Effect of Base, Pentose, and Phosphodiester Backbone Structures on Binding and Repair of Pyrimidine Dimers by *Escherichia coli* DNA Photolyase

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ABSTRACT: Photolyases reverse the effects of UV light on cells by converting cyclobutane dipyrimidine photoproducts (pyrimidine dimers, Pyr<>Pyr) into pyrimidine monomers in a light-dependent reaction. Previous work has suggested that, based on substrate preference, there are two classes of photolyase: DNA photolyase as exemplified by the *Escherichia coli* enzyme, and RNA photolyases found in plants such as *Nicotiana tabacum* and *Phaseolus vulgaris*. In experiments aimed at identifying substrate determinants, including the pentose ring, for binding and catalysis by *E. coli* DNA photolyase we tested several Pyr<>Pyr. We found that the enzyme has relative affinities for photodimers of T<>T ≥ U<>T > U<>U ≫ C<>C and that the E–$FADH_2$ form of the enzyme repairs these dimers at 366 nm with absolute quantum yields of 0.9 (T<>T), 0.8 (U<>T), 0.6 (U<>U), and 0.05 (C<>C). The enzyme also repairs an isolated thymine dimer and the synthetic substrate, 1,1′-trimethylenebis(thymine) cyclobutane dimer. Unexpectedly, we found that this enzyme, previously thought to be specific for DNA, repairs uracil cyclobutane dimers in poly(rU). The affinity of photolyase for a uracil dimer in RNA is about 10^4-fold lower than that for a U<>U in DNA; however, once bound, the enzyme repairs the photodimer with the same quantum yield whether the dimer is in ribonucleoside or deoxyribonucleoside form.

DNA photolyases repair cyclobutane dipyrimidines (Pyr<>Pyr) in DNA by splitting the cyclobutane ring joining the two pyrimidines in a light-dependent reaction (Rupert et al., 1958; Sancar & Sancar, 1988). In addition to these well-characterized enzymes, photolyases specific for Pyr<>Pyr in RNA have been known for some time (Gordon et al., 1976). RNA photolyases have been found only in plants so far, and even though the paucity of these enzymes has prevented extensive study, these enzymes appear to be inactive on Pyr<>Pyr in DNA in contrast with DNA photolyases, which are, reportedly, inactive on Pyr<>Pyr in RNA (Rupert & Harm, 1966;Gordon et al., 1976).

Escherichia coli photolyase, which is available in large quantities, has been investigated in considerable detail with regard to substrate binding and catalysis [see Sancar (1991)]. The enzyme binds a T<>T-containing substrate by contacting the first phosphate 5′ and the three phosphates 3′ to T<>T on the damaged strand and the phosphate opposite the T<>T across the minor groove on the undamaged strand of a duplex. However, all these contacts are not essential for binding as the enzyme binds with equal affinity to a T<>T on single-stranded DNA and even to a dinucleotide photodimer, albeit at much lower affinity (Witmer et al., 1989). Catalysis occurs through photoinduced electron transfer. The enzyme has two chromophores (Jorns et al., 1984), methenyltetrahydrofolate (MTHF; Johnson et al., 1988) and flavin adenine dinucleotide (Sancar & Sancar, 1984), which is in $FADH_2$ form in vivo and is oxidized to the catalytically inert $FADH^0$ neutral blue radical form during purification. MTHF is the major chromophore, absorbs a 350–450-nm photon, and transfers energy to $FADH_2$, which then transfers an electron to Pyr<>Pyr to initiate ring splitting by a radical mechanism (Okamura et al., 1991) with a relatively high quantum yield (Payne & Sancar, 1990).

In vivo studies have shown that not all Pyr<>Pyr are repaired with equal efficiency, with T<>T being repaired more efficiently than T<>C, which is repaired more efficiently than C<>C (Setlow & Carrier, 1966). It was not clear whether the reduced rate of repair was due to lower affinity of the enzyme for cytosine-containing dimers or to lower quantum yield of repair. An in vitro study (Myles et al., 1987) provided qualitative evidence that both low affinity and low efficiency of photolysis contributed to the slow rate of repair of C<>C. Here, we have undertaken a comprehensive approach to define the roles of all three components (the phosphodiester backbone, the pentose, and the bases) of the substrate in binding and photolysis. We found that C<>C binds with 10-fold lower affinity and is photolyzed with about 20-fold lower quantum yield compared to T<>T. The intradimer phosphodiester bond increases the affinity but is not essential for binding or photolysis. Quite unexpectedly, we also found that this DNA photolyase binds to and repairs uracil dimers in RNA and therefore might be considered an RNA photolyase as well.

Materials and Methods

Materials. Deoxyribose dinucleotides TpT, UpU, and CpC were obtained from Sigma Chemical Co. Poly(dT), oligo-$(dT)_{12-18}$, oligo $(dC)_{12-18}$, and poly(rU) were purchased from Pharmacia. Poly(dU) was obtained form Midland Certified Reagents Co. The average chain lengths of poly(dT), poly(dU), and poly(rU) are approximately 200 bases. Thymine was from Sigma, and [*methyl*-^{3}H]thymine (1 Ci/mmol) was puchased from New England Nuclear–Du Pont. 1,3-Dibromopropane, dimethyl sulfoxide (DMSO), hexamethyldisilazane, potassium carbonate, and trimethylsilyl chloride, which are reagents for the synthesis of 1-(3-bromopropyl)-thymine (Browne et al., 1968), were from Aldrich Chemical Co. (Milwaukee, WI). DMSO was purified by treatment with sodium hydroxide followed by vacuum distillation. Potassium carbonate was dried by pulverizing and slightly flaming the solid prior to use.

Instrumentation. UV absorption measurements were performed on a Hewlett-Packard 8451A spectrophotometer. NMR spectra were recorded on a Varian XL-400 NMR spectrometer. Signal positions are reported in parts per million relative to tetramethylsilane as an internal standard. Melting

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points were determined with an electrothermal melting point apparatus. Fluorescence measurements were carried out as described previously (Kim et al., 1991) with use of a Model RF5000 Shimadzu spectrofluorometer.

