Active Site of Escherichia coli DNA Photolyase: Asn378 Is Crucial both for Stabilizing the Neutral Flavin Radical Cofactor and for DNA Repair †

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Active Site of *Escherichia coli* DNA Photolyase: Asn378 Is Crucial both for Stabilizing the Neutral Flavin Radical Cofactor and for DNA Repair[†]

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ABSTRACT: *Escherichia coli* DNA photolyase repairs cyclobutane pyrimidine dimer (CPD) in UV-damaged DNA through a photoinduced electron transfer mechanism. The catalytic activity of the enzyme requires fully reduced FAD ($FADH^-$). After purification in vitro, the cofactor $FADH^-$ in photolyase is oxidized into the neutral radical form $FADH^•$ under aerobic conditions and the enzyme loses its repair function. We have constructed a mutant photolyase in which asparagine 378 (N378) is replaced with serine (S). In comparison with wild-type photolyase, we found N378S mutant photolyase containing oxidized FAD (FAD_{ox}) but not $FADH^•$ after routine purification procedures, but evidence shows that the mutant protein contains $FADH^-$ in vivo as the wild type. Although N378S mutant photolyase is photoreducable and capable of binding CPD in DNA, the activity assays indicate the mutant protein is catalytically inert. We conclude that the Asn378 residue of *E. coli* photolyase is crucial both for stabilizing the neutral flavin radical cofactor and for catalysis.

Cyclobutane pyrimidine dimer (CPD)[1] is the major lesion in UV-damaged DNA. *Escherichia coli* DNA photolyase (EC 4.1.99.3) catalyzes the repair of CPD in DNA under near-UV or blue light irradiation. It is a monomeric protein of approximately 55 kDa containing two noncovalently bound chromophore cofactors (*1*). One cofactor is flavin adenine dinucleotide (FAD) (*2, 3*). This molecule is bound in the core of the protein (*4*) and is essential for the functions of the enzyme (*5–7*). Photolyase contains anionic two-electron reduced FAD ($FADH^-$) in vivo (*5, 6*), and $FADH^-$ is required for catalysis both in vivo and in vitro (*8, 9*). However, in the freshly isolated enzyme, the cofactor is in the blue neutral radical ($FADH^•$) state due to O_2 oxidation (*2*). The cofactor is to be slowly oxidized to the FAD_{ox} state during storage under aerobic conditions (*8*). The catalytically inert radical and oxidized photolyase forms can regain their activity by chemical reduction with dithionite (*2*) or by photoreduction in the presence of external electron donors such as dithiothreitol (DTT) (*10*). The second cofactor was identified as 5,10-methenyltetrahydropteroylpolyglutamate (MTHF) (*11*). This pterin derivative is located at the surface of the protein which can act as an antenna molecule transferring energy to the flavin during the photoreaction (*4, 12*). It is usually present in substoichiometric amounts in the purified enzyme (*11*) and not necessary for catalysis (*1, 5*).

A model for the catalytic reaction of photolyase is proposed: the reduced flavin cofactor is excited by a photon or by the photoexcited MTHF cofactor and then transfers an electron to CPD to generate a charge-separated radical pair ($FADH^• + CPD^{•-}$). The CPD radical anion then cleaves, and the excess electron returns to the flavin radical to restore the reduced form and close the catalytic photocycle (*1, 13*). There are many works that support the hypothesis. Recently, this photocycle was directly mapped by the femtosecond-resolved spectrometric method (*13*). Although the radical repair mechanism has been perfectly elucidated, it is necessary to further examine the protein structure to reveal why the enzyme is more efficient than normal chemical systems (*14*). The essential residues that function in catalytic steps, such as intermediate stabilization, substrate splitting, and back electron transfer, need to be clarified.

From the crystal structure of *E. coli* photolyase, Asn378 was shown to be located near the N(5) position of the FAD cofactor (*4*), which may form a hydrogen bond with the

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[1] Abbreviations: CPD, cyclobutane pyrimidine dimer; $FADH^-$, reduced anionic FAD; $FADH^•$, neutral FAD radical; WT, wild type; FAD_{ox}, oxidized FAD; MTHF, methenyltetrahydrofolate; DTT, dithiothreitol; SPR, surface plasmon resonance.

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N(5)H group via the carbonyl in its side chain. We have constructed a mutant photolyase in which Asn378 was replaced with serine. In contrast to WT photolyase, N378S mutant photolyase is purified with its FAD cofactor in the oxidized state instead of the radical state under aerobic conditions, but the FAD binding properties of the mutant protein suggest that it contains $FADH^-$ in vivo as WT photolyase. The MTHF cofactor binding of N378S mutant photolyase is indistinguishable from that of WT. Nevertheless, the mutant is catalytically inert, though it is photoreducable and capable of binding CPD in DNA as WT. These results reveal that Asn378 is crucial for stabilizing the flavin radical, and this stabilization might be significant for catalysis.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids. Chelating Sepharose Fast Flow was from Pharmacia. Oligothymidylates [oligo$(dT)_{16}$] and primers were purchased from Sangon. The substrate [UV-oligo$(dT)_{16}$] was made by irradiating oligo$(dT)_{16}$ under a 300 W high-pressure Hg lamp to generate pyrimidine dimers (*15*). Solutions of sodium dithionite in oxygen free water (flushed with argon) were prepared immediately before being used.

