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Analysis of the Role of Intraprotein Electron Transfer in Photoreactivation by DNA Photolyase *in Vivo*†

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ABSTRACT: *Escherichia coli* DNA photolyase contains $FADH^-$ as the catalytic cofactor. The cofactor becomes oxidized to the $FADH^\bullet$ blue neutral radical during purification. The $E-FADH^\bullet$ form of the enzyme is catalytically inert but can be converted to the active $E-FADH^-$ form by a photoreduction reaction that involves intraprotein electron transfer from Trp306. It is thought that the $E-FADH^\bullet$ form is also transiently generated during pyrimidine dimer repair by photoinduced electron transfer, and it has been suggested that the $FADH^\bullet$ that is generated after each round of catalysis must be photoreduced before the enzyme can engage in subsequent rounds of repair. In this study, we introduced the Trp306Phe mutation into the chromosomal gene and tested the non-photoreducible W306F mutant for photorepair *in vivo*. We find that both wild-type and W306F mutant photolyases carry out at least 25 rounds of photorepair at the same rate. We conclude that photoreduction by intraprotein electron transfer is not part of the photolyase photocycle under physiological conditions.

Photolyase repairs UV-induced cyclobutane pyrimidine dimers (Pyr<>Pyr)[1] in DNA by photoinduced electron transfer using near-UV blue light (350−450 nm) as a cosubstrate (*1*, *2*). The *Escherichia coli* enzyme contains two chromophores or cofactors: methenyl tetrahydrofolate (MTHF) and FAD in the two-electron-reduced and deprotonated form ($FADH^-$). During purification of the enzyme under aerobic conditions, the flavin is oxidized to the FADH° blue neutral radical (*3*). The enzyme containing this form of the cofactor is inactive. However, if the $FADH^\bullet$ is chemically reduced by dithionite to $FADH^-$, the activity is restored (*4*). Alternatively, if the repair reaction is carried out in the presence of a reducing agent, such as dithiothreitol, the enzyme is capable of photorepair even though DTT does not reduce $FADH^\bullet$ in the ground state. Analysis of the repair reaction with the $E-FADH^\bullet$ form of the enzyme by a variety of methods revealed that the reaction catalyzed by this form of the enzyme proceeds in two steps (*5*, *6*). First, absorption of a photon by $FADH^\bullet$ directly or by MTHF followed by energy transfer to $FADH^\bullet$ generates the flavin radical excited state $^2(FADH^\bullet)^*$ that abstracts an electron from a Trp residue in the apoenzyme, resulting in formation of $FADH^-$ and $TrpH^{+\bullet}$. The tryphophan radical is reduced by electron uptake from the solvent. For repair to occur, the $FADH^-$ must absorb a second photon (or be excited by Förster energy transfer from MTHF that has been excited by a second photon). The resulting $^1(FADH^-)^*$ is highly reducing and

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[1] Abbreviations: PL, photolyase; Pyr<>Pyr, cyclobutane pyrimidine dimer; MTHF, methenyl tetrahydrofolate; WT, wild type.

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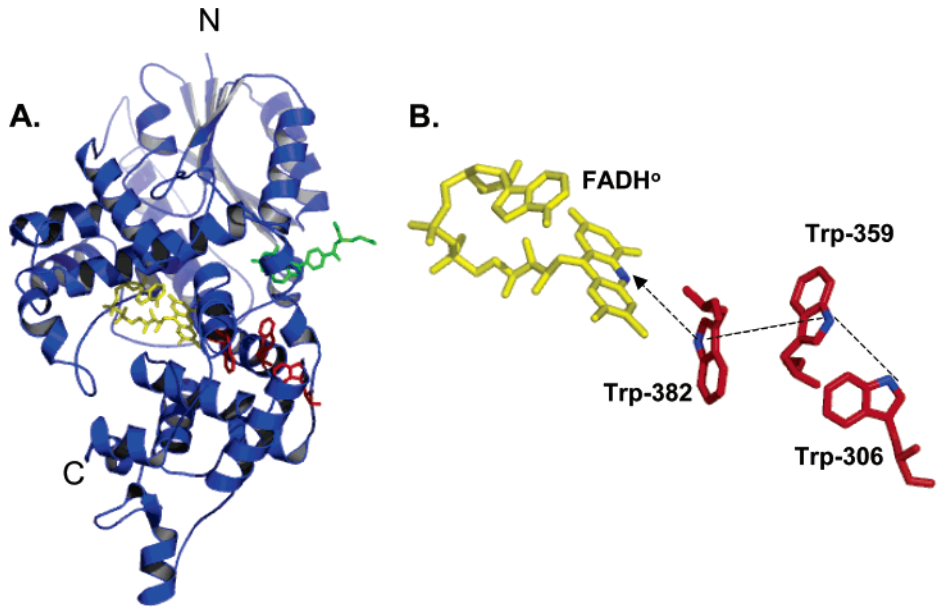

FIGURE 1: Structure of *E. coli* photolyase and location of the tryptophan triad implicated in photoreduction. (A) Ribbon diagram of the crystal structure (*10*): yellow for FADH•, green for MTHF, and red for the Trp triad. (B) Spatial relationship between the Trp triad and FADH• with a proposed pathway for electron transfer from Trp306 to ^{2}(FADH•)*.

transfers an electron to the Pyr<>Pyr to generate a charge-separated radical pair (FADH•···Pyr<>Pyr•⁻); the cyclobutane ring is split, and the flavin radical is presumed to be restored to the FADH⁻ form by back electron transfer.

