification of W277 at the DNA binding site of *E. coli* photolyase (Li & Sancar, 1990). Therefore, we decided to replace each of the 15 Trp residues with the sterically similar Phe in an effort to find the potential electron donor in the photoreduction reaction.

The 15 mutant genes were constructed and expressed in the overproduction vectors pMS1310 and pMS725, and the mutant proteins were characterized in vivo and in vitro. The results are summarized in Table I. With the exception of PL-W6F, all mutants complement the photoreactivation-deficient strain *E. coli* CSR603. More significantly, all other mutant photolyases have the same photolytic cross section ($\epsilon\phi$) in vivo as the wild-type enzyme. This means that the replacement of any single Trp with Phe does not affect the

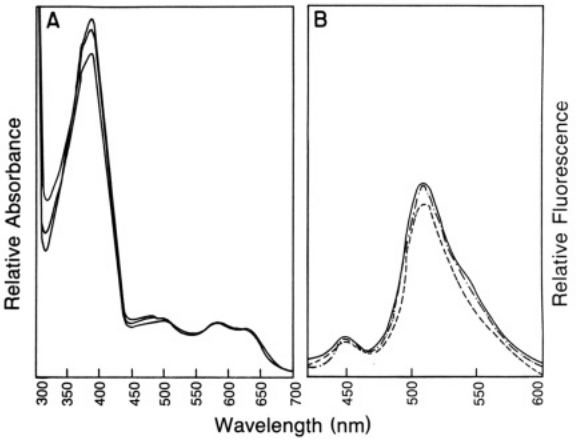

FIGURE 2: Absorption and fluorescence spectra of mutant photolyases. (A) Absorption of E–FADH•–MTHF (supplemented) form. The spectra have been normalized to the same magnitude at 580 nm. The actual absorbances at 580 nm were 0.1 for PL-WT, 0.12 for PL-W306F, and 0.14 for PL-W306Y. (B) Fluorescence spectra of E–$FADH_2$ forms. Defolated enzymes in storage buffer (20 μM) were reduced with 23 μM dithionite under anaerobic conditions. The excitation wavelength was 390 nm. Dashed line, PL-WT; dot–dashed line, PL-W306F; solid line, PL-W306Y.

photocatalytic properties of the enzyme, that is, the rate and efficiencies of interchromophore energy and intermolecular (enzyme–substrate) electron transfer. This is consistent with the fact that, in vivo, photolyase contains $FADH_2$ and therefore photoreduction is not a factor in the overall repair process.

Of the 15 Trp → Phe mutant genes that were constructed, 8 overproduced photolyase and were therefore purified and characterized, especially with regard to photoreducibility, in order to identify the H-donor Trp residue. All but PL-W306F were photoreducible, and with the exception of PL-W384F (which had lower affinity to DNA), all were similar to wild-type photolyase in all other aspects. PL-W306F was tentatively identified as the Trp residue acting as H donor in photoreduction. Other amino acid replacements were also made at this position, and the mutant proteins were characterized in greater detail.

Steady-State Spectral Properties of PL-W306F and PL-W306Y. Lack of photoreducibility of FADH• in PL-W306F is a "negative test" (Sivaraja et al., 1989); in order to gather more evidence for this residue as the electron donor, we wished to substitute W306 with other redox-active amino acids and find out whether we could achieve photoreduction with these amino acids as donors. We substituted tryptophan with either tyrosine or histidine at position 306 to obtain photolyases PL-W306Y and PL-W306H, respectively. PL-W306H complemented Phr^- phenotype in vivo but was not overproduced, and therefore its photochemical properties could not be investigated in vitro. Thus, we characterized PL-W306F and PL-W306Y only, with regard to their spectroscopic, photochemical, and biochemical properties.

First of all, PL-W306Y, like PL-W306F, was not photoreducible, and thus it failed to provide the "positive test" (Sivaraja et al., 1989) for the residue at position 306 being the electron donor. It was then necessary to characterize these mutants with regard to all other properties to ensure that the lack of photoreducibility was not the result of some overall conformational change.