The N378S mutant gene was obtained by site-directed PCR mutagenesis. The mutant gene was cloned into pET22b(+) to generate the pN378S plasmid. DNA sequence analysis of the plasmid confirmed the mutant gene had been successfully constructed. The clone procedures had added a C-terminal six-histidine tag coding sequence to the gene, and thus, the protein was expressed as a His-tagged fusion protein. The tag is convenient for purification and has no discernible effect on the functions of the protein.

Expression and Purification of the Protein. The pN378S plasmid was transformed into the competent cells of *E. coli* strain BL21(DE3). The cultured cells were induced with isopropyl β-D-thiogalactoside (ITPG) at 25 °C. The induced cells were collected by centrifugation, resuspended, and then disrupted with a French press. The lysate supernatant was purified via Ni^{2+}-activated chelating Sepharose to give a purity of ~95%. The purification should be quickly completed within 2 h at 4−8 °C to prevent the release of the FAD cofactor. The protein buffer contained 50 mM NaCl, 10% glycerol, 1 mM EDTA, and 50 mM Tris-HCl (pH 7.2).

We obtained defolated protein by washing the mutant protein binding on the Ni^{2+} chelating column with deionized water with the WT method (*16*).

Absorption Spectroscopy. Absorption spectra were recorded from 200 to 700 nm on a Beckman DU640 spectrophotometer. Quartz cuvettes (300 μL) were used, and the temperature was held at 15 °C. The molar extinction coefficient of protein-bound FAD_{ox} ($\varepsilon_{450} = 11.2\ mM^{-1}\ cm^{-1}$) was determined for calculation of the holoprotein concentrations.

Determination of the K_D of P−FAD$_{ox}$. Fluorescence measurements were taken on an Aminco-Bowman series 2 luminescence spectrometer (Thermo electron). The excitation wavelength was set at 450 nm, and the emission wavelength was 520 nm.

The K_D of the defolated N378S mutant protein (P−FAD_{ox}) was derived from the equation as previously reported (*16*):

$$I/I_f = I_b/I_f + \frac{-K_D + \sqrt{K_D^2 + 4K_D[P_0]}}{2[P_0]}(1 - I_b/I_f) \quad (1)$$

where I/I_f is the ratio of the fluorescence intensity of the protein solution and free FAD_{ox}, I_b/I_f is the ratio of the fluorescence intensity of protein-bound and free FAD_{ox}, and $[P_0]$ is the concentration of the protein solution. I/I_f was obtained from the P−FAD_{ox} solution at various concentrations, and then 0.2% SDS was added to the solution to release FAD_{ox}. I_b/I_f and K_D were obtained by the nonlinear fitting method.

Differential Scanning Calorimetric Studies. Micro DSC measurements were carried out on a VP-DSC instrument from MicroCal. The volume of the sample cell was 0.509 mL. The samples were degassed before being loaded into the cell to prevent bubbles from occurring during heating. The scans were performed at a rate of 1 °C/min from 10 to 75 °C. Data were analyzed with ORIGIN with the VP-DSC add-on.

Photoreduction of the Mutant Protein. The buffer in the photoreduction experiment was the protein buffer supplemented with 10 mM dithiothreitol (DTT). The protein solution was flushed with argon gently for 10 min and then injected into the cuvette, and the surface was sealed with mineral oil. The solution was irradiated with an 11 W white light lamp in 4 °C. The spectra had been recorded after certain periods until the sample had been completely reduced.

Fluorescence Quenching Study. A fluorescence quenching study was performed with the Aminco-Bowman luminescence spectrometer. The excitation wavelength was 360 nm. The samples contained folate or defolated mutant photolyase reduced by sodium dithionite under anaerobic conditions. The emission spectra of the samples were recorded before and after addition of excess UV-oligo$(dT)_{16}$ at 20 °C.

Surface Plasmon Resonance Measurements. These measurements were taken on a BIAcore 3000 instrument (Biacore AB). The 5′-biotinylated CPD-containing 15-mer AGAG-CAGT<>TGACACG was immobilized on the sensor chip SA (Biacore AB). The dissociation constants were obtained from the fitting analysis with a 1:1 binding model using BIAevaluation, version 3.0 (Biacore AB).

Activity Assays in Vitro. The activity of the mutant and WT protein in vitro was evaluated by the SE-HPLC assays as described previously (*15*). For ease of comparison, the samples of N378S and WT photolyase were both defolated and reduced by dithionite. The 100 μL assay system contained 1 μM protein, 5 μM UV-oligo$(dT)_{16}$, 1 mM DTT, and 3 mM $Na_2S_2O_4$ under anaerobic conditions. The mixtures were incubated for 15 min and irradiated with a black light lamp (365 nm) for different periods of time. Then, the solution was boiled for 5 min and centrifuged at 16000*g* for 10 min. A 20 μL portion was subjected to SE-HPLC. The detection wavelength was 260 nm. The activity was evaluated from the increment of the peak area.