Photolyase. *E. coli* photolyase was purified as described previously (Sancar et al., 1984). Enzyme purified by this method contains stoichiometric $FADH^0$ and substoichiometric (20–50%) MTHF. Photolyase containing only $FADH_2$ and no MTHF was prepared by selective photodecomposition on MTHF with the use of black light at a rate of 70 J m^{-2} s^{-1} (UV Products black-ray longwave lamp) under an argon atmosphere at 5 °C. MTHF decomposition was monitored by the decrease in fluorescence emission at 470 nm with a Model RF5000 Shimadzu spectrofluorometer. Complete photodecomposition was usually achieved with a dose of 2×10^5 J m^{-2}. Following photodecomposition, the folate degradation products were removed by passing the sample through a Sephadex G-25 column (PD-10, Pharmacia-LKB Biotechnology Inc.). Photolyase containing stoichiometric amounts of both chromophores was prepared by supplementing the defolated enzyme with the monoglutamate form of MTHF (Hamm-Alvarez et al., 1989, 1990; Kim et al., 1991).

Substrates. (a) DNA 20-mer duplex with a centrally located T<>T was a kind gift of Drs. J.-S. Taylor and D. Svoboda (Washington University, St. Louis, MO). (b) Di-, oligo-, and polynucleotides and *cis,syn*-T<p>T were prepared as described previously (Kim et al., 1991). The other substrates were prepared by acetone-photosensitized irradiation. Each of poly(dT), oligo$(dT)_{12-18}$, poly(dU), poly(rU), UpU, oligo$(dC)_{12-18}$, and CpC were dissolved in 10% aqueous acetone to concentrations of 1–2 mg/mL. Portions of each sample (3 mL) were placed in a quartz cuvette held at 5 °C, flushed with argon, and irradiated with 313-nm light from a 75-W xenon lamp equipped with a f/4 grating monochrometer (1200 L/mm; 300-nm blaze). The fluence rate at the inner surface of the incident beam side of the cuvette was 23 J m^{-2} s^{-1} as monitored by an AMKO-Quantacount (Quantacount-Photon Technology International, Inc.) calibrated by ferrioxalate actinometry (Hatchard & Parker, 1956). The samples were irradiated with $(3–5) \times 10^5$ J m^{-2}, and then the solvent was removed by flash evaporation. The residues were resuspended in diethyl ether [except poly(rU), which was resuspended in 100% ethanol] and centrifuged to collect the products. For thymine and uracil oligo- and polynucleotides the yield was about 75% conversion to dimers while about 40% of the cytosines were converted to dimers in cytosine-containing samples. Since C<p>C deaminates to U<p>U at pH 7.0 with a half-life of 10 h at 25 °C and 100 h at 0 °C (Freeman et al., 1965), the cytosine photodimers were used immediately after preparation. Other substrates were stored at −20 °C until use.

Irradiation of these substrates at 366 nm in the presence of photolyase resulted in recovery of 70–80% of 250–270-nm absorption typical of pyrimidine monomers. We consider this to be quantitative conversion to monomers as even though photosensitized radiation is not known to generate 6–4 photoproducts (Wang, 1976; Rahn, 1983) it does generate other isomers of the cyclobutane dimers: the yield of *cis,syn*-Pyr<>Pyr in photodimers from acetone-photosensitized irradiation of UpU has been reported to be 70% (Rycyna & Alderfer, 1988) and 80% in the case dTpdT (Liu & Yang, 1978). No figures are available for the distribution of cytosine isomers after photosensitized irradiation. We assumed 70% *cis,syn*-Pyr<>Pyr for substrates containing uracil or cytosine. Since photolyase is specific for *cis,syn*-Pyr<>Pyr, calculations for substrate concentrations are based on the concentration of this isomer. Purified *cis,syn*-dU<p>dT photodimer was a kind gift of Dr. J.-S. Taylor and was free of impurities as determined by NMR spectroscopy. (c) Base dimers: *cis,syn*-thymine dimer was prepared by a modified procedure described by Wulff and Fraenkel (1961). Approximately 1 g of thymine was dissolved in 1 L of H_2O and filtered. Portions of the filtrate (200 mL) were frozen in an aluminum tray (40 × 25 cm) at −75 °C. The frozen solution was placed in a dry ice bath in a temperature-controlled room at 5 °C and irradiated with two 15-W germicidal lamps placed about 10 cm from the surface of the solution. The dose rate of 254-nm light was 30 J m^{-2} s^{-1} as determined by a black-ray ultraviolet meter from UV products. After 1 h the solution was thawed, frozen, and irradiated again. This procedure was repeated until the sample received a total fluence of 8×10^5 J m^{-2}. After irradiation, the solution was concentrated to one-fourth of the original volume by use of a rotary evaporator under reduced pressure. The concentrated solution was heated to 100 °C, filtered while hot, and allowed to crystallize overnight at 5 °C. The crystalline precipitate was collected and recrystallized once more from 95% ethanol. The thymine dimer purified by this procedure was >98% free from thymine, judged by ^1H NMR in DMSO-d_6: ^1H-NMR (400 MHz, DMSO-d_6) δ 1.29 (6 H, s), 3.66 (2 H, s), 7.64 (2 H, s), 9.99 (2 H, s). The crystalline product was further purified by chromatography on thin-layer (100 μM) silica plates (R_f = 0.38 in the top phase of ethyl acetate 1-propanol/water, 4:1:2 by volume) prior to use. Dimer concentration was determined on the basis of its absorbance at 230 nm (ϵ = 2100 M^{-1} cm^{-1}). Preparation of the *cis,syn*-[*methyl*-^3H]thymine dimer (1 Ci/mmol) was carried out essentially as described above, except that the frozen solution of [*methyl*-^3H]thymine was irradiated in an anaerobic quartz tubing (5.0 × 100 mm i.d.). The synthetic photodimers, T<1$(CH_2)_3$1>T and mT<1$(CH_2)_3$1>mT were prepared by acetone-photosensitized irradiation as described previously (Kim, 1990; Young et al., 1990).