Time-resolved absorption (*7*) and EPR (*8*) spectroscopy identified the intrinsic electron donor as a tryptophan. By site-specific mutagenesis, it was found that Trp306 (W306) was necessary for photoreduction of flavin, and therefore, W306 was identified as the primary electron donor in photoreduction (*7*). The photolyase W306F mutant (PL-W306F) has normal DNA binding properties *in vitro* but is catalytically inert and cannot repair the Pyr<>Pyr even in the presence of 25 mM DTT. However, the mutant enzyme can be activated by reducing FADH• with dithionite, demonstrating that the loss of activity in this mutant under standard assay conditions is due to its inability to be photoreduced (*7*). Because of such drastic effects of the W306F mutation on photoreduction and photorepair, it was concluded that Trp306 must be in the vicinity of FADH• (*7*). Indeed, electron abstraction from neighboring amino acids upon excitation of flavin by light is a common phenomenon in flavoproteins (*9*). Therefore, it was rather surprising when the crystal structure of photolyase revealed that Trp306 is located on the surface of the enzyme 13 Å from the flavin which is located in the core of the protein (*10*) (Figure 1A). Two potential pathways for electron transfer from Trp306 to FADH• were proposed (*10*). One pathway consisted of Trp306 → Trp359 → Trp382 → FADH• transfer and involved three electron hops through the "Trp triad" (Figure 1B); the other consisted of α-helix 15 between residues 358 and 366 and the side chain of Phe366. We suggested that photolyase constituted a good model system for studying long-range electron transfer reactions in proteins. However, we also suggested that this intraprotein electron transfer in photolyase had no physiological relevance because the W306F mutant, which is completely inactive under standard assay conditions *in vitro*, exhibited wild-type activity *in vivo* (*7*, *11*). We concluded that photolyase repairs the Pyr<>Pyr by a nonreductive cyclic electron transfer reaction (Figure 2A). Recent work has shed new light on the flavin radical and the intraprotein electron transfer involved in photoreduction (*12*−*17*), and some studies have ascribed a prominent role to this intraprotein electron transfer reaction in the actual repair reaction of photolyase and indeed in the photocycle of the entire photolyase/cryptochrome family (*14*, *15*, *18*). In fact, the conservation of the Trp triad in other members of the photolyase/cryptochrome family was taken to be evidence that these flavoproteins with no known photocycle possess a photosensory activity (*14*, *19*, *20*). These new studies necessitated a re-evaluation of the role of the Trp triad in the photolyase photocycle.

Previous work, which demonstrated that the W306F, W359F, and W382F mutants repaired the Pyr<>Pyr *in vivo* with the same quantum yield as the wild-type enzyme, was conducted with strains that overproduced photolyase (*7*). Because in that study the experiments were conducted under enzyme excess conditions, the repair rates and the quantum yields calculated from them were those of single-turnover reactions. Therefore, those experiments did not eliminate the possibility that after each repair reaction the enzyme was converted to the E−FADH• form. Thus, the following was a plausible scenario (Figure 2B). The FAD cofactor binds to the enzyme in the form of FAD_{ox} and is reduced to the FADH⁻ form by a flavin reductase. The E−FADH⁻ form of photolyase can carry out a single repair reaction only, and it is converted to the catalytically inactive FADH• form at the end of a repair cycle. With the wild-type enzyme, under standard assay conditions where the enzyme/substrate mixture in a buffer containing a reducing agent such as dithiothreitol or in the reducing environment of the cell is exposed to continuous illumination, the FADH• that is generated at the end of each photocycle is photoreduced through intraprotein electron transfer from Trp306. The continuous oxidation−photoreduction cycles enable the wild-type enzyme to carry out multiple rounds of repair. In contrast, according to this model, after the first round of