Activity Assays in Vivo. The activity of N378S mutant photolyase in vivo was analyzed by testing whether the mutant gene could complement the *phr* defect. Because the genes under the T7 promoter of pET22b(+) could not be expressed in the normal strains without T7 RNA polymerase, we constructed two plasmids, pTrcphr and pTrcN2S, by subcloning *phr* and the N378S mutant gene (without the histidine tag coding sequence) into the pTrcHisA plasmid

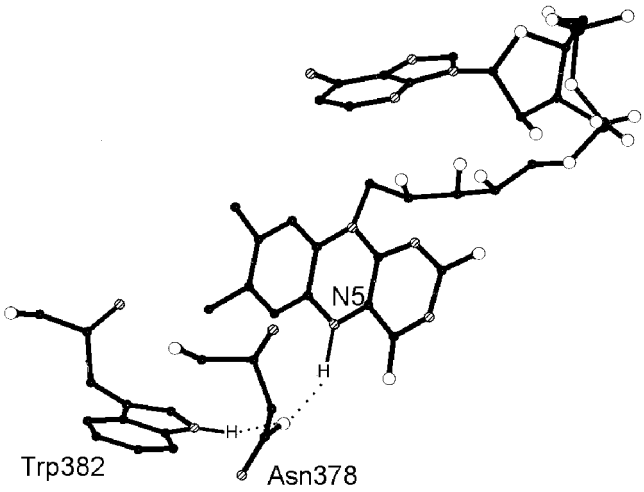

FIGURE 1: Conformations of the FAD cofactor and vicinal residues Asn378 and Trp382 in *E. coli* DNA photolyase.

under the *trc* promoter, respectively. The plasmids were transformed into the competent cells of *E. coli* K12 strain UNC1085 (*recA*, *uvrA*, *phr1*), which was generously provided by *E. coli* Genetic Stock Center (Yale University, New Haven, CT). The transformants were selected and inoculated into 2 mL of LB cultured at 25 °C. The induction of ITPG could have been omitted, because we had found that without induction there were enough molecules of the expressed proteins. The stationary phase cells (A_{600} = 3.0−4.5) were harvested, washed, and diluted with saline. For UV irradiation, the cell suspension (10 mL) was irradiated with 254 nm light from a germicidal lamp. The UV doses were measured by the iodide−iodate chemical actinometer (*17*). For photoreactivation, a daylight lamp (30 W) at a distance of 10 cm was adopted as the light source and glass covers were used to filter out <300 nm wavelengths. Then aliquots of the irradiated samples were plated onto LB plates, and colonies were counted after incubation at 37 °C for 36−48 h. The null UNC1085 strain was also tested as a negative control.

RESULTS

Construction of the N378S Mutant of E. coli Photolyase. From the crystal structure of *E. coli* photolyase, several residues such as Arg344, Asp372, Asp374, and Asn378 were shown to interact with the isoalloxazine ring of the FAD cofactor. Among these residues, Asn378 is located near the N(5) position of FAD (*4*), which may form hydrogen bonds with the N(5)H group of $FADH^-$ or $FADH^\bullet$ and the indole ring of Trp382 by its carbonyl group in the side chain (Figure 1). The Asn residue is highly conserved among most species of photolyases. The location and conservation imply that the Asn residue might be important for the function of photolyase. To reveal the role of the residue, we have constructed a mutant photolyase in which the asparagine residue was replaced with serine; thus, the carbonyl group in the side chain was changed to hydroxyl. This substitution alters the immediate environment of FAD, especially that of N(5). N378S mutant photolyase has been successfully overexpressed and exhibits many differences from the WT enzyme indeed. In this paper, the mutant is characterized in detail.

Absorption Spectra of N378S Mutant Photolyase. Wild-type *E. coli* photolyase was usually purified with FAD in the blue neutral radical form and a substoichiometric amount of MTHF [E−$FADH^\bullet$−(MTHF)] (*2*). Although the flavin radical cofactor becomes slowly oxidized under aerobic conditions, considerable amounts of $FADH^\bullet$ remain present in photolyase even after it has been stored for a long time (*7*). Stable neutral radical flavin hardly exists free in aqueous solutions because the dismutation of the radical is favored. Neutral flavin radicals can be formed under some conditions when bound to certain flavoproteins, but *E. coli* DNA photolyase is one of the unusual flavoproteins in which the radical is extremely stable (*2*).

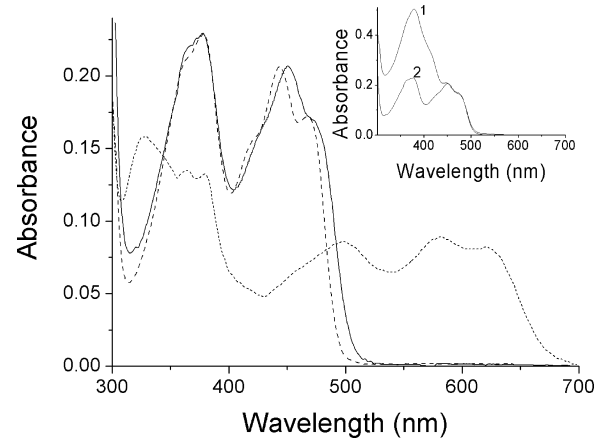

FIGURE 2: Absorption spectra of N378S mutant and WT photolyase. The solid curve shows the spectrum of the defolated N378S mutant. The dotted curve is for defolated WT photolyase (E−$FADH^\bullet$). The dashed curve is for FAD_{ox} reconstituted WT photolyase (E−FAD_{ox}). The curves in the inset represent the fresh (curve 1, with MTHF) and defolated (curve 2, the same as the solid curve in the main panel) N378S mutant protein.