Assays. The various substrates were tested for both affinity (binding) and photolysis (repair). (*a*) *Binding.* The gel retardation assay (Husain & Sancar, 1987) was used to determine the equilibrium binding constants of radiolabeled substrates directly and the nonradioactive substrates by competition. The radiolabeled substrate was a 20-bp duplex with a T<>T in the middle and was labeled at the 5′ termini by kinasing. To measure the affinity of nonlabeled substrates, a known amount of 20-mer was mixed with cold substrates at various concentrations and binding to the 20-mer was measured by gel retardation (Figure 2). The competing substrate concentration that reduces binding of radiolabeled DNA by 50% under enzyme-limiting conditions is equal to K_D, the equilibrium dissociation constants of the nonradioactive substrate. The concentration of all substrates were determined spectrophotometrically. The gel retardation assay was conducted as follows. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 100 μg/mL bovine serum albumin, and enzyme and substrate at the indicated amounts. The mixture was incubated in the dark at 5 °C for 30 min; glycerol was added to a final concentration of 5%. The samples were loaded onto a 5% polyacrylamide gel (30:1 acrylamide/bisacrylamide) and electrophoresed at a constant voltage of 110 V for 1 h. Following electrophoresis the free and enzyme-bound DNAs were located by autoradiography, excised from the gel, and quantified by Cerenkov counting.

Thin-Layer Chromatography. Analytical thin-layer chromatographhy (TLC) was performed to isolate photolysis products (*cis,syn*-thymine dimer and thymine) with the use of glass plates (10 × 10 cm) precoated with a 250-μM thickness of silica gel (Analtech) with 254-nm fluorescence indicator or cellulose sheets (Brinkman-MN-Polygram Cel 300).

After photolysis, 80% aqueous ethanol was added to the photolysis reaction mixture. Ethanol-insoluble material was separated by centrifugation, and the ethanol-soluble material was dried under vacuum and resuspended in ethanol. The samples were spotted onto silica gel plates (or cellulose sheets), and chromatographs were developed with the upper phase of ethyl acetate/1-propanol/water (4:1:2).

For the assay of [*methyl*-^{3}H]thymine from photolysis of the labeled thymine dimer, regions coeluting with the authentic thymine were outlined and scraped into a scintillation vial containing 1.0 mL of water. After standing for 20 min, 5.0 mL of scintillation cocktail (Scintiverse II, Fisher Scientific) was added and radioactivity was determined by liquid scintillation counting.

Quantum Yield of Pyr<>Pyr Repair. The photolysis apparatus used in quantum yield measurements consisted of a 75-W xenon lamp and an f/4 grating monochromator (1200 L/mm; 300-nm blaze) equipped with an electronically operated shutter. The beam of the lamp, after emerging from the monochromator, was filtered through a BP370 broad bandpass and neutral density glass filters to reduce stray light and adjust intensity and then passed through a quartz beam splitter. The transmitted light was directed to the photolysis cell (90%), and the reflected light was directed to an actinometer reference cell, which allowed the light intensity to be monitored. The ratio of transmitted to reflected light was determined by potassium ferrioxalate actinometry (Calvert & Pitts, 1966).

For photolysis, E-FADH^0 (2.5×10^{-5} M) was mixed with substrate [(1.0×10^{-4})-(5×10^{-3}) M, depending on the substrate] in 350 μL of photolyase reaction buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 50 mM dithiothreitol). The reaction mixture was placed in an anaerobic cuvette with rubber septa. The cuvette was deoxygenated for 30 min by blowing argon through the photolyase reaction buffer and the cuvettes through small needles placed in the septa. Following deoxygenation, photolyase was photoreduced by filtered camera flashes ($\lambda > 500$ nm). Following photoreduction a 25-μL sample was removed from the cuvette with a gas-tight syringe. After the absorbance at 366 nm (irradiation wavelength) was determined from the UV spectrum, the cuvette was placed in a photolysis cell holder and irradiated for specific time intervals at 12 °C. After each irradiation, a 25-μL sample was removed, and 20 μL of this sample was diluted in the reaction buffer to monitor the progress of the reaction by recording absorption spectra in the wavelength region of 220–320 nm. After each sampling the absorption spectrum of the photolysis cuvette was also monitored to ascertain that $FADH_2$ had not been reoxidized or denatured during sampling. If the enzyme denatured or oxidized during sampling the data was discarded. The quantum yield of dimer repair was determined from the initial slope of a plot of the total absorbance increase at 265 nm vs the total absorbed light intensity at 366 nm using the relation

$$\Phi_{spl} = \frac{(6.023 \times 10^{23})V(\Delta A_{265}/\Delta I_{366})}{\Delta\epsilon_{265}}$$

where V is the volume of irradiated dimer solution, ΔA_{265} is the absorbance change at 265 nm during a certain irradiation time interval, and ΔI_{366} is the total amount of light absorbed by $FADH_2$ at 366 nm as determined using ferrioxalate actinometry (Hatchard & Parker, 1956). $\Delta\epsilon_{265}$ is the molar absorption coefficient difference between the two pyrimidines and the corresponding dimer. The values of $\Delta\epsilon$ at 265 nm [ϵ(Pyr-Pyr) - ϵ(Pyr<>Pyr)] used for calculating repair were as follows (in M^{-1} cm^{-1}): 19000 (TpT), 18500 (UpT), 18000 (UpU), and 17000 (CpC).