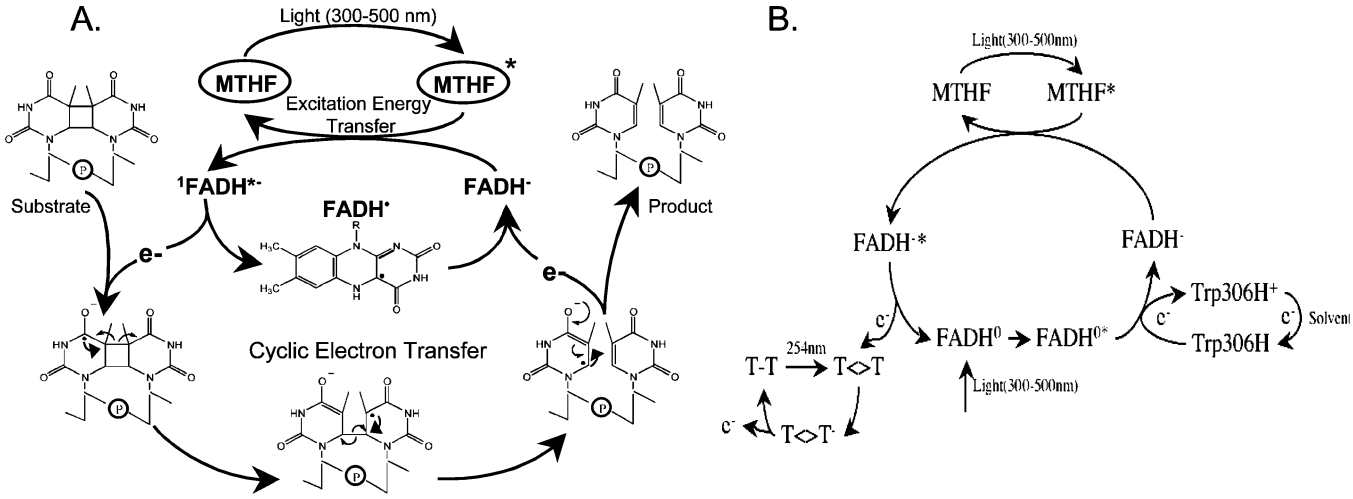

FIGURE 2: (A) Model for Pyr<>Pyr repair by cyclic electron transfer by photolyase (*2*). Following light-independent binding of substrate, a near-UV blue light photon is absorbed by the MTHF and the excitation energy is transferred to $FADH^-$ by fluorescence resonance energy transfer. The $^1(FADH^-)^*$ transfers an electron to Pyr<>Pyr, generating a charge-separated radical pair. The pyrimidine dimer radical undergoes bond rearrangement to monomers concomitant with back electron transfer to the flavin radical to generate the catalytically competent $FADH^-$. (B) Single-turnover model for Pyr<>Pyr repair by photolyase (*33*). In this model, the flavin is converted to the catalytically inert $FADH^•$ form at the end of each photocatalytic repair cycle. For subsequent repair reactions, the $E-FADH^•$ form of the enzyme must absorb a second photon and the excited-state flavin is reduced by long-range electron transfer from Trp306 to $FADH^•$ to generate the catalytically competent $E-FADH^-$ form of the enzyme. The tryptophan radical picks up an electron from reducing agents in the solvent.

catalysis, the PL-W306F−$FADH^-$ protein would be converted to the non-photoreducible PL-W306F−$FADH^•$ protein, which is incapable of further photorepair.

To address the issue of whether the $FADH^•$ photoreduction is an integral part of the photolyase photocycle, we introduced the W306F and W382F mutations into the *E. coli phr* gene and inserted the mutated *phr* genes into the bacterial chromosome of an *E. coli* strain with a *phr* deletion. The inserted genes carry their own promoters and therefore produce the mutant enzymes at a low constitutive level. Using this system, we show that *E. coli* K-12 contains 16−17 molecules of photolyase and that these 16−17 molecules repair at least 400 Pyr<>Pyr at the same rate whether the photolyase is the wild type or carries the W306F mutation. These results suggest that intraprotein electron transfer from W306 to $FADH^•$ is not a part of the normal photolyase photocycle.

MATERIALS AND METHODS

Bacterial Strains. E. coli K-12 strain UNC3112 (Δ*phr uvrA*::*Tn10*) carries a deletion that removes the entire *phr* gene and in addition has a *Tn*10 insertion into the *uvrA* gene rendering the strain totally defective in Pyr<>Pyr removal in both the dark and light (*21*, *22*). The strain is also deleted in the *kdp* operon that encodes potassium transport proteins, and therefore, this strain and its derivatives are grown in Luria broth or Luria agar that contains 5 g of KCl per liter. *E. coli* BW24304 which carries the *pir-116* mutation that enables plasmids with R6Kγ replication origin to replicate at high copy numbers was used as a host for cloning wild-type and mutant *phr* integration plasmids (*23*).

The pMS2 plasmid (5) was used as the source of the *phr* gene. The CRIM (conditional-replication, integration, modular) plasmid pAH153 was the vehicle for integrating Flag-tagged wild-type and mutant *phr* genes into the *E. coli* chromosome (*23*), and the CRIM helper plasmid pAH123 was used to integrate the CRIM plasmid into the Φ80 *att* site at 27 min of the *E. coli* chromosome (*23*).

Construction of E. coli Strains Expressing Epitope-Tagged Wild-Type and Mutant Photolyases. We wished to have *E. coli* strains containing either a wild-type or a mutant *phr* gene expressed from its own promoter to produce enzyme-limiting reaction conditions *in vivo*. We also wished to know the exact number of photolyase molecules in the cell so we could determine the number of Pyr<>Pyr repaired by a photolyase molecule under various experimental conditions. To this end, we used appropriate PCR primers to amplify from pMS2 a DNA fragment that carries 525 bp upstream of *phr* and terminates with the last codon of *phr* fused to the Flag tag epitope-expressing sequence. The sequences of the two primers are as follows: *phr*-F, 5′-GAGCGAGTGGGTACCTAAACCFCTCAGGAGCAGTAATAG; and *phr*-R, 5′-CTCTTGGTCTGCAGTTACTTGTCGTCATCGTCTTTGTAGTCTTTCCCCTTCCGCGCCGCCTCATACGC.