However, the N378S mutant displays distinguishable absorption spectral features in comparison with WT photolyase (Figure 2). In contrast with WT, N378S photolyase has a bright yellow color when purified. The peak at 380 nm of the fresh sample indicates that N378S photolyase is still capable of binding MTHF as WT (curve 1 in the inset of Figure 2). The MTHF cofactor in N378S photolyase can be removed by washing the protein with deionized water, suggesting that the binding of MTHF by the mutant protein occurs via hydrophobic interaction as in WT (*16*). The spectra of the defolated protein are shown in Figure 2 (curve 2 in the inset and the solid curve in the main panel). However, there is no absorption above 550 nm but a peak at 450 nm with a shoulder at 475 nm, of either the defolated or the folate-containing protein. These show N378S photolyase is purified with FAD in the oxidized state but not the blue radical state as WT (the dotted curve of Figure 2, defolated WT, E−$FADH^\bullet$). When the spectrum of fully oxidized WT photolyase obtained by the reconstitution method (the dashed curve of Figure 2, E−FAD_{ox}) is compared, a significant bathochromic effect of FAD was found in oxidized N378S photolyase, where the 443 nm peak in WT shifted to 450 nm. These spectral features reflect the fact that the FAD binding environment is altered in N378S photolyase.

FAD Binding Properties of N378S Mutant Photolyase. Although the fresh purified mutant protein is in the oxidized form, we need to know whether N378S photolyase contains FAD_{ox} in vivo or is only a purification artifact under aerobic

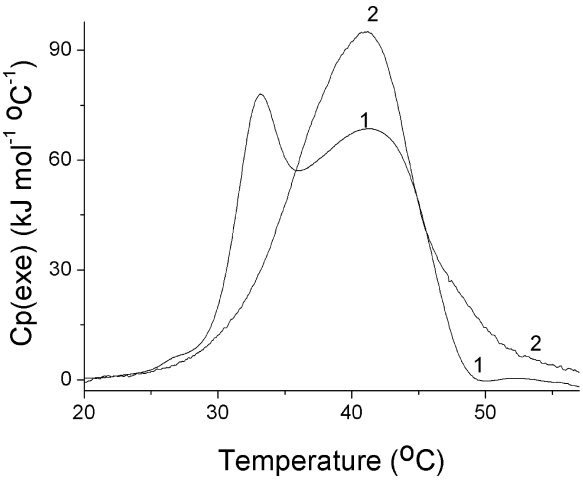

FIGURE 3: DSC thermograms of the N378S mutant protein in the oxidized form at 40 μM (trace 1) and the dithionite-reduced sample at 28 μM (trace 2). The scan rates were 1 °C/min.

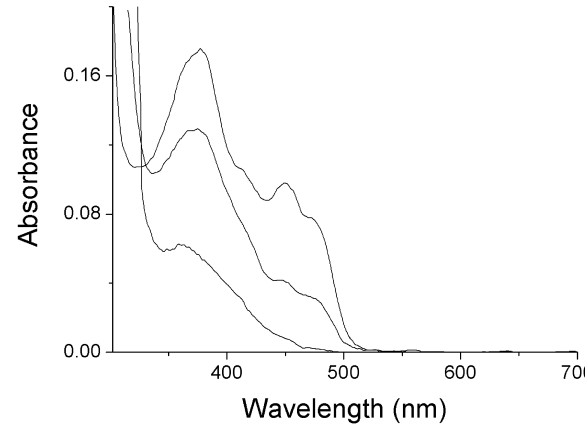

FIGURE 4: Photoreduction of N378S mutant photolyase. During photoreduction, the 380 nm peak and the 450 nm peak of the mutant protein decreased in magnitude and only a broad peak at 360 nm was left finally.

conditions. Directly detecting the redox state of the protein in vivo is not easy, but we can deduce it from the FAD binding properties in vitro.

First, the molar ratio of FAD and apoprotein in N378S photolyase (purified by a single step of Ni^{2+}-activated chelating Sepharose) was estimated. The protein was defolated and denatured with 0.2% SDS. From the molar absorption coefficients of free FAD_{ox} ($\varepsilon_{450} = 11.3$ mM^{-1} cm^{-1}, and $\varepsilon_{280} = 21.2$ mM^{-1} cm^{-1}) and that of the apoprotein ($\varepsilon_{280} = 103.6$ mM^{-1} cm^{-1}), we found that 1 mol of protein contained approximately 0.9 mol of FAD_{ox}.

The K_D value of P−FAD_{ox} was determined by the method described previously (*16*). We measured the fluorescence intensities of solutions of P−FAD_{ox} at different concentrations. The fluorescence intensities of the solution, after addition of 0.2% SDS to release free FAD_{ox}, were also measured. The following I/I_f values were determined: 12.5 μM (79.2%), 7.5 μM (83.3%), 6.25 μM (86.2%), 5 μM (86.6%), 3.13 μM (91.8%), 1.56 μM (97.0%), and 0.78 μM (98.9%). By plotting I/I_f versus $[P_0]$ and fitting the data to eq 1, we obtained the following values: $I_b/I_f = 0.24 \pm 0.22$ μM and $K_D = 22.7 \pm 10.1$ μM.