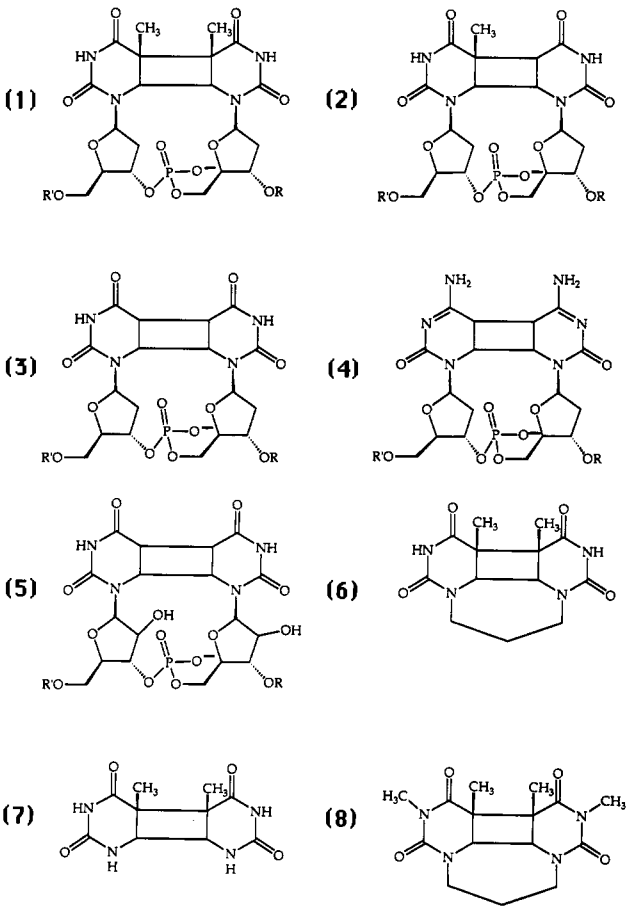

FIGURE 1: Structures of the substrates used in the present study. (1) UV-irradiated poly(dT), UV-irradiated oilgo(dT)$_{12-18}$, and *cis,syn*-T<p>T; (2) *cis,syn*-T<p>dU; (3) UV-irradiated poly(dU) and UV-irradiated dU<p>dU; (4) UV-irradiated oligo(dC)$_{12-18}$ and UV-irradiated C<p>C; (5) UV-irradiated poly(rU) and UV-irradiated U<p>U; (6) T<1(CH_2)$_3$1>T; (7) T<>T; (8) mT<1(CH_2)$_3$1>mT.

RESULTS

We wished to investigate the effects of the three components of Pyr<>Pyr in DNA, the bases, the pentose, and the phosphodiester bond on binding and catalysis by *E. coli* DNA photolyase to better define the structural requirements for binding and the effect of binding strength and base composition on quantum yield of photorepair. The structures of photodimers tested are shown in Figure 1.

(*a*) *Substrate Length and Primary Structure.* We compared the binding and photorepair of T<>T in DNA, in poly(dT), in oligo(dT)$_{12-18}$, and as a dinucleotide. Binding of various substrates was quantified by performing a competition assay with the use of gel retardation of radiolabeled DNA and by varying the competing substrate concentrations (Figure 3). The results summarized in Table I reveal that while the affinity of a T<>T in a homopolymer is about 10-fold lower than that of a T<>T in DNA and that the affinity of a thymine dinucleotide dimer is about 10^4-fold lower the quantum yield

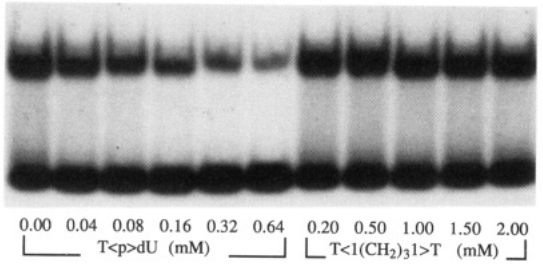

FIGURE 2: Equilibrium competition by dinucleotide photodimer, T<p>dU, and synthetic photodimer, T<1$(CH_2)_3$1>T, with ^{32}P-labeled 20-bp single-stranded substrate that contained a thymine dimer in a central location. The labeled substrate (10 nM) was mixed with increasing concentrations of T<p>dU (or T<1$(CH_2)_3$1>T), and then a fixed amount of photolyase was added. After 30 min at 5 °C, the mixture was subjected to the gel retardation to separate the E–S complex from unbound substrate. Even though synthetic T<1-$(CH_2)_3$1>T was repaired by photolyase, no competition was observed up to a 2 mM concentration.

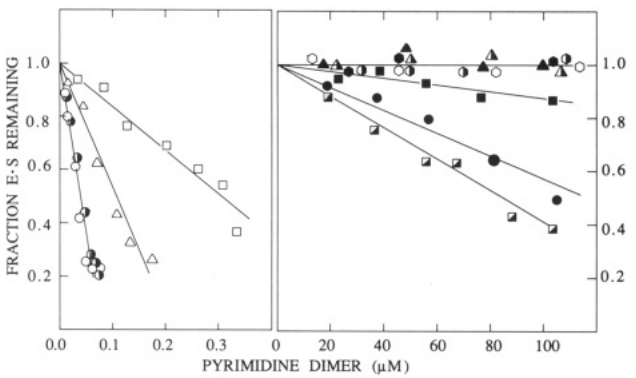

FIGURE 3: Combined plots showing the equilibrium competition by UV-irradiated nucleic acids and synthetic dimers. The experimental procedure for gel retardation was that described in the legend to Figure 2. Following the gel retardation, the bands corresponding to E–S complexes were excised and quantized by Cerenkov counting. (○) UV-irradiated poly(dT); (◑) UV-irradiated oligo(dT)$_{12-18}$; (△) UV-irradiated poly(dU); (□) UV-irradiated oligo(dC)$_{12-18}$; (◪) *cis,syn*-T<p>T; (●) *cis,syn*-T<p>dU; (■) UV-irradiated dC<p>dC; (▲) UV-irradiated poly(rU); (△) UV-irradiated U<p>U; (○) *cis,syn*-T<1$(CH_2)_3$1>T; (◑) *cis,syn*-mT<1$(CH_2)_3$1>mT; (●) *cis,syn*-T<>T.

of repair is—within experimental error—the same for all these substrates. This result is at variance with an earlier report (Rupert & To, 1976) that indicated that T<>T in poly(dT) was repaired at approximately 1/10th the quantum yield of a T<>T in DNA by yeast photolyase, an enzyme structurally and photochemically very similar to the *E. coli* photolyase (Sancar et al., 1987). We suspect that the earlier results were inaccurate because of the difficulties of conducting quantum yield measurements by competition assay using cell-free extract or partially purified enzyme preparations. The important conclusion that emerges from this study is that the quantum yield of repair is independent of the binding constant. This conclusion, in turn, leads to the suggestion that a T<>T dinucleotide has all the essential structural determinants to position the substrate in the enzyme active site in the optimal conformation. Electron transfer rate and efficiency are very sensitive to the orientations of and the distance between the donor and acceptor (Closs & Miller, 1988). Therefore, these results show that the geometry of the electron donor ($FADH_2$) and the acceptor (T<>T) is maintained constant with substrates of widely differing affinities. This follows from the fact that the rate of electron transfer from excited singlet state of $FADH_2$ is 5×10^9 s^{-1} (Kim et al., 1991) while the rate of dissociation of T<p>T from the enzyme is $\sim 10^2$ s^{-1} and therefore the probability of electron transfer is far higher than