The PCR product was digested with PstI and KpnI and inserted into integration vector pAH153. The resulting plasmid carrying the wild-type *phr* gene was named pAH153-*phr*. This plasmid was grown in *E. coli* BW24304, purified using the Maxi prep kit (Qiagen), and used as a template for site-specific mutagenesis using the Strategene Quickchange kit to obtain plasmids with the Trp → Phe mutations in the apical and distal tryptophans of the tryptophan triad: pAH153-*phr*W306F and pAH153-*phr*W382F.

Strains containing a single copy of the *phr* gene expressing wild-type or mutant photolyases from the *phr* promoter were constructed using the CRIM plasmid/host system (*23*). First, the helper plasmid pAH123 carrying *IntΦ80* and *fir* genes was inserted into *E. coli* UNC3112. Then, competent cells of UNC3112/pAH123 were transformed with pAH153 (negative control), pAH153-*phr*WT, pAH153-*phr*W306F, and pAH153-*phr*W3882F. Following transformation by electroporation, cells were grown at 37 °C for 90 min and then at 42 °C for 30 min in Luria broth to stimulate integration of the plasmid at the single Φ80 attachment site in the UNC3112 chromosome. Cells were plated on LB agar containing tetracycline and gentamycin at 5 μg/mL each, and

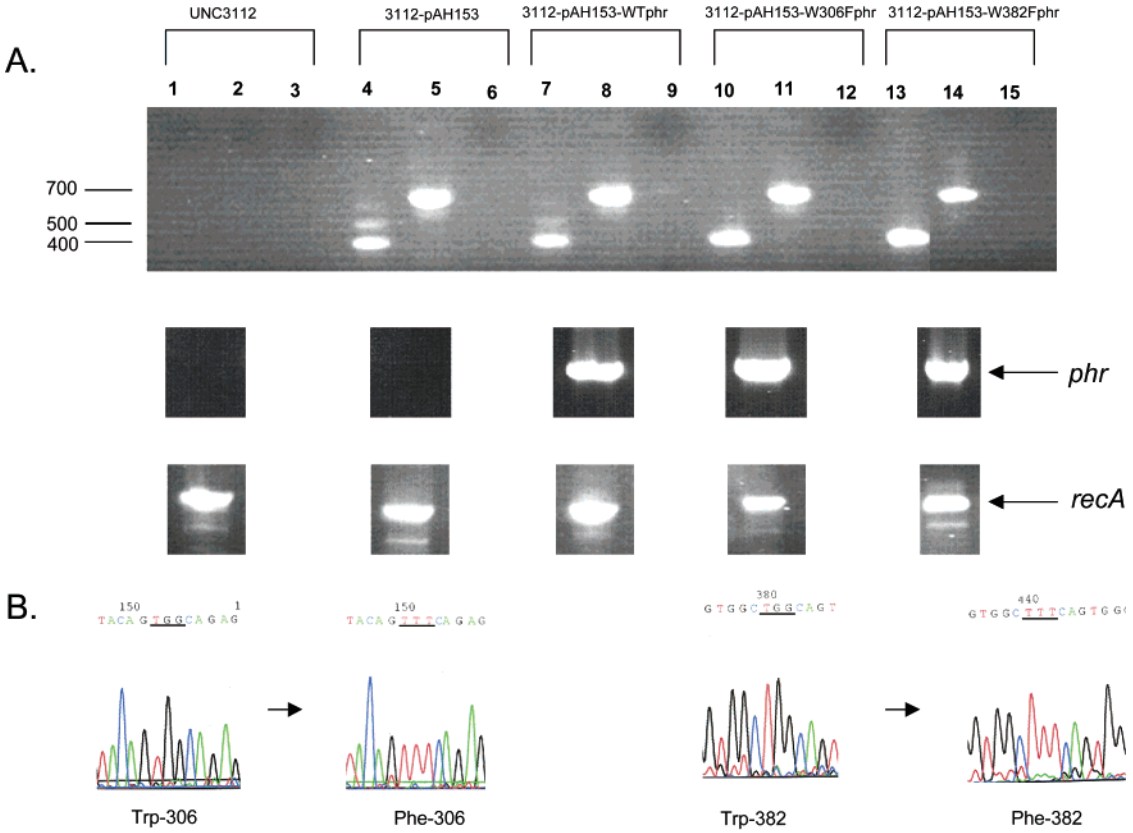

FIGURE 3: Single-copy integrations of WT and mutant *phr* genes into UNC3112 (Δ*phr uvrA::Tn10*). (A) PCR analysis of the integrated genes. At the top, primers P1 and P2 (lanes 1, 4, 7, 10, and 13), P3 and P4 (lanes 2, 5, 8, 11, and 14), and P2 and P3 (lanes 3, 6, 9, 12, and 15) reveal the presence of 400 and 750 bp fragments, respectively, with the first two sets of primers and no PCR product with the third set of primers, consistent with single *phr* integrants in the indicated strains (*23*). In the middle panel, genomic DNAs from the same strains were used as PCR templates to amplify the entire *phr* or *recA* genes to demonstrate the absence of *phr* in UNC3112 and its vector integrant and its presence in the other constructs; the *recA* gene was used as a positive control for the PCRs. (B) Sequence changes in the mutant strains. The ABI 3730 Genetic Analyzer sequencer readout for the regions around Trp306 and Trp382 for the wild-type and mutant integrant DNAs isolated by PCR in panel A is shown.

grown at 37 °C overnight. Single colonies were picked and analyzed for single *phr* integrants as described previously (*23*). In addition, two pairs of primers were used to amplify *phr* and *recA* (positive control), respectively, to confirm the lack of a *phr* gene in UNC3112 and its presence in pAH153-*phr* and its mutant derivative integrants.