Nearly stoichiometric FAD_{ox} in the isolated protein and the relatively high dissociation constant of P−FAD_{ox} suggest that N378S photolyase should not associate with FAD_{ox} in vivo. If the concentration of the overexpressed protein in *E. coli* cells was ≥100 μM (>10000 molecules per cell), there was still only approximately 65−70% protein in the holo form if $K_D \sim 20$ μM. Further support for this suggestion is provided by the scanning calorimetric studies. Figure 3 shows the thermograms of oxidized and dithionite-reduced N378S photolyase (P−FAD_{ox} and P−$FADH^-$). There are two endothermic peaks (33 and 41 °C) of P−FAD_{ox} at 40 μM, while the sample of P−$FADH^-$ exhibits only one endothermic peak at 41 °C even at a lower concentration of 28 μM. From the K_D value of P−FAD_{ox}, we expected that an amount of apoprotein was presented in its solution at approximately 40 μM. We concluded that the low-temperature peak at 33 °C of P−FAD_{ox} was for the transition of the apoprotein. The two resolved heat absorption peaks also indicate that the apo and holo forms of N378S photolyase exhibit an extremely slow rate of interconversion on the time frame of the calorimetric scan (*18*), but no endothermic peak of the apoprotein was detected in P−$FADH^-$, indicating that P−$FADH^-$ has a much higher flavin binding affinity (K_D estimated to be $<10^{-7}$ M from the thermogram). [Similar phenomena have been observed. Oxidized WT photolyase exhibits two endothermic peaks (33 and 48 °C), but fully reduced WT exhibits only one peak (48 °C), which is consistent with the observation that apophotolyase binds much tighter with $FADH^-$ than FAD_{ox} (*16*). However, the holoprotein of WT photolyase has a peak at approximately 48 °C, but that of the N378S mutant is at 41 °C which indicates the thermal stability of the mutant holoprotein is dramatically decreased (unpublished data).]

From the results described here, we tentatively conclude that N378S mutant photolyase contains fully reduced FAD in vivo as WT photolyase but is oxidized during purification under aerobic conditions. [A less likely possibility that N378S photolyase containing radical $FADH^\bullet$ in vivo was ruled out by electron paramagnetic resonance experiments in which no strong signal was found with the cells containing the overexpressed mutant protein (data not shown).]

N378S Mutant Photolyase Is Photoreducible. Many flavoproteins, including photolyase, can be photoreduced under illumination in the presence of external electron donors, which is caused by light-induced electron transfers. The pathway for electron transfer in *E. coli* photolyase was proposed through three hops (W306 → W359 → W382) or through an α-helix by a superexchange mechanism (*1*).

In our experiments, we found that N378S photolyase could also be photoreduced with DTT as the electron donor. The progressive spectra of N378S photolyase during illumination are presented in Figure 4. The spectra show that the 380 nm peak and the 450 nm peak were decreasing in magnitude and only a broad peak at 360 nm was left finally. These results indicate that MTHF was photodecomposed and FAD_{ox} was photoreduced to $FADH^-$, which is similar to the case for WT (*10*), but the flavin radical intermediate (characterized by long wavelength absorption) was not observed in steady state absorption spectra. The results are reasonable, since no change has been made in N378S photolyase in the primary structures of the proposed electron pathways. However, because of the high dissociation constant of oxidized N378S photolyase, the possibility that free FAD_{ox} is photoreduced

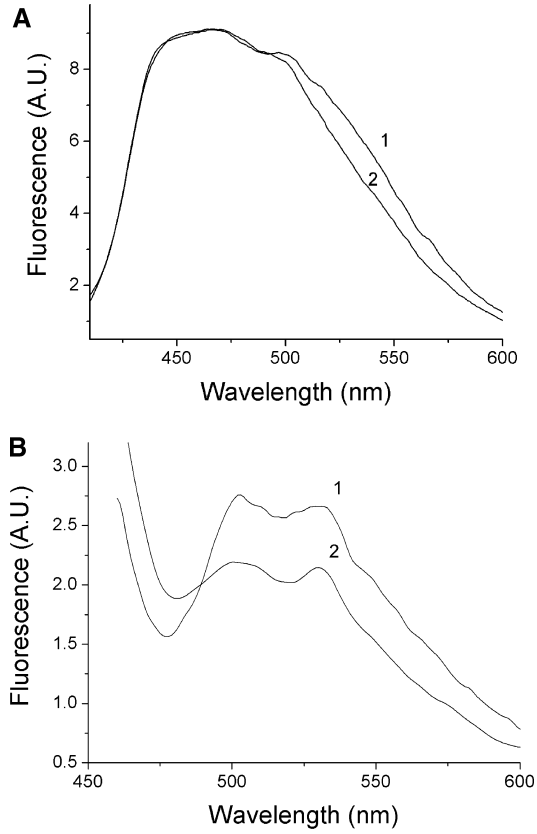

FIGURE 5: Effect of substrate on the fluorescence of N378S mutant photolyase. The excitation wavelength was 360 nm. (A) Fluorescence emission spectra of the reduced mutant protein containing MTHF [P−FADH$^-$−(MTHF)] in 10 μM before (curve 1) and after addition of excess UV-oligo(dT)$_{16}$ (2.5 mol of dimer/mol of protein) (curve 2). (B) Emission spectra of the reduced and defolated mutant protein (P−FADH$^-$) at 12.5 μM before (curve 1) and after addition of excess UV-oligo(dT)$_{16}$ (2.0 mol of dimer/mol of protein) (curve 2).

and then free FADH$^-$ couples with the apoprotein cannot be excluded.