The absorption spectra of the wild-type and mutant photolyases are shown in Figure 2A. The absorption at the 325–425-nm region is mostly due to MTHF and at $\lambda > 425$ nm is exclusively FADH•. As is apparent from the figure, the absorption spectra of all three enzymes are superimposable,

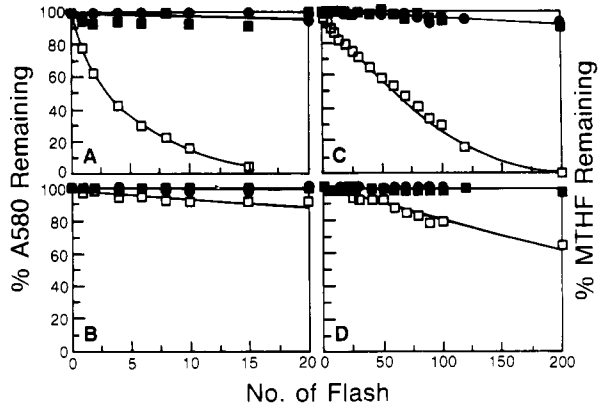

FIGURE 3: Photoreduction and photodecomposition. Photolyase in storage buffer at 2.5×10^{-5} M ($A_{580} = 0.11$) was exposed to camera flashes at 4–5-s intervals, and the absorbance at 384 and 580 nm was followed to monitor photodecomposition of folate and photoreduction of flavin, respectively. In the quantitation of photodecomposition, the contribution of FADH• and $FADH_2$ to the absorbance at 384 nm was taken into account by assuming that 20% of the absorbance at this wavelength is contributed by the flavin. This is the level of absorbance remaining at 384 nm after 200 flashes to wild-type enzyme in the presence of DTT. (A and C) The storage buffer contained 25 mM DTT. (B and D) Storage buffer without a reducing agent. Open squares, PL-WT; solid squares, PL-W306Y; solid circles, PL-W306F.

indicating that there is no gross conformational change in mutant enzymes which would have been expected to cause some change in the absorption of either or both chromophores. Similarly, the MTHF fluorescence spectra of all three enzymes (supplemented) were identical (data not shown). A quantitative comparison of the MTHF fluorescence of nonsupplemented enzymes is not reliable as the amount of MTHF in the enzyme varies widely between different preparations of the same mutant (Hamm-Alvarez et al., 1989). However, a quantitative comparison of the flavin fluorescence is feasible as this cofactor is associated with the apoenzyme at 1:1 stoichiometry under standard purification conditions. Furthermore, the flavin absorption and fluorescence spectra are quite sensitive to the environment, and a conformational change, should it exist, would be expected to affect these spectra. Therefore, we defolated the enzyme, reduced the flavin with dithionite, and compared the fluorescence properties of E–$FADH_2$ forms of the three enzymes. The results presented in Figure 2B show that the $FADH_2$ fluorescence is identical both qualitatively and quantitatively in the mutant and wild-type proteins. It appears that there are no gross changes in the conformations of mutant proteins and that any changes in photochemical and catalytic properties must be due to the specific effect of the W306 residue.

Photoreduction and Photodecomposition. The purified *E. coli* DNA photolyase is in the form of E–FADH•–MTHF. Exposure of this form of the enzyme to intense light results in photoreduction of FADH• with a quantum yield of ca. 0.1, and photodecomposition of MTHF to species which no longer bind to the enzyme, with a quantum yield of ca. 0.01 (Heelis et al., 1987; Li & Sancar, 1990). Figure 3 shows the effect of mutations at W306 on these two processes. In the presence of DTT, the FADH• radical of the wild-type enzyme is completely reduced with 15 camera flashes while no significant change in the FADH• absorption is seen with either mutant with up to 200 flashes (Figue 3A). Photoreduction involves electron abstraction by FADH• from an internal donor followed by reduction of the donor radical by reducing agents in the medium (Heelis et al., 1987, 1990). As the mutants fail to be reduced in the presence of DTT, we conclude that W306 is the electron donor in photoreduction.