Determination of the Number of Photolyase Molecules per Cell. Integrants of *phr*-Flag were used to determine the number of photolyase molecules per *E. coli* cell using Western blotting. *E. coli* UNC3112 derivatives containing Flag-tagged wild-type or mutant *phr* were used. Cells were grown in 1 L of LB containing 5 g of KCl at 37 °C for 16 h. Cells were diluted, and appropriate dilutions were plated to obtain the titer of each culture. The entire 1 L culture of each strain was centrifuged and resuspended in 20 mL of lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 10% (w/v) sucrose. Cells were lysed by sonication, and the cell debris was removed by centrifugation at 30000*g* for 30 min. The cell debris contained only a trace amount of photolyase that could be detected by Western blotting. The cell free extract was incubated with 100 μL of Flag resin (Sigma) for 1 h at 4 °C with gentle shaking. The resin was washed with lysis buffer (3 × 100 μL), and then the bound protein was eluted with 1 mL of lysis buffer containing 200 μg of Flag peptide. The PL-Flag was quantitatively eluted as there was only a trace amount of Flag-reactive material when the resin was probed by Western hybridization. To determine the amount of PL-Flag yield for the 1 L cultures, we used known quantities of Vc-Cry1-Flag protein (*24*) as a standard. The concentration of Vc-Cry1 can be accurately determined from its extinction coefficients at 278 and 380 nm and thus provides a reliable standard. The isolated PL-Flag proteins were separated on SDS−PAGE alongside the Vc-Cry1-Flag standards and subjected to Western blotting followed by detection by enhanced chemiluminescence. Standard curves were generated using ImageQuant. The amounts of PL-Flag per microliter of sample were estimated from the averages of the values obtained for each volume applied. From these values, the total amounts of PL-Flag per liter of culture were calculated. This amount was divided by the number of cells in 1 L of culture (7×10^{11} cells for the wild type and 5×10^{11} cells for W306F) to obtain the amount of photolyase per cell which was then converted to the number of photolyase molecules per cell.

Photoreactivation. We conducted dose-dependent UV killing and dose- and time-dependent photoreactivation experiments. A General Electric germicidal G75 lamp emitting mainly at 254 nm was the UV source, and photoreactivation was carried out with a Sylvania F15T8/BLB black light emitting mainly at 366 nm. The UV and black light fluence rates were measured using a UVX digital radiometer (Ultraviolet Products Inc.) fitted with the appropriate sensors. For UV radiation, 16 h cultures with a