N378S Mutant Photolyase Is Capable of Binding CPD in DNA. The fluorescence of bound FADH$^-$ is quenched when photolyase associates with CPD in DNA. This is evidence that singlet state FADH$^-$ functions as an intermediate in catalysis (*19*). The fluorescence quenching experiments were performed with N378S photolyase. The fluorescence spectra of fully reduced N378S photolyase (MTHF-containing or defolated) were recorded before and after addition of excess UV-oligo(dT)$_{16}$ at 20 °C (Figure 5). In Figure 5A, the sample was P−FADH$^-$−(MTHF); the fluorescence of FADH$^-$ was quenched, but that of MTHF was less affected. In Figure 5B, the fluorescence of FADH$^-$ is characterized in detail with the defolated sample. There is a 505 nm emission peak, which mimics that of WT, but a 530 nm peak is also observed in the spectrum (curve 1, Figure 5B). This emphasizes the fact that the FAD cofactor is in a slightly different environment. After addition of UV-oligo(dT)$_{16}$, the fluorescence was quenched, but the spectrum still has significant character (curve 2, Figure 5B). The spectrum did not change after more UV-oligo(dT)$_{16}$ was supplied, indicating that the quenching was complete. The fluorescence quenching reveals that N378S photolyase is capable of binding CPD in DNA.

Table 1: Activity Assays of WT and N378S Mutant Photolyase in Vitro

	CPD repaired (μM) (repaired percent)		
	0 min[a]	10 min[a]	40 min[a]
WT	0 (0%)	5.03 (18.29%)	13.18 (47.93%)
N378S	0 (0%)	0.27 (0.98%)	−0.05 (−0.18%)

[a] Photorepair time.

Preliminary studies with the surface plasmon resonance method were conducted to investigate the binding affinity of N378S photolyase for CPD in DNA. The dissociation constant of fully reduced N378S photolyase binding CPD in single-stranded DNA was estimated to be approximately 2×10^{-8} M ($k_a \sim 10^6$ M^{-1} s^{-1}, and $k_d \sim 0.02$ s^{-1}), which is in the same range of that of WT photolyase.

N378S Mutant Photolyase Is Catalytically Inert. From the experimental results given above, N378S photolyase is photoreducible and capable of binding CPD in DNA, properties similar to those of WT photolyase. To gain insight into N378S photolyase, we have determined its catalytic activity.

We used the SE-HPLE method to analyze the catalytic activity of N378S and WT photolyase in vitro, on the basis of the fact that CPD in UV-irradiated oligo(dT)$_{16}$ has no absorption at 260 nm but restores the absorbance when repaired (*15*). This method is convenient, and the interference of other chemicals is avoided. The samples of N378S and WT photolyase were both defolated and reduced with dithionite. The results show that a significant amount of CPD was photorepaired in the system of WT photolyase, but there was almost no detectable change in that of the N378S mutant (Table 1), indicating that the mutant protein had negligible activity in vitro.

To determine whether N378S mutant photolyase is active in vivo, two plasmids, pTrcphr and pTrcN2S, harboring the *phr* and N378S mutant genes under the *trc* promoter, respectively, were transformed into the UNC1085 strain (*recA*, *uvrA*, *phr1*). The cells containing different plasmids were harvested after the cells reached the stationary phase. The stationary phase cells of the null UNC1085 strain were set as the negative control. They were tested for photoreactivation. The results are shown in Figure 6. UNC1085 was found to be very sensitive to UV irradiation. After the cells were given a UV dose of approximately 1 J/m^2, the survival rates decreased to $<10^{-5}$. There was a residual photoreactivation in which the surviving fraction had a several-fold increase after illuminattion under white light for 1 h. The pTrcphr plasmid complemented the *phr* defect well so that the rate of survival increased approximately 5 orders of magnitude after photoreactivation. However, the pTrcN2S plasmid did not improve the photoreactivation of the strain much. The kinetics of photoreactivation in them tells the same story (Figure 6B). The UNC1085/pTrcphr strain almost reached its maximal photoreactivation under white light for 10 min, which implied relatively large numbers of photolyase molecules in the cells. The UNC1085/pTrcN2S strain had weak photoreactivation as the null UNC1085 strain. These results demonstrate N378S photolyase is almost catalytically inert in vivo.