Quite interestingly, the mutant enzymes fail to undergo folate photodecomposition as well (Figure 3C). This would indicate that folate photodecomposition involves a reductive reaction mediated by reduced flavin. This is in agreement with the suggestion made by Jorns et al. (1990), who arrived at this conclusion by comparing the rate of photodecomposition in enzyme containing folate only or both folate and flavin either in the radical form or in the $FADH_2$ state. In the absence of reducing agents, we did not observe photoreduction with wild-type or mutant enzymes as expected (Figure 3B). The folate photodecomposition in the absence of DTT is also interesting. With wild-type enzyme, the process is about 20% as efficient compared to that in buffers with DTT, whereas with the mutants photodecomposition is not detectable (Figure 3D). We interpret these data as follows: in the wild-type enzyme, flavin photoreduction by abstraction of a H atom from W306 generates $FADH_2$, which absorbs a second photon and leads to reductive photodecomposition of folate; because of the absence of reducing agents, the back-reaction from $FADH_2$ to Trp• is rapid, and therefore the photodecomposition is inefficient. In the mutants, because of lack of the internal H-atom donor, there is no transient formation of $FADH_2$ and, therefore, no photodecomposition.

This observation leads to the conclusion that $FADH_2$ is the obligatory electron donor in photodecomposition of MTHF. This conclusion differs from that of Jorns et al. (1990), who suggested that $FADH_2$ is not the obligatory electron donor but simply accelerates the reaction. Taken together, the results presented in Figures 2 and 3 are consistent with the notion that W306 is the internal electron donor involved in photoreduction of FADH•, an essential step for further photochemical reactions by the flavin: the physiologically important repair, and the perhaps physiologically irrelevant but experimentally significant photodecomposition of folate. Further evidence for the role of W306 came from nanosecond flash photolysis studies.

Laser Flash Photolysis. It has previously been shown that upon absorbing a photon the excited doublet state of FADH• is the earliest detectable species which is converted to the FADH• excited quartet state by intersystem crossing within 100 ps (Okamura et al., 1989). The quartet is long-lived ($\tau \sim 1$ μs) and abstracts an H atom from a Trp residue (Heelis et al., 1987, 1990). To ascertain that the photophysical properties of FADH• in mutant proteins were unchanged, we conducted transient absorption spectroscopy with a nanosecond flash photolysis system. Figure 4 shows the transient absorption spectra of PL-WT and PL-W306Y 500 ns after an 8-ns 532-nm laser pulse. (The transient spectrum of PL-W306F was also obtained, which showed essentially the same general features as the other two, but was of lower resolution and therefore has not been included in the figure.) As is apparent, the two spectra are quite similar and are typical of the FADH• quartet (Heelis et al., 1990). The differences at 420–500 nm are real and probably reflect the differences in the microenvironment as a result of the amino acid substitution. Indeed, the prominent 420-nm band observed in the wild-type enzyme has not been seen with model flavin neutral radicals (unpublished observation).

With wild-type enzyme, the quartet spectrum is followed by permanent depletion of absorption in the 450–650-nm region as a result of FADH• → $FADH_2$ conversion. This is seen in the oscilloscopic trace of the transient spectrum monitored at 580 nm shown in Figure 5A. The initial depletion due to the flavin quartet is followed by further depletion due to FADH• → $FADH_2$ at a rate of about 10^6 s^{-1} (Heelis et al.,

Table II: Binding, Spectroscopic, and Catalytic Properties of PL-W306F and PL-W306Y

photolyase	K_A (M^{-1})	relative rate: photodecomposition (MTFH)	relative rate: photoreduction ($FADH_2$)	relative rate: fluorescence ($FADH_2$)	quantum yield at 366 nm: PL-$FADH_2$	PL-$FADH^{\bullet}$-MTHF (DTT, mM): 0	10	25
WT-PL	2.9×10^8	1.00	1.00	1.00	0.48	3×10^{-2}	4.7×10^{-2}	6.4×10^{-2}
PL-W306F	3.2×10^8	0.06	0.00	1.16	0.42	$<10^{-5}$	1×10^{-3}	2.6×10^{-3}
PL-W306Y	2.5×10^8	0.07	0.05	1.19	0.41	$<10^{-5}$	6.6×10^{-4}	1.5×10^{-3}