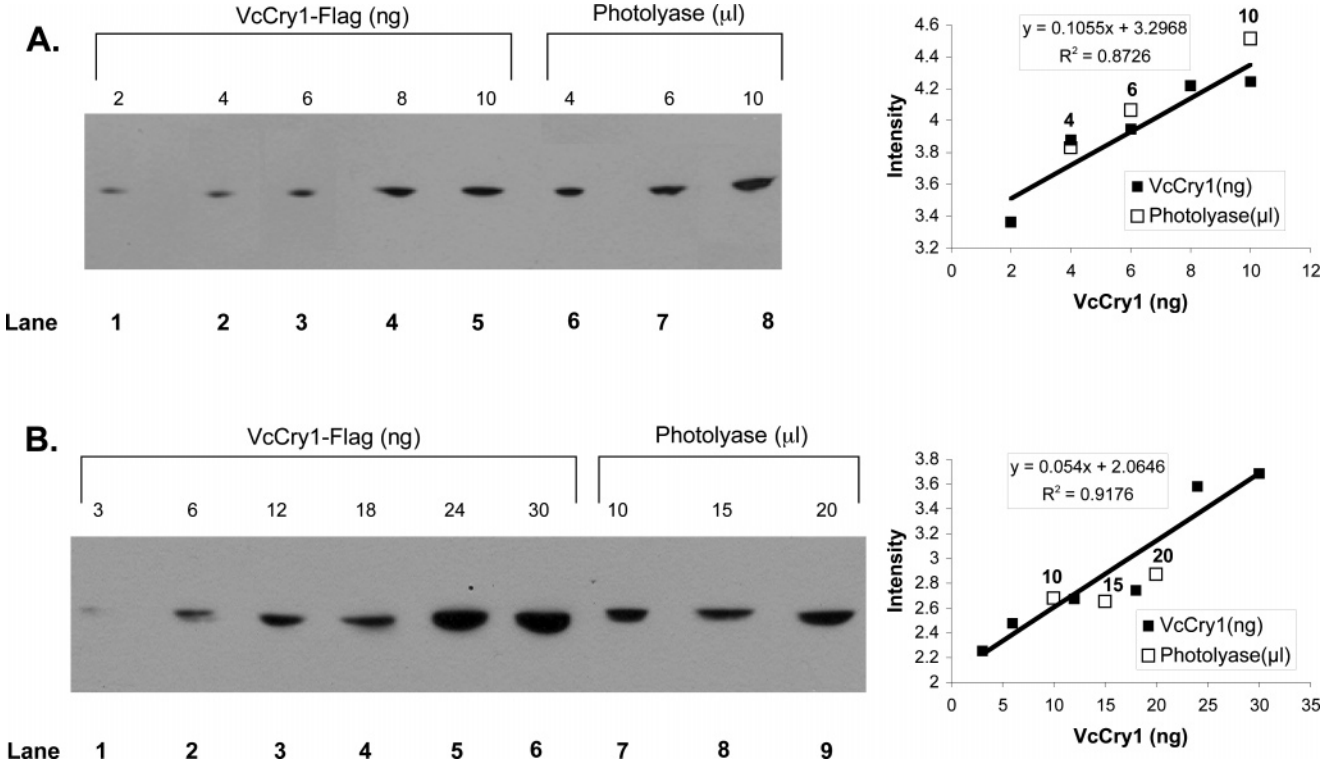

FIGURE 4: Quantitation of the number of photolyase molecules in an *E. coli* cell. Photolyase was isolated from cells expressing the Flag-tagged enzyme from the *phr* promoter, and the enzyme was affinity purified. The indicated volumes of the purified protein were separated by SDS−PAGE along with known amounts of Flag-tagged *V. cholerae* cryptochrome 1 and subjected to Western blotting using ECL: left, ECL of SDS−PAGE; right, quantitative analysis. The symbols represent the following: (■) standard in nanograms and (□) photolyase in microliters, indicated by numbers above the symbols. (A) PL-WT and (B) PL-W306F.

typical titer of 5−10 × 10^8 cells/mL were collected by centrifugation, washed with PBS, and resuspended in PBS at a 1/10 dilution. The cells were placed in a Petri dish and irradiated with appropriate doses of UV radiation with constant stirring on a magnetic plate. Samples were taken for determining survival at each dose. The fluence rate of 254 nm UV was 0.5 J m^{-2} s^{-1}. For photoreactivation, the plates were covered with the plastic cover of the Petri dish and two layers of window glass to filter out radiation below 300 nm. Photoreactivation was carried out at a fluence rate of 2.5 J m^{-2} s^{-1} of 366 nm for the indicated periods; appropriate dilutions of the photoreactivated samples or the "dark" controls (which consisted of cell suspensions in PBS in Petri dishes that were wrapped with aluminum foil and kept under the light along with the test samples) were made and plated. Following incubation for 28−30 h at 37 °C, the plates were counted and survival was calculated. All dilutions for survival determination were plated in triplicate. The UV killing and photoreactivation experiments were carried out under yellow light from General Electric "Gold" fluorescent light bulbs to prevent uncontrolled photoreactivation.

Calculation of the Number of Pyr<>Pyr Repaired by the Photoreactivation Treatment. It has been determined that 1 J/m^2 of 254 nm radiation introduces 50−65 photoproducts in the *E. coli* genome (*22*, *25*−*27*). At the moderate UV doses used in our experiments, 85% of these photoproducts are Pyr<>Pyr (*28*). Thus, the number of Pyr<>Pyr repaired in an *E. coli* cell after photoreactivation can be calculated from

$$\Delta D = D_{PR} - D_D$$

where ΔD is the dose decrement or the amount of the UV dose whose effect has been negated by photoreactivation, D_D is the UV dose that reduces cell viability to a certain level, and D_{PR} is the dose that is necessary to give the same viability as D_D but for cells that were exposed to photoreactivating light following UV irradiation (*26*). Because within the moderate UV dose range used in our experiments there is a linear relationship between dose and UV photoproducts induced (*22*, *25*, *27*, *28*), the approximate number of Pyr<>Pyr repaired per *E. coli* cell (assuming 1 J/m^2 produces 50 Pyr<>Pyr per cell) after a certain photoreactivation treatment is readily calculated from

$$\text{Number of Pyr<>Pyr repaired} = \Delta D\ (\text{J/m}^2) \times 50.$$

RESULTS AND DISCUSSION

Construction of E. coli Strains with Epitope-Tagged Wild-Type and Mutant phr Genes. To determine whether the Trp triad and $FADH^{\bullet}$ photoreduction played a role in the photolyase photocyle *in vivo*, we first Flag tagged the *phr* gene so that we could readily measure the number of photolyase molecules per cell by Western blotting. Then, we integrated both the wild-type and Trp triad mutant *phr*-Flag genes into the chromosome using the CRIM plasmid system (*23*). The genes were integrated into UNC3112, which is defective in excision repair and carries a deletion that removes the entire *phr* gene (*21*), to prevent problems that may arise from *phr* gene conversion and removal of Pyr<>Pyr by nucleotide excision repair. Thus, derivatives of this strain that contain wild-type or mutant *phr* gene are totally dependent on light-induced elimination of Pyr<>Pyr for UV survival.