Table I: Equilibrium Binding Constants and Repair Quantum Yields of Pyrimidine Dimers by *E. coli* Photolyase

substrate	K_A (M^{-1})	Φ_{366}[a]
(a) DNA		
20-mer-T⟨p⟩T	3.5×10^8	0.88
(b) homopolymers		
UV-poly(dT)	5.0×10^7	0.91
UV-oligo(dT)$_{12-18}$	4.6×10^7	0.92
UV-poly(dU)	1.8×10^7	0.60
UV-oligo(dC)$_{12-18}$	6.0×10^6	0.05
UV-poly U	7.5×10^2	0.54[b]
(c) dinucleotides		
T⟨p⟩T	1.8×10^4	0.89
T⟨p⟩dU	2.2×10^4	0.80
C⟨p⟩C	5.6×10^3	0.04[b]
U⟨p⟩U	$<5.0 \times 10^2$	+
(d) base dimers		
T⟨1$(CH_2)_3$1⟩T	$<5.0 \times 10^2$	+
mT⟨1$(CH_2)_3$1⟩mT	$<3.0 \times 10^2$	–
T⟨⟩T	$<1.0 \times 10^3$	+

[a] A plus (+) indicates repair was detectable; a minus (–) indicates repair was not detectable under conditions employed. [b] Corrections were made for the percentage of free enzyme.

that of dissociation from the substrate within the lifetime of the flavin excited state.

(*b*) *Effect of Base Composition of Pyr<>Pyr.* The binding and photolysis of T<>T, U<>T, and U<>U, and C<>C were investigated. All these photodimers are generated by UV irradiation of DNA, thymine, and cytosine dimers directly, and U<>T and U<>U are also generated by accelerated deamination of cytosines in C<>T and C<>C compared to cytosine monomer (Setlow & Carrier, 1966). By comparing the affinity of C<>C in oligo(dC)$_{12-18}$ to that of a T<>T in oligo(dT)$_{12-18}$, we observe a 4-fold lower affinity of C<>C compared to T<>T; a similar difference is found at the dinucleotide level as well (Table I). The most drastic effect of base composition, however, is seen in photolysis. The quantum yield for repairing C<>C by E–$FADH_2$ is 0.04–0.05, about 20-fold lower than that for T<>T. The low quantum yield of C<>C photorepair reported here is in qualitative agreement with that found by Rupert and To (1976) with a crude preparation of yeast photolyase. As in the case of the thymine dimer, the dinucleotide form of cytosine dimer was also bound with an affinity about 10^3-fold lower than that of a dimer in an oligonucleotide; but, once bound it was repaired with a nearly constant quantum yield regardless of the structural context.

The U<>T dinucleotide bound photolyase with an affinity the same as that of the T<>T dinucleotide, but it was repaired with a somewhat lower quantum yield of $\phi = 0.80$ compared to $\phi = 0.89$ for the T<>T dinucleotide (Table I). While these values are subject to some experimental error, we have consistently found that U<>T is repaired less efficiently and therefore are reasonably certain that the quantum yield for splitting U<>T is lower than that of T<>T.

The uracil photodimer in poly(dU) bound with about 3-fold lower affinity than a thymine dimer in poly(dT) and was repaired with a quantum yield of 0.6 or of about 63% the efficiency of T<>T. These lower quantum yields for U<>U and C<>C repair compared to that of T<>T are not the consequence of experimental artifact resulting from lower affinities of U<>U and C<>C because T<>T dinucleotide has a 10^4-fold lower affinity to photolyase than a thymine dimer in poly(dT), yet the two are repaired with nearly identical quantum yields. Thus, was conclude that Pyr<>Pyr are repaired with the following hierarchy of quantum yields