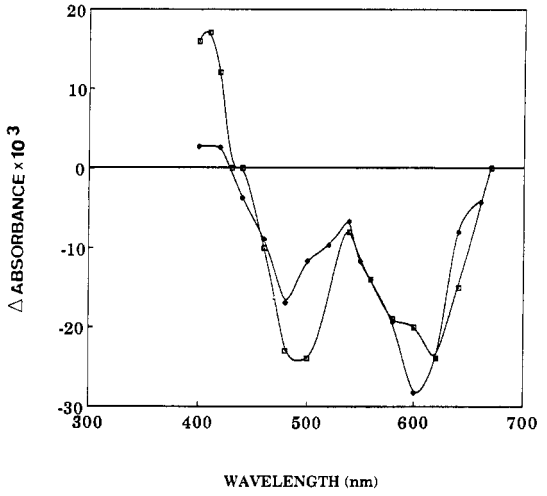

FIGURE 4: Laser flash photolysis of PL-WT and PL-W306Y. The spectra were generated by measurements made at the indicated wavelengths 500 ns after the laser pulse and have been normalized to the same absorption at 650 nm. The excitation wavelength was 532 nm, and the pulse duration was 8 ns. Open squares, PL-WT; solid diamonds, PL-W306Y.

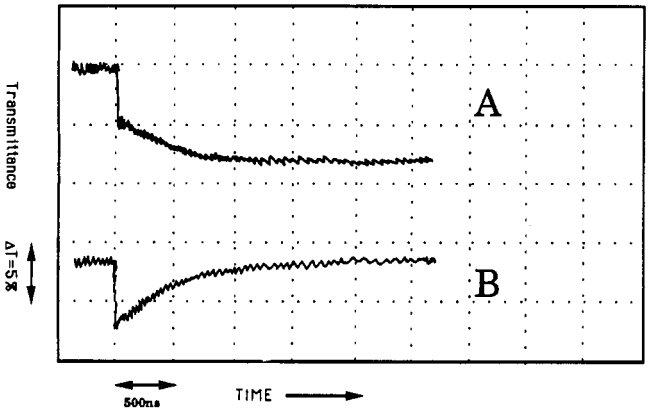

FIGURE 5: Oscilloscope traces of the absorbance at 580 nm for (A) PL-WT and (B) PL-W306F.

1990). In contrast, the PL-W306F transient spectrum shows that following depletion to a similar level as the wild type, the transient decayed back to the base line with a first-order rate constant $k \sim 2 \times 10^6$ s^{-1} without any further reaction (Figure 5B). Thus, the transient spectra provide evidence that quartet flavin, which abstracts an electron (H atom) from the internal donor, is produced and that the lack of photoreduction is solely due to the replacement of the H-atom donor W306 with amino acids which are incapable of doing so.

Repair with E–FADH• and E–$FADH_2$. Earlier work has suggested that photoreactivation observed with photolyase in vitro proceeds through two steps: first, photoexcited $FADH^{\bullet}$ is reduced with an internal electron donor which in turn is rapidly reduced by an electron donor such as DTT in the solvent; then, $FADH_2$ is exicted by a second photon either directly or via energy transfer from MTHF, and only then it repairs Pyr()Pyr by charge transfer. The availability of

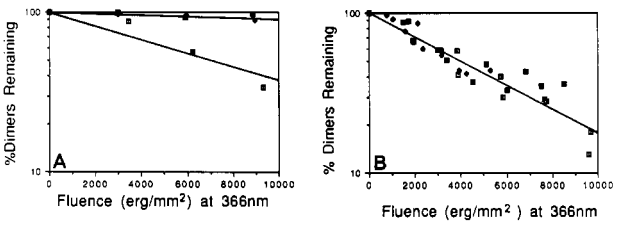

FIGURE 6: Rate of T()T repair by (A) E–$FADH^{\bullet}$–MTHF (in the presence of 10 mM DTT) and (B) E–$FADH_2$ forms of wild-type and mutant photolyases. Reaction mixtures containing 1 nM T()T substrate and 5 mM E–$FADH_2$ were exposed to the indicated fluences at a rate of 20 erg mm^{-2} s^{-1}, and the fraction of repaired DNA was quantified by digesting with T4 endonuclease V following deproteinization of the DNA by phenol extraction. Open squares, PL-WT; solid squares, PL-W306Y; solid diamonds, PL-W306F. Note that the extinction coefficients at 366 nm for E–$FADH^{\bullet}$–MTHF and E–$FADH_2$ are 2.9×10^4 and 5.7×10^3 M^{-1} cm^{-1}, respectively.

nonphotoreducible mutants made it possible to test this model by comparing the mutants with wild type.