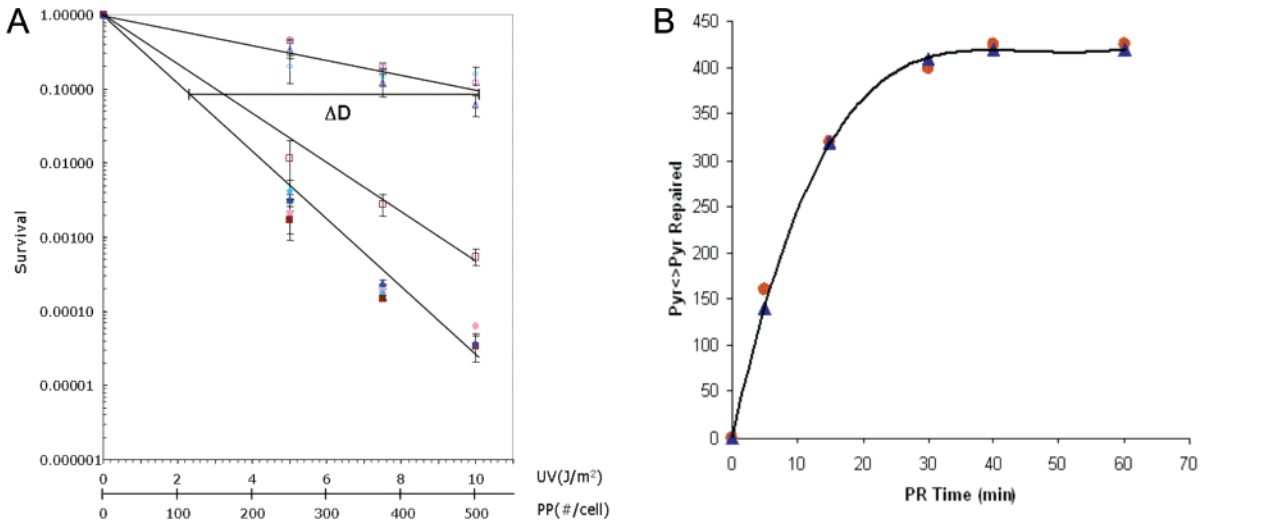

FIGURE 5: Photoreactivation by two of the Trp triad mutant strains. (A) Dose response. Cells from stationary phase cultures were collected, suspended in PBS, and exposed to 5, 7.5, or 10 J/m^2 of 254 nm UV radiation and photoreactivated for 60 min with black light emitting mostly at 366 nm. Note that because 1 J/m^2 of 254 nm radiation introduces ~50 photoproducts [~85% Pyr<>Pyr and ~15% (6−4) photoproducts], the units of the *x*-axis are given both in joules per square meter and in photoproducts per cell so that the number of photoproducts repaired by photolyase at various UV doses can be directly estimated from the graph: circle, WT; triangle, W306F; diamond, W382F; and square, UNC3112 (Δ*phr uvrA::Tn10*). Filled symbols represent data for no photoreaction; empty symbols represent data for 60 min photoreactivation. The residual photoreactivation seen in W3112 *(*Δ*phr*) is caused by the effect of light on cellular physiology by an unknown mechanism and is not associated with elimination of photoproducts from the chromosome (*22*). There was no statistically significant difference between the killing and photoreactivation of WT, W306F, and W382F strains, and therefore, a single line is drawn for the dark survival and a single line for the survival after photoreactivation of these strains. Δ*D* (dose decrement) is the number of joules per square meter of UV dose whose killing effect has been erased by photoreactivation and corresponds to 8 J/m^2 which is equivalent to ~400 Pyr<>Pyr per cell. (B) Photoreactivation kinetics. Cells were irradiated with 10 J/m^2 and photoreactivated for the indicated times. From the increase in the level of survival after photoreactivation, the number of dimers that were repaired was calculated (after correcting for the residual photoreactivation in the *phr* null mutant) and plotted as a function of photoreactivation time: circle, wild type; triangle, W306F.

Figure 3A shows the genetic characterization of UNC3112 and its *phr* integrants as analyzed by PCR. The top panel shows single-copy integration of the CRIM plasmid (*23*). The middle panel reveals the presence of the *phr* gene in the appropriate strains, and the bottom panel shows PCR of the *recA* gene as an internal control for the PCR conditions in the corresponding strains.

Figure 3B shows the sequences of the appropriate regions of *phr*-WT, *phr*-W306F, and *phr*-W382F in the integrated *phr* genes shown in Figure 3A. These data established that we have constructed strains that express the Flag-tagged PL-WT, PL-W306F, and PL-W382F photolyases from *phr*'s own promoter.

Wild-Type and Mutant Photolyases Are Expressed at a Level of 16−17 Molecules per Cell. To determine whether the photolyase Trp triad mutants can carry out only a single round or multiple rounds of repair, we sought to determine the number of photolyase molecules per *E. coli* cell. It has been reported that *E. coli* B contains 20 photolyase molecules per cell (*26*). However, that determination was based on flash photolysis which measures the number of active photolyase molecules only, and as a consequence has limitations with respect to the total number of fully active, partially active, and inactive photolyase molecules. Hence, in the *E. coli* UNC3112 derivatives we constructed, we determined the total number of apoenzymes, and thus the theoretical maximum number of photolyase active enzyme molecules, by Western blotting of Flag-tagged photolyase purified from strains expressing the protein from its own promoter.