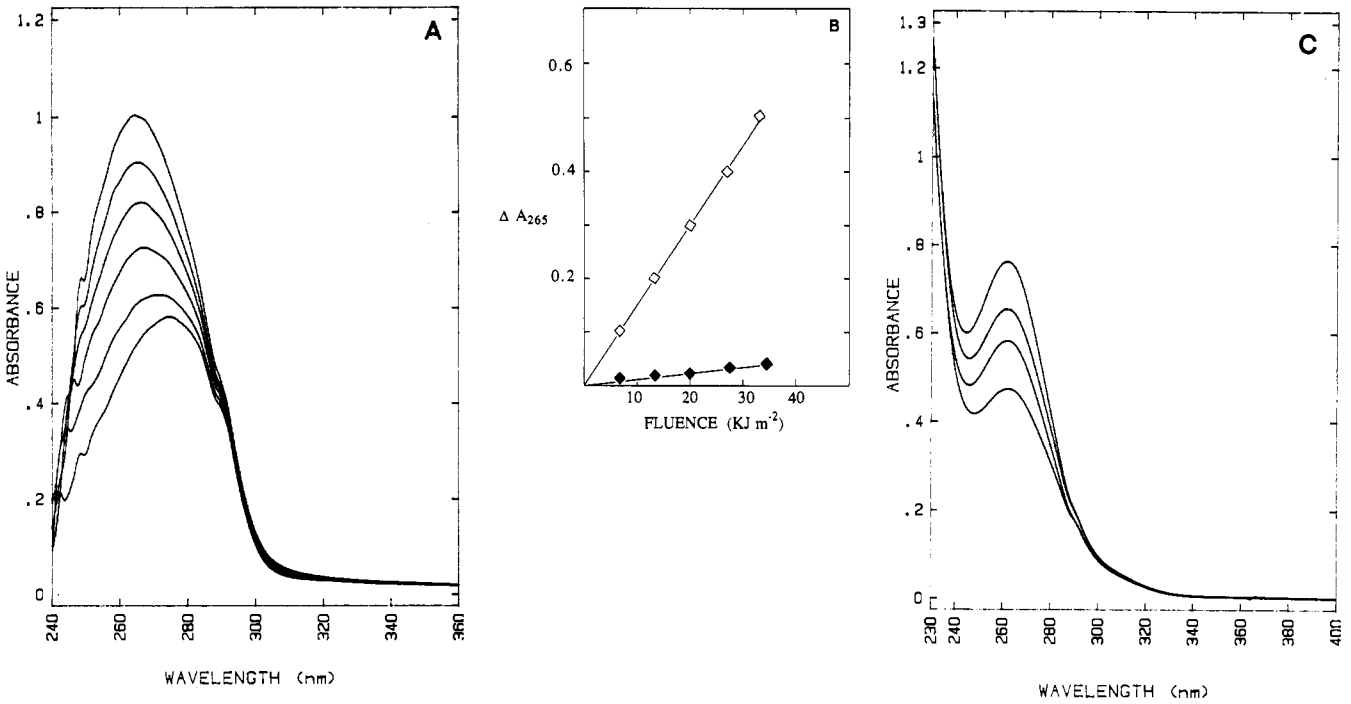

FIGURE 4: Photorepair of UV-irradiated RNA by *E. coli* DNA photolyase. (A) Absorbance increase as a result of dimer splitting upon irradiation of UV-irradiated poly(rU) (1.5×10^{-4} M dimers), with enzyme-bound $FADH_2$ (4.0×10^{-6} M) at 366 nm. The curves were obtained after irradiation with 0–33.3 kJ m^{-2} dose at 6.7 kJ m^{-2} intervals. (B) Absorbance increase (□) observed with UV-irradiated poly(rU) upon irradiation was replotted along with that of U<p>U for comparison. The latter was obtained under the same conditions as described for UV-irradiated poly(rU) except that a 4-fold higher concentration of U<p>U (6.0×10^{-4} M dimer) was used. (C) Absorption spectra taken from 50-fold diluted solutions of aliquots of reaction mixture containing enzyme-bound $FADH_2$ (2.5×10^{-5} M) and UV-irradiated poly(rU) (2.0×10^{-3} M dimer) with increasing irradiation time. Control experiments without enzyme did not cause any detectable absorbance change in the wavelength region of 320 nm. The quantum yield was determined from the slope of a plot of total absorbance increase at 265 nm vs the total absorbed light intensity at 366 nm.

(Table I): T<>T > U<>T > U<>U ≫ C<>C.

(*c*) *Effect of the Pentose Moiety.* Previous attempts to demonstrate photoreversal of Pyr<>Pyr in RNA by DNA photolyases failed to detect any repair (Rupert & Harm, 1966; Gordon et al., 1976). Instead, photolyase activity specific for ribonucleotide cyclobutane dimers was found in some plants (Gordon et al., 1976). We wished to test the susceptibility of Pyr<>Pyr in RNA to *E. coli* DNA photolyase to find out whether the lack of activity on RNA reported in older literature was due to lack of binding or of photolysis. We measured the binding to and repair of U<>U in poly(rU) by photolyase. It appears that rU<>rU in poly(rU) is a very poor substrate for binding; its affinity for photolyase is nearly 5 orders of magnitude lower than that of U<>U in poly(dU) and is even lower than the affinity of a T<>T dinucleotide (Table I). However, DNA photolyase does repair ribonucleotide cyclobutane dimers (Figure 4) and does so with essentially the same quantum yield as the deoxynucleotide dimer (Table I). The failure to detect this activity in earlier studies stems from the fact that crude enzyme preparations of much lower activities were used in those investigations.

(*d*) *Effect of the Phosphodiester Backbone.* Previous work indicted that photolyases were not inhibited by ethylation (Husain et al., 1987; Kiener et al., 1989; Baer & Sancar, 1989) or cleavage by β-elimination (Weinfeld et al., 1989) of the intradimer phosphodiester bond, suggesting that this bond did not play a significant role in photoreactivation. However, these previous studies were conducted with a T<>T in an oligonucleotide or DNA context and therefore the photodimer could have maintained its unique configuration even when the intradimer phosphodiester bond was broken or ethylated. In the present study we tested the repairability of T<>T at the base level to better quantify the contribution of the phosphodiester backbone to photolyase activity. The enzyme bound T<>T very poorly, and at the solubility limit of T<>T (~10^{-3} M) we were unable to observe any competition with T<>T in DNA at 1×10^{-9} M. However, the photodimer was repaired by the enzyme in a dose-dependent manner upon illumination with camera flashes and monitored spectrophotometrically. As this was very low efficiency repair we wished to eliminate the possibility of an experimental artifact such as an increase in 265-nm absorbance of the reaction mixture due to enzyme denaturation, photodecomposition of $FADH_2$, or direct photoreversal of T<>T by stray light of short wavelengths. To directly demonstrate photolyase-induced dimer splitting experiments were conducted with ^{3}H-labeled T<>T and the products were analyzed by thin-layer chromatography. It was found that 5×10^3 and 1×10^4 flashes converted 12.7% and 21.6%, respectively, of T<>T to monomers when photolyase was present in the reaction, but had no effect in its absence (Table II). Thus, clearly, photolyase repairs T<>T at the base level. However, due to the low efficiency of the reaction we are unable at present to determine the quantum yield of this particular repair reaction.

Table II: Thymine Dimer Splitting by *E. coli* Photolyase[a]

no. of flash × 10^{-3}	no. of count T⟨⟩T × 10^{-6}	no. of count Thy × 10^{-6}	% of T⟨⟩T repaired
0	3.57	0.02[b]	0
5.0	3.08	0.45	12.7
10.0	2.80	0.86	21.6

[a] Irradiation was performed with filtered camera flash (λ > 345 nm) for buffered solution containing 1.0×10^{-3} M *cis,syn*-[*methyl*-^{3}H]thymine dimer and 1.0×10^{-4} M enzyme-bound $FADH_2$. [b] This activity results from a small amount of contamination of the monomer region with dimer.