For such a comparison, it was essential that we first characterized the binding properties of the mutant proteins. As shown in Table II, PL-WT, PL-W306F, and PL-W306Y have essentially the same specific binding constant to a T()T-containing substrate. Then, the relative rates of repair by the wild-type and mutant enzymes were measured in the presence and absence of DTT under conditions of enzyme excess so as to exclusively look at the photochemical step. In the absence of DTT, the PL-WT repaired T()T with a quantum yield which was dose-rate-dependent (biphotonic) and was $\phi = 0.03$ at a fluence rate of 100 erg mm^{-2} s^{-1} (M. A. Phillips and A. Sancar, unpublished observation). Thus, it appears that $FADH^{\bullet}$ does repair T()T, the possible sequence of events being that the $FADH^{\bullet}$ excited-state quartet abstract a H atom from W306 and the resulting $FADH_2$ absorbs a second photon before the back-reaction occurs, and repairs the dimer. In contrast, with the mutant enzymes, no photorepair was observed with steady-state illumination. About 15% of substrate was repaired with 600 camera flashes under conditions where a single flash repairs about 80% of the DNA with wild-type enzyme. Thus, the quantum yield of repair is $<10^{-5}$ with mutant enzymes under these conditions.

In the presence of DTT, the quantum yield of the wild-type enzyme increased with DTT concentration, consistent with the idea that reduction of the Trp radical by DTT prevents back-reaction and thus increases the efficiency of electron transfer to substrate by photoexcited $FADH_2$. In the presence of DTT, some low efficiency of repair is observed with mutant proteins as well (Figure 6A). However, the mechanism of repair is different. Excited-state $FADH^{\bullet}$ is directly reduced by DTT, and a second photon absorbed within the lifetime of $FADH_2$ results in photorepair. The more efficient repair observed with higher DTT concentrations is consistent with a pseudo-first-order reaction of DTT with the flavin quartet. Finally, the nearly 2-fold difference observed between the quantum yields of repair by PL-W306F and PL-W306Y is reproducible, and may be taken as further evidence that Tyr does not act as an electron donor in this system and perhaps

interferes with reduction of excited flavin by reducing agents in the medium, by a subtle effect on the flavin's accessibility to the solvent.

In contrast to these differences observed between the wild-type and mutant proteins, no such difference was observed when the flavin cofactor was reduced chemically by treatment with dithionite (Figure 6B). These data along with the identical efficiencies of in vivo repair (Table I) further support our conclusion that W306 is the tryptophan involved in photoreduction of flavin radical in vitro and that the activity changes observed with the purified enzyme are strictly due to the specific role of this residue in photoreducing radical flavin.

Discussion

The main goals of our work were to provide independent evidence for the conclusion based on flash photolysis that a Trp residue was the internal electron donor in photoreduction of photolyase FADH• chromophore, and to identify the specific Trp residue which becomes oxidized in the process. Our results show that the internal electron donor is indeed a Trp residue and that W306 is the specific Trp which donates the H atom to FADH•. Recently, redox-active amino acids have been implicated in a number of enzymatic and electron-transfer processes (Stubbe, 1988, 1989; Prince, 1988; Prince & George, 1990). It has been suggested that the most convincing way to identify the redox-active amino acid in a particular system is to use a combination of several approaches: spectroscopic (EPR and transient absorption spectroscopy where possible); the "negative test" by site-specific mutagenesis whereby replacement of the suspected redox-active amino acid with redox-inactive amino acid would lead to functional inactivation and disappearance of the spectroscopic signal; and finally the "positive test" of site-specific mutagenesis where a redox-active amino acid is replaced with another redox-active amino acid (Tyr → Trp) and the resulting protein retains activity but now exhibits a spectroscopic (e.g., EPR) signal typical of the replacement (Sivaraja et al., 1989; Babcock et al., 1990).