DISCUSSION

E. coli DNA photolyase is an important enzyme that functions in the photorepair of UV-induced CPD lesions in

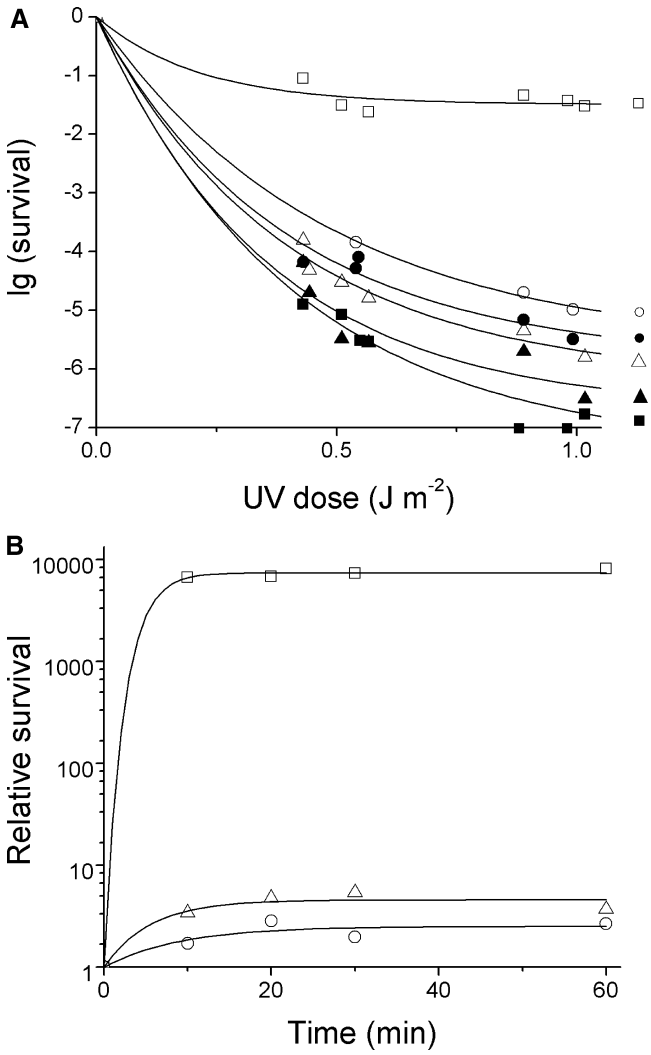

FIGURE 6: Effect of the WT and N378S mutant genes on photoreactivation of UNC1085 (*recA*, *uvrA*, *phr1*). (A) Survival of UNC1085 (circles), UNC1085/pTrcphr (squares), and UNC1085/pTrcN2S (triangles) after UV (black symbols) and maximal photoreactivation (white symbols). In some cases of UNC1085/pTrcphr, UV survival rates decreased to $<10^{-8}$, which are indicated on the abscissa. (B) Kinetics of photoreactivation of UNC1085 (○), UNC1085/pTrcphr (□), and UNC1085/pTrcN2S (△) with UV doses of approximately 0.5 J/m^2.

DNA. Usually, the enzyme is purified with its flavin cofactor in the blue neutral radical state, and the radical cofactor has great resistance to air oxidation and dismutation (*2*). The features of the neutral flavin radical cofactor in photolyase have been described in theoretical and experimental studies (*20–22*). The electron density of the isoalloxazine moiety was considered to be concentrated mostly at the C(4a) and N(5) positions (*21*). The electron of the H(5) atom might be attracted by those proximal atoms which made it bear a significant positive partial charge, and the structure of *E. coli* photolyase shows that the carbonyl group in the side chain of Asn378 sticks toward the N(5) position of the FAD cofactor (*4*). A strong hydrogen bond can thus be formed between the flavin N(5)H group and the side carbonyl oxygen of Asn378 which would decrease the free energy of the radical. It is intriguing that *Clostridium beijerinckii* flavodoxin, which is also able to hold a neutral flavin radical cofactor (FMNH$^\bullet$), has a similar feature: the backbone carbonyl oxygen of Gly57 points toward the flavin ring which accepts a hydrogen bond from the flavin N(5)H group in its semiquinone or reduced form (*23, 24*). This hydrogen bond in flavodoxin has been suggested to be important for the modulation of the redox potentials of the cofactor and the stabilization of the radical form (*23*), which were supported by recent studies (*24, 25*). In principle, we suspect that the interaction between the side carbonyl of Asn378 and the flavin N(5)H group in *E. coli* photolyase plays a role mimicking that in *C. beijerinckii* flavodoxin. The hydrogen bond might increase the difference between the redox potentials of oxidized/radical ($E_{ox/rad}$) and radical/reduced ($E_{rad/re}$) pairs. The formation constant (K_f) of the radical can be calculated with the formula (*24*)

$$E_{ox/rad} - E_{rad/re} = \frac{RT}{F} \ln K_f = \frac{RT}{F} \ln \frac{[rad]^2}{[ox][re]}$$

The larger K_f value is, the greater the proportion of the radical that can be reached. Because *E. coli* photolyase is stable in the radical form in vitro, the K_f value should be very large, but because the enzyme is readily in the reduced form in vivo, $E_{rad/re}$ would not be too negative; hence, $E_{ox/rad}$ must be relatively high (that is, smaller ΔG between them), which should be a consequence of the carbonyl−N(5)H interaction. Destroying the interaction would lower the $E_{ox/rad}$ and K_f values, making the radical formation limiting. The interaction might also provide a significant kinetic barrier to stabilize the flavin radical toward oxidization. Here, we present our data to show that substituting serine for Asn378 of *E. coli* photolyase abolishes its ability to stabilize the radical flavin, which supported the suppositions. However, although Asn378 might form a hydrogen bond with the reduced flavin, the N378S mutant still shows the strong binding preference for FADH$^-$ but not FAD$_{ox}$, which is similar to that of WT photolyase (*16*). It shows the binding preference of photolyase is determined by the entire structure that interacts with the flavin cofactor rather than a single hydrogen bond between the carbonyl and the flavin N(5)H group.