(*e*) *Synthetic Substrates.* Having discovered that a T<>T without the phosphodiester bond is bound and repaired very poorly, we wished to find out whether conformational restraints imposed on the two halves of the dimer were the important factor for the much higher affinity of a T<>T dinucleotide compared to a T<>T base dimer. We used the synthetic substrate T<1$(CH_2)_3$1>T, in which a thymine dimer is joined with a trimethylene bridge (Figure 1) and its 3-methyl derivative mT<1$(CH_2)_3$1>mT. Both compounds were tested for binding and photolysis. At the limit of solubility for both compounds we failed to detect binding to photolyase by the competition assay. On the basis of the concentrations used in our studies, we estimate the binding constants to be $< 5 \times 10^2$ and $< 3 \times 10^2$, respectively (Table I). Thus, the synthetic substrates are at least 2 orders of magnitude less efficient in binding photolyase compared to the deoxynucleotide thymine dimer. Thus, the "immobilization" of the pyrimidine rings in the dimer by the trimethylene bridge does not appear to improve the affinity. When the synthetic substrates were tested for repair we found that T<1$(CH_2)_3$1>T was repaired at a rate comparable to T<>T by repeated camera flashes, but we were unable to detect any repair of the 3-methyl derivative even with extensive irradiation. We cannot tell at present whether this lack of repair is due to lack of binding or photolysis.

Discussion

Substrate Recognition. Photolyases are structure-specific DNA binding proteins that specifically bind to *cis,syn*-Pyr<>Pyr in DNA. Enzymatic and chemical footprintings from three evolutionarily distant organisms, *E. coli*, *Saccharomyces cerevisiae*, and *Methanobacterium thermoautotrophicum* revealed very similar binding modes and led to the suggeston that a substrate of the structure pT<>TpNpNp would be necessary and sufficient for high-affinity binding by photolyses. Furthermore, thermodynamic considerations led to the conclusion that of the four phosphates that appeared to be contacted by chemical footprinting only one to two were bound through salt bridges that have no directionality while the others were presumed to make H-bonds (Sancar et al., 1987). In addition, site-specific mutagenesis has shown that Trp277 of *E. coli* photolyase contributes to specificity either by van der Waals or stacking interactions (Li & Sancar, 1990).

The work of Begley and colleagues (Husain et al., 1987; Witmer et al., 1989) has shown that photolyase recognizes and repairs a dinucleotide photodimer, albeit at a reduced rate. This perhaps explains the results from an earlier study with yeast photolyase where T<>T in oligomers shorter than nine nucleotides failed to be repaired with low concentrations of photolyase (Setlow & Bollum, 1968). The present study further expands and defines the structural requirements for binding of photolyase. In light of the results presented in this paper it may be of some use to consider every atom of the pyrimidine dimer with regard to its contribution to binding affinity. The N(1) position is in the form of a glycosylic bond in all Pyr<>Pyr. This position cannot act as an H-bond donor or acceptor but may make van der Waals contacts with the enzyme. We have used a synthetic substrate where N(1) is joined to a trimethylene bridge by a σ bond instead of the glycosylic bond; this substrate was repaired by photolyase although the low binding affinity precluded any conclusion regarding the contribution of N(1) per se to the stability of the enzyme–substrate complex. Similarly, an isolated thymine dimer where N(1) is in the form of a secondary amide and could act as a H-bond donor was bound and repaired by photolyase; again, the low affinity of this substrate precluded a quantitative evaluation of N(1) in binding to the enzyme, separate from its role in making the glycosylic bond, which must be important for maintaining the proper geometry of the two halves of the pyrimidine dimer.

C(2)=O is common to all pyrimidines and therefore two O(2)s positioned in a unique configuration imposed by the cyclobutane ring are potential candidates for H-bond formation with photolyase. However, O(2) protrudes into the minor groove in B-DNA yet chemical footprinting suggests mostly major groove interactions (Husain et al., 1987). Nevertheless, minor groove interactions have not been ruled out, nor is there definitive proof that DNA maintains the normal B-form in the immediate vicinity of the dimer; it is quite conceivable, then, that photolyase makes H-bonds with the O(2)s of the photodimers.

N(3) and C(4) are on the Watson–Crick face of pyrimidines, that is the substituents at these positions are H-bonded to the complementary bases in normal B-DNA. N(3) is in the form of imide in T and U and secondary imine in C; it acts as a H-bond donor in the former two and as a H-bond acceptor in the latter. It would therefore be an unlikely candidate for recognition by photolyases especially in light of recent studies (Kemmink et al., 1987; Taylor et al., 1990) suggesting that H-bonding properties of pyrimidines do not change appreciably upon pyrimidine dimer formation. However, formation of pyrimidine dimer shifts the equilibrium for tautomerization of cytosine in the direction of enol form conferring H-bonding properties similar to those of T and U at positions 3 and 4. The fact that the synthetic substrate with a methyl group at the N(3) of T failed to be repaired could be taken as evidence that this position might be involved in H-bonding, though it is also possible that a methyl group at N(3) interferes with binding by steric hindrance.

The C(4)=O group is common to T and U dimers that bind to photolyase with a higher affinity than C-containing dimers. Cytosine has an NH_2 at position 4 that is an H-bond donor in contrast to the H-bond acceptor C(4)=O found in T and U. It is possible that photolyase donates an H-bond to C(4)=O, hence the lower affinity to C dimers that cannot make this H-bond. Indeed the decrease in affinity of the C<>C dimer compared to the U<>U dimer corresponds to the loss of two H-bonds ($\sim$2 kcal/mol).

The C(5) and C(6) positions are tetrahedral in all Pyr<>Pyr. However, there is a methyl group at C(5) of Thy, and this group appears to contribute to binding either by van der Waals interactions or hydrophobic bonding as evidenced by the higher affinity of thymine dimers compared to all the others. However, it is clear that the major driving force in binding is the contacts made with the sugar–phosphate backbone of DNA as evidenced by the 5–6-kcal loss in binding affinity in going from pT<p>TpNpNp to T<p>T and a more drastic decrease in affinity upon substitution of the hydroxyl group (RNA) for the C(2′) position for the deoxyribose.