Our previous work (Heelis et al., 1990) yielded a transient spectrum consistent with a Trp radical and in addition provided evidence against other redox-active amino acids by the pH independence of the transient radical spectrum. In this study, we have conducted the "negative test" which has confirmed our earlier conclusion and identified W306 as the H-atom donor. However, our "positive test", which consisted of searching for a sign of the Tyr radical upon substitution of W306 with Tyr, failed to produce supporting evidence. We have previously shown that Tyr can reduce the Trp radical generated during photoreduction and therefore is thermodynamically capable of reducing FADH• as well (Heelis et al., 1990); this is to be expected as the redox potentials of TyrOH/TyrO• and TrpH/Trp• pairs are 0.94 and +1.04 V, respectively (DeFilippis et al., 1989).

We think that the failure of FADH• to be reduced by Tyr in PL-W306Y is due to some subtle steric effect which requires precise geometry for H-atom transfer. This is evident in the quartet spectra of the mutant proteins; although their general features are similar to those of the wild-type enzyme, their fine structures are different. Therefore, even though our "positive test" failed, we believe that all our data taken together make a very strong case for W306 being the H-atom donor. If that is the case, then W306 must be quite close to the flavin for such an efficient transfer, and therefore we could justifiably state that W306 is at the active site of the enzyme. Aromatic amino acids are known to be involved in binding flavins in flavoproteins (Karplus et al., 1991), and W306 might function as such. However, we have no evidence that it binds FADH• via H bonds or van der Waals contacts, as it does not appear to perturb the absorption spectrum of the radical; the FADH• spectra are nearly identical in PL-WT, PL-W306F (Phe has no H-bonding ability), and PL-W306Y. In addition to this main conclusion, the mutants we have generated have enabled us to make the following interesting observations regarding the structure–function of photolyase.

First, the availability of nonphotoreducible photolyases has made it possible to observe a "pure" quartet spectrum of FADH•. Our previous spectra were compounded by the relatively rapid quenching by W306, and, therefore, even the spectra recorded at earliest time points were partially quenched and contained some component of the Trp• spectrum. Since no reductive decay occurs in PL-W306Y, the spectrum of the FADH• quartet is closer to the "pure" flavin quartet spectrum, though such spectra are always going to be affected by the specific protein context and will differ from that obtained with a model FADH• compound (P. F. Heelis and A. Sancar, unpublished results).

Second, the nonreducible mutants enabled us to definitively answer the question of whether repair by the E–FADH• form of photolyases was possible. We showed that repair by the "radical" did occur only in the presence of W306, which provided unambiguous evidence to our previous contention (Sancar et al., 1987a; Payne et al., 1987) that repair by E–FADH• was actually photoreduction of E–FADH• followed by repair by E–$FADH_2$. The mutants PL-W306Y and PL-W306F, which cannot be photoreduced, cannot repair DNA. This is partly overcome by high concentrations of a reducing agent in the medium which further supports the original model for the sequence of reactions leading to repair by the E–FADH• form of the enzyme.

Third, the nonphotoreducible mutants have also helped us understand a little better the events leading to photodecomposition of folate. This phenomenon is characterized by conversion of folate to species which do not absorb in the near-UV (Heelis et al., 1987), but the products are not 5- or 10-formyltetrahydrofolate which result from reducing the 5,10-methenyl bridge such as observed with cyanoborohydride (Hamm-Alvarez et al., 1989). However, photodecomposition appears to be involved in a reductive reaction because it occurs more slowly in the E–FADH•-MTHF form of the enzyme compared to the E–$FADH_2$–MTHF form, even though it did occur in the complete absence of $FADH_2$ at 20% of the rate observed with holoenzyme, leading to the conclusion that $FADH_2$ was not essential for but accelerates photodecomposition (Jorns et al., 1990). It could be argued that the less efficient photodecomposition with E–FADH•–MTHF was due to the extremely high efficiency of energy transfer from MTHF to FADH• in this form of the enzyme compared to the E–$FADH_2$–MTHF form. We find no photodecomposition with the mutants and therefore conclude that $FADH_2$ is an obligatory intermediate in reductive photodecomposition of MTHF. (Naturally, MTHF, like any other chromophore, will eventually photodecompose by extreme exposure to light by other pathways.) This is interesting as it provides evidence for electron transfer from $FADH_2$ to MTHF, contrary to what we originally suggested as a possible role of MTHF when the roles of the two chromophores were first addressed (Sancar et al., 1987a). It is now well established that MTHF transfers energy to flavin (Okamura et al., 1989) and the excited-state flavin decays mainly through electron transfer to Pyr⟨⟩Pyr and therefore in all likelihood the electron transfer from $FADH_2$ to MTHF is a low quantum yield side reaction with no mechanistic significance. Indeed, this phenomenon does