The active photolyase form contains FADH$^-$ (*8, 9*). The catalytic process was proposed to be a photoinduced electron transfer mechanism (*1, 9*). There are many photophysical and photochemical processes in the photorepair of the enzyme as follows. FADH$^-$ is excited, producing an excited state of FADH$^-$. The excited state has relaxation pathways: fluorescence (k_f), internal conversion (k_{ic}), and electron transfer to the dimer (k_{et}). The charge-separated species (FADH$^\bullet$ + CPD$^{\bullet-}$) formed via electron transfer can undergo two processes, splitting (k_{spl}) or back electron transfer (k_{bet}), resulting in an unproductive reversal. There are two pairs of competitions in these processes: (1) the photophysical process (k_f and k_{ic}) and electron transfer (k_{et}) and (2) the splitting (k_{spl}) and back electron transfer (k_{bet}). In the two pairs of competitions, k_{et} and k_{spl} contribute to the observed quantum yield of dimer splitting.

Stabilizing the neutral radical FAD is very important for achievement of high repair efficiency. Splitting and charge recombination (back electron transfer) constitute one pair of competition. Observation of active site solvation by Zhong et al. suggested that it appears to be critical to strategically slow the charge recombination and stabilize the charge-separated intermediate, leaving enough time for cleavage of the cyclobutane ring to reach a high repair efficiency (*13*). The apophotolyase, especially the side carbonyl group of

Asn378, should provide the stabilization during the splitting process of CPD. When the stabilizing effect was disrupted by the mutation, the transient flavin radical would become more labile and back electron transfer might be accelerated, leading to low repair efficiency. Besides the stabilization, the active sites including Asn378 should also suppress the internal conversion (k_{ic}) of the excited $FADH^-$, which is key factor for highly efficient repair. The internal conversion is dominant among deactive pathways of the excited $FADH^-$ in aqueous solution, due to the lack of various nonbond interactions at the active sites of the protein (*26*).

Asn378 is a crucial residue of *E. coli* photolyase, and the residue is highly conserved in other photolyases. In most class I CPD photolyases and (6−4) photolyases [a group of photolyases which split the UV-induced (6−4) adduct but not CPD in DNA], the residues are unchanged. Only in rare cases such as photolyase in *Halobacterium halobium* is asparagine replaced with the similar residue aspartic acid (*27*), and in all of the available crystal structures of photolyases, the asparagine/aspartic acid residues mostly adopt the structures resembling that in *E. coli*, expect for one of the structures of *Anacystic nidulans* photolyase (Protein Data Bank entry 1QNF): the side NH_2 group of the asparagine residue but not the carbonyl group faces toward flavin N(5) (*28*). Some blue light receptors or named cryptochromes in plants and animals are homologues of photolyases (*1, 29–32*). In those proteins, the residues are also mostly conserved to be Asn or Asp (*1, 27*), which implies a similar radical mechanism in their functions [an exception is *Drosophila* cryptochrome, where it is replaced with Cys (*32*)]. However, in many class II CPD photolyases, the asparagine residues are replaced with lysine (*27*). There is no doubt that class II photolyases also use the radical mechanism. However, how the lysine residues are located and function in those enzymes needs their structures to be determined. Nevertheless, the possibility that they are artificial for the scoring rules used cannot be excluded, because many conserved residues of class I photolyases are replaced in the alignments with the class II ones (*27, 33*).

During the complementation experiments in vivo, we found a weak but substantial tendency that the strains carrying the WT or N378S mutant photolyase genes are more sensitive to UV than the null UNC1085 strain in the dark (Figure 6A). Especially in the strain with the WT photolyase gene, extremely low survival rates ($<10^{-8}$) were observed in some cases. These show that the WT or mutant gene has negative effects on the dark repair of those strains. It was reported that photolyase could aid the dark repair in *recA* strains (*34, 35*). Nevertheless, our results are consistent with the observation that the *phr* gene increased the UV sensitivity of *uvr* strains (*35*). These might due to the fact that photolyase can stimulate uvrABC excision nuclease when it binds to CPD lesions in the dark (*36*), but UNC1085 is a triple mutant (*recA*, *uvrA*, *phr1*) which is defective in excision repair. WT or N378S mutant photolyase binding to CPD lesions would not enhance excision repair but might block some leaky translesion synthesis in UNC1085 (which is also rare in the strain because the full process needs recA to be activated) (*37*). Leaving the cells of the transformed strains in room temperatures without shaking for several hours in the dark sometimes could slightly increase the rate of UV survival, which more often occurred in the strain carrying the N378S mutant gene (data not shown). Perhaps it was because vigorous growth of the cells was needed to maintain the reduced state of the flavin cofactors in those proteins; when growth became slow, some of them could be oxidized. The oxidized proteins might have a lower affinity for the lesions, and then the translesion synthesis could save a few of the cells.

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