DNA Photolyase vs RNA Photolyase. E. coli DNA photolyase binds very poorly to U<>U in RNA, yet once bound it repairs it efficiently. Considering the fact that there are photolyases that repair Pyr<>Pyr in RNA (Gordon et al., 1976), the question arises as to whether these two activities and the two classes of photolyases are evolutionarily and structurally related. Assuming that an "RNA world" preceded the "DNA world", we are inclined to think that the two classes of enzymes are structurally related and that DNA photolyase resulted from a mutation at the binding site that generated an enzyme that no longer accommodated the C(2′)-OH group of RNA. Once binding occurs, DNA photolyase repairs the

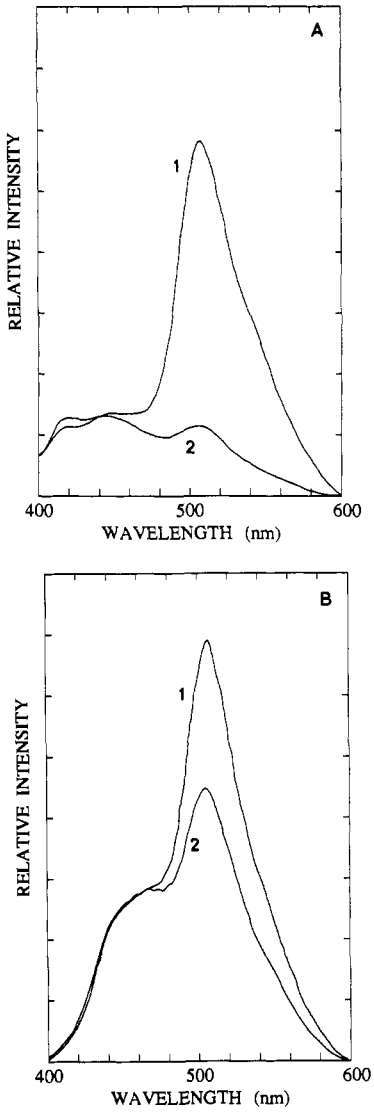

FIGURE 5: Effect of substrate on the fluorescence emission (noncorrected) spectra of enzyme-bound $FADH_2$. The shoulder at 460 nm is due to residual MTHF. The excitation wavelength was 360 nm with a 2-nm bandwidth. (A) Curve 1: $FADH_2$ (5.0×10^{-5} M) only. Curve 2: $FADH_2$ + UV-irradiated oligo(dT)$_{12-18}$ (4.0×10^{-4} M dimer). (B) Curve 1: $FADH_2$ (5.0×10^{-5} M) only. Curve 2: $FADH_2$ + UV-irradiated oligo(dC)$_{12-18}$ (8.0×10^{-4} M dimer).

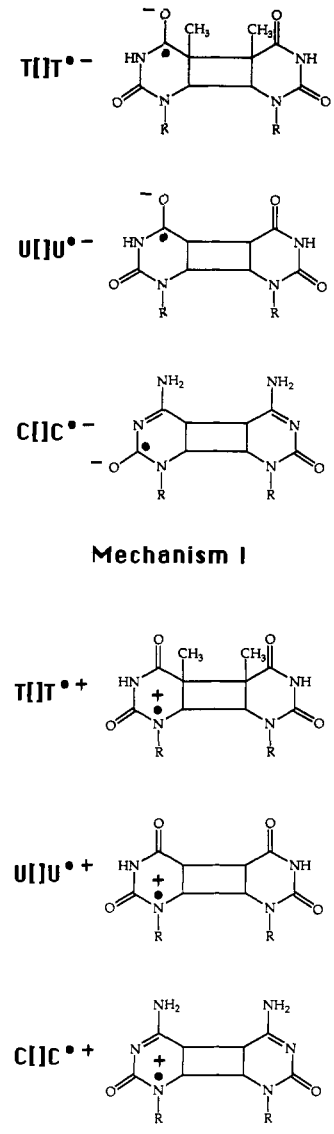

FIGURE 6: Two possible types of pyrimidine dimer radical intermediates associated with the pyrimidine dimer repair pathway by photolyase.

substrate with the same quantum yield regardless of whether it is RNA or DNA. Purification and characterization of genes and proteins of plant RNA photolyases should help in answering these questions.

Effect of Substrate on Quantum Yield. E. coli photolyase repairs Pyr<>Pyr by an electron transfer mechanism that involves radical intermediates (Okamura et al., 1991). There is no direct experimental evidence regarding the direction of electron transfer. However, thermodynamic considerations as well as work with a model system in which a Pyr<>Pyr was split by electron donation from a covalently linked indole (Van Camp et al., 1987; Kim et al., 1990) favor a mechanism in which the excited singlet state of the electron-rich $FADH_2$ donates an electron to the dimer to initiate concerted cleavage of the cyclobutane ring. The results presented in this paper provide further evidence for this mechanism.

If the $FADH_2$ excited-state singlet donates an electron to the substrate, the intermediate in Figure 6 (mechanism I) are expected to be produced from T<>T, U<>U, and C<>C, which would then undergo ring splitting. If flavin abstracts an electron then the intermediates in Figure 6 (mechanism II) are expected. Comparison of these two mechanisms would suggest that if the dimer radical anion is an intermediate (mechanism I), T<>T (and U<>U), in which the lone-pair electron is localized at the C(4)=O position, would split more efficiently than C<>C, in which the lone-pair electron would be localized at the C(2)=O position, which is incapable of initiating ring splitting except by low probability delocalization. However, the splitting efficiencies for all Pyr<>Pyr should be similar in a dimer radical cation mechanism (mechanism II) because of charge localization at N(1) position in all photodimers. As we see an 18-fold higher quantum yield of splitting of T<>T than C<>C, our data is consistent with a dimer anion mechanism. Furthermore, since about 50% $FADH_2$ fluorescence quenching is observed with the C<>C substrate (and >95% quenching with T<>T, Figure 5) it is clear that the low efficiency of repair of C<>C is only slightly due to reduced efficiency of initial electron transfer but mostly is due to nonproductive competing deactivation processes imposed on cytosine dimer radical anion. Indeed, it is possible that photolyase repairs only the C<>C when the cytosine moiety is in the enol form, which would result in charge localization at the C(4)=NH and subsequent ring cleavage.

In fact, the reported frequency of the enol form of C in C<>C (about 4%; Brown & Hewlins, 1968) is comparable to the quantum yield of repair of this photodimer. Finally, the 39–40% higher quantum yield observed with T<>T compared to U<>U could be ascribed to the strained ring configuration imposed on the photodimer by the C(5)-methyl groups in T<>T, which favors rapid and synchronous opening of the cyclobutane ring (Pac et al., 1982).

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