not occur in the yeast DNA photlyase which in all other aspects is very similar to the *E. coli* enzyme (Sancar et al., 1987b; Payne & Sancar, 1990).

Third, in our search to identify the Trp responsible for photoreduction, we previously identified W277 as a residue important for DNA binding. Mutations at this site lowered k_{cat}/K_m (the specificity constant) drastically under limiting light. It is interesting to compare the W277 mutants with those of W306 as an illustration of how the specificity constants can be reduced by affecting one or the other of the reaction parameters. In W277R, k_{cat}/K_m is 300-fold lower than for wild type in the presence of DTT and under limiting light ($K_s = K_m$) because of the 300-fold higher K_s of the mutant. In contrast, in W306, the specificity constant, under the same conditions, is about 100-fold lower than that for wild type because of this level of decrease in the overall photochemical efficiency. Thus, one could say that PL-W277R is a pure K_m mutant while PL-W306Y is a pure k_{cat} mutant, though it should be kept in mind that the k_{cat} of photolyase is light intensity dependent and, therefore, the specificity constant of PL-W306Y may approach that of PL-WT at high enough light intensity.

Fourth, of 15 Trp residues in *E. coli* photolyase, 5 are conserved among all photolyases sequenced to date (5), yet replacements of any of these residues with Phe do not change the activity of the enzyme in vivo. However, our results indicate that these conserved residues have either structural or functional roles. Thus, photolyases with mutations in W316, W359, or W382 which do not affect the activity in vivo apparently have a structural role (Radzicka & Wolfenden, 1988) because the mutant proteins are not overproduced presumably because of decreased stability. In contrast, W277 and W306 appear to have mainly functional roles in the enzyme as mutations at these positions do not affect overproduction but interfere with either binding (W277) or photorepair in vitro (W306). In the same vein, we found the following observation quite interesting. PL-W384F had normal photochemical properties yet had a specific activity 100-fold lower than PL-WT as a consequence of lower affinity to substrate. Of five photolyases sequenced, only the yeast photolyase contains Phe instead of Trp at this position. Yet, the yeast enzyme has the same binding constant as the *E. coli* enzyme and appears to make the same contacts with DNA as probed by chemical footprinting (Baer & Sancar, 1989). Clearly, it cannot be said that W384 is at the DNA–protein interface in yeast photolyase. Thus, caution must be exercised in assigning specific functions to amino acids based on site-specific mutagenesis alone.

Finally, recently there has been an increased awareness in the roles of redox-active amino acids in catalysis (Stubbe, 1989; Prince & George, 1990). In addition to the redox function of Tyr in photosystem II, ribonucleotide reductase, and prostaglandin synthase, recent studies have revealed that Trp participates in the redox reaction of cytochrome *c* peroxidase (Sivaraja et al., 1989) and that the D2-Y160W mutation in the D2 polypeptide of photosystem II creates a redox-active Trp in the photosystem II core polypeptide D2 (Babcock et al., 1990). Our discovery of W306 as the electron donor in photoreduction of $FADH^{\bullet}$ in photolyase adds to the list of redox-active amino acids and provides only the second known redox-active Trp in a naturally occurring system, and should provide a useful system for investigating the paramagnetic properties of Trp in a protein context. As W306 is conserved in all photolyases, and as all extensively characterized photolyases appear to undergo flavin photoreduction, we expect this residue to perform the same function in other photolyases as well.

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