Quantitative analyses of affinity-purified WT-PL and PL-W306F by Western blotting are shown in Figure 4. From the data in this figure, we calculate that UNC3112-Flag *phr* contains 1.1×10^{-6} g of photolyase per 7×10^{11} cells. Using a molecular mass of 5.4×10^4 Da for photolyase (*2*), we estimate (1.1×10^{-6} g of PL/5.4×10^4 g/mol of PL) × (6.023×10^{23} molecules/mole/7×10^{11} cells) = 17.2 molecules of photolyase per cell. In a similar manner, for PL-W306F, we obtained 7.3×10^{-7} g of photolyase per 5×10^{11} cells, from which we calculate 16.0 photolyase molecules per cell. We obtained similar values for PL-W382F; however, this mutant protein was prone to proteolytic degradation during cell lysis, precluding an accurate determination of the copy number. Nevertheless, the available data for PL-W382F suggest a number comparable to those of PL-WT and PL-W306F. It is interesting to note that Harm *et al.* (*26*) using flash photolysis as a functional assay reported that *E. coli* B has 20 molecules of photolyase per cell whereas *E. coli* K-12 contained 14 molecules per cell. UNC3112 is an *E. coli* K-12 derivative, and therefore, we find the value of 14 found by the functional assay and the value of 16−17 found by direct protein level measurement in remarkably good agreement. We consider the minor difference found by the two methods to be within experimental error and conclude that *E. coli* K-12 contains 16−17 molecules of photolyase per cell and that all enzyme molecules are active. For simplicity, we will use the number of 17 photolyase molecules per cell in the following analysis.

Catalytic Turnover of Wild-Type and Trp Triad Mutant Photolyases. Ultraviolet radiation (254 nm) introduces two major photoproducts in DNA, cyclobutane pyrimidine dimers and the (6−4) photoproducts, which constitute approximately 85 and 15% of the total photoproducts, respectively (*28*, *29*).

Furthermore, in *E. coli*, both lesions can be repaired by nucleotide excision repair, but photoreactivation can repair only Pyr<>Pyr (*29*). As a consequence, in a strain lacking nucleotide excision, only ~85% of the UV-induced damage can be repaired by photoreactivation. In other words, in such a strain, photoreactivation, at maximum, can erase only 85% of UV-induced damage and increase cell survival proportionally. Finally, according to several independent measurements, 1 J/m^2 of 254 nm radiation introduces ~40−50 Pyr<>Pyr in the genome of *E. coli* (*22*, *25*−*28*). Thus, Pyr<>Pyr repair can be accurately estimated from UV survival curves. Hence, to determine the number of Pyr<>Pyr that can be repaired by wild-type and Trp triad mutant photolyases, we subjected *E. coli* cultures of UNC3112 (*phr*$^-$ *uvrA*$^-$), UNC3112-*phr*WT, UNC3112-*phr*W306F, and UNC3112-*phr*W382F to UV irradiation and photoreactivation. The results are shown in Figure 5A. Photoreactivation for 60 min erases the effect of ~80% of the photoproducts (10 J/m^2 UV + photoreactivation yields approximately the same survival as 2 J/m^2 with no photoreactivation) in all three strains. This means that 17 photolyase molecules are capable of repairing nearly all of the Pyr<>Pyr caused by 10 J/m^2, which is ~400−500 Pyr<>Pyr, regardless of whether the enzyme is the wild-type form or mutated in the Trp triad tryptophans. If photoreduction were part of the catalytic cycle, the maximum number of Pyr<>Pyr repaired by the mutants would be equal to the number of photolyase molecules, or 16−17 per cell which is equivalent to ~0.3 J/m^2. This is clearly not the case, and these results, therefore, suggest that photoreduction of FADH$^\bullet$ through the Trp triad or by any other means is not a part of the photolyase photocycle.

We considered the possibility that photoreduction of FADH$^\bullet$ was in fact an integral part of the photocycle, but FADH$^-$ could also be regenerated by a flavin reductase in an alternate pathway. Such an alternate pathway is expected to be considerably slower than the photochemical reaction which is essentially a first-order unimolecular reaction. Hence, we performed photoreactivation kinetics with UNC3112 derivatives containing PL-WT and the PL-W306F Trp triad mutant. As seen in Figure 5B, wild-type and W306F mutant photolyases repair Pyr<>Pyr at identical rates of ~2 Pyr<>Pyr per photolyases per minute under our reaction conditions. Similar kinetics were also observed with the W382F mutant (data not shown). Thus, the data, which do not formally eliminate the possibility of an E−FADH$^\bullet$ intermediate that is reduced by a flavin reductase, strongly indicate that the photolyase photocycle does not generate FADH$^\bullet$ as an end product and that photoreduction of photolyase by intraprotein electron transfer from Trp306 is not part of the photolyase photocycle *in vivo* under physiological conditions. In line with this conclusion, we have found that an *E. coli* mutant lacking the two major flavin reductases, Fre (*30*, *31*) and NADPH-sulfite oxidoreductase (*32*), has normal capacity for overproducing the reduced form of photolyase.[2] Taken together with other *in vivo* and *in vitro* data on photoreactivation kinetics (*2*), these results indicate that photolyase repairs Pyr<>Pyr by a cyclic electron transfer reaction.

[2] S. Özgür and A. Sancar, unpublished observation